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DISSERTATION / DOCTORAL THESIS

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„Standardization and Harmonization in Lipidomics“

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Abstract

Lipidomics, which aims to study pathways and networks of cellular lipids in biological systems, is a continuously growing research field. Several analytical challenges accumulate especially in quantitative analysis by mass spectrometry (MS). Only suitable standardization can overcome its limitations and isotopically labeled internal standards (ISTD) are without question the “Gold Standard” for accurate and reproducible quantification.

Within the first part of this thesis, the implementation of a quantification method based on lipidome isotope labeling of yeast (LILY), an *in vivo* ^{13}C labeled *Pichia pastoris* yeast lipidome, as ISTD showed the potential of this cost-saving ISTD alternative. A novel fully automated workflow for lipidomics based on flow injection (FI), followed by reversed-phase- liquid chromatography-high resolution MS (RP-LC–HRMS) achieved absolute quantification of the sample lipidome by using a large set of species-specific and/or retention time-matched, class-specific calibrants. Due to the independence of the quantitative values from common deuterated or non-endogenous ISTDs, the novel workflow offered cross-validation of different organic MS-based lipid methods.

The second part cross-validated lipid class concentrations via multiple quantification platforms including common lipidomics workflows but also alternative approaches such as nuclear magnetic resonance (NMR) and inductively coupled plasma (ICP). Quantitative values for seven different polar lipid classes enabled traceable absolute lipid class concentrations, which are rare in a field without certified reference materials.

The final part further tried to optimize the use of yeast-based ISTD by fractionating the lipidome in its lipid classes via semi-preparative supercritical fluid chromatography (SFC). This powerful tool offered separation of a wide polarity range covering neutral and polar lipids in one 26 min run. The workflow doubled the identified lipids in *Pichia pastoris* to 400, due to the enriched and purified fractions and enabled concentration and composition adoptions of LILY to the sample of interest.

Zusammenfassung

Lipidomics, die biochemische Wissenschaft die sich mit der Untersuchung von Lipiden und deren Stoffwechselwege beschäftigt, steht seit einigen Jahren im Fokus vieler Forschungen, da die zentrale Funktion von Lipiden in vielen Bereichen eine Rolle spielt. Die Analyse dieser Verbindungen ist allerdings oft eine Herausforderung, speziell bei der Quantifizierung mittels Massenspektrometrie (MS). Nur die Verwendung von geeigneten Standards kann mögliche Fehlerquellen vermeiden und isotopenmarkierte interne Standards (ISTD) gelten hierbei als „Goldstandard“ für genaue und reproduzierbare Quantifizierung.

Der erste Teil dieser Arbeit beschäftigte sich daher mit der Implementierung von „lipidome isotope labeling of yeast“ (LILY) für die Quantifizierung von Lipiden. Dabei wurde ein *in vivo* ^{13}C markiertes *Pichia pastoris* Hefe Lipidome als kostengünstige ISTD Alternative in einen neuartigen vollautomatisierten Workflow integriert. Mittels Fließinjektion wurde der LILY ISTD on-Demand quantifiziert und anschließend bei der Probenanalyse mittels Umkehrphasen-Flüssigchromatographie angewendet. Dadurch ist eine große Abdeckung von Spezies-spezifischen und/ oder Retentionszeit angepassten Klassen-spezifischen Kalibratoren möglich, was eine genaue absolute Quantifizierung für viele Lipid-Analyten garantiert. Die Unabhängigkeit von herkömmlichen kommerziellen deuterierten und nicht-endogenen ISTDs eröffnet außerdem eine Vergleichsprüfung für unterschiedliche organische MS Methoden.

Im zweiten Teil wurden verschiedene Quantifizierungsmöglichkeiten vergleichsgeprüft, um eine rückverfolgbare Lipidklassenquantifizierung der Hefe zu garantieren. Klassische MS Lipidomics Methoden wie Shotgun Lipidomics und Chromatographie-basierte Methoden wurden mit alternativen Methoden wie der Kernresonanzspektrometrie (engl. NMR) oder der induktiv gekuppelten Plasma-MS verglichen und validiert. Für sieben verschiedene Lipidklassen konnten dadurch rückverfolgbare absolute Konzentrationen bestimmt werden. Da für Lipidomics Methoden noch keine zertifizierten Referenzmaterialien vorhanden sind, ist dies ein wichtiger Schritt in Richtung Standardisierung und Harmonisierung.

Im letzten Teil sollte der Hefe-basierte ISTD weiter optimiert werden. Um mögliche Unterschiede zwischen ISTD und Probe fallspezifisch adaptieren zu können, wurde eine Fraktionierung des Lipidomes in die einzelnen Lipidklassen durch semi-präparative überkritische Fluidchromatographie (engl. SFC) ermöglicht. Diese Methode bietet den Vorteil sowohl neutrale als auch polare Lipide in nur 26 min zu trennen. Durch das besser aufgetrennte und angereicherte Lipidome konnten 400 Lipide in *Pichia pastoris* identifiziert werden, die nach Bedürfnis in ihrer Konzentration und Zusammensetzung wieder verändert zusammengefügt werden können.

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1. Introduction

1.1. Lipids, lipidome and lipidomics

Lipids play a crucial role in our life and are a prerequisite for living organisms.[1] Their roles involve cellular membrane formation, energy storage as well as signal transduction, and many other functions, which lead to their important roles in diseases such as diabetes, obesity, atherosclerosis, stroke, cancer, psychiatric and neurological disorders, neurodegenerative diseases, and infectious diseases.[2] As small biomolecules they belong to the metabolome, the entity of small molecules in a biological system. Due to their hydrophobic or amphiphilic nature, a sub-ome has been introduced in 2001 termed lipidome.[3] Additionally, around 80% of the compounds forming the human metabolome consist of lipids.[4] Lipids themselves are nowadays defined according to their biosynthesis. The consortium of lipid metabolites and pathways strategy (Lipid MAPS) defines: "lipids based on the origin of the lipid structures are hydrophobic or amphipathic small molecules that may originate entirely or, in part, by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.)."[5]

Following other -omics disciplines of systems biology such as genomics or proteomics, lipidomics studies the pathways and networks of lipids and their interacting partners in a large-scale manner.[6] Down the omics cascade from the genotype to the phenotype, lipidomics as part of metabolomics is the closest to the phenotype and tries to answer what has happened and is happening right now.[7,8] It is a continuously growing research field with over 40 000 lipid structures in the biggest lipid database, the lipid MAPS structure database.[5] This high number of compounds leads to analytical challenges such as a high diversity and the occurrence of many isomers and isobars but at the same time, their similarity offers simplified workflows pre- and post- analytically.[9]

Within this thesis, the principles of the possible analytical techniques for lipid analysis will be introduced as it is the backbone for accurate lipid quantification. Further, common quantification strategies are shown and the connection to standardization and harmonization should highlight the importance of the work.

1.2. Analytical techniques in lipidomics

Analytical techniques in mass spectrometry (MS)-based lipidomics are multiple,[10] but can be summarized in three fundamental principles, when focused on liquid-based, electrospray ionization (ESI): (1) an MS analysis without chromatographic separation, called shotgun lipidomics[11], where the sample is directly infused into the MS, (2) the use of lipid class separation techniques such as normal phase liquid chromatography (NP-LC), hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC) and finally (3) the use of reversed-phase liquid chromatography (RP-LC) to separate lipids by their hydrophobicity.

1.2.1. Shotgun lipidomics

A shotgun approach is the simplest sample introduction as the sample solution is directly infused into the MS. This can be conducted by a syringe pump[11], flow injection (FI),[12], or by the use of a nanospray devise[13] offering different benefits in terms of availability of instruments, costs, throughput, avoidance of carrying over, and sensitivity (see Table 1). Together with RP-LC, it is the most common lipidomics technique,[14] due to its rapid nature for quantitative screening of the most abundant lipids. Especially, the use of FI and nanospray devices enable fully automated analysis with high reproducibility.[15]

Table 1 Comparison of direct infusion methods. The three possible sample introduction systems nanospray device, flow injection, and syringe pump are exemplarily shown on three commercially available instruments and rated for different important factors.

	Nanospray device	**Flow injection**	**Syringe Pump**
			
Required instrument	chip-based nano electrospray ionization	LC-system, no column	Syringe + syringe pump
Acquiring cost	+	+	+++
Maintenance cost	+	++	+++
Throughput	+++	+++	+
Carry over	+++	++	+
Sensitivity	+++	+	+
Possible flow rate	100-200 nL/min	5µL-5 mL/min	0.1nL-90 mL/min
Example Instrument	Advion TriVersa NanoMate	Thermo Fisher Scientific Vanquish Horizon	Chemyx Fusion 101
Picture source (18.08.2021)	https://www.advion.com/wp-content/uploads/photo_products_triversa-nanomate.png	https://assets.thermofisher.com/TFS-Assets/CMD/product-images/Vanquish-TCC-Open-1920x1080.jpg-650.jpg	https://cdn.chemyx.com/wp-content/uploads/2017/01/fusion-100-classic.png

In shotgun lipidomics, all analytes are exposed to the same matrix effects as they are in the same solution while entering the MS. This offers class-specific quantification as only one non-endogenous synthetic standard per lipid class is routinely used to quantify other lipid molecules from the same class that exhibit the same ionization efficiency.[16] This reduces the needed amount of internal standards (ISTD) to a minimum compared to hundreds if each analyte would have its own ISTD. However, the assumption of the same ionization efficiency is limited to polar lipid classes and other work arounds are needed for neutral lipids such as a higher number of ISTDs[17] or response factor correction.[12,18–20]

Compared to LC methods, shotgun lipidomics is limited in terms of sensitivity and selectivity. The constant ion suppression that simplifies quantification also leads to a constant decrease in sensitivity, which hardly can be overcome within one analytical run and might need derivatization[21] or further sample clean-up.[22] This might also help in terms of selectivity as isomers and isobars are hard to distinguish. As analysis times of up to 50 min with 10µL sample consumption can be achieved with nanospray devises,[23] different

fragmentation modes can be applied in this rather unlimited time increasing identification capabilities. It is common to use data-independent acquisition (DIA) by covering the whole mass range in 1 Da steps to enable full fragmentation coverage of the sample.[24] The combination with MS^n, double bond indicating fragmentation techniques or ion mobility spectrometry (IMS) offers further possibilities to enlarge the qualitative information of shotgun lipidomics.[10]

1.2.2. Lipid class separation

Complex lipid mixtures can be separated into lipid classes by chromatographic methods that separate analytes by their polarity. As the polarity of lipids is mostly defined by the polar head group, which further defines the lipid class itself, lipid class separation can be achieved. This trend can be observed in three methods: NP-LC, HILIC, and SFC (see Table 2). They might have different separation principles, but in most cases, polar materials are used as stationary phases.[25]

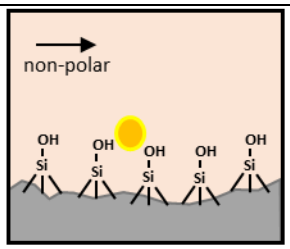
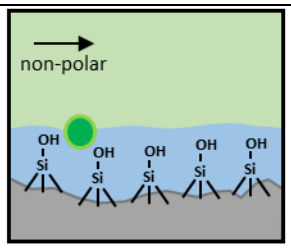
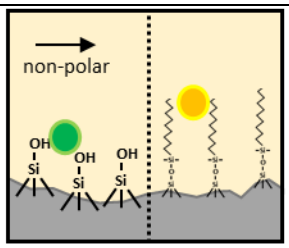
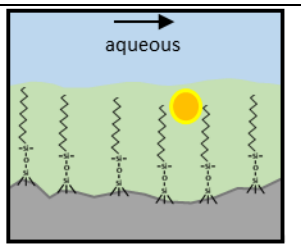
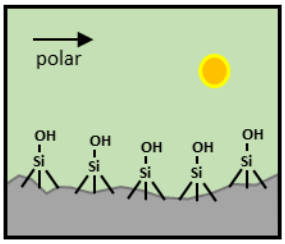
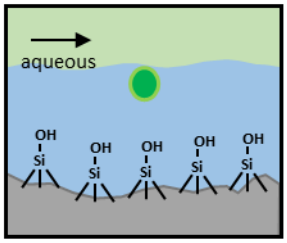
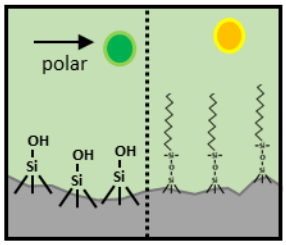
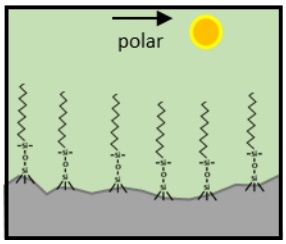
In NP-LC non-polar solvents are used as mobile phase without any water. This method is useful for the separation of non-polar lipid classes, but polar lipids cannot be separated from each other. Furthermore, the eluents, typical alkanes or chlorinated solvents, are not compatible with ESI-MS which hampers detailed lipid identification and makes this method unfavorable in nowadays lipidomics approaches.[21]

On the contrary, HILIC is a suitable tool for phospholipid (PL) analysis as the retention increases with polarity. It separates classes from each other but still enables lipid-class specific internal standardization as lipid species from the same lipid class elute close to each other. Eluents in HILIC contain water and polar organic solvents e.g. methanol (MeOH) or acetonitrile (ACN). In contrast to NP-LC, non-polar lipids are not retained e.g.: glycerolipids, cholesterol esters, or sterols. In blood-derived samples these lipid classes are often strongly influenced by the recent diet of the patients and quantification with single-lipid-per-class internal standards is not ideal as lipids of these classes have not the same ionization efficiency. This shifts the focus of many types of research to polar lipids only, making HILIC an appropriate tool.[26] However, also the analysis of polar lipids is often hampered as acidic lipid classes suffer from insufficient retention behavior on most HILIC columns. These lipids include classes such as phosphatidic acid (PA), phosphatidylserine (PS), and their lysospecies, which contain two negative charges in the head group. Therefore, they have several potential dissociation states, broadening the peaks and reducing the sensitivity. It makes the selection and control of the pH highly important. Previously, good peak shapes have been achieved by the use of additives, e.g. alkyl ammonium salts as ion-pairing agents [27] but these additives are often not well compatible with MS measurements. Alternatively, the use of columns, in which the acidic silanol groups are replaced, has been successfully applied for the analysis of acidic lipids.[28]

The third method, SFC, can separate both, non-polar and polar lipid classes in one analytical run if gradients go from pure supercritical CO_2 to subcritical CO_2/MeOH mixtures, therefore overcoming the limitation of NP-LC and HILIC.[29] Supercritical carbon dioxide has been used as eluent since the 1980s but has been limited due to low chromatographic efficiency and insufficient reproducibility. [30–32] With recent developments, including improved injection systems, a better backpressure regulation and pressure up to 600 bar that subsequently allowed smaller particle sizes (<2µm), a tremendous improvement in efficiency and sensitivity has been achieved.[33] Moreover, SFC benefits from the use of "green" CO_2, making it especially suitable for preparative applications in pharmaceutical sciences. [34,35] While SFC has been

originally coupled to ultra violet (UV) detectors or evaporative light scattering detectors (ELSD), the use of MS is nowadays widely common in lipidomics studies.[29,36] A clear benefit compared to other chromatographic methods is the rather constant low chromatographic plate height with increased flow rate (defined by the Van Deemter equation) as the mass transfer is less dependent on the flow. This enables very fast run times (e.g. 6 min for whole lipid polarity spectrum) without a compromising separation.[29]

Table 2 Comparison of the three lipid class separation techniques with RP-LC. [37,38] The green or yellow dot indicate rather non-polar or polar analytes, respectively while the background colors symbolize non-polar (organic solvents e.g. heptane, diethyl ether: red, supercritical CO_2: orange), polar (organic solvents e.g. ACN, MeOH, IPA: green) and aqueous solutions (blue). *SFC separations with non-polar columns separate lipids also according to their fatty acyl chain composition.

	NP-LC	**HILIC**	**SFC**	**RP-LC**
Loading				
Elution				
Column	Polar	Polar	Polar or non-polar	Non-polar
Classical mobile phase	A: Heptane B: IPA	A: ACN B: water	A: CO_2 B: MeOH	A: water B: ACN
LogP Analyte	5 - 10	-5 - 0	-5 - 10	0 - 5
Type of separation	NL **class** separation	PL **class** separation	NL and PL **class** separation*	NL and PL **species** separation
Pro			Fast "green" eluent	Reproducible Isomer separation
Cons	Not MS-compatible	Long regeneration	Special equipment	Limited co-elution

1.2.3. Reversed-phase liquid chromatography

RP-LC columns are non-polar stationary phases and use polar eluents to separate analytes according to their hydrophobicity. In lipids, the length and position of the fatty acyl chains, the number of double bounds, and their configuration are the main factors defining hydrophobicity. Thus, only RP-LC as a chromatographic method can theoretically separate isomers from the same lipid class. The run time is normally the longest compared to shotgun, HILIC, or SFC and can reach up to 120 min to achieve high-resolution power.[39] As the retention time cannot be easily determined with a single lipid class standard as in lipid class separation methods, identification of each lipid species is especially important. Retention time matching with standards, identification in positive and negative mode as well as class-specific fragments help to identify a lipid. Furthermore, false-positive annotations can be filtered according to the equivalent carbon number (ECN) model.[40] This regression model determines homologous lipid series within one class by the retention time vs. ECN= number of carbons in the chain – 2*number of double bonds in the chain.

The higher degree of separation has the benefits of improved selectivity as well as decreased matrix and subsequently higher sensitivity. However, the non-coelution of a single-lipid-per-class ISTD with the lipid species of the same class hampers the simplified quantification approach normally applied in lipidomics as different solvent compositions and different matrix effects cause changes in ionization efficiency.[17] Only a higher number of ISTDs can lead to a higher coverage in accurate quantification, which prerequisites are further described below.

1.3. Standardization and quantification of lipids

In the analytical field, it is common to follow certain guidelines for accurate, absolute quantification (European Medicines Agency (EMA),[41] United States Food & Drug Administration (US.FDA)[42] or other national institutes worldwide) but they are not always fully applicable to the omics-type analysis of endogenous compounds. Either the theoretically unlimited number of analytes within one measurement or the fact that an analyte-free matrix is not possible when all substances in a sample are also analytes, are contradicting the recommended guidelines for method validation. The search for accurate and applicable workarounds is therefore still ongoing.

1.3.1. Relative quantification

In general, two types of quantification can be distinguished, relative and absolute quantification. Research in relative quantification always tries to indicate the true representation of the actual fold change between certain groups e.g. healthy vs. diseased, which needs normalization across the sample files. The gold standard here is the use of ISTD but other normalization strategies have also been summarized.[43–45] To cover the high number of analytes, the use of labeled biomass has been shown to reduce the relative standard deviation (RSD) in GC metabolomics experiments compared to TIC or single ISTD normalization.[46] Because of the relatively high costs of fully labeled substances, partially labeling has been also suggested. Kim et al. 2019[47] added 5% deuterium oxide to the medium to partially label lipids from HeLa cells. A deconvolution algorithm has been used following classical isotopic dilution approaches to obtain fold-change ratios for each lipid analyte. Another approach is the use of partially ^{13}C-labeled mouse tissue, obtained from a mouse fed a ^{13}C-labeled *Ralstonia eutropha* bacterial protein-based rodent diet.[48] An enrichment between 6-75% has been observed, depending on the bio-pathway of the metabolite. With

this ISTD, the coefficient of variation has been reduced from 27% to 10%. Both approaches profit from the ideal sample matched ISTD, which cannot be achieved with relatively simple produced fully labeled biomasses, but they suffer from a higher complexity during MS analysis and data processing. Another way to use a sample-matched ISTD is the application of derivatization. By using isotopically labeled and unlabeled derivatization reagents, the sample itself or a sample pool can be used to compensate for differences in ionization. However, as derivatization is often the final step in sample preparation, this method cannot overcome differences in sample collection or extraction. It is, therefore, necessary to fully control the sample preparation by experienced conductors or automatized workflows.[49]

The limitation of relative quantification is the usually unaddressed accuracy of the fold change. For example, if a signal reaches saturation, a two-fold increase in concentration might only increase the signal intensity by 10%. The measured fold change would therefore not correctly represent the actual fold change important for biological interpretation. This is concentration and substance-dependent as it has been highlighted in a comparative study between relative, one-point, and multi-point quantification.[50] Only multi-point calibrants can determine the actual fold change and should also be integrated into relative experiments. However, as shown by Yu *et al.*[51] also a QC sample injected in serial injection volumes can be used for fold change determination to improve biological hypotheses generation.

1.3.2. Absolute quantification in lipidomics and their calibration strategies

On the contrary, absolute quantification determines the concentration of an analyte with an appropriate unit e.g. ppm, mol/L, or mg analyte/mg protein. This value is determined by a reference point with a known concentration. Although widely used, the term itself is not defined either by the IUPAC gold book,[52] the FDA guideline[42], or other common institutes. Especially in lipidomics, this has led to a broad use of the term for multiple methods as soon as a unit has been somehow connected to concentration. The crucial parameter to distinguish these methods is the accuracy, the "closeness of the agreement between the result of a measurement and a true value of the measurand."[52] Sometimes also the term trueness is used. The different workflows will be presented below and should highlight their accuracy, limitations, and benefits in lipid quantification (see Table 3).

Table 3 Comparison of the different absolute quantification strategies applied in lipidomics. Uncertainty calculation defines the theoretical accuracy of a quantification strategy and follows an approach defined in Schoeny *et al.* 2021.[17] The factors I, E and F in the formulas represent associated uncertainties to a value of 1 due to ionization efficiency (30% only for neutral lipids)[19], extraction efficiency (10%)[91] and fragmentation efficiency (40%, only for quantification on MS2 level)[71], respectively. It is assumed that all standards are species-specific except for surrogate standards, which still are from the same lipid class and co-elute with the analyte. An additional retention factor is necessary if co-elution is not given. k-slope, d-intercept, PC – phosphatidylcholine, TG – triacylglyceride.

Strategy	Formula	Uncertainty	Pro	Cons
External Calibration intern. standardized	$C_{Analyte} = \frac{\frac{Area_{Analyte}}{Area_{ISTD}} - d}{k}$	4%	• perfect control (LOD, linearity, etc.)	• Long sequence • No matrix-matched ESTDs
Standard addition	$C_{Analyte} = \frac{d}{k}$	3%	• no ISTD needed	• multiple runs per sample • limited by saturation
Isotope Dilution	$C_{Analyte} = \frac{Area_{Analyte}}{Area_{ISTD}} \cdot c_{ISTD}$	5%	• no ESTD needed • Matrix matched	• Defined ISTD • Use Reverse isotope dilution • LOD and linearity in an additional experiment
Surrogate Standard (one-point)	$C_{Analyte} = \frac{\frac{Area_{Analyte}}{Area_{ISTD}} - d}{k} \cdot I \cdot E\ (\cdot F)$	TG: 32% (50%) PC: 11% (41%)	• Reduced number of ISTD • no ESTD needed • Matrix matched	• Limited compensation • LOD and linearity in an additional experiment

Guideline recommended external calibration internal standardized

Both, the US FDA[42] and the European counterpart EMA[41] defined a similar approach for the MS-based analysis with the highest metrological order: matrix-matched multi-level external calibration with internal standardization. While this is common and applicable for exogenous compounds, it suffers severe limitations in omics-approaches of endogenous compounds as the global detection of all compounds within an extract contradicts the needed matrix removal for external calibrations and QC samples. The only alternatives are selective clean-ups, work-intense knockout experiments for selected metabolites or simplifying the process by using extraction blanks as the standard matrix. Further, the high number of analytes subsequently leads to a high number of external and internal standards, which are often not available or succeed the feasible budget of an analysis. Therefore, there are only a few examples following exactly this guideline in lipidomics for a selected panel of analytes.[53–57] Nevertheless, it is common to rely on or cite the FDA guideline to establish validation and quantification strategies without explaining the differences an omics-approach has to take.[26] In our recent review,[24] we defined four major parts of the guideline-recommended calibration and also their task, the challenge, and the solution for each of them. An important point in lipidomics is the use of ISTDs, which are either structurally similar substances or in the best case isotopically labeled analogs. Due to their diverse types, application, and tasks, prerequisites of ISTD in lipidomics are well-defined.[16] Summarized, the compound should not be in the sample, it needs to be added as early as possible in the sample preparation, it has to co-ionize with the analyte, and, as mentioned, it should be structurally similar or in the best case the isotopically labeled analog. The use of biotechnological produced labeled lipid extracts as ISTD offers a less cost intense alternative and will be discussed in a chapter below. External standards are easier and cheaper in production but also request a high number for global lipidomics approaches. The leading lipid standard producing company Avanti (Alabaster, USA)[58] can nowadays deliver over 500 unlabeled reference standards for the categories glycerolipids, glycerophospholipids, and sphingolipids but still lack important lipids of the human lipidome. An interesting alternative has been suggested for metabolomics studies.[59] The use of post-column infused ISTDs helps to normalize external calibration with only three deuterated metabolites for in total 17 endogenous compounds. With a matrix normalization factor, the limitation of non-matrix-matched calibration standards can be tackled together with the reduction of ISTDs. However, correction factors add additional uncertainties and the consumption of the ISTD is higher than single injected mixtures.

Due to the different possibilities, it is of utmost importance to indicate how quantitative values are achieved. Questions such as: “which standard is the concentration reference? how is the accuracy controlled? and which estimations (e.g. similar ionization or fragmentation efficiency) are necessary to obtain the absolute value?” can help to harmonize lipidomics workflows. Uncertainty calculations have been suggested[17] to translate these questions into clear and easily comparable numbers and are applied to compare the different absolute quantification strategies (see Table 3).

Standard addition

Another method fulfilling the need for accuracy in absolute quantification is standard addition. By adding the analyte substance itself at different concentration levels directly to the sample, a calibration curve

can be generated in which the intercept of the x-axis corresponds to the negative value of the sample concentration. There is no need for internal standardization, and it always applies matrix-matched analysis. However, multiple runs for one sample are necessary, making it impractical in daily high-throughput use. Nevertheless, it is a simple benchmarking possibility for new ways of quantification or a validating control for a representative QC sample. In lipid analysis, standard addition is often applied in spectroscopic methods[60–63] quantifying a class or sub-type of lipids. It has been also applied in the recovery control of cholesterol in GC-MS experiments.[64] However, to the best of our knowledge, only one study compared different quantification strategies in LC-MS-based lipidomics also with the classical standard addition approach for a selected panel of phosphatidylcholine (PC) species.[20] This study acts as a best-practice example in lipidomics and more studies should follow this workflow.

Isotope dilution

In analytical chemistry, other approaches with a high metrological order are known but limited in lipidomics application. Especially in elemental mass spectrometry, isotope dilution is a common technique for accurate quantification,[65] in which isotopically labeled ISTD with traceable concentrations and known isotopic ratios are added in known amounts to the sample. Via multiple linear regressions, considering all these factors, concentration values of the analyte can be obtained. Translated to organic MS both partially and fully labeled ISTDs can be used but with the possibility that each carbon in the compound structure is a potential label site, overlaps are easily avoidable, resulting in a simplified one-point calibration. The "single spike" nature of this approach accelerates analysis time, but linearity and limit of quantifications must be determined in additional validation experiments. Recently, we suggested the use of reversed, isotope dilution to recalibrate the ISTD, overcoming oxidation or degradation processes.[17] However, most labs rely on commercially available ISTD mixtures or kit solutions.[9]

It has to be in mind that isotope dilution is only possible with compound-specific ISTDs. If a lipid of the same class is used instead, surrogate calibration is applied. This might be similar in data processing as it is common for both, to use one-point calibration but differ significantly in terms of metrological order. For example, the group of Markus Wenk could show that the variability is significantly better if compound-specific analogs are used for normalization.[66] Interestingly, the lipid panel of Avanti (Alabaster, USA)[58] cover already more than 70 deuterated ISTD but their class-specific quantification design with both deuterated and odd fatty acids is hampering the application as compound-specific ISTDs in most biological samples.

Surrogate standards as internal standards

As an ISTD for each analyte (in lipidomics several hundred analytes are the norm) is considered as not feasible[14] alternatives are widely common. As these methods cannot compete with the high metrological order of guideline-recommended methods or isotope dilution different names have been suggested for example semi-quantification,[67] accurate quantification,[68] or further splitting in Level 1, 2, and 3 quantification.[69] By using a surrogate calibrant, one ISTD with a known concentration can be used to quantify a high number of similar analytes (e.g. one non-endogenous or isotopically labeled lipid species is used for the whole lipid class),[16] which reduces the number of standards to an applicable and cost-

saving amount. Furthermore, the use of ISTD as a reference standard precludes the need for external standards (ESTD) and further saves time and costs. However, this simplified surrogate one-point calibration delivers absolute concentrations but neglects necessary figures of merit or validation methods. Therefore, applying the surrogate class-specific standard in a multi-point calibration has been recently suggested for better control of the linear range and the limits of quantification.[20,26] Unfortunately, this is still not coping with the fact that not all lipids within one lipid class ionize similarly. Either the number of ISTDs are increased,[70] which also increases costs, or response factor corrections are included, which has been suggested years ago[18] but undergoes a revival as otherwise accurate values cannot be obtained for neutral lipid classes (cholesterol ester (CE), triacylglyceride (TG), diacylglyceride (DG), etc.).[12,19,20] On the MS2 level also variations in signal intensities between the different fatty acyl chain fragments must be corrected if lipid surrogate quantification is performed. Schuhmann et al. 2019[71] published a model, based on commercially available lipid standards to correct errors (up to 60%), resulting from the differences for glycerophospholipids in the sn-1/2 positions at the glycerol backbone, length of the hydrocarbon chain, and number and location of double bonds.

Quantitative alternatives

As all the methods have their pro and cons in omics measurements, further suggestions outside the "classical" approaches have been made in metabolomics suitable also for lipidomics approaches. For example, the combination of nuclear magnetic resonance (NMR) and MS can first quantify a reference sample with NMR, derivatize it with labeled propyl-chloroformate and apply it to the samples of interest, which is derivatized with an unlabeled agent. This has been applied to 20 amino acids without the need of any amino acid standards as NMR is traceable to the internal reference 3-(trimethylsilyl) propionic acid-2,2,3,3-d_4. The lower sensitivity in NMR can be compensated by using higher amounts of a reference sample.[72,73]

Our group further suggested the combination of lipid class quantification methods (^{31}P-NMR and HILIC-inductively coupled plasma (ICP)-MS) and the common lipid species methods (shotgun lipidomics, RP-LC-MS, and HILIC-MS) for the analysis of the phospholipidome in yeast. Due to the traceable nature of NMR and ICP, a concentration reference for a whole class can be used to cross-validate the sum parameter of the lipid species methods. Another novel lipid class method based on all ion fragmentation (AIF) in HILIC-ESI-MS experiments has been introduced. As lipids from the same class produce the same (head group fragments) or similar (fatty acyl chain fragments) fragments in this DIA approach of fragmenting all co-eluting masses at once, a defined mass and retention time range represents the total lipid class signal and external calibration can be applied. This method benefits from the wide availability of the instruments in the lipidomics community and benchmark results with ^{31}P-NMR and HILIC-ICP-MS have shown a similar accuracy.

1.3.3. Labeled biomasses as ISTD and the benefit of *Komagataella phaffii*

As mentioned, the use of labeled biomass as ISTD can be beneficial in relative but also in the different types of absolute quantification. Compared to the chemical synthesis of isotope-labeled standards it benefits from a fast, work- and cost-efficient production of a high number of compounds. While the isotope labeling degree and the composition are often well-studied, the concentration remains unknown

and the actual concentration reference is external calibration.[53–56,74] This approach improved the precision, trueness, and linearity of the analysis - important figures of merit in lipidomics and metabolomics.[75–78]

Different labeled organisms covering bacteria, yeast, plants, or mammalian cells have been labeled with different elements (^{13}C, ^{2}H, ^{15}N, ^{34}S) achieving different labeling degrees.[24] Especially controlled growth conditions have led to commercial availability of yeast, bacteria, and different types of algae. Higher organisms are of great interest as the overlap of analytes would increase to near perfection but the complex feed or media only has allowed partial labeling so far.[47,48]

Workflows in our group are built on *Komagataella phaffii* (often referred to by its obsolete name *Pichia pastoris*).[53] This well-known cell factory in biotechnology, a eukaryotic yeast microorganism, is used to produce a uniformly ^{13}C labeled lipidome. The organism can be fermented by only one ^{13}C source: glucose, leading to complete isotope labeling (carbon enrichment > 99%). Although *Saccharomyces cerevisiae* is the most studied yeast organism also in terms of lipidomics,[79–81] its distant cousin *Komagataella phaffii* is of big interest as it can grow on methanol or glucose as the sole carbon source and plays an important role as a host for recombinant protein production.[82] Subsequently, also the lipidome of *K. phaffii* and especially the differences in the cell compartments have been well-studied by the Daum group.[83–86] The Daum group has also published several quantitative data for hydrolyzed fatty acids and phospholipids obtained by gas chromatography (GC)-MS and thin-layer chromatography (TLC), respectively.[83,87] PC is the most abundant lipid class compromising around 50% of all quantified PLs. Phosphatidylethanolamine (PE) follows with appr. 30% and phosphatidylinositol (PI) & PS with appr. 7% each. Other lipid classes are below 2%. Also, PL species have been identified and relatively quantified,[85] however, a comprehensive lipid species quantification method has not been performed. To the best of our knowledge, for *K. phaffii* this only has been performed once after lipid class prefractionation by preparative supercritical fluid chromatography (prep-SFC) and subsequent quantification by shotgun lipidomics.[22] In this study, also a deep profiling method lead to the identification of more than 400 molecular lipid species. A possible outlook for *K. phaffii* lipid complexity can be seen in recent nanoLC-based works by the Ahrends lab,[88,89] where they have identified almost 900 lipid species in total[89] and already 447 lipids only of PL classes alone.[88]

As labeled biomass for internal standardization, *K. phaffii* not only profits from the possible high enrichment degree due to the use of sole labeled carbon sources[53] but also from the fact that its membrane composition is more similar to multicellular eukaryotes than in *S. cerevisiae.* [82] In detail, *K. phaffii* contains polyunsaturated lipids (linolenic acid FA 18:3) and it produces both (mannosyl)inositol phosphoceramides ((M)IPC) and glucosyl ceramide (GlcCer), in which the latter is also present in mammalians but not in *S. cerevisiae.* Differences between *K. phaffii* and human plasma include different sphingolipid classes (IPC, MIPC, and mannose-bis(inositolphospho)ceramide (M(IP)2C) instead of sphingomyelin (SM), ceramide 1-phosphate (Cer-P) and gangliosides), a different main sphingoid base (SPH 18:0;3 instead of SPH 18:1;2), no other higher poly unsaturated fatty acids (PUFA) (e.g. arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA)), ergosterol as the main sterol and cholesterol as not present and the absence of ether lipids (plasmanyl (ether bond), plasmenyl (vinyl bond)). Nevertheless, a wide variety of not occurring lipid species can still

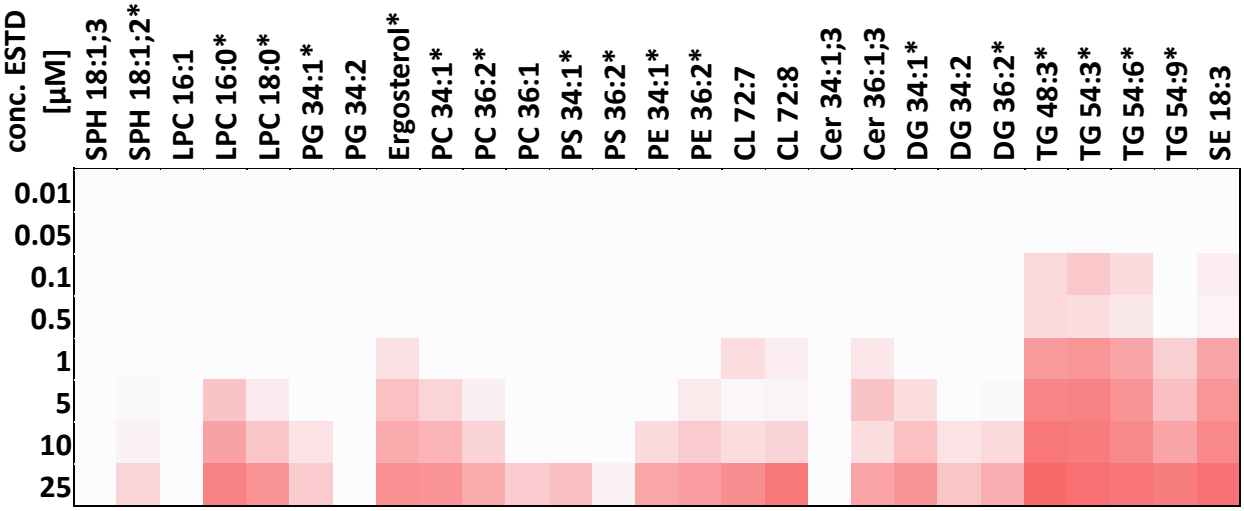
be quantified if a reference standard is available and a structurally similar lipid is present in LILY as ISTD (e.g. cholesterol, SM, ceramide (Cer), ether lipids and PUFA containing PLs and glycerolipids (GLs)).[17] Further, *K. phaffii* can cover the majority of the most promising cancer biomarkers as summarized in a review by Wolrab et al.[90]including lipids from the classes PC, PE, PI, PS, lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidic acid (LPA), free fatty acids (FFA), TG and DG and only misses CE, SM, gangliosides, and sulfatides.

1.3.4. The quantification bias- influence of ionization and fragmentation efficiency

While compound-specific quantification (external calibration internal standardized, standard addition, isotope dilution) benefits from the same chemical behavior of analyte and standards, a bias can occur if surrogate standards are used. Here analytes and internal standards have different compositions, which leads to three differing factors: the extraction efficiency,[91] the ionization efficiency,[92] and the fragmentation efficiency[71] (see surrogate standards in Table 3). All factors are influenced by the structure of the lipid including the length and position of the fatty acyl chains, the alkyl chain bond (ester, ether, vinyl ether), the number of hydroxy groups, the number of double bonds, their position, and their configuration. Due to the severe influence of the head group, quantification with standards from another lipid class is not recommended or considered as uncertain.[69] Another important point is the prerequisite of co-ionization.[16] While this is always the case in shotgun lipidomics, it can limit LC applications, especially RP-LC as this method separates lipid species according to their hydrophobicity, a factor mainly influenced by the lipid tail.

Also, the matrix or solvent composition influences the extraction and ionization efficiency. However, the fragmentation is rather unaffected leading to the possibility of library and fragmentation rule identification independent from the sample type. The influence of the matrix on ionization efficiency is shown in Table 4. Controlled matrix concentrations (here different calibration levels of an ESTD) have been applied on lipids with a constant concentration (here internal standard compounds from the LILY extract). Depending on the class, ion suppression occurs at a certain concentration level, in most cases in the µmolar range. Especially neutral lipids have been affected as they elute in a rather narrow time window and structurally, they miss a polar head group which leads to a higher influence on the ionization efficiency of the non-polar lipid tail.[16,92] Further, lipids with a co-eluting unlabeled analog in the ESTD mix (indicated by *) are stronger affected than lipids of the same class with no analog and subsequently no co-eluting lipid, seen at SPH, LPC, phosphatidylglycerol (PG), PC, and DG. While this is not hampering quantification per se as the ratio between the analyte and internal standard in the same sample stays constant, the signal of one of these compounds might be totally suppressed and the ratio cannot be determined anymore. Therefore, a balance of a low total lipid concentration to avoid saturation and a sufficient concentration to reach values above LOQ is crucial in lipid analysis.

Table 4 Ion suppression in RP-LC of internal standard compounds in external calibration levels. Each box indicates the ratio between the signal of the internal standard (columns) in the external standard mix (rows) in relation to the signal of the internal standard in an internal standard blank. The darker the red the lower the signal in the external standard mix compared to the blank (e.g. lightest red min. 10%, dark red >80% suppressed). * Marked internal standards with a compound-specific unlabeled equivalent in the external standard mix. CL- cardiolipin, SE – sterol ester.



Similar considerations are necessary for extraction. While a lower plasma volume than 10 µL hampers homogeneity,[93] higher plasma volumes negatively influence extraction recovery.[9,93,94] The right ratio between plasma volume and total extraction solvent has been determined[94] for Folch,[95] Bligh-Dyer,[96] and Matyash extraction.[97] Further, dilution of the reconstituted extract in chloroform/methanol (1:1, v/v) to maximum 100 pmol/µL (100µM) total lipid per solvent volume has been suggested for linear dynamic ranges in shotgun lipidomics. [98] These experiments are useful reference points, but it should be in mind that other samples, solvents, analytes, or analytical workflows need a reevaluation to avoid misleading biases.

As surrogate internal standardization is the state-of-the-art method in lipidomics, solutions towards these problems deal more with correction factors than a more expansive increase of standard compounds. As neutral lipids show the biggest differences in ionization efficiency within the lipid class, they are of highest interest for response factor correction. [12,18–20] Either the response of each lipid species in ratio to the ISTD is determined with reference standards, again requiring a high number of standards or the species-specific response is expressed as a mathematical model with dependencies of carbon and double bond numbers. The second approach has been applied for CE and validated with the enzymatic determination of total cholesterol.[12] While these effects are less prominent in polar lipid classes, especially short-chain fatty acyl chain analogs can have tremendously different signal responses compared to the naturally high abundant lipids with 16-20 carbon chains.[99] These lipids are often either very low abundant or not present in a sample but can be used as a low-cost alternative to isotopically labeled surrogate ISTDs if the signal response is corrected. The suggested model for the lipid class PG can also be used if short-chain fatty acyl chain analogs are present in a sample and it shows the limitation of surrogate standardization also in PLs.

Differences in fragmentation efficiency can also be corrected with mathematical models but as even the position of the double bonds in PC play a role, a more complex model for bottom-up shotgun lipidomics (quantification on the MS2 level) is needed. Corrected results have been compared to quantification on the MS1 level, showing that cross-validation with differently obtained values is key in lipidomics.[71] Also the commercial Lipidyzer™ platform from Sciex (Framingham, MA, USA) and its further improvement[100] corrects for different fatty acyl chain fragments. With over 50 labeled ISTDs of 13 lipid classes, all abundant lipid acyl chains are available for each class always combined with a second labeled FA. With that, each analyte is matched to the best fitting ISTD according to the FA composition. In combination with ion mobility spectrometry (IMS) and ready-to-use workflows it offers an accurate absolute lipid quantification, only limited by the relatively high cost per sample.[101]

1.4. Harmonization of lipidomics workflows

Without harmonization of lipid results, data are not transferable, reusable, or comparable to validation studies. Therefore, it is a huge community effort to set certain rules to harmonize the field including validation studies, guidelines for sample collection, storage, and preparation as well as reference samples to increase interchangeability.[102]

1.4.1. Validation approaches, stability of samples, and their limits

In the last few years, the working group of Michal Holčapek has worked intensively on validation,[26] profile stability of healthy volunteers,[103] and intra-laboratory repeatablilty[104] of quantitative lipid data. Their focus lies on the lipid class separation techniques HILIC and SFC and, by including a standard reference material (SRM 1950) in their workflow, useful reference data has been obtained. They cover important information about extraction,[26] the difference of serum, heparin, and ethylenediaminetetraacetic acid (EDTA) plasma samples,[26,103] matrix effects on calibration curves,[26] variations of lipid profiles of healthy donors over one year,[103] signal drifts, and retention time stability[103] and the influence of different analytical platforms or laboratory sites.[104] The covered lipid classes are monoacylglceride (MG), DG, TG, CE, Cer, PC, LPC, and SM with ca. 70 lipids covered by both HILIC and SFC. [104] Also within this studies slight differences compared to the intra-laboratory comparison[105] have been found (half of SFC data and one-third of HILIC data have RSDs >25%).[103] By including normalization to SRM 1950, bias has been reduced in the intra- and interlaboratory studies as it also had been shown previously.[66]

Crucial for reproducible data is the stability of lipids themselves during storage and analysis. A recent review[106] summarizes best practices to prevent hydrolysis, oxidation, or interspecies conversation. Already three lipids (sphingadienine 1-phosphate, spingosine-1-phosphate, and LPC 18:2) have been proposed as stability markers in human plasma samples and several techniques such as additives and antioxidants are known to prevent undesired changes. Further studies, investigated the long-term stability of plasma metabolites including lipids during -80°C storage[107] over five years, analyzed with the biocrates p180 kit. Interestingly, an increase for PLs with chain length above 40 carbons (+18 %) have been observed, while acylcarnitines (-12.1%), LPC (-15.1%), PC (-17%) plasmenylcholine (PC P) (-13.3%) and SM (-14.8%) decrease over time. Other lipid classes have not been tested in this study and the possible reasons for these findings can be various according to the authors. Yang et al.[108] also tested the 24h stability of blood at 4°C, important if samples are not immediately processed. They have observed an increase of LPC and the metabolite choline, both degradation products of the highly abundant PC lipid

class, while another lysolipid, LPE, even decreased. The performance stability is an important topic as Heiskanen et al.[93] concluded that shotgun is more stable than LC-MS as Yang et al.[108] observed instability over 5 years but not in their 3.5 years long shotgun study. Their conclusion is that shotgun is less prone to changes in matrix effects as it is always affected not like LC-MS.

These are all important steps towards validation but i.e. the US FDA guideline requests in total 6 types of stability tests, including the autosampler stability, the bench-top stability, the extract (or processed sample) stability, the freeze-thaw stability, the long-term stability, and the stock solution stability. This normally exceeds the capacity of research projects and is therefore limited available for lipidomics.

1.4.2. Reference materials and the way to get them

Standardization in lipidomics has evolved as the key step towards harmonized quantitative lipid data. The highest metrological order is fulfilled if certified reference materials are included in the workflow, which would guarantee the accuracy and traceability of the data. However, certification is a complex process, which includes compositional and quantitative data but also a characterized stability and uncertainty for each analyte. Until 2021, this stage has not been reached in lipidomics yet.[24]

Alternatively, international ring trials or intra-laboratory comparison can offer consensus values for the analytes of interest in biological matrices, whose results can be used for benchmarking novel or in-house workflows. The SRM 1950 of human plasma from the National Institute of standards and technology (NIST) is the most common reference material in lipidomics workflows since an inter-laboratory comparison in 2017 provided consensus values for 339 lipids.[105] An international ring trial has further delivered consensus values for 250 metabolites, which also include lipids, based on the Biocrates AbsoluteIDQp400HR kit.[109] However, all these trials offer guidance and no certified reference values and even more highlight the still present problems in reproducibility. Even with the use of isotopically labeled ISTDs, the reproducibility of quantitative data obtained from different platforms is a bottleneck in lipid quantification.[66] This study has suggested normalization to SRM 1950 or another QC sample to harmonize the quantitative results. Accurate absolute values are then dependent on the consensus values of these materials. More human plasma reference material by NIST has been recently examined covering hypertriglyceridemic, diabetic, and African-American plasma pools in comparison to SRM 1950.[110] A nontargeted lipidomics approach has revealed changes in composition such as the unsurprising elevation of TG in hypertriglyceridemic samples or oxidized fatty acid-containing phospholipids in African Americans. However, it should be emphasized that only for the African American pool K2 EDTA has been used as anticoagulant and lithium heparin for SRM 1950, the hypertriglyceridemic and the diabetic pool. In this study, they have also compared the semi-quantitative values of SRM 1950 to the intra-laboratory comparison,[105] which is not in agreement for all lipid species highlighting the need for future quantitative studies for benchmark material.

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2. Research objectives

The primary research question of my PhD was the establishment of a quantification method based on LILY to optimize accuracy, coverage, and throughput within one analysis.[17] A novel fully automated workflow for lipidomics was proposed based on flow injection (FI), followed by reversed-phase liquid chromatography (RP-LC)–HRMS.[17] The workflow combined in-depth characterization of the lipidome achieved *via* RP-LC–HRMS with absolute quantification by using a large number of lipid species-specific and/or retention time-matched, class-specific calibrants. Overall, the workflow enables a streamlined analysis, with a limit of detection in the low femtomolar range and provides validation tools together with absolute concentration values for >350 lipids in human plasma on a species level. Based on the selected standard panel, lipids from 7 classes (LPC, LPE, PC, PE, PI, DG, TG) have passed stringent quality filters, which included QC accuracy, a precision and recovery bias of <30%, and concentrations within the 99% confidence interval of the international laboratory comparison of SRM 1950, NIST, USA.

Due to the independence of the quantitative values from common deuterated or non-endogenous ISTDs, the novel workflow offers cross-validation of different lipid methods. Compared to other analytical fields, this is often a neglected topic in omics-approaches and was, therefore, further investigated by cross-validating our results via multiple quantification platforms including non-organic-MS-based methods such as NMR and ICP-MS. The lipid standard independent nature of FI-ICP-MS and ^{31}P-NMR enabled quantification based on other available certified, traceable, phosphor-containing substances, accelerating and simplifying accurate lipid class quantification also of newly identified lipid species. This approach acts as an important step towards the traceability of quantitative lipid values obtained by different lipidomics platforms.

Finally, an automatable fractionation method was needed to enlarge the versatility of LILY in terms of concentration and composition. We have implemented an offline semi-preparative lipid class-specific fractionation by supercritical fluid chromatography (SFC) that can be followed by high-resolution mass spectrometry methods such as shotgun lipidomics and RP-LC-MS-based lipidomics for in-depth characterization. The powerful SFC approach offers separation of a wide polarity range for lipids, enables enrichment (up to 3 orders of magnitude) of lipids, selective fractionation of 14 lipid classes/subclasses, and increases dynamic range. A significantly increased coverage of low abundant lipids improving lipid identification by numbers and degree (species and molecular level) has been obtained in *K. phaffii* when comparing high-resolution mass spectrometry-based lipidomics with and without prior fractionation. In total, 400 lipids have been detected in the *K. phaffii* yeast lipidome which enables now a more specific application than a global lipidomics approach and paves the way to biotechnological production of single ^{13}C-labeled molecules.

3. Results

Within the cumulative doctoral thesis three peer-reviewed publications, which appeared or will appear in international journals are listed in thematical order:

I. A combined flow injection/reversed-phase chromatography-high resolution mass spectrometry workflow for accurate absolute lipid quantification with ^{13}C-internal standards.

Schoeny, H., Rampler, E., El Abiead, Y., Hildebrand, F., Zach, O., Hermann, G., Koellensperger, G. Analyst, 2021, 146, 2591-2599.

II. Hunting absolute molar lipid concentrations: A phospholipidomics cross-validation study

Schoeny, H., Rampler, E., Binh Chu, D., Schoeberl, A., Galvez, L., Blaukopf, M., Kosma, P., Koellensperger, G. Submitted.

III. Preparative supercritical fluid chromatography for lipid class fractionation—a novel strategy in high-resolution mass spectrometry-based lipidomics.

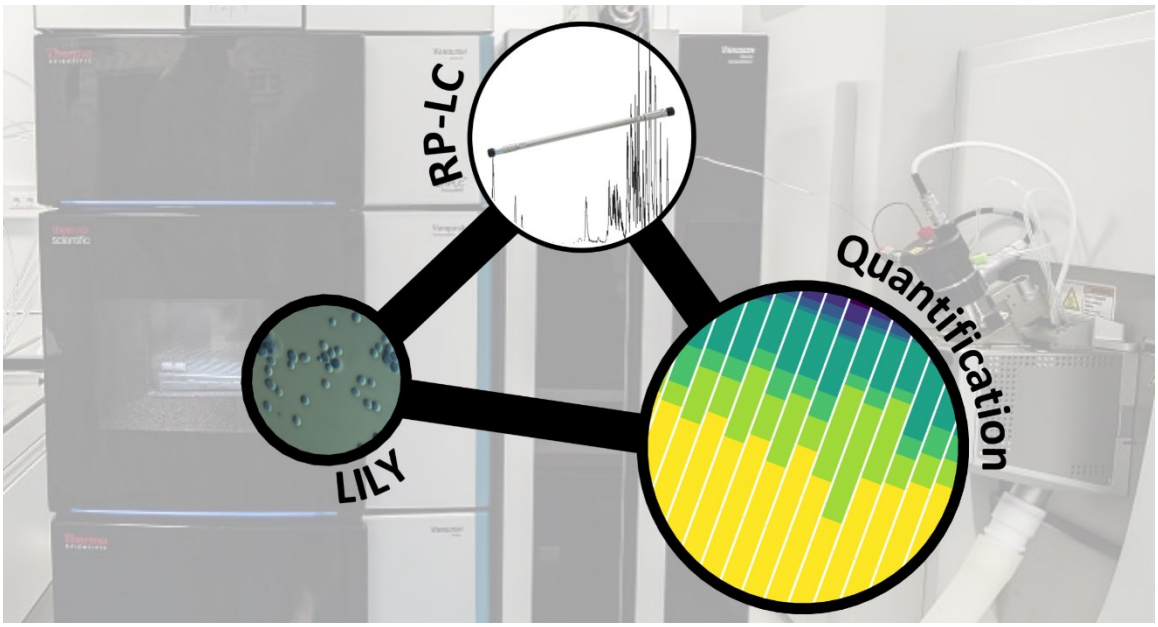
Schoeny, H., Rampler, Hermann, G., Grienke, U., Rollinger, J.M., Koellensperger, G. *Analytical Bioanalytical Chemistry, 2020,* 412, 2365-2374.

3.1. Publication I

A combined flow injection/reversed-phase chromatography-high resolution mass spectrometry workflow for accurate absolute lipid quantification with ^{13}C-internal standards.

Harald Schoeny, Evelyn Rampler, Yasin El Abiead, Felina Hildebrand, Olivia Zach, Gerrit Hermann and Gunda Koellensperger

Analyst, 2021, 146, 2591-2599.



A novel fully automated workflow for lipidomics has been proposed based on flow injection (FI), followed by reversed phase liquid chromatography (RP-LC)–high-resolution mass spectrometry (HRMS). The workflow combines in-depth characterization of the lipidome achieved *via* RP-LC–HRMS with absolute quantification by using lipid species-specific and/or retention time-matched, class-specific calibrants. The workflow enables a streamlined analysis, with a limit of detection in the low femtomolar range and provides validation tools together with absolute concentration values for >350 lipids in human plasma on a species level. Based on the selected standard panel, lipids from 7 classes (LPC, LPE, PC, PE, PI, DG, TG) have passed stringent quality filters, which included QC accuracy, precision and recovery bias of <30% and concentrations within the 99% confidence interval of the international laboratory comparison of SRM 1950, NIST, USA.

I have developed the method including sample preparation, analysis, and data processing. I have conducted the experiments, have been responsible for data evaluation and writing of the manuscript.

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A combined flow injection/reversed-phase chromatography–high-resolution mass spectrometry workflow for accurate absolute lipid quantification with ^{13}C internal standards†‡

Harald Schoeny, [a] Evelyn Rampler, [a,b,c] Yasin El Abiead, [a] Felina Hildebrand, [a] Olivia Zach,[a] Gerrit Hermann[a,d] and Gunda Koellensperger *[a,b,c]

We propose a fully automated novel workflow for lipidomics based on flow injection, followed by liquid chromatography–high-resolution mass spectrometry (FI/LC–HRMS). The workflow combined in-depth characterization of the lipidome achieved *via* reversed-phase LC–HRMS with absolute quantification by using a large number of lipid species-specific and/or retention time (RT)-matched/class-specific calibrants. The lipidome of ^{13}C-labelled yeast (LILY) provided a large panel of cost-effective internal standards (ISTDs) covering triacylglycerols (TG), steryl esters (SE), free fatty acids (FA), diacylglycerols (DG), sterols (ST), ceramides (Cer), hexosyl ceramides (HexCer), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidic acids (PA), cardiolipins (CL), phosphatidylinositols (PI), phosphatidylserines (PS), phosphatidylcholines (PC), lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE). The workflow in combination with the LILY lipid panel enables simultaneous quantification *via* (1) external multi-point calibration with internal standardization and (2) internal one-point calibration with LILY as a surrogate ISTD, increasing the coverage while keeping the accuracy and throughput high. Extensive measures on quality control allowed us to rank the calibration strategies and to automatically select the calibration strategy of the highest metrological order for the respective lipid species. Overall, the workflow enabled a streamlined analysis, with a limit of detection in the low femtomolar range, and provided validation tools together with absolute concentration values for >350 lipids in human plasma on a species level. Based on the selected standard panel, lipids from 7 classes (LPC, LPE, PC, PE, PI, DG, TG) passed stringent quality filters, which included QC accuracy, a precision and recovery bias of <30% and concentrations within the 99% confidence interval of the international laboratory comparison of SRM 1950, NIST, USA. The quantitative values are independent of common deuterated or non-endogenous ISTDs, thus offering cross-validation of different lipid methods and further standardizing lipidomics.

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Introduction

LC–MS-driven lipidomics strives to quantify lipids in biological entities comprehensively. However, to date, validation of quantitative omics-type analysis has remained a challenge.[1,2] Appropriate standardization strategies regarding different analytical platforms and their compliance with guidelines are currently under debate. Internal standardization has been propagated as the method of choice for absolute quantification, and the selection criteria of internal standards (ISTDs) in lipidomics are well defined.[3] Ideally, the lipid selected as the ISTD is added as early as possible to the sample in the analytical process. In the best case, it is an isotopically labeled analog of the investigated lipid. Otherwise, at least the prerequisites of co-ionization (*e.g.*, co-eluting compounds when using liquid

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†Raw data available on MetaboLights[43] http://www.ebi.ac.uk/metabolights/MTBLS1876.

‡Electronic supplementary information (ESI) available: Abbreviations, extended materials and methods, additional figures and tables; XLSX—quantitative results, quantification strategy comparison, figures of merit, quantified LILY lipids with FI-HRMS, the MS sequence list; ZIP—MFQL files for LipidXplorer. See DOI: 10.1039/d0an02443k

chromatography) and structural similarity have to be met. Different calibration approaches have been developed to minimize the number of required ISTDs by using a class-specific surrogate standard.[4] However, co-elution is a must and only possible in direct infusion mass spectrometry and chromatographic techniques separating lipids according to their head group chemistry (as enabled *via* hydrophilic interaction liquid chromatography (HILIC), normal-phase liquid chromatography (NP-LC) or supercritical fluid chromatography (SFC)). However, nowadays, reversed-phase liquid chromatography–mass spectrometry (RP-LC–MS), which separates lipids according to their fatty acyl chain chemistry, is the most widely used analytical lipidomics technique as revealed by a recent survey[1] among expert laboratories and a comprehensive literature review.[5] It provides efficient matrix separation[6] and excellent chromatographic selectivity and retentivity for lipids due to the possibility of separating lipids with different chain lengths and different numbers/positions of double bonds, resulting in high sensitivity and dynamic range when combined with MS detection. While the method is unrivaled in terms of lipid separation and thus identification (combined with high-resolution MS (HRMS)), appropriate standardization approaches imply the use of multiple standards per lipid class; otherwise, quantification accuracy is compromised (see ESI Fig. S6‡).[3,6–9] Only quantification based on external calibration with internal standardization by an (isotopically labeled) analog[10–14] is the method of the highest metrological order and complies with the FDA guideline for Bioanalytical Method Validation,[15] commonly used in clinics and biomarker evaluation. In fact, recent large-scale clinical studies have resorted to targeted analysis of a small panel of lipids.[16]

In this work, we want to increase the number of quantified lipids within one analytical run on RP-LC–HRMS without compromising accuracy. A lipid extract provided by ^{13}C fully labeled biomass (*Pichia pastoris*), coined as lipidome isotope labeling of yeast (LILY),[10] is used as the ISTD, providing over 250 uniformly ^{13}C-labeled lipid species from 19 lipid classes. However, the number of accurately quantified lipid species is also limited by the number of external calibrants.[11,12] As the quantitative information is lacking for LILY, a workflow has been designed to quantify LILY on a day-to-day basis with reverse isotope dilution prior to RP-LC–HRMS analysis of samples. This strategy enables implementing different calibration strategies within one lipidomics workflow: (1) external multi-point calibration with internal standardization and (2) surrogate internal standardization without external standardization. The reverse isotope dilution of LILY coped with the fact that comprehensive lipidome stability and storage conditions are still ill-defined for such cost-effective materials. It is well known that certain lipid species are prone to oxidation and degradation, making frequent recalibration a prerequisite.[17–19]

Human plasma lipidomics serves as a prime example to show the presented workflow's validity as it represents the most frequently analyzed sample matrix in the field.[1,5] Quantification on the lipid species level will simplify the calculation and serve as a proof of concept as the implementation of quantification on the MS2 level was proven elsewhere.[10] The presented validation will capitalize on healthy donor samples[20–24] and reference materials[23] with published consensus values of an interlaboratory comparison and the results of the workflow will be compared to shotgun lipidomics data.

Materials and methods

Materials

Human plasma samples were purchased from Innovative Research (Novi, Michigan). Standard reference material (SRM) 1950 from the National Institute of Standards and Technology (NIST, Gaithersburg, USA) was used. Reference standards (endogenous compounds for external calibration) and SPLASH® LIPIDOMIX® Mass Spec Standard were obtained from Avanti (Alabaster, USA) and Merck KGaA (Darmstadt, Germany). The internal standard LILY was obtained *via* the procedure described by Neubauer *et al.*[25] and Schoeny *et al.*[26]

Methods

Two different methods (flow injection (FI) and RP-LC) were applied in the same analytical sequence as enabled by a 6-port valve controlled using MS software (see ESI Fig. S1‡). A detailed description of extraction, analysis, and data processing can be found in the ESI's‡ Extended Materials and Methods section.

Briefly, a Vanquish Horizon HPLC system and a high-field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (both from Thermo Fisher Scientific) were used. For RP chromatography of lipids, an Acquity HSS T3 column (2.1 mm × 150 mm, 1.8 μm, Waters) with a VanGuard Pre-column (2.1 × 5 mm, 100 Å, 1.8 μm) was used. The column temperature was set to 40 °C and the flow rate was set to 250 μL min^{-1}. Acetonitrile (ACN)/H_2O (3 : 2, v/v) was used as solvent A and isopropanol (IPA)/ACN (9 : 1, v/v) as solvent B, both containing 0.1% formic acid and 10 mM ammonium formate. A gradient of 23 min was applied. MS1 acquisition was used for quantification. An injection volume of 2 μL was selected, and polarity switching was performed. For data-dependent acquisition (DDA), the LC method was identical, but the injection volume was increased to 5 μL, the positive mode and negative mode were acquired separately, and only the pooled sample together with the extraction blank and a highly concentrated external standard were analyzed. For FI, the column was by-passed *via* a 6-port valve. 25 μL was injected at a 5 μL min^{-1} flow rate to obtain a constant signal for around 5 min. The eluents were kept constant at 50% A/50% B. Each FI measurement lasted 10 min, including washing. Polarity switching was triggered after 2.5 min (afterwards 10 s for equilibration). For each polarity, 200 data-independent acquisition (DIA) scans (precursor isolation width m/z 1) were alternated with an MS1 scan for quantification. MS1 RP-LC lipid data were processed using Skyline (version 20.1), data-dependent acquisition (DDA) files were processed using LipidSearch 4.2 from Thermo Scientific, and FI data were evaluated using LipidXplorer (version 1.2.8).

All final data processing was performed in the R/R studio environment.

Results and discussion

RP-LC–HRMS is the gold standard for the in-depth characterization of lipidomes.[1,5] While the superiority of this method with regard to sensitivity and dynamic range is widely accepted, its quantification capability is currently under debate. It becomes increasingly evident that a large number of lipid standards is mandatory for accurate omics-type quantification by RP-LC-based methods.[3,6–9] In this work, an FI/LC–HRMS workflow integrated isotopically labeled yeast (LILY) as an internal standard (see Fig. 1) to simultaneously quantify a large number of lipids either *via* external multi-point calibration with internal standardization or internal one-point calibration with LILY as the surrogate ISTD. This increased the coverage while keeping the accuracy and throughput high. Several proof-of-concept studies showed the potential of LILY.[10–12] However, the applied internal standardization strategies lacked the quantitative characterization of the ^{13}C yeast lipidome. As the stability data for such an omics-type quantitative standard were beyond the possibility of this study, the integration of FI into the RP-LC workflow enables on-demand control of the ISTD for each sequence with only a slight increase in time (10%). This approach follows reverse isotope dilution, as it is often performed in quantitative atomic spectrometry to improve the accuracy by characterizing the spike material on a day-to-day basis.[27] Internal standardization was accomplished by spiking samples and external standards with known amounts of LILY.

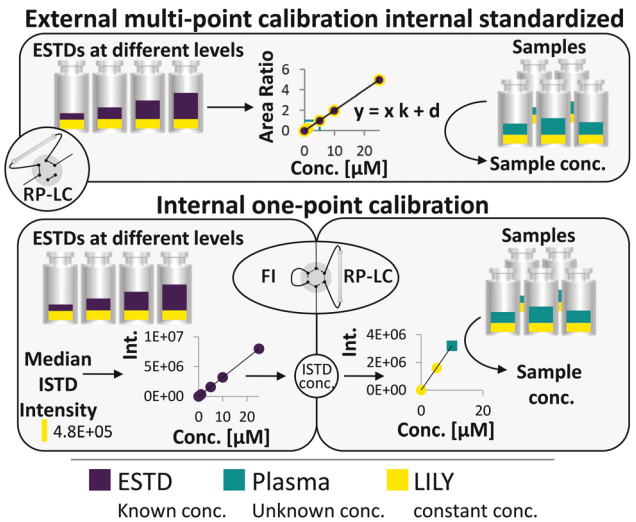

Fig. 1 The two combined quantification strategies: (1) external multi-point calibration with internal standardization and (2) internal one-point calibration. LILY (yellow) was added to the plasma sample (blue) and the ESTDs (purple) in the same concentration. ESTDs were measured *via* RP-LC–HRMS and FI-HRMS. Samples were only measured *via* RP-LC–HRMS.

The combined FI/RP-LC–HRMS workflow for lipid quantification

An FI analysis step preceded RP-LC–HRMS analysis and enabled LILY's quantitative characterization on a day-to-day basis by reverse isotope dilution. For this purpose, FI was selected as a direct infusion method,[28] given the possibility of automated switching to subsequent RP-LC-based lipidomic analysis (see ESI Fig. S1‡). An acquisition time of 5 min per sample was obtained by injecting 25 µL at a flow rate of 5 µL min^{-1} so that polarity switching and DIA MS/MS can be applied. As a prerequisite, the UHPLC system used in this work delivers highly precise flow rates in the flow regime from 1 µL min^{-1} to 5 mL min^{-1}. Regarding sensitivity and precision, a comparable performance was observed for the optimized FI-HRMS approach and the chip-based infusion nano-ESI-MS[29] (limits of detection (LOD) comparable to shotgun data were found in this work; see also Table S1‡).

Fig. 1 shows the two quantification strategies that are possible with the suggested workflow. For (1), the area ratio of ESTD/ISTD was used for linear regression models, and the area ratio of analyte/ISTD in samples was used to calculate the final analyte concentration. In (2), ESTDs measured *via* FI generated a linear regression model with their intensities. The median intensity value of the ISTD (present in all ESTDs) was used to calculate the ISTD concentration in a reverse isotope dilution manner. This calculated concentration was used in samples for analytes, which could not be analyzed with strategy 1, to quantify them *via* one-point calibration. Therefore, the accuracy and coverage can be combined *via* RP-LC with only a small increase in time (+10% of total run time) even when a vast number of standards and different blanks were included (see ESI Fig. S4 and ESI Excel table/Sequence‡). By tailoring the calibration levels, the measurement time could be reduced even further. The measurement sequence starts with FI analysis of external standards spiked with LILY. Fully automated switching from FI analysis to RP-LC–HRMS is accomplished *via* a 6-port valve. A typical lipidomics RP-LC–HRMS run in 23 min was performed to analyze samples and external standards spiked with LILY lipids. Excellent retention time (RT) stability (see ESI Fig. S3A‡) supported lipid identification and quantification across samples. Despite the short chromatographic separation time, for some lipid species, isomer separation was achieved for LPC, LPE, PC, PE and DG. Exemplarily, baseline separation of two lipid species (PC 18:1(9Z)/18:1(9Z) and PC 18:1(9E)/18:1(9E)) is shown in ESI Fig. S3B.‡ However, quantification was based on the sum integral to simplify data evaluation. The streamlined workflow involved RP-LC–HRMS analysis of all samples and standards in full MS mode (MS1, mass resolution 120 000, dynamic polarity switching) and a pooled sample in data-dependent MS/MS for quantification and identification, respectively. The measured calibration dilution series covered 4 orders of magnitude (low nM to low µM range). For data evaluation, only the linear working range was considered.

As a key advance, the large panel of external and internal standards paved the way for different applicable calibration strategies expanding the number of lipid species amenable to

absolute quantification. Moreover, an extensive dilution series of external calibrants allowed tailored calibration levels for lipid species evaluation, both in FI-HRMS and RP-LC–HRMS analyses. Thus, the method allowed accurate quantification without an initial screening of every single analyte's linear range. Following stringent quality criteria, an automatic selection of the calibration strategy for each lipid species was implemented. Wherever applicable, species-specific standardization (denoted as level 1, according to the definition of LSI,[30] see ESI Fig. S5‡) was preferred over class-specific calibration (denoted as level 2 in the case of RT matching, otherwise level 3). The ISTD and ESTD were chosen in the following decreasing preference order: level of standard,[30] number of hydroxy groups (necessary only for sphingolipids), number of double bonds, and number of carbons in the fatty acyl chain. The quantification approach of the highest metrological order is lipid species-specific standardization by external multi-point calibration with internal LILY standardization. Evidently, this calibration approach is limited to the selection of external standards and lipids present in LILY. Otherwise, level 2 (RT match was accepted for maximal ±0.5 min RT shift) external calibration using the species-specific (level 1) LILY ISTD was applied, followed by one-point calibration using the quantified level 1 LILY ISTD. If no species-specific calibration was available, lipid class-specific RT-matched standardization was applied. The remaining species were assessed using level 3 standards.

Quantification of LILY by FI-HRMS

169 of the 250 ^{13}C fully labeled lipid species (identified on the lipid species level) were present in all samples with an intensity high enough for internal standardization (>10% of ISTD Blank). Out of these, 67 lipids were accurately quantified by reverse isotope dilution on an FI-HRMS routine by using either level 1 or 2 type standardization. Depending on the lipid class, MS quantification was based on the precursor, the head group fragment or the fatty acyl chain fragments in either positive or negative mode. Lipids from 8 classes (DG, TG, ST, PC, PE, PG, LPC, HexCer; see the ESI‡ for lipid class abbreviations) ranging in concentrations from 3 to 1500 nM were quantified (see ESI Excel table/LILY-lipids_FI‡). For all obtained LILY standard concentrations, stringent quality criteria were met in the FI-HRMS data. A detailed description of the quality criteria used for filtering is given in the ESI.‡ Overall, experimental uncertainty in QC samples, signal stability, linear dynamic range, and LOQ were considered. Thus, out of the 169 LILY lipids, 67 compounds were potential one-point ISTDs with experimentally assessed concentrations. All other LILY lipids served as ISTDs in external calibrations requiring no quantitative information to compensate for variations in sample preparation and instrument performance (see strategies 1 and 2, respectively, in the ESI‡/absolute lipid quantification).

Evaluation of different calibration strategies

The two possible quantification strategies in this workflow, external multi-point calibration with internal standardization and internal one-point calibration in combination with the three different levels[30] for the ISTD and ESTD, enable multiple calibration strategies that need to be evaluated. Fig. 2 shows concentration values for selected lipid species obtained by these different calibration strategies in SRM 1950 with respect to the published consensus values.[23,31] More specifically, the quantification of 4 lipid species, *i.e.* DG 34:1, TG 48:3, PE 36:2 and PC 36:2, is addressed. The isomers of PC 36:2 (PC 18:1_18:1 and PC 18:0_18:2) were separated by RP-LC. However, quantification was performed with the sum integral over all isomers. The importance of species-specific calibration or at least RT matching in class-specific calibration can be readily observed, emphasizing that a small number of standards is not practical in RP-LC analysis. Regardless of whether level 1 or level 2 calibration was applied, all concentrations were within the 99% confidence interval as published for the SRM 1950 material. However, the international lipidomics interlaboratory comparison revealed a rather broad distribution of measured concentration values for single lipid species[32] (DG 34:1: <1–22 μmol L^{-1}, TG 48:3: 1–10 μmol L^{-1}, PE 36:2: 2–30 μmol L^{-1}, PC 36:2: 75–350 μmol L^{-1}), making it difficult to validate the different calibration strategies based on these consensus values only. Only certified reference material would allow an actual accuracy assessment.[21,33]

As already mentioned, species-specific external calibration and internal standardization is the method of the highest metrological order. The isotope dilution strategy ensures accuracy by compensating for losses during sample preparation and for variations in MS measurement, provided that (1) high-purity external standards with certified concentrations are used and (2) equilibration of the LILY spike material and the sample is given. Considering typical experimental uncertainties of measured MS intensity ratios and sample preparation, the latter governed by extraction efficiencies and recoveries, typical total combined uncertainties of 4–7%, were expected (see Fig. 3). In fact, the experimentally observed uncertainties for biological replicates ranged from 2–7% when using level 1 ESTD and ISTD calibrations. When using level 2 external standardization with level 1 internal standardization, the ionization bias between the class-specific standard (ESTD) and investigated species contributes to the total combined uncertainty, while the contribution of sample preparation and MS detection was still minimized due to the species-specific ISTD. Introducing a correction factor – as an estimate of the contribution of the ionization efficiency distribution within the lipid class – in the model equation of the uncertainty budgets results in estimated uncertainties of up to 30% for neutral lipids. However, for polar lipids, this contribution was significantly lower, resulting in calculated uncertainties ranging at 10% (ESTD level 2, ISTD level 1), in accordance with the widely accepted hypothesis that the ionization efficiency is determined through the head group.[3,34] When using level 2 ESTD and level 2 ISTD, for polar lipids, total combined uncertainties of 12–15% were estimated, while for neutral lipids, uncertainties of again up to 35% were calculated. The combined uncertainty of species-specific internal standardization (one-point calibration) by LILY was mainly determined by the uncertainty of the LILY quantification based on FI-HRMS.

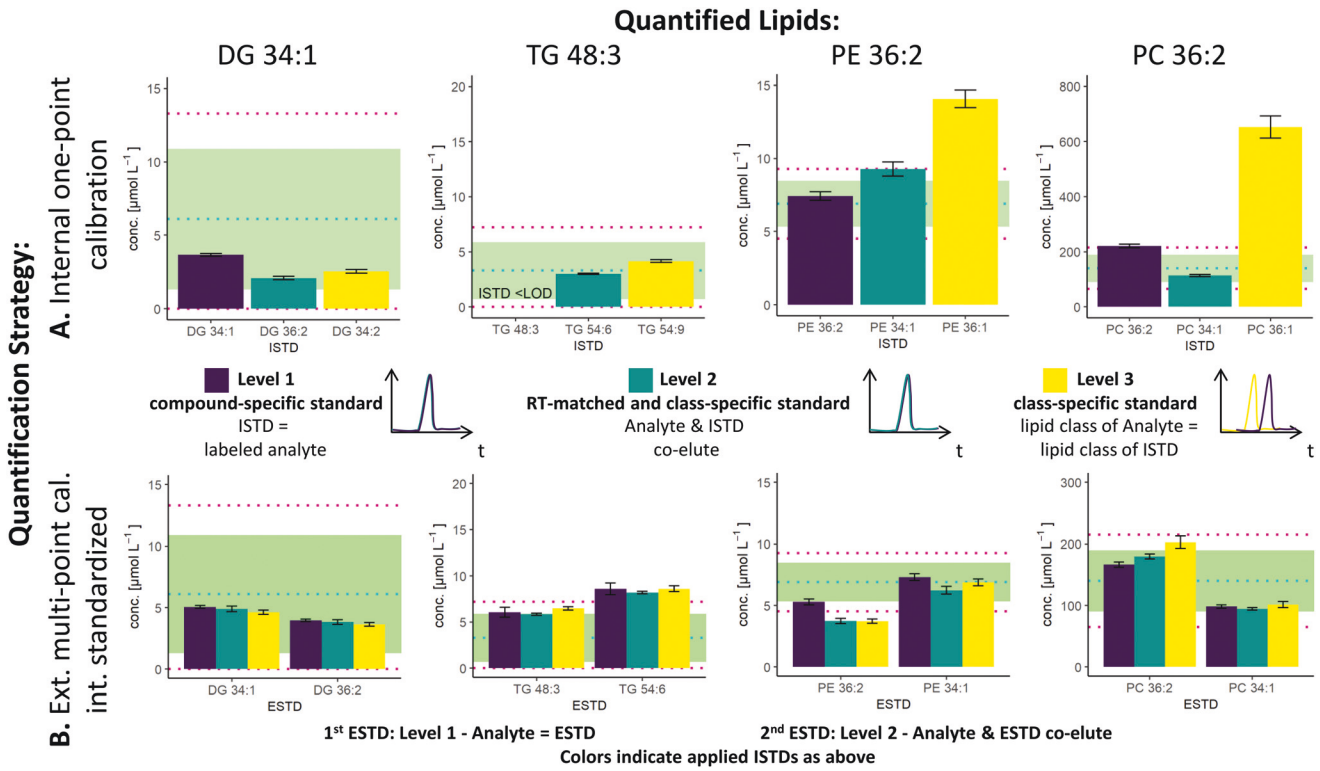

Fig. 2 Influence of the selected ESTD and ISTD on (A) internal one-point calibration and (B) external multi-point calibration with internal standardization. Different results were obtained with different levels of ESTD and ISTD. Exemplarily, the quantitative results of the lipids DG 34:1, TG 48:3, PE 36:2 and PC 36:2 in SRM 1950 are shown. The blue dotted line shows the median of the mean (MEDM), the light green region shows the 95% confident interval (CI) of the interlaboratory comparison and the red dotted line shows the 99% CI. (A) The *x*-axis and the color for the one-point calibration show the applied ISTD. (B) The *x*-axis for the multi-point calibration shows the applied ESTD (left: level 1, right: level 2) and the colors show the applied ISTD (purple: level 1, blue: level 2, yellow: level 3). The color code for the ISTD in (B) is the same as that in (A).

Error propagation results in estimated combined uncertainties ranging at 14% (assuming that the measured ratios are within the dynamic range and level 1 standardization). Again, in the case of quantifying LILY *via* level 2 standardization, the uncertainty for neutral lipids increased to 33% as the differences in ionization efficiency cannot be overcome without correction. When performing species-specific ISTD calibration (one-point calibration) with certified standards, 5–7% uncertainty has been estimated. It has to be mentioned that the estimated uncertainties of level 2 ISTD calibration is comparable regardless of whether synthetic certified standards or cost-saving LILY ISTDs were used due to the fact that the correction factor for ionization bias is the major contribution to the total combined uncertainty. Consequently, the quantitative output of the workflow was ranked according to these considerations and labeled accordingly. Quantification resorted to level 3 standardization only when level 1/2 ESTD and ISTD were not available. Again, multi-point calibration was preferred over one-point calibration.

Application to human plasma lipidomics

Although the yeast lipidome might be less complex, retention time windows matching the respective plasma lipid classes were obtained (see Fig. 4A). Co-elution and thus co-ionization were supported by the inverse retention order observed with respect to increased carbon number *versus* increased double bond number, also described in the equivalent carbon number (ECN) model.[36] For example, TG 48:3 (ECN: 42) co-eluted with TG 50:4, TG 52:5, TG 54:6, *etc*. (all ECN: 42, exemplarily, the TG elution profile is shown in ESI Fig. S3C,‡ also true for DG, LPC, LPE, PC, PI). This phenomenon increased the number of co-eluting LILY and plasma lipid species. For example, when analyzing the human plasma sample SRM 1950, LILY provided a species-specific ISTD (level 1) for 26% out of the 351 quantified lipid species (see Fig. 4B). For the great majority of lipid species (54%), a class-specific and RT-matched ISTD (level 2) was offered. Quantification of 9% of plasma lipids had to be based on a class-specific ISTD (level 3) only. Lipids from classes not present in LILY (SM, Hex2Cer and AcCa) could be quantified with external calibration, only resampling to 11% of the total quantified lipid species. These lipids followed a rather relative quantification approach and were excluded from accuracy assessments.

The FI/LC–HRMS lipidomics workflow was applied to the analysis to human plasma samples from 20 healthy donors striving to provide high lipidome coverage and accurate quantification. Lipid extraction of human plasma is well established.[8,37–40] Spiking plasma with LILY increased the overall lipid amount of the sample by approximately 10%.

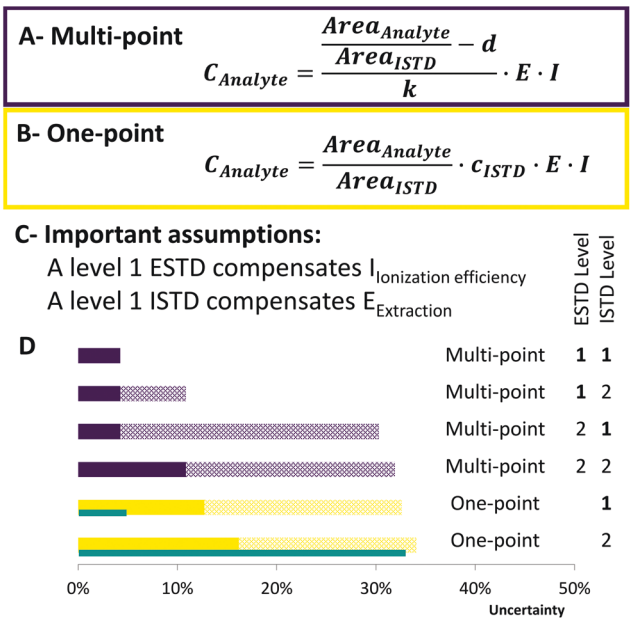

Fig. 3 Uncertainty calculation of the applied methods. Concentration formula for (A) multi-point calibration and (B) one-point calibration. $Area_{Analyte}/Area_{ISTD}$: standard uncertainty of 3% in RP-LC, d – intercept, k – slope, E – extraction factor: E = 1, associated uncertainty 10%,[35] I – ionization efficiency factor: I = 1, associated uncertainty 30% (only for neutral lipids). (C) Assumptions that have been made to estimate the uncertainty of each calibration. (D) Ranking of the applied quantification strategies. Purple bars indicate multi-point calibration and yellow one-point calibration. The blue bars next to one-point calibration show the uncertainty if synthetic certified standards (uncertainty of 2%) had been used. Full color bars show the uncertainty for polar lipids, whereas patterned stacked bars show the uncertainty that can occur for neutral lipid classes (*e.g.* TG, CE and DG). One-point calibration considers LILY quantified upon level 2 standardization.

Thus, the established Matyash protocol[41] was adopted by reducing the plasma sample amount to a minimum of 10 μL, which still ensures homogeneity,[17] and increasing the sample *versus* solvent ratio to 1 : 325. By automatizing the workflow and streamlining the data evaluation, manual curation of data was reduced to a minimum (for more details, see the ESI‡). The reference material for human plasma SRM 1950 – providing consensus values for 339 lipid species based on an international interlaboratory comparison – was used for method validation and data filtering.[23]

Three independent methods (QC accuracy and precision tests, recovery test considering standard addition of non-endogenous standards and *Z*-score calculation of SRM 1950) were implemented as quality control measures of the workflow next to the filter criteria described in the extended methods part in the ESI.‡ In total, quantitative values for 351 lipid species were obtained. 86 lipids from 7 lipid classes (LPC, LPE, PC, PE, PI, DG, TG) passed all quality filters. More specifically, accurate quantification was based on ESTD and ISTD panels passing the QC accuracy (accepting <30% trueness bias) and precision tests (accepting <30% relative standard deviation (RSD)) and the recovery test with bias <30% for the respective lipid class and obtaining a calculated *Z*-score within the 99% CI.

The total quantitative output of the novel workflow was compared to the state-of-the-art shotgun lipidomics strategy measured previously[26] with regard to the calculated *Z*-score based on SRM 1950 data. As can be readily observed in Fig. 5A, the quantitative performance was comparable after both data sets were filtered by strict criteria to avoid misidentifications, including signal stability (RSD < 30%), mass accuracy (<3 ppm) and LOQ. However, the novel workflow benefits from increased sensitivity, leading to a higher number of quantified

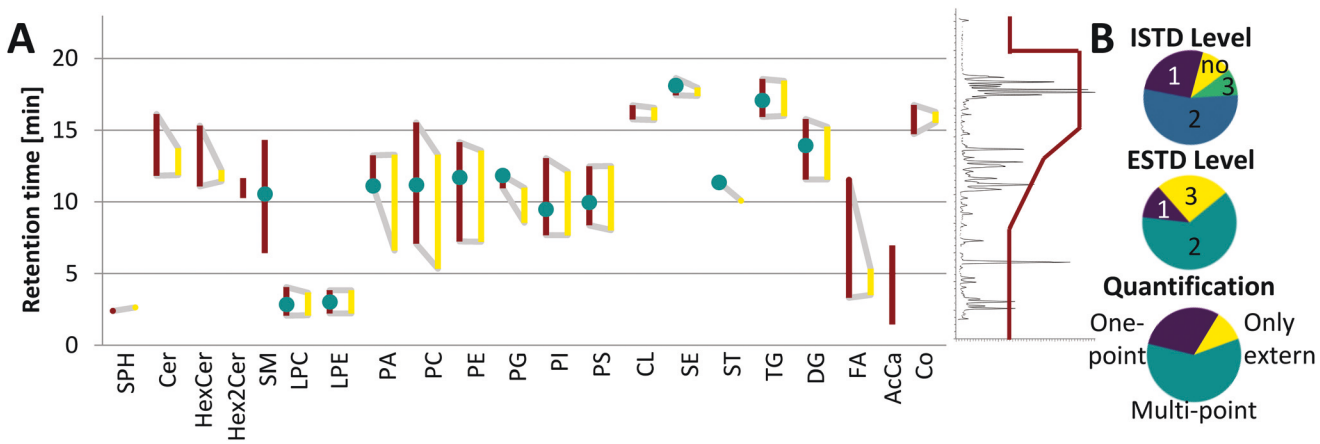

Fig. 4 Application of LILY with RP-LC and its benefits. (A) Broad RT coverage: the RT coverage for different lipid classes (each polygon corresponds to one lipid class) is shown, the left red side of the polygon shows the elution range (1st to last eluting lipid of each class) of human plasma lipids, the right yellow side shows the elution range of LILY lipids, lipid classes shown as the vertical line are only present in plasma, and green dots show the elution time of the deuterated single-lipid-per-class mixture SPLASH® LIPIDOMIX® Mass Spec Standard. A total ion chromatogram and the gradient starting at 55% B and going up to 100% B are shown on the right side as visual help. (B) Distribution of the used ISTD, quantification strategies and the ESTD. ISTD: 80% of analytes have RT-matched ISTD; the level (defined by LSI)[30] of the ISTD is shown. Quantification strategies: 59% of analytes are quantified *via* multi-point calibration; three different quantification strategies have been applied: multi-point, external multi-point calibration with internal standardization; one-point, surrogate internal standardization without external standardization; only external, multi-point calibration without the use of the ISTD and ESTD; 12% of analytes are available as the ESTD and 63% have co-ionized ESTD.

lipids, an increased selectivity, a higher number of standards and the possibility to control accuracy by the mentioned tests. Moreover, the quantitative values of the proposed workflow are traceable to external standards, offering an additional method independent of the widely used ISTD (deuterated lipids or lipids with odd, short or long chains, respectively).[8] This enables cross-validation of different lipid methods and brings lipidomics a step forward in terms of standardization and harmonization.

The obtained relative lipid class distribution resembling the biological variance of the 20 healthy donors can be seen in Fig. 5B.[21,42] On average, state-of-the-art lipidomics methods mentioned in the literature reported 50–300 annotated lipid species when quantification was mentioned.[5] In this work, 351 lipids out of 429 identified lipids were quantified in human plasma providing new tools for data validation and ranking quantitative values after a stringent metrological order. Additionally, new tools enabling data validation have been introduced, offering a new way of method control and improving the reliability of all quantitative results.

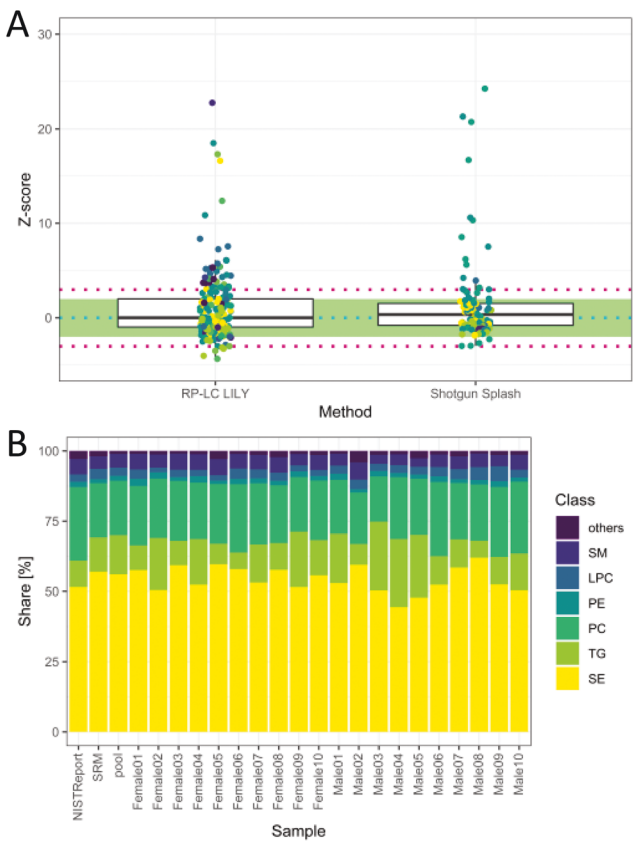

Fig. 5 (A) *Z*-Score distribution of the presented workflow (RP-LC LILY) covering 208 lipids in comparison with the state-of-the-art quantification using SPLASH® LIPIDOMIX® in a shotgun nano-ESI experiment covering 99 lipids (coverage is determined by the NIST report). In ESI Fig. S6 and Table S2,‡ a more detailed version can be found. Data for shotgun experiment taken from a previous publication (Schoeny *et al.*[26]). (B) Relative lipid class distribution of the major lipid classes in human plasma without cholesterol. "Others" contains all lipid classes with a smaller contribution to the total lipid content than 2%. The NIST report shows the distribution from the consensus values, whereas SRM is the analyzed SRM 1950 sample.

Conclusions

Lipidome wide absolute quantification and validation were enabled *via* high-resolution mass spectrometry coupled with reversed-phase chromatography. As a prerequisite, the implementation of a reversed isotope dilution step increased the number of available lipid standards provided by fully labelled ^{13}C yeast extracts in a cost-effective manner. Indeed, our experiments showed that the designed workflow provided a comparable analytical performance with regard to accuracy, uncertainty and number of absolute quantifications compared to established lipidomics platforms with the additional possibility to control accuracy *via* actual analyte QC accuracy and precision tests, recovery test considering standard addition of non-endogenous standards and *Z*-score calculation of SRM 1950. Hence, all acknowledged benefits of RP-LC–HRMS-based lipidomics, including possible isomer separation and superior sensitivity and selectivity, are now amenable without compromising the aspect of quantification. We think that the workflow increases the option of quantifying in the field of lipid analysis, offering an independent calibration strategy relying on different calibrants and standard resources, which is important regarding harmonization and standardization. Furthermore, due to the implementation of reversed isotope dilution *via* FI, this workflow can be used for any isotopically labeled biomass as the ISTD, enabling adaptation or self-production. Our study was restricted to quantification on a lipid species level based on MS1 measurements. In the future, the quantification capability could be extended to the molecular species level (*i.e.* known fatty acyl chain composition) by integrating MS2 measurements and the LC-separated isomers in the workflow.

Author contribution

Conceptualization: Schoeny, H.; Koellensperger, G.; data curation: Schoeny, H.; formal analysis: Schoeny, H.; funding acquisition: Koellensperger, G.; Rampler, E.; investigation: Schoeny, H.; Zach, O.; methodology: Schoeny, H.; Koellensperger, G.; project administration: Schoeny, H.; Koellensperger, G.; resources: Hermann, G.; Koellensperger, G.; software: Schoeny, H.; El Abiead, Y.; Hildebrand, F.; supervision: Koellensperger, G.; validation: Schoeny, H.; visualization: Schoeny, H.; writing—original draft preparation: Schoeny, H.; Koellensperger, G.; writing—review and editing: Schoeny, H.; Koellensperger, G.; El Abiead, Y.; Rampler, E.; all authors have read and agreed to the published version of the manuscript.

Conflicts of interest

This work is supported by the University of Vienna, the Faculty of Chemistry, the Vienna Metabolomics Center (VIME; http://

metabolomics.univie.ac.at/) and the research platform Chemistry Meets Microbiology. The project was funded by the aws PRIZE prototype from the Austrian BMWFW Federal Ministry. ISOtopic solutions supplied the yeast cells for internal standardization.

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A combined flow injection/reversed phase chromatography – high resolution mass spectrometry workflow for accurate absolute lipid quantification with ^{13}C- internal standards

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Table of content

1. Abbreviations of lipid classes

PL	phospholipid	NL	neutral lipid
PC	phosphatidylcholine	LPC	lysophosphatidylcholine
PE	phosphatidylethanolamine	LPE	lysophosphatidylethanolamine
PS	phosphatidylserine	LPS	lysophosphatidylserine
PG	phosphatidylglycerol	LPG	lysophosphatidylglycerol
PI	phosphatidylinositol	LPI	lysophosphatidylinositol
PA	phosphatidic acid	LPA	lysophosphatidic acid
CL	cardiolipin	ST	sterol
FA	fatty acid	DG	diglyceride
Cer	ceramide	TG	triglyceride
HexCer	hexosyl ceramide	MG	monoglyceride
Hex2Cer	dihexosyl ceramide	Co	coenzyme
SE	Sterol ester	CE	cholesterol ester
AcCa	Acyl carnitine		

2. Extended Materials and Methods

2.1. Material

Human plasma samples were purchased from Innovative Research (Novi, Michigan). To prove the concept of the method 20 single plasma donors (10 male and 10 female) were analyzed. Standard reference material (SRM) 1950 from National Institute of Standards and Technology (NIST, Gaithersburg, USA) was used. Reference standards (endogenous compounds for external calibration) and SPLASH® LIPIDOMIX® Mass Spec Standard were obtained from Avanti (Alabaster, USA) and Merck KGaA (Darmstadt, Germany). An external standard mix produced from reference standards and used for quantification was produced and regularly controlled if lipids had been degraded by observing the ratio to SPLASH® LIPIDOMIX® lipids over time. The concentration was adopted if possible or the mixture was produced freshly. The internal standard lipidome isotope labeling of yeast (LILY) was obtained according to the procedure described in Neubauer *et al*[1]. and Schoeny *et al.*.[2] Other used chemicals were of LC-MS grade and ordered at Fisher Scientific (Vienna, Austria), VWR International (Vienna, Austria) or Sigma Aldrich (Vienna, Austria).

2.2. Extraction

Extraction follows Matyash, *et al.*.[3] To a 10 µL plasma sample aliquot, which was placed at room temperature in a 5 mL microcentrifuge tube, 746 µL methanol (MeOH, cooled to -20°C), 4 µL SPLASH® LIPIDOMIX® Mass Spec Standard, 2 mL methyl tert-butyl ether (MTBE) (cooled to 4°C) and 0.5 mL of ^{13}C LILY extract (obtained from 1.6 10^7 cells) in MTBE were added. The mixture was shaken for 1h at room temperature. Phase separation was induced by adding 625 µL of MS-grade water. Upon 10 min of incubation, the sample was centrifuged at 1,000 rcf for 10 min. The upper (organic) phase was collected in a 5 mL microcentrifuge tube. The phase was dried under nitrogen and stored at -20°C if necessary. The dried lipids were dissolved in 200 µL IPA, centrifuged at 1000 rcf for 10 min and transferred into an insert

in a HPLC vial. 10µL of each sample was pooled in a glass vial with insert to obtain a sample pool for lipid identification. An extraction blank was produced with the same protocol but 10 µL water instead of the plasma.

2.3. Flow injection (FI) and reversed phase- liquid chromatography (RP-LC) high resolution mass spectrometry (HRMS) conditions

Two separate methods (flow injection (FI) and reversed phase- liquid chromatography (RP-LC)) can run in the same sequence as a fast switching was enabled by a 6-port valve controlled from the MS (see figure S1). Several parameters stayed constant during all measurements on the Vanquish Horizon and a high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer equipped with an electrospray both form Thermo Fisher Scientific. The injector needle was always washed with 75% isopropanol (IPA), 25% H_2O, 0.1% formic acid for 10 s prior and after each injection. This solution was also used for piston seal wash. Standards were measured in increasing concentration order, samples were randomized.

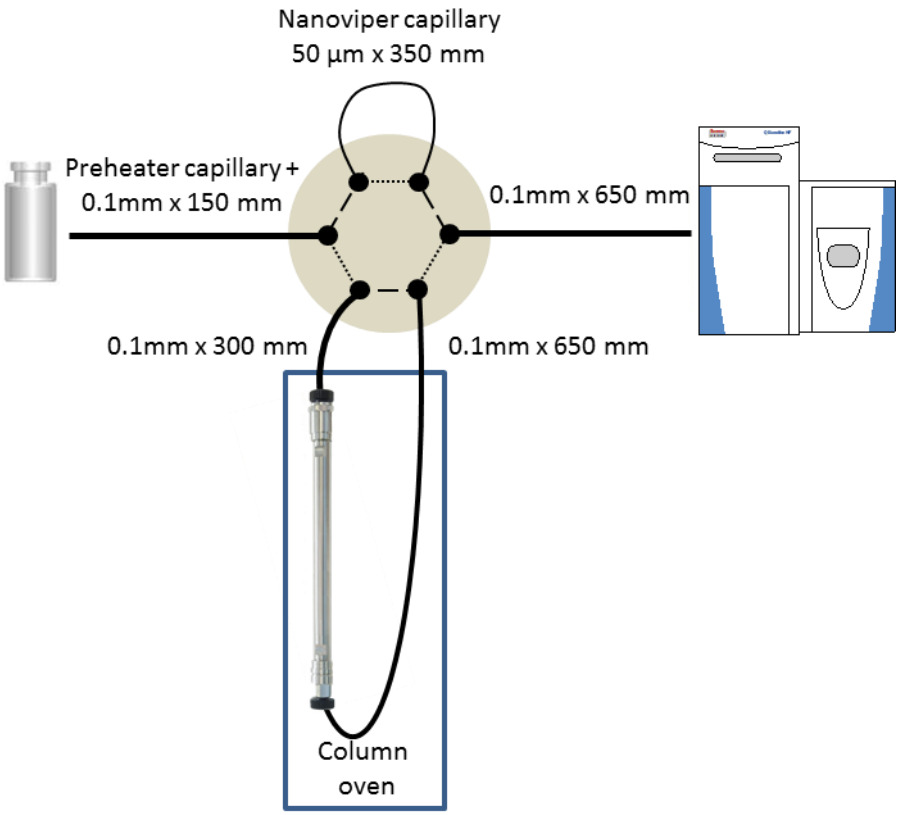

Figure S1 6-port valve for the switch between RP-LC (dotted line) and DI (dashed line) with the dimensions of the used capillaries

For RP chromatography of lipids, an Acquity HSS T3 (2.1 mm x 150 mm, 1.8 µm, Waters) with a VanGuard Pre-column (2.1 x 5 mm, 100 Å, 1.8 µm) was used. The column temperature was set to 40°C and the flow rate to 250 µL min^{-1}. Acetonitrile (ACN)/H_2O (3:2, v/v) was used as solvent A and IPA/ACN (9:1, v/v) as

solvent B, both containing 0.1% formic acid and 10 mM ammonium formate. The following gradient was applied: start at 55% B, 0−8.0 min ramp to 65% B, 8.0-13.0 min ramp to 85% B, 13.0-15.0 min ramp to 100% B, 15.0-20.0 min 100% B, 20.0 min fast switch to 55% B and equilibrated at the starting conditions for 3 min (20.0-23.0 min 55% B).

MS1 acquisition was used for quantification. The injection volume of 2 µL was selected and polarity switching was performed. The electrospray ionization (ESI) source parameters were the following: sheath gas 35, auxiliary gas 5, spray voltage 3.1 kV in both modes, capillary temperature 220 °C, S-Lens radio frequency (RF) level 30 and auxiliary gas heater 300 °C. Spectral data were acquired in profile mode. The full MS runs in positive and negative mode were acquired in the range of *m/z* 200 – 2000 at a resolution of 120,000, an automatic gain control (AGC) target at 1e6 and a maximum injection time (IT) of 200 ms.

For data dependent acquisition (DDA) the LC method was identical, but the injection volume was increased to 5 µL, positive mode and negative mode were acquired separately and only the pooled sample together with the extraction blank and a high concentrated external standard were analyzed. The MS parameters were the following: For both polarities, a Top8 method with normalized collision energy (NCE) of 25 (+)/28 (-) and an isolation window of *m/z* 1 was applied. In MS1 the range of *m/z* 200-2000 at a resolution of 120,000 was used with an AGC target at 1e6 and a maximum IT of 200 ms. The resolution in the MS2 was set to 30,000, the AGC target to 2e5 (minimum 8e3) and the max IT to 60 ms. The dynamic exclusion of triggered *m/z* was set to 15 s. An inclusion list was used for all possible lipids in human plasma. An exclusion list was generated by acquisition of a solvent blank run.

For FI, the column was by-passed via 6-port valve. 25 µL were injected in a 5 µL min^{-1} flow to get a constant signal for around 5 min. The eluents were kept constant at 50% A/50% B. The flow starts at 100 µL min^{-1} for 0.1 min, after a fast switch to 5 µL min^{-1} this flow was kept until 5.5 min. The flow was switched to 250 µL min^{-1} for flushing until 9 min at which it was switched back to 100 µL min^{-1}, which was kept until the end at 10 min.

Following tune settings were crucial for a stable flow: ionization voltage 3.5 kV (+)/-2.8 kV (-); sheath gas flow rate of 5; aux gas heater temperature 50°C; aux gas flow rate of 10; sweep gas flow rate of 4 capillary temperature 200°C; S-Lens RF level 50.

Each standard mixture was measured for 10 min and polarity switching was triggered after 2.5 min (afterwards 10 sec for equilibration). For each polarity, only MS1 spectra were acquired at the beginning before 200 data independent acquisition (DIA) scans alternated with a MS1 scan for quantification. In MS1, the resolution was set to 240,000, the AGC target to 1e6 and the maximum IT to 150 ms. For the DIA scans, a resolution of 60000 was applied and the AGC target and the max IT was set to 2e5 (+)/-5e5 (-) and 130 ms, respectively. NCE of 25 was used in positive mode and 28 in negative mode. The scan range was set to *m/z* 250-1200 in both modes and isolation window to *m/z* 1.

2.4. RP-LC-MS1 data pre-processing by Skyline

Quantitative RP-LC lipid data was processed by Skyline (version 20.1). Raw files were converted by MSConvert (Proteowizard) into mzML (centroided). A transition list was uploaded to Skyline in the

molecule interface containing all possible analytes, external standards (ESTDs) and internal standards (ISTDs). Selected lipids were based on identification software outputs, elution order, main adduct abundance, polarity matching, the awareness of common misidentifications (see website of the Lipidomics Standard Initiative (LSI)).[4] All MS1 files were imported and peak boundaries were chosen manually in order to obtain accurate peak integration. The results were exported as csv-report (columns: File Name, Acquired Time, Precursor Ion Name, Molecule Formula, Isotope Label Type, Best Retention Time (RT), Total Area, Total Area Ratio). ISTDs with severe ion suppression were excluded from the RP-LC-HRMS data by comparing the signal in the spiked sample and the spiked extraction blank by setting a threshold (max. 90% intensity loss).

2.5. FI-MS data pre-processing by LipidXplorer

Data evaluation for identification and quantification of FI data was performed with LipidXplorer (version 1.2.8). Raw files were converted by MSConvert following the guidelines of the LipidXplorer wiki. The converted mzML files were imported via LipidXplorer into a Master Scan database using following settings: mass tolerance 5 ppm (MS1)/ 0.02 Da (MS2), min. occupation of 0, intensity threshold 0 (MS1)/ 0 (MS2), resolution 230000 (MS1)/ 60000 (MS2), resolution gradient -90 (MS1)/ -20 (MS2) in positive mode and -170 (MS1)/ -40 (MS2). The molecular fragmentation query language (MFQL) files used for identification can be found in the Supporting Information. The Reporting of all MFQL files needs to be equal for further R data evaluation. The following information was reported:

MASS (*m/z* value), CHEMSC (formula of ion), FORMULA (formula of neutral lipid species), ERROR (mass error between accurate and exact mass), CLASS (lipid class), NAME (shorthand notation), SPECIES (shorthand notation on fatty acyl chain level), ISOBARIC (occurrence of isobaric overlaps with other identified lipids), PRINTENS (intensity of precursor ion), depending on lipid class: Head intensity/fatty acid (FA) intensity, Headmass/FAmass, Headerror/FAerror. The data was exported as csv-file.

2.6. RP-LC-HRMS lipid identification by LipidSearch

A fast lipid screening was performed with LipidSearch 4.2 from Thermo Scientific, by analyzing the DDA files only. Following filters were applied: RT tolerance 0.25 min, m-score threshold 2, ID quality filter A,B,C (D- only for free fatty acids and cardiolipins), calculate unassigned peak area TRUE and toprank filter TRUE. The identifications were curated manually following the criteria published elsewhere.[2] The data was exported as txt-file.

2.7. Absolute lipid quantification

By automatizing the workflow and streamlining the data evaluation, manual curation of data was reduced to a minimum. All data processing was performed in R/ R studio. A brief summary is given here.

RP-LC-HRMS data: From the Skyline export, information about the file and the analyte were added from the file name or the formula, blank values were subtracted and the stability of area and RT of the quality control (QC) standards (10, 100, 1000 and 10000 nM) was calculated and filtered by certain thresholds (area relative standard deviation (RSD) < 30%, RT RSD < 3%). The area was also deisotoping Type 1

corrected[5] and compounds with a mass error above 3 ppm were removed. To control the RT and the lipid identification, LipidSearch data was integrated and mismatching lipids were reported for manual control. The lipid identification output of the utilized software packages, was curated considering elution order, main adduct abundance, polarity matching, and finally being aware of common misidentifications (see website of the LSI).[4]

FI-HRMS data: The csv export from LipidXplorer was transformed, information was added as with the Skyline data, average blank values for each analyte were calculated and subtracted, the intensity stability of the lipids in the QC standards (10, 100, 1000 and 10000 nM) were controlled (RSD < 30%), compounds with a mass error above 3 ppm were removed and deisotoping Type I[5] and Type II (adopted script published by Triebl *et al.*[6]*)* were performed. For data evaluation only the linear working range of the standard addition dilution series was considered and only LILY concentrations above the lower limit of the linear range (see Figure 2) were reported. We defined the lower limit of quantification (LOQ) and the upper one (ULOQ) according to FDA guideline recommendations[7] as it was deduced from the linear working range. Figure 2 also shows the automatized solution to determine the linear range by calculating the slope between all neighboring points and remove of all points at the ends until the single slopes are higher than 10% of the average slope. The limit of 10% was chosen according to visual inspection.

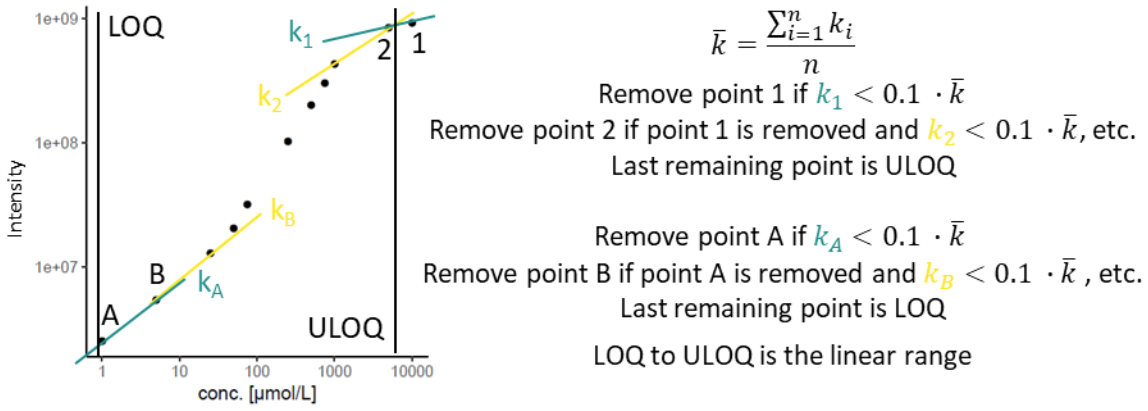

Figure S2 Determination of the linear range and the LOQ/ULOQ in FI-HRMS measurements.

For an easier nomenclature, the 3 levels definition of the LSI[4] (see figure S5) was used for ESTD and ISTD. Only the standards containing ESTD at a minimum of 6 concentration levels were used and LILY lipids were matched to the available ESTD based on the level of standard, number of hydroxy groups, number of double bonds and number of carbons in the fatty acyl chain. Always the closest 6 concentration levels were finally used for calibration. On top of that, for the final selection of LILY standards, only species with area RSD < 30% and RT RSD < 3% in the RP-LC-HRMS data as assessed in replicate QCs were accepted. The remaining quantified LILY lipids can be used as one-point calibrators for the plasma sample (second quantification strategy in the following paragraph).

Three quantitative strategies were applied:

(1) Multi-point calibration using the ESTDs measured via RP-LC-HRMS internal standardized with LILY lipids

Each LILY ISTD was matched to an ESTD of the same class based again on the level of standard, number of hydroxy groups, number of double bonds and number of carbons in the fatty acyl chain. The area ratios of ESTD/ISTD were calculated with the RP-LC-HRMS data only (one for each ISTD), and the linearity was determined as above. Area ratios between analyte and ISTD were calculated selecting the ISTD according to the previously described rules. Linear regression was calculated considering for analyte/ISTD ratio the six closest calibration points of the corresponding ESTD/ISTD (see equation 1). The LOQs of RP-LC-HRMS data were calculated by multiplying the standard deviation of low ESTDs (at conc. 1, 10 or 100 nM, n=5) with a factor of 10 and calculating a concentration based on the multi-point calibration. Concentrations below LOQ were discarded.

$$C_{Analyte} = \frac{\frac{Area_{Analyte}}{Area_{ISTD}} - d}{k} \quad (1)$$

(2) One-point calibration with the on-demand quantified LILY lipids as ISTD

Within this strategy, the via FI-HRMS quantified LILY ISTDs were applied as one-point calibrators. Again, the ISTD and the analytes were matched according to the rules above, concentration was calculated (see equation 2) and concentrations below LOQ were discarded.

$$C_{Analyte} = \frac{Area_{Analyte}}{Area_{ISTD}} \cdot c_{ISTD} \quad (2)$$

(3) Only external standardization based multi-point calibration using the ESTDs measured via RP-LC-HRMS (for lipid classes not present in LILY)

This strategy follows the same principle as strategy 1 but no ISTD were used. Therefore, the ESTD were directly matched with the analytes following again the rules above. The area of the six closest ESTDs were used to calculate the regression equation and the final lipid concentration of each analyte (see equation 3). LOQs were determined as strategy 1 and concentrations below LOQ were discarded.

$$C_{Analyte} = \frac{Area_{Analyte} - d}{k} \quad (3)$$

The different quantification strategies were calculated independently. Quantitative values were accepted provided that the recovery test of a non-endogenous standard was accurate for each class and the Z-score calculation of the SRM 1950 NIST report was within the 99% confident interval (CI) for each lipid species. For each analyte, concentration values based on different calibration strategies were obtained (see Supporting Information Excel table) and the final value was chosen based on the order following the uncertainty calculation.

3. Limit of detection (LOD) comparison

Table S1 LOD comparison of shotgun methods (FI and chip based Nanomate robot system) and RP-LC in positive mode and during pos/neg switching

		LOD [nmol L^{-1}]			
Class	**Name**	**FI**	**Nano-mate**	**LC pos**	**LC posneg**
DG	DG 34:1	5.0	-	6.3	3.5
DG	DG 36:2	21	-	1.2	1.7
LPC	LPC 16:0	-	-	1.4	17
LPE	LPE 18:0	-	-	2.1	13
PC	PC 32:0	-	-	17	56
PC	PC 34:1	55	-	2.8	6.5
PC	PC 34:2	64	12	0.6	2.2
PC	PC 36:2	-	-	1.0	9.2
PE	PE 34:1	95	60	2.0	21
PE	PE 36:2	19	-	1.0	8.7
TG	TG 48:3	22	13	2.0	1.0
TG	TG 54:3	9.5	-	-	49
TG	TG 54:6	25	25	-	16
TG	TG 54:9	10	6.1	0.1	3.3
TG	TG 60:3	-	-	0.4	22

4. Chromatographic figures

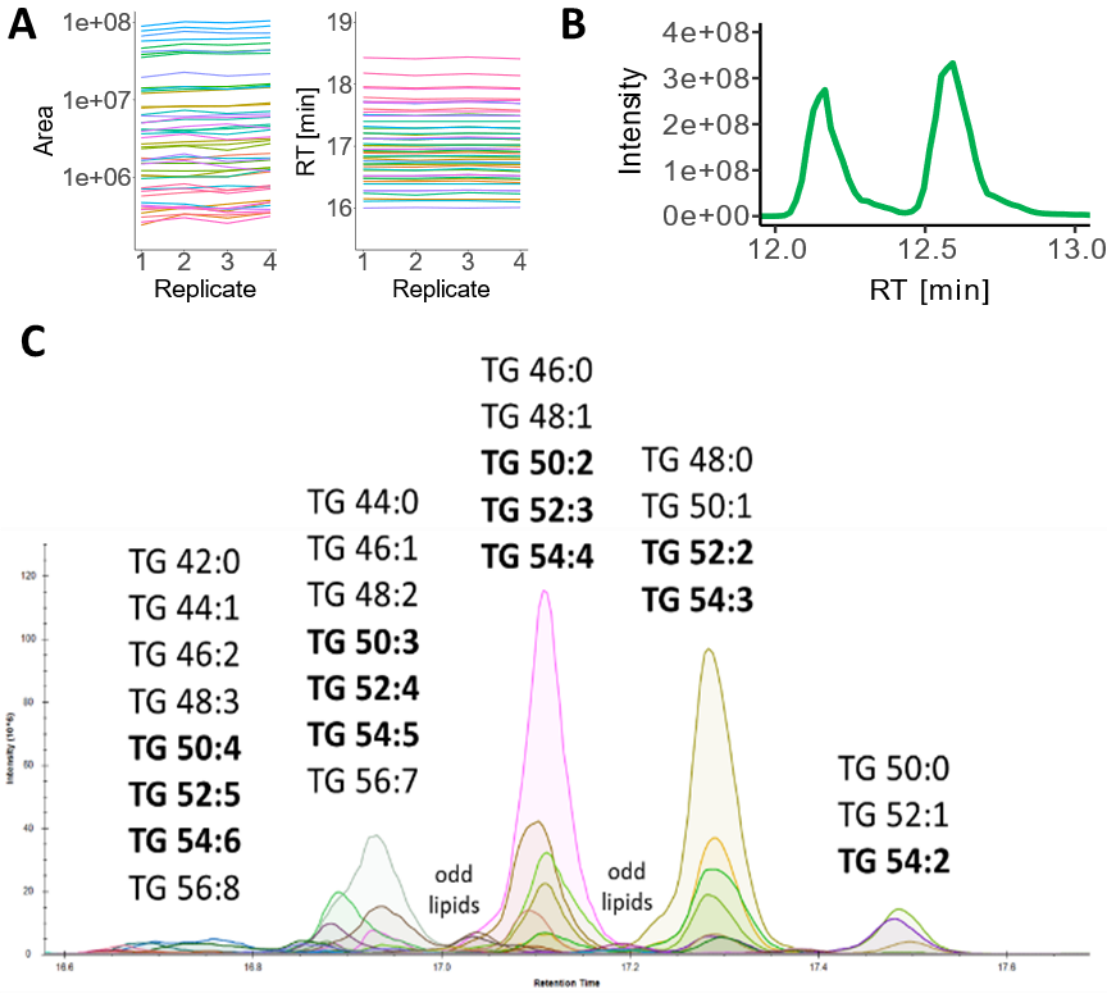

Figure S3 (A) stable analysis: RT and area stability over the run (3 days). **(B) Isomer separation:** baseline separation of PC 18:1(9Z)/ 18:1(9Z) and PC 18:1(9E)/ 18:1(9E). **(C) Retention time coverage of plasma lipids and LILY-ISTD.** shows the elution profile of TG plasma lipid species. Lipids, which occur also in yeast, are written in bold.

5. Sequence overview

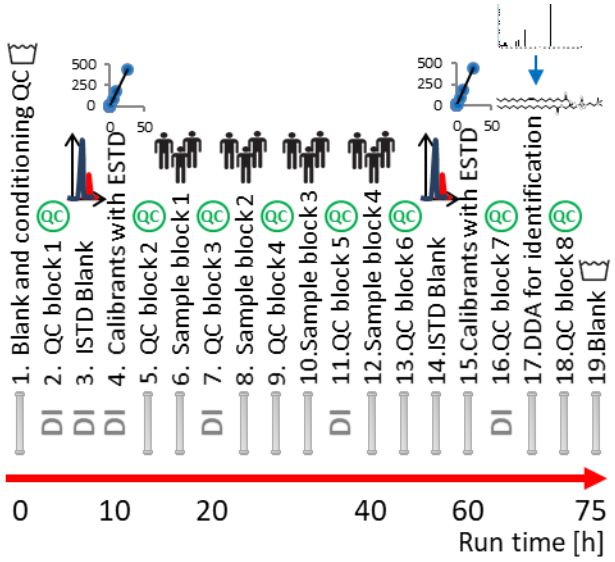

Figure S4 Sequence overview. Should give an overview of a suggested sequence for sample measurement. A detailed list can be found in the Supporting Information Excel file. Briefly, the column was washed and conditioned at the beginning followed by the calibrants measured via DI-MS, the samples divided in four blocks and the calibrants measured via RP-LC-MS. Between each block or after 10 injections, QC samples were measured alternately via DI or RP-LC. At the end, the pooled sample was measured with DDA for lipid identification and the column was washed with blank injections.

6. Levels of quantification

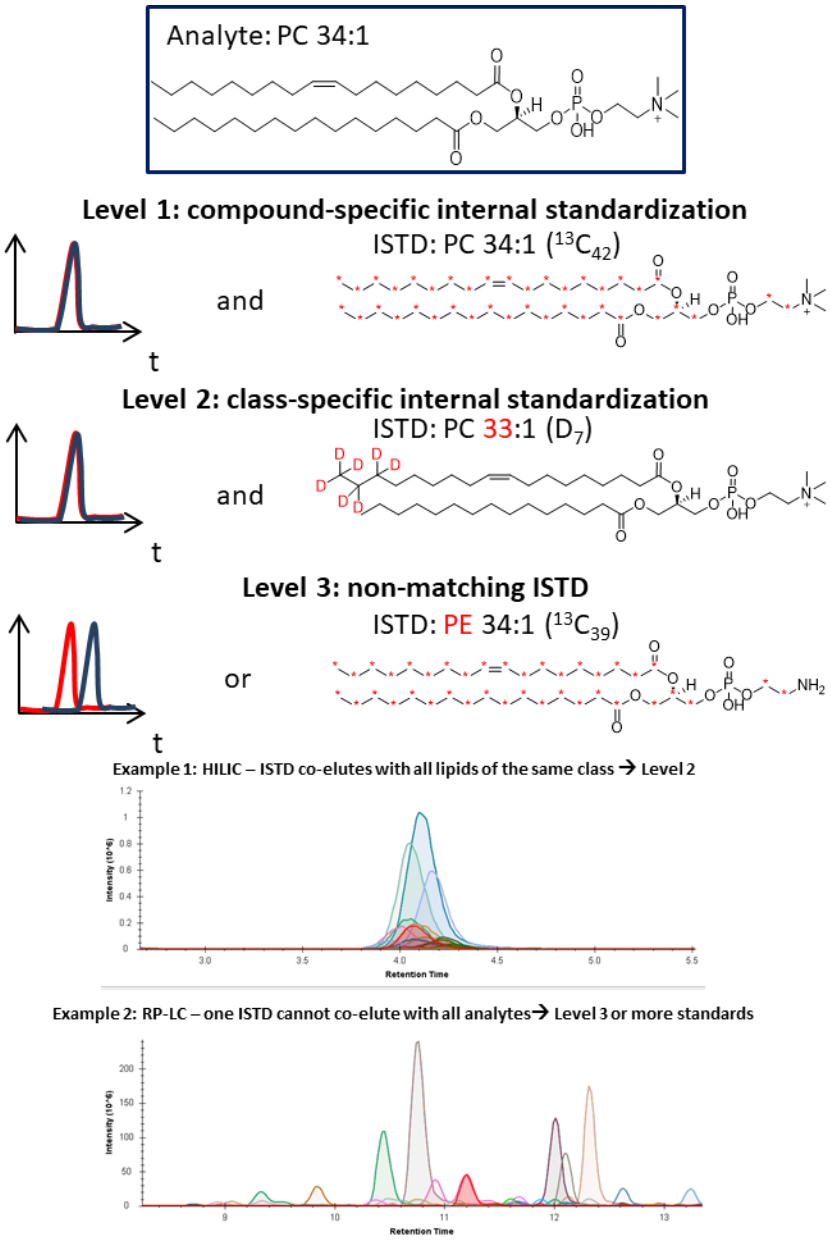

Figure S5 Levels of Quantification as defined by the LSI and examples on HILIC and RP-LC. In the example chromatograms, the ISTD (PC 33:1 (D7)) is highlighted in red. Co-elution is only given for HILIC. For RP-LC measurements, more standards are needed to cover whole elution window.

7. SRM control

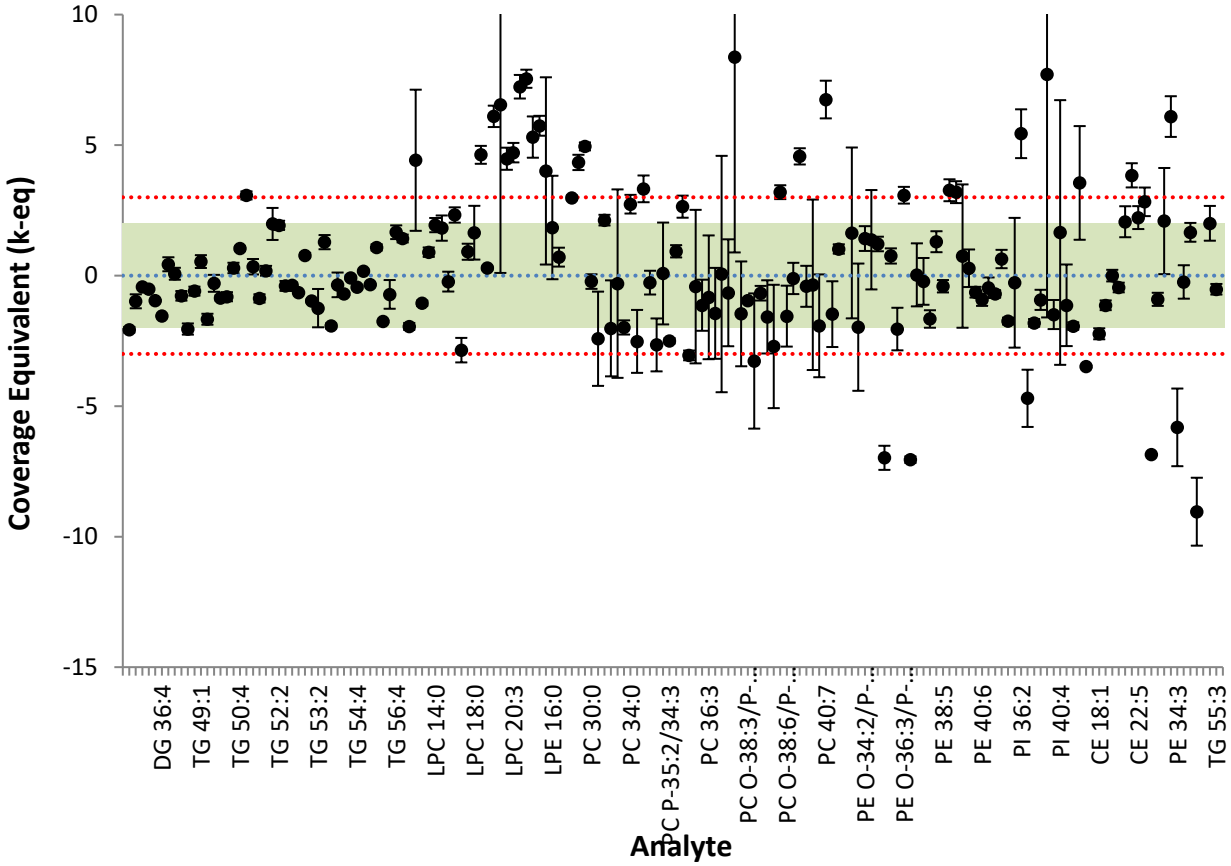

Figure S6 SRM control for the presented workflow (RP-LC LILY): Accuracy assessment for SRM 1950 - "Metabolites in Frozen Human Plasma".[8] Values are presented as normalized coverage equivalents at the mean (dots) and stdev (error bars, N=2) of measurements, overlaid onto the consensus mean value (blue line) and uncertainty (95% coverage-green region, 99% coverage-red region).

Table S2 SRM control for the presented workflow (RP-LC LILY): Measurement accuracy summary compared against SRM 1950 - "Metabolites in Frozen Human Plasma"

Lipid Species	Measurement*	Consensus Value**	Units	No. of labs	Notes
CE 16:0	416 ± 126	210 ± 58	nmol/mL	13	
CE 16:1	5,9 ± 1,4	100 ± 27	nmol/mL	11	
CE 18:0	79 ± 22	15 ± 3,7	nmol/mL	7	
CE 18:1	205 ± 23	450 ± 110	nmol/mL	14	
CE 18:2	1211 ± 80	1 700 ± 430	nmol/mL	14	
CE 18:3	84 ± 5,3	84 ± 24	nmol/mL	13	
CE 20:3	30 ± 2,2	35 ± 12	nmol/mL	13	
CE 20:4	470 ± 34	350 ± 58	nmol/mL	14	
CE 20:5	71 ± 4,0	38 ± 8,6	nmol/mL	12	
CE 22:5	7,7 ± 0,70	4,1 ± 1,6	nmol/mL	6	
CE 22:6	64 ± 5,2	37 ± 9,5	nmol/mL	11	
Cholesterol	16 ± 1,2	770 ± 110	nmol/mL	8	
DG 30:0	0,48 ± 0,018	0,83 ± 0,17	nmol/mL	7	
DG 32:0	1,4 ± 0,32	2,6 ± 1,2	nmol/mL	11	
DG 34:1	5,1 ± 0,12	6,1 ± 2,4	nmol/mL	16	
DG 36:2	5,1 ± 0,06	6,2 ± 2,2	nmol/mL	16	
DG 36:3	5,2 ± 0,09	8,4 ± 3,3	nmol/mL	15	
DG 36:4	1,3 ± 0,017	2,8 ± 1,0	nmol/mL	12	
LPC 14:0	1,4 ± 0,05	1,0 ± 0,20	nmol/mL	16	
LPC 15:0	0,72 ± 0,053	0,52 ± 0,11	nmol/mL	9	
LPC 16:0	70 ± 4,2	73 ± 11	nmol/mL	20	
LPC 16:1	1,4 ± 0,16	2,4 ± 0,35	nmol/mL	19	
LPC 17:0	1,6 ± 0,07	1,4 ± 0,24	nmol/mL	6	
LPC 18:0	32 ± 3,4	27 ± 3,3	nmol/mL	20	
LPC 18:1	29 ± 0,79	18 ± 2,3	nmol/mL	19	
LPC 18:2	23 ± 0,22	22 ± 2,9	nmol/mL	19	
LPC 18:3	1,2 ± 0,05	0,44 ± 0,13	nmol/mL	18	
LPC 20:1	0,35 ± 0,15	0,19 ± 0,024	nmol/mL	13	
LPC 20:2	0,43 ± 0,019	0,23 ± 0,044	nmol/mL	9	
LPC 20:3	3,0 ± 0,10	1,8 ± 0,26	nmol/mL	18	
LPC 20:4	10 ± 0,27	6,0 ± 0,60	nmol/mL	20	
LPC 20:5	1,0 ± 0,032	0,33 ± 0,092	nmol/mL	15	
LPC 22:4	0,34 ± 0,032	0,12 ± 0,041	nmol/mL	8	
LPC 22:5	1,2 ± 0,050	0,43 ± 0,13	nmol/mL	12	
LPC 22:6	1,3 ± 0,50	0,77 ± 0,14	nmol/mL	17	
LPC O-16:0	0,92 ± 0,047	0,55 ± 0,16	nmol/mL	10	
LPC O-18:1	0,29 ± 0,033	0,41 ± 0,13	nmol/mL	3	
LPE 16:0	1,4 ± 0,54	0,91 ± 0,27	nmol/mL	14	

LPE 18:0	2,0 ± 0,20	1,6 ± 0,55	nmol/mL	15	
LPE 18:1	6,9 ± 0,10	1,4 ± 0,47	nmol/mL	14	
LPE 18:2	3,6 ± 0,05	1,9 ± 0,56	nmol/mL	16	
LPE 20:4	2,9 ± 0,12	1,1 ± 0,41	nmol/mL	14	
LPE 22:6	1,4 ± 0,027	0,52 ± 0,18	nmol/mL	12	
PC 30:0	1,5 ± 0,09	1,6 ± 0,32	nmol/mL	11	
PC 32:0	4,8 ± 1,8	7,2 ± 1,0	nmol/mL	18	
PC 32:1	9,2 ± 3,5	13 ± 1,9	nmol/mL	18	
PC 34:0	3,1 ± 0,13	2,1 ± 0,37	nmol/mL	12	
PC 34:1	67 ± 25	120 ± 21	nmol/mL	19	
PC 36:1	38 ± 1,9	26 ± 4,6	nmol/mL	17	
PC 36:2	129 ± 74	140 ± 25	nmol/mL	18	
PC 36:3	88 ± 33	100 ± 14	nmol/mL	17	
PC 36:5	9,8 ± 3,7	11 ± 1,8	nmol/mL	16	
PC 38:2	4,0 ± 1,5	2,3 ± 0,20	nmol/mL	15	
PC 38:3	18 ± 10	26 ± 5,2	nmol/mL	14	
PC 38:4	38 ± 36	84 ± 14	nmol/mL	18	
PC 38:5	30 ± 11	42 ± 7,9	nmol/mL	18	
PC 38:6	55 ± 1,2	41 ± 4,4	nmol/mL	18	
PC 40:4	4,6 ± 0,12	2,9 ± 0,37	nmol/mL	18	
PC 40:5	6,3 ± 3,6	6,7 ± 1,1	nmol/mL	18	
PC 40:6	9,0 ± 5,1	14 ± 2,6	nmol/mL	17	
PC 40:7	5,3 ± 0,19	3,5 ± 0,26	nmol/mL	16	
PC 40:8	0,93 ± 0,036	0,73 ± 0,20	nmol/mL	14	
PC O-32:0/31:0	2,4 ± 0,08	1,5 ± 0,41	nmol/mL	11	Includes only PC
PC O-32:1/P-32:0/31:1	1,5 ± 0,87	1,6 ± 0,24	nmol/mL	11	Includes only only
PC O-34:1/P-34:0/33:1	7,8 ± 0,44	4,9 ± 0,86	nmol/mL	17	Includes only PC P-34:
PC O-34:2/P-34:1/33:2	4,9 ± 0,59	5,2 ± 1,3	nmol/mL	17	Includes only PC P-34:
PC O-34:3/P-34:2/33:3	2,4 ± 0,89	4,7 ± 0,88	nmol/mL	12	Includes only only
PC O-35:4/34:4	1,2 ± 0,06	1,0 ± 0,25	nmol/mL	9	Includes only P
PC O-36:1/P-36:0/35:1	0,47 ± 0,19	3,5 ± 0,99	nmol/mL	16	Includes only onl
PC O-36:2/P-36:1/35:2	5,5 ± 1,7	7,4 ± 1,7	nmol/mL	17	Includes only PC P-36:
PC O-36:3/P-36:2/35:3	2,5 ± 1,4	3,7 ± 0,82	nmol/mL	12	Includes only only
PC O-36:4/P-36:3/35:4	12 ± 6,3	12 ± 1,4	nmol/mL	17	Includes only PC P-36:
PC O-38:3/P-38:2/37:3	1,0 ± 0,037	1,5 ± 0,51	nmol/mL	14	Includes only only
PC O-38:4/P-38:3/37:4	6,1 ± 0,56	7,4 ± 2,0	nmol/mL	12	Includes only PC P-38:3 and
PC O-38:5/P-38:4/37:5	6,7 ± 3,8	11 ± 1,6	nmol/mL	16	Includes only only
PC O-38:6/P-38:5/37:6	2,0 ± 1,2	3,6 ± 1,0	nmol/mL	12	Includes only only PC P-38:
PC O-40:4/P-40:3/39:4	0,79 ± 0,30	0,95 ± 0,38	nmol/mL	8	Includes only only PC P-40:
PC O-40:7/P-40:6/39:7	0,76 ± 0,29	1,1 ± 0,23	nmol/mL	9	Includes only only
PC O-42:5/P-42:4	3,0 ± 0,13	0,79 ± 0,12	nmol/mL	7	Includes only PC

PC O-42:6/P-42:5	0,75 ± 0,28	0,46 ± 0,14	nmol/mL	4	Includes only PC P-42:5 res
PC O-44:5/P-44:4	3,1 ± 0,23	1,3 ± 0,30	nmol/mL	3	Includes only PC P-44:4 res
PC P-33:1/32:2	1,9 ± 0,10	2,6 ± 0,37	nmol/mL	16	Includes only PC 32:2 result
PC P-35:1/34:2	244 ± 92	240 ± 47	nmol/mL	18	Includes only PC 34:2 result
PC P-35:2/34:3	7,8 ± 0,17	12 ± 1,7	nmol/mL	18	Includes only PC 34:3 result
PC P-38:6/36:0	1,2 ± 0,23	1,2 ± 0,39	nmol/mL	10	Includes PC P-38:6 and PC 3
PE 34:1	1,5 ± 0,56	1,2 ± 0,17	nmol/mL	14	
PE 34:2	1,7 ± 0,63	2,2 ± 0,26	nmol/mL	16	
PE 34:3	0,024 ± 0,030	0,14 ± 0,020	nmol/mL	4	
PE 36:1	1,6 ± 0,07	1,3 ± 0,26	nmol/mL	14	
PE 36:2	1,2 ± 0,36	6,7 ± 0,79	nmol/mL	16	
PE 36:3	1,6 ± 0,31	2,4 ± 0,38	nmol/mL	16	
PE 36:4	0,35 ± 0,049	3,1 ± 0,39	nmol/mL	16	
PE 38:4	6,1 ± 0,40	8,1 ± 1,2	nmol/mL	16	
PE 38:5	2,5 ± 0,11	2,7 ± 0,47	nmol/mL	12	
PE 38:6	5,1 ± 0,25	3,2 ± 0,59	nmol/mL	15	
PE 40:6	1,5 ± 0,08	1,8 ± 0,36	nmol/mL	14	
PE O-34:2/P-34:1	1,0 ± 0,08	0,78 ± 0,17	nmol/mL	11	Includes only PE
PE O-34:3/P-34:2	2,1 ± 0,78	1,5 ± 0,41	nmol/mL	11	Includes only PE P-34:2 res
PE O-36:2/P-36:1/35:2	1,1 ± 0,06	0,93 ± 0,22	nmol/mL	12	Includes only only
PE O-36:3/P-36:2/35:3	5,5 ± 0,24	3,2 ± 0,76	nmol/mL	15	Includes only only PE P-36:
PE O-36:4/P-36:3	1,6 ± 0,35	1,6 ± 0,29	nmol/mL	14	Includes only PE
PE O-36:5/P-36:4	4,5 ± 1,7	4,9 ± 1,9	nmol/mL	15	Includes only PE P-36:4 res
PE O-38:4/P-38:3/37:4	1,2 ± 0,07	0,94 ± 0,18	nmol/mL	9	Includes only only PE P-38:
PE O-38:5/P-38:4	12 ± 0,80	5,8 ± 1,9	nmol/mL	17	Includes only PE
PE O-38:6/P-38:5	5,8 ± 3,3	4,9 ± 1,2	nmol/mL	16	Includes only PE P-38:5 res
PE O-38:7/P-38:6	3,8 ± 0,71	3,5 ± 0,98	nmol/mL	8	Includes only PE
PE O-40:5/P-40:4/39:5	0,65 ± 0,027	0,73 ± 0,13	nmol/mL	12	Includes only only
PE O-40:6/P-40:5/39:6	1,2 ± 0,12	1,3 ± 0,31	nmol/mL	14	Includes only only
PE O-40:7/P-40:6/39:7	2,0 ± 0,10	2,5 ± 0,72	nmol/mL	14	Includes only only PE P-40:
PG 34:1	0,14 ± 0,086	1,3 ± 0,60	nmol/mL	5	
PI 34:1	2,7 ± 0,15	2,4 ± 0,42	nmol/mL	14	
PI 34:2	2,1 ± 0,05	2,8 ± 0,38	nmol/mL	14	
PI 36:1	1,9 ± 1,5	2,1 ± 0,59	nmol/mL	13	
PI 36:2	13 ± 0,87	7,7 ± 0,93	nmol/mL	15	
PI 36:3	0,84 ± 0,32	2,2 ± 0,29	nmol/mL	14	
PI 36:4	2,1 ± 0,07	3,0 ± 0,48	nmol/mL	14	
PI 38:3	2,9 ± 0,21	3,4 ± 0,54	nmol/mL	14	
PI 38:4	36 ± 20	19 ± 2,2	nmol/mL	17	
PI 38:5	1,8 ± 0,25	2,5 ± 0,44	nmol/mL	15	
PI 40:4	0,37 ± 0,21	0,30 ± 0,042	nmol/mL	7	

PI 40:6	0,66 ± 0,25	0,84 ± 0,16	nmol/mL	12
PS 36:2	0,34 ± 1,02	0,73 ± 1,6	nmol/mL	4
TG 42:0	0,70 ± 0,069	0,38 ± 0,19	nmol/mL	5
TG 44:3	0,095 ± 0,012	0,18 ± 0,0094	nmol/mL	4
TG 46:2	4,2 ± 0,35	3,6 ± 1,3	nmol/mL	8
TG 47:2	0,66 ± 0,044	0,21 ± 0,027	nmol/mL	3
TG 48:0	4,6 ± 0,27	4,5 ± 1,2	nmol/mL	10
TG 48:1	11 ± 0,59	13 ± 3,2	nmol/mL	16
TG 48:2	10 ± 0,60	16 ± 2,8	nmol/mL	15
TG 48:4	1,2 ± 0,040	1,3 ± 0,23	nmol/mL	5
TG 49:0	0,42 ± 0,037	0,31 ± 0,055	nmol/mL	3
TG 49:1	2,2 ± 0,10	2,0 ± 0,42	nmol/mL	9
TG 49:2	0,86 ± 0,12	1,8 ± 0,56	nmol/mL	6
TG 50:0	3,6 ± 0,27	3,8 ± 0,83	nmol/mL	11
TG 50:1	29 ± 0,94	38 ± 10	nmol/mL	14
TG 50:2	37 ± 2,1	47 ± 12	nmol/mL	15
TG 50:3	25 ± 1,3	23 ± 6,6	nmol/mL	16
TG 50:4	12 ± 0,25	8,7 ± 2,9	nmol/mL	15
TG 50:5	3,6 ± 0,10	1,6 ± 0,64	nmol/mL	7
TG 51:1	2,0 ± 0,14	1,8 ± 0,48	nmol/mL	7
TG 51:2	3,8 ± 0,18	4,8 ± 1,1	nmol/mL	8
TG 51:3	5,2 ± 0,36	4,8 ± 1,9	nmol/mL	5
TG 52:1	20 ± 1,8	14 ± 2,9	nmol/mL	11
TG 52:2	71 ± 2,5	44 ± 14	nmol/mL	16
TG 52:3	89 ± 5,5	100 ± 29	nmol/mL	16
TG 52:4	42 ± 1,8	48 ± 17	nmol/mL	15
TG 52:5	11 ± 0,19	15 ± 5,7	nmol/mL	13
TG 52:6	5,1 ± 0,13	4,0 ± 1,4	nmol/mL	8
TG 52:7	0,26 ± 0,009	0,39 ± 0,13	nmol/mL	5
TG 53:2	1,4 ± 0,30	1,9 ± 0,41	nmol/mL	9
TG 53:3	5,1 ± 0,30	3,7 ± 1,1	nmol/mL	6
TG 53:4	0,94 ± 0,072	2,4 ± 0,76	nmol/mL	6
TG 54:1	2,9 ± 0,43	3,2 ± 0,91	nmol/mL	10
TG 54:2	6,4 ± 0,14	8,2 ± 2,6	nmol/mL	13
TG 54:3	25 ± 0,71	26 ± 9,8	nmol/mL	15
TG 54:4	30 ± 1,0	36 ± 13	nmol/mL	15
TG 54:5	29 ± 0,64	27 ± 11	nmol/mL	15
TG 54:6	12 ± 0,22	14 ± 5,1	nmol/mL	16

* Measurement mean ± 1 s
** Consensus mean ± stand

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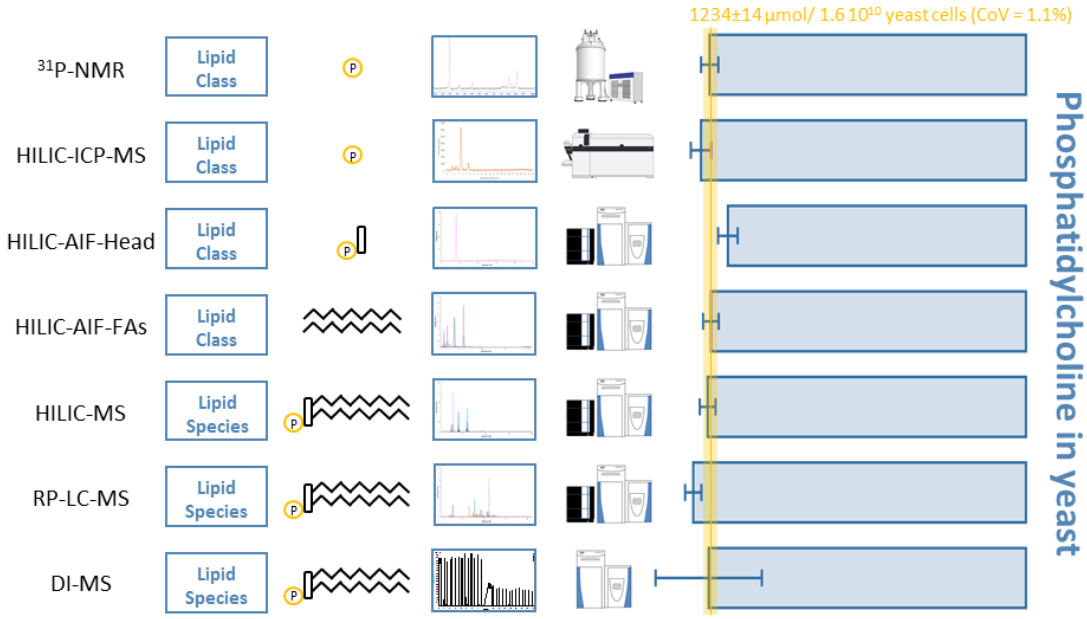
3.2. Publication II

Hunting absolute molar lipid concentrations: A phospholipidomics cross-validation study

Harald Schoeny, Evelyn Rampler, Dinh Binh Chu, Anna Schoeberl, Luis Galvez, Markus Blaukopf, Paul Kosma and Gunda Koellensperger

Submitted manuscript



A cross-validation of different common lipid mass spectrometry (MS)-based methods (hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RP-LC), direct infusion (DI)) has been achieved by including alternative methods such as nuclear magnetic resonance (NMR) and inductive coupled plasma (ICP). The lipid standard independent nature of flow injection (FI)-ICP-MS and ^{31}P-NMR enables quantification based on other available certified, traceable, phosphor containing substances, accelerating, and simplifying accurate lipid class quantification also of newly identified lipid species. A novel lipid class quantification based on all ion fragmentation (AIF) offers an additional tool for benchmarking quantitative results on organic MS-based instruments. These approaches act as important steps towards traceability of quantitative lipid values obtained by different lipidomics platforms.

I have prepared the samples, measured the organic MS-based methods, and calculated the quantitative values of each method. I have also written the main part of the manuscript.

Hunting absolute molar lipid concentrations: A phospholipidomics cross-validation study

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ABSTRACT

Standardization is essential in lipidomics, and part of a huge community effort. However, with the still ongoing lack of reference materials benchmarking quantification is hampered. Here, we propose traceable lipid class quantification as an important layer for validation of quantitative lipidomics workflows. ^{31}P-nuclear magnetic resonance (NMR) and inductively coupled plasma (ICP)-mass spectrometry (MS) can use certified species-unspecific standards to validate shotgun or liquid chromatography (LC)-MS-based lipidomics approaches. We further introduce a novel lipid class quantification based on lipid class separation and all ion fragmentation (AIF). Normally, dedicated software solutions are necessary to resolve the chimeric spectra for lipid species quantification but the integration of class-specific fragments over a mass range typical for a lipid class can be used to quantify the concentration of the whole lipid class. The concept is particularly interesting as low absolute limits of detection in the fmol range can be achieved and LC-MS platforms are widely used in the field of lipidomics, while the accessibility of NMR and ICP-MS is limited. On one hand, the study of the yeast *Komagataella phaffii* (former: *Pichia pastoris*), with its simple and reproducible production and its more similar lipid profile to multicellular eukaryotes than *S. cerevisiae*, enables the production of suitable long-term system quality controls. While on the other hand the comparison of the different lipid class workflows paves the way for quantitative lipidomics studies and supports standardization and harmonization in the field of lipidomics.

KEYWORDS

Lipidomics, Lipid class quantification, All ion fragmentation, Standardization, Traceability, Phospholipids

INTRODUCTION

Up to date, accurate absolute quantification remains a grand challenge in lipidomics.[1] Standardization is inherently difficult in omics type of analysis as the number of lipid species in a biological sample is high (typically several hundred) and the concentration ranges can cover several orders of magnitude (for example 8 in plasma[2] or 7 in platelets[3]). We have seen huge progress in standardization,[2,4–6] driven by valuable community efforts, however, the highest metrological order methods requiring traceable, certified reference materials are not yet routine. Certification is a complex process, which includes compositional and quantitative data but also a characterized stability and uncertainty for each analyte. Until 2021, this stage has not been reached in lipidomics.[7] Diverse international ring trials were of

paramount importance for harmonization in the field. In 2017, an inter-laboratory comparison with more than 30 participants established consensus values for 339 lipids in the human plasma standard reference material (SRM) 1950 material provided by the National Institute of Standards and Technology (NIST).[8] An international ring trial has further delivered consensus values for 250 metabolites, including lipids, based on the Biocrates AbsoluteIDQp400HR kit.[9]

In lipidomics, it is common practice to calibrate by class-specific internal standards (ISTDs) using a limited set of non-endogenous lipid species [10] (e.g. short, long, or odd fatty acyl chains to avoid overlaps). Species-specific internal standardization by stable isotope-labeled analogs is the method of choice when aiming at accurate absolute quantification.[11] However, comprehensive lipidome analysis by species-specific isotope dilution covering several hundreds of lipids is challenging. Despite progress (also regarding the availability of lipid standard panels),[7] even when using isotopically labeled ISTDs, assay commutability (reproducibility of quantitative data obtained from different platforms) is still a bottleneck in lipid quantification.[11] Normalization to SRM 1950 or to quality control (QC) samples was suggested to ameliorate harmonization and finally lead to assay commutability. As a drawback, no traceability is achieved by this strategy, as concentrations are traced back to consensus values that are not traceable themselves. This problem is highlighted in different seminal studies,[12–14] which emphasize the challenge of integrating validation schemes and cross-platform comparisons.

In this work, we introduce a validation scheme based on traceable orthogonal quantification of lipid classes. Lipid class quantification has a long-standing tradition in the science of lipids,[15] however with the emergence of omics tools this application became less important. Table 1 summarizes methods and their analytical figures of merit.

Table 1 Summary of published phospholipid class quantification strategies. Limit of detection (LOD) values have been converted to the same unit (nmol) in absolute amount corresponding to LOD on column in chromatography. If only masses (e.g. ng) of lipid classes are given, an average molar mass of 700 g/mol was estimated. TLC- thin layer chromatography, IC- ion chromatography, SFC- supercritical fluid chromatography, CE- capillary electrophoreses, RI- refractive index, CAD- charged aerosol detector, QTOF- quadrupole time-of-flight, IT- ion trap, HG- head group, TIC- total ion chromatogram, RF- response factor, PL- phospholipid, PA- phosphatidic acid, SM- sphingomyelin.

Separation technique	Analyzer/ detector	LOD [nmol]	Analyte	Reference standard	Paper
-	^{31}P-NMR	600	^{31}P	P containing ISTD	Kato 2018[16]
-	2D-^{31}P,^{1}H-NMR	4	^{31}P	P containing ISTD	Kaffarnik 2013[17]
-	colorimetry	14-140	total PL	P containing ESTD	Stewart 1980[18]
-	fluorometry	0.5	total PL	P containing ESTD	Nanjee 1991[19]
enzymatically	UV/VIS	2.8	PA	class-specific ESTD	Dippe 2009[20]
enzymatically	UV/VIS	0.02	SM	class-specific ESTD	He 2002[21]

TLC	autoradiography	0.9	^{32}P	P containing ESTD	Stephens 1989[22]
TLC	densitometry	0.3	Lipid class	class-specific ESTD	Weerheim 2002[23]
TLC	colorimetry	0.2	P	P containing ESTD	Zhou 1992[24]
TLC	RP-LC-UV/VIS	0.01	Lipid class, derivatized	class-specific ESTD	Rastegar 1990[25]
IC	conductivity	0.1	Lipid class, deacyl. HG	class-specific ESTD	Nasuhoglu 2002[26]
offline-IC	autoradiography	0.09	^{32}P, deacyl. HG	P containing ESTD	Stephens 1989[22]
NP-LC	RI	1.0	universal	class-specific ESTD	Grit 1991[27]
HILIC	ELSD	0.00014	Non-volatile comp.	class-specific ESTD	Giuffrida 2013[28]
HILIC	CAD	0.007	Non-volatile comp.	class-specific ESTD	Kielbowicz 2015[29]
SFC	CAD	0.0009	Non-volatile comp.	class-specific ESTD	Takeda 2019[30]
NP-LC	ICP-MS	0.007-0.04	P	P containing ISTD	Kovacevic 2004[31]
HILIC	ICP-MS	0.003-0.009	P	P containing ISTD	Vosse 2020[32]
HILIC	ESI-MS	0.07	Lipid class, TIC+RF	retained ISTD	Cifkova 2012[33]
SFC	ESI-QTOF-MS	0.007	Lipid class, TIC+RF	retained ISTD	Bartosova 2021[34]
CE	ESI-IT-MS	0.0015	Lipid class, deacyl. HG	class-specific ESTD	Warren 2020[35]

We revisit class-specific quantification and explore its potential for mass balancing and thus benchmarking current lipidomics workflows such as shotgun high-resolution mass spectrometry (HRMS), hydrophilic interaction liquid chromatography (HILIC)-HRMS and reversed phase liquid chromatography (RP-LC)-HRMS. While triglycerides and total cholesterol can been validated with clinical enzymatic tests,[36,37] phospholipids (PLs) lack these widespread possibilities. Here, we address traceable PL class quantification in yeast by ^{31}P-nuclear magnetic resonance (NMR) and elemental mass spectrometry. The orthogonal methods allow the use of species-unspecific quantification by phosphorus, traceable to SI. While ^{31}P-NMR requires high sample amounts and long analysis time, which in turn demands stabilizing agents, inductively coupled plasma (ICP)-MS requires selective chromatographic separation of lipid classes. Recently, Vosse *et al*.[32] discussed the analytical figures of merit of HILIC-ICP-MS analysis of PLs. Excellent absolute limits of detection < 10 pmol were reported outperforming ^{31}P-NMR by at least 3 orders of magnitude.[16,17] As a drawback, matrix dependence of ICP-MS analysis must be considered in species-unspecific quantification.

Additionally, we introduce LC-electrospray ionization (ESI)-MS strategies for lipid class quantification. Only a few reports on lipid class quantification by LC-ESI-MS exist.[33,34,38] Cifkova *et al.*[33] have used the intensities of the total ion chromatogram (TIC) to generate lipid class peaks. To further reduce the number of necessary standards, only one ISTD (sphingosyl phosphoethanolamine) has been used together with a response factor approach to compensate for differences in ionization efficiency. Here, an all ion fragmentation (AIF) approach will be combined to a class-specific chromatographic separation and

will be validated for lipid class quantification by ^{31}P-NMR and ICP-MS analysis. AIF is considered as data independent acquisition (DIA) method as all ions are fragmented intensity independently in a certain mass range in contrary to data dependent acquisition (DDA), which is limited to identification. In lipidomics, AIF is either used for the determination of the fatty acyl chain distribution of each class[39] or dedicated software solutions are necessary to resolve the chimeric spectra of DIA for lipid species quantification.[40] In this project, the disadvantage has been used as a benefit by integrating a class-specific fragment or a mass range typical for a lipid class over a certain retention time range.

We apply the validation scheme, to the analysis of PLs in the yeast *Komagataella phaffii* (often referred to by its obsolete name *Pichia pastoris*), which is well-known in biotechnological industries as it has an important role as host for recombinant protein production, can grow on methanol or glucose as sole carbon source and is, therefore, a favorable organism for labeled biomass production as internal standard.[41] Its membrane composition is even more similar to multicellular eukaryotes than in *Saccharomyces cerevisiae,* as *K. phaffii* also contains polyunsaturated fatty acids (FA) (linolenic acid FA 18:3) and the lipid class glycosylceramides (GlcCer).[42] The extensive knowledge on the phospholipidome of *K. phaffii*[43–46] was the ideal starting point for our validation study, ultimately aiming at getting a more profound understanding of limitations in lipid species quantification by introducing mass balances.

EXPERIMENTAL SECTION

Methods

Nine different quantification methods using different platforms were used (see Table 2) and described according to the use of separation and analyzer in detail in the supplementary.

Table 2 Summary of the applied quantification methods. PC- Phosphatidylcholine, PE- phosphatidylethanolamine, PI- phosphatidylinositol, PS- phosphatidylserine, PG- phosphatidylglycerol, LPC- lysophosphatidylcholine

Nr.	Abbreviation	Level	Separation	Analyzer	Mode	Quantification	Quantified classes
1	P-NMR	Class	-	NMR	-	ISTD	PC, PE, PI, PS
2	HILIC-ICP-MS-ext.cal.	Class	HILIC	ICP-MS	-	ESTD	PC, (PE), PG
3	HILIC-ICP-MS-std.add.	Class	HILIC	ICP-MS	-	Std. add.	Validated Method 2
4	FI-ICP-MS	Total		ICP-MS	-	ESTD	Total P
5	HILIC-ESI-AIF-Head	Class	HILIC	ESI-MS	AIF	ESTD	PC, (PE), PG, LPC
6	HILIC-ESI-AIF-FAs	Class	HILIC	ESI-MS	AIF	ESTD	PC, (PE), PG, LPC
7	HILIC-ESI-MS1	Species	HILIC	ESI-MS	MS1	ISTD	PC, PE, PG, LPC
8	Shotgun	Species	-	nESI-MS	MS1/DIA	ISTD	PC, PE, PI, PS, PG, LPC

9	RP-LC-MS	Species	RP-LC	ESI-MS	MS1	ISTD	PC, PE, PI, PS, PG, LPC

Briefly, ^{31}P-NMR analysis followed the protocol of Kato *et al.* 2018.[16] The sample was prepared in a surfactant solution and mixed with phosphoserine as ISTD. The sample was pH adjusted to 6.9±0.04 and measured on a Avance III 600 MHz (Bruker, Billerica, MA, USA). The peaks of the obtained spectra were integrated manually.

ICP-MS analysis was used for total PL content (by flow injection (FI)) and lipid class (by HILIC separation) quantification with external standards (ESTDs) via phosphorus tracer. An Agilent 1260 Infinity Bio-Inert HPLC system was coupled with an Agilent 8800 Triple Quadrupole ICP-MS (both Agilent Technologies, Santa Clara, CA, USA). An iHILIC P column (2.1 mm x 150 mm, 5 µm, HILICON, Upsala, Sweden) was used to obtain class separation of polar lipids. SPLASH® Lipidomix® Mass Spec Standard (Avanti Polar Lipids, AL, USA) was used for both external calibration and standard addition via HILIC-ICP-MS. FI experiments were conducted without a column and tributyl phosphate acted as the reference standard for external calibration. The obtained chromatograms were smoothed before integration and integrated manually by using MassHunter 4.6 (Agilent Technologies).

HILIC-ESI-MS analysis was based on the same column but connected to a Vanquish Horizon HPLC coupled to a high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (both Thermo Fisher Scientific, Waltham, MA, USA). Lipid class quantification was conducted with ESTDs in AIF mode and lipid species quantification with SPLASH® Lipidomix® Mass Spec Standard as ISTDs in MS1 mode. Skyline (version 20.2) was used for MS1 data processing and AIF fatty acyl chain or product ion head fragments whereas neutral loss head fragments in the AIF files were integrated manually with Qual Browser Thermo Xcalibur (version 4.0.27.19, Thermo Fisher Scientific).

Shotgun analysis was conducted as previously described elsewhere[46] on a high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific), connected with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY, USA). SPLASH® Lipidomix® ISTD was added as ISTD and data processing was performed with LipidXplorer 1.2.8.

A Vanquish Horizon HPLC (Thermo Fisher Scientific) with an Acquity HSS T3 (2.1 mm × 150 mm, 1.8 µm, Waters, Milford, MA, USA) was coupled to a high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer and used for RP-LC-HRMS. Skyline (version 20.2) was used for peak integration and R/ R studio for final data processing.

Determination of concentration locations across multiple platforms

Determination of concentration locations across multiple lipid class platforms followed the CCQM Guidance note of the Bureau International des Poids et Mesures (BIPM).[47] No extreme outlier values were expected but the uncertainty might differ between the applied values. Hence the recommended use of uncertainty-weighted mean $\bar{x}_u$ was chosen (see Formula 1).

$$Formula\ 1.\ \bar{x}_u = \frac{\sum_{i=1}^{m} x_i/u^2(x_i)}{\sum_{i=1}^{m} 1/u^2(x_i)}$$

For each lipid class value, the inconsistency was checked via a chi-squared test (see Formula 2). Values above the critical chi-squared value $\chi^2_{0.05,m-1}$ were considered as inconsistent and the uncertainty (see Formula 3) was corrected over observed dispersion (see Formula 4). Standard deviation $u^2(x_i)$ and mean x_i of technical replicates were used for calculation.

Formula 2. $\chi^2_{obs} = \sum_{i=1}^{m} \frac{(x_i-\bar{x}_u)^2}{u^2(x_i)}$ 3. $u^2(\bar{x}_u) = \frac{1}{\sum_{i=1}^{m}\frac{1}{u^2(x_i)}}$ 4. $u^2_{corr}(\bar{x}_u) = \frac{\chi^2_{obs}}{m-1}u^2(\bar{x}_u)$

RESULTS AND DISCUSSION

Quantification of the total phospholipidome

Both ^{31}P-NMR and ICP-MS enable traceable species-unspecific standardization with the highest metrological order based on the phosphorus content of PLs.[16,31] In this work, both methods were applied to assess the phospholipidome of *K. phaffii* quantitatively. Exemplarily, Figure 1 in the Supplementary gives a ^{31}P-NMR spectrum of the yeast extract. To control the pH of the measurement solution, the dried extract was reconstituted in a surfactant-containing solution.[48] A recovery of 92% was assessed using the standard PC 36:2 (0.5 mmol/L) for this reconstitution protocol, dedicated to ^{31}P-NMR analysis only. The complete phospholipidome (the sum of all PL classes) of *K. phaffii* was addressed by the entirely orthogonal methods of traceable phosphorus quantification, (1) by flow injection –ICP-MS analysis of the yeast lipid extract, and (2) by calculating the sum of ^{31}P-NMR spectral lipid class peaks. A certified standard of phosphoserine and tributyl phosphate was used for calibration in ^{31}P-NMR and flow injection ICP-MS, respectively. The obtained values were in excellent agreement with concentrations at 2340±150 (6.4%) and 2250±30 (1.3%) µmol/ 1.6 10^{10}cells for ^{31}P-NMR and FI-ICP-MS, respectively. The selectivity of the FI-ICP-MS method relied on a sample clean-up introduced by the tailored Folch extraction,[49] selectively extracting lipids and removing otherwise abundant phosphorus-containing salts,

macromolecules, and metabolites. NMR suffers from reduced sensitivity compared to MS methods (see Figure 1B) but as saturation cannot occur, the sample can be enriched until sufficient signal intensity is achieved.

Lipid class quantification: HILIC-ICP-MS and a promising fragment-based approach HILIC-ESI-AIF

In mass spectrometry, lipid class quantification strategies demand chromatographic separations, due to potential overlaps in the mass traces. HILIC is the established class separation method of polar lipids as retention is governed by the chemistry of the head group.[7] HILIC is very versatile, but there is no single separation method, which is ideally suitable for the simultaneous measurement of acidic and neutral lipid classes. Therefore, compromised conditions are selected accepting severe peak tailing for acidic classes. As a result, high limits of detection, reduced column recoveries, and thus high uncertainties in quantification are observed for the classes of PI, PS, and phosphatidic acid (PA).[50] When designing ICP-MS approaches, the selectivity of class separation is a must, as the quantification is based on phosphorus measurements only (see Supplementary Figure 2). Gradient and matrix dependencies increase the uncertainty and have to be considered involving correction strategies by response factor [31] or the implementation of isocratic conditions using counter gradient systems.[32] In this work, the problem was overcome by lipid class-specific external calibration. Prior to analysis of the sample, these standards could be quantified by their phosphorus content using FI-ICP-MS. This way, traceability was ensured, and standard stability (see supplementary Figure 3) was monitored. External calibration was validated by standard addition proofing the validity of the method.

HILIC-ESI-MS-based all ion fragmentation (AIF)

The combination of HILIC and the ESI-MS-based AIF was only used for the determination of fatty acyl chain compositions so far.[39] Here, we introduce HILIC-ESI-AIF as a novel strategy for lipid class quantification, relying on class-specific fragments and defined retention time ranges. Figure 1A exemplarily shows the selection of different fatty acyl chain fragments and the use of the neutral loss for PE and the product ion head group fragment for PC, while fragments for further classes can be found in Supplementary Table 1. Thus, one peak with a defined mass and retention time window represents one lipid class either as a head group or a fatty acyl chain fragment (Supplementary Figure 4+5). In general, triple quadrupole instruments are as applicable as high-resolution instruments as neutral loss scans or product ion scans are common modes in tandem mass spectrometry. A clear limitation for MS2 based quantification strategies is that the fragmentation efficiency correlates to fatty acyl composition

(especially concerning the number of double bonds[51]). Hence, the fatty acyl composition difference between standard and the lipid species needs to be considered. In the case of the investigated *K. phaffii*, the rather simple fatty acid profile facilitates the AIF strategy as the fatty acids FA 16:0, FA 18:1, FA 18:2 and FA 18:3 make up to 90% of the total fatty acid content in the cell homogenate. Only, minor contributions were found for FA 14:0, FA 16:1, FA 18:0, and FA 26:0.[45] The HILIC-ESI-AIF concept is particularly interesting as low absolute limits of detection in the fmol range can be achieved (see Figure 1B) and LC-MS platforms are widely used in the field of lipidomics, while the accessibility of NMR and ICP-MS is limited at the same time having compromised sensitivity.

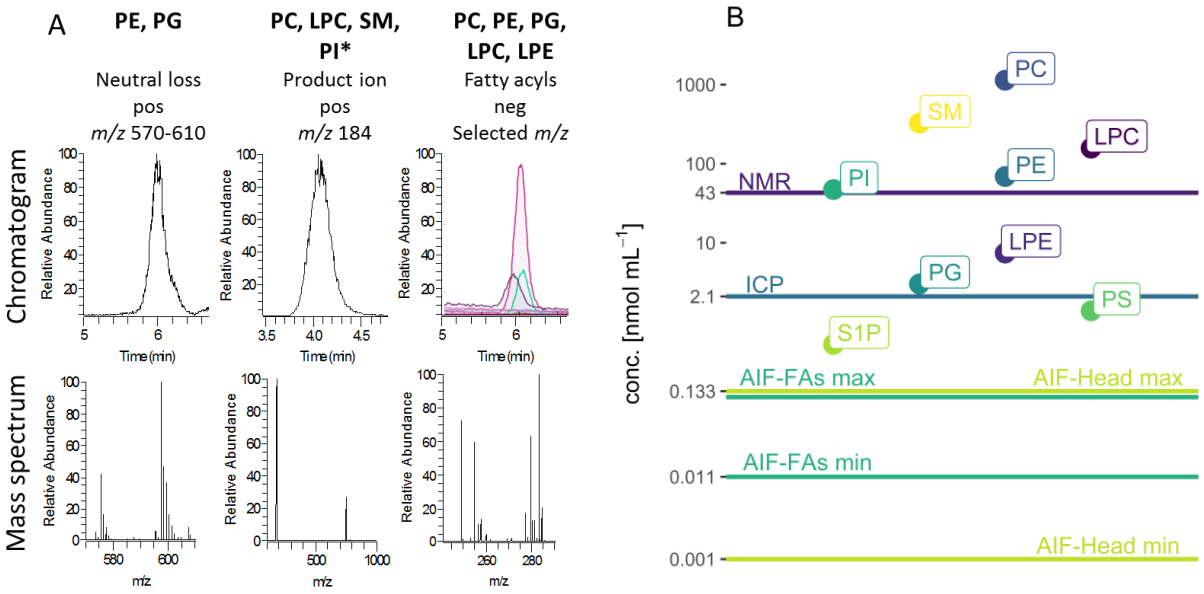

Figure 1 (A) Chromatograms and mass spectra of the three possible types in HILIC-ESI-AIF: Neutral loss, Product ion (both head group fragments), and the sum of fatty acyl fragments. *PI functions after a similar approach but the head group fragment can be detected in negative mode at *m/z* 241. A detailed list can be found in Supplementary Table 1 **(B) Limits of detection in the solution of four lipid class quantification methods in comparison with lipid class concentrations in human plasma.** Lines indicate LOD values for ^{13}P-NMR (purple, 42.5 nmol mL^{-1}, 85 nmol in tube), FI-ICP (blue, 2.13 nmol mL^{-1}, 21.3 pmol), maximal and minimal value for HILIC-AIF with the sum of FAs (turquoise, 0.011-0.112 nmol mL^{-1}, 55-560 fmol) the head group fragments (green, 0.001-0.133 nmol mL^{-1}, 5-665 fmol). Exact values for all lipid classes can be found in Supplementary Table 1. Lipid class conc. in human plasma represents the sum conc. for each class taken from the interlaboratory comparison of SRM 1950, NIST.[8] LPE- lysophosphatidylethanolamine, S1P- sphingosine-1-phosphate.

Benchmarking the lipid class concentration of *K. phaffii*

The different class-specific strategies, namely ^{31}P-NMR, HILIC-ICP-MS, and HILIC-ESI-AIF were cross-validated calculating the uncertainty-weighted mean[47] of the PL classes of the yeast *K. phaffii*. The implemented HILIC separation was not optimized for acidic lipid classes. Consequently, HILIC analysis of

PA, PI, and PS was compromised showing a biased lower total PL concentration across all HILIC methods (1890±35 µmol/ 1.6 10^{10}cells), which amounts to roughly 80 % as assessed by ^{31}P-NMR and FI-ICP-MS. Supplementary Table 2 summarizes the obtained lipid class methods results, while Figure 2 also benchmark the data with lipid species methods. PC is the most abundant PL class followed by PE. For PC, all methods were above LOQ and could be included showing excellent agreement. Across all lipid class platforms, values for PC were consistent with a coefficient of variation (CoV) of 1.3% and a mean concentration of 1218±17 µmol/ 1.6 10^{10}cells. The implemented HILIC method failed to selectively separate PE from PA, therefore HILIC based lipid class methods are not shown in Figure 2. However, the amount of PA present in *K. phaffii* is rather low compared to PE (one study reported 2% PA vs. 31% PE of total PL content[45]), therefore it was neglected, which overall results in good agreement between the platforms with a mean value of 594±8 µmol/ 1.6 10^{10}cells and a CoV of 1.3% (Supplementary Figure 6). HILIC approaches were excluded for the acidic lipid classes PA, PI, and PS as the classes were found < LOQ. However, the latter classes could be quantified with ^{31}P-NMR as column saturation is not an issue and further preconcentration was possible as enough sample material was available. For the low-abundant class of PG, only HILIC-ICP-MS enabled traceable lipid class quantification. A mean concentration of 18±1.6 µmol/ 1.6 10^{10}cells, CoV of 9.4% was assessed. For the lipid classes of PG, PE, and PC, the novel HILIC-ESI-AIF-MS was validated successfully via ^{31}P-NMR and HILIC-ICP-MS. Therefore, for low-abundant LPC (<LOD for ^{31}P-NMR/ICP-MS assessment), the two HILIC-ESI-AIF values were accepted as a reference point for mass balancing lipid species quantification. In summary, the appropriate method for lipid class quantification depends on the sample type, the available sample amount, the analytes of interest, and their lipid concentration, while a general rating according to the metrological order of the methods is as follows: (1) ^{31}P-NMR, (2) HILIC-ICP-MS, (3) HILIC-ESI-AIF with head group fragments and (4) HILIC-ESI-AIF with FA fragments.

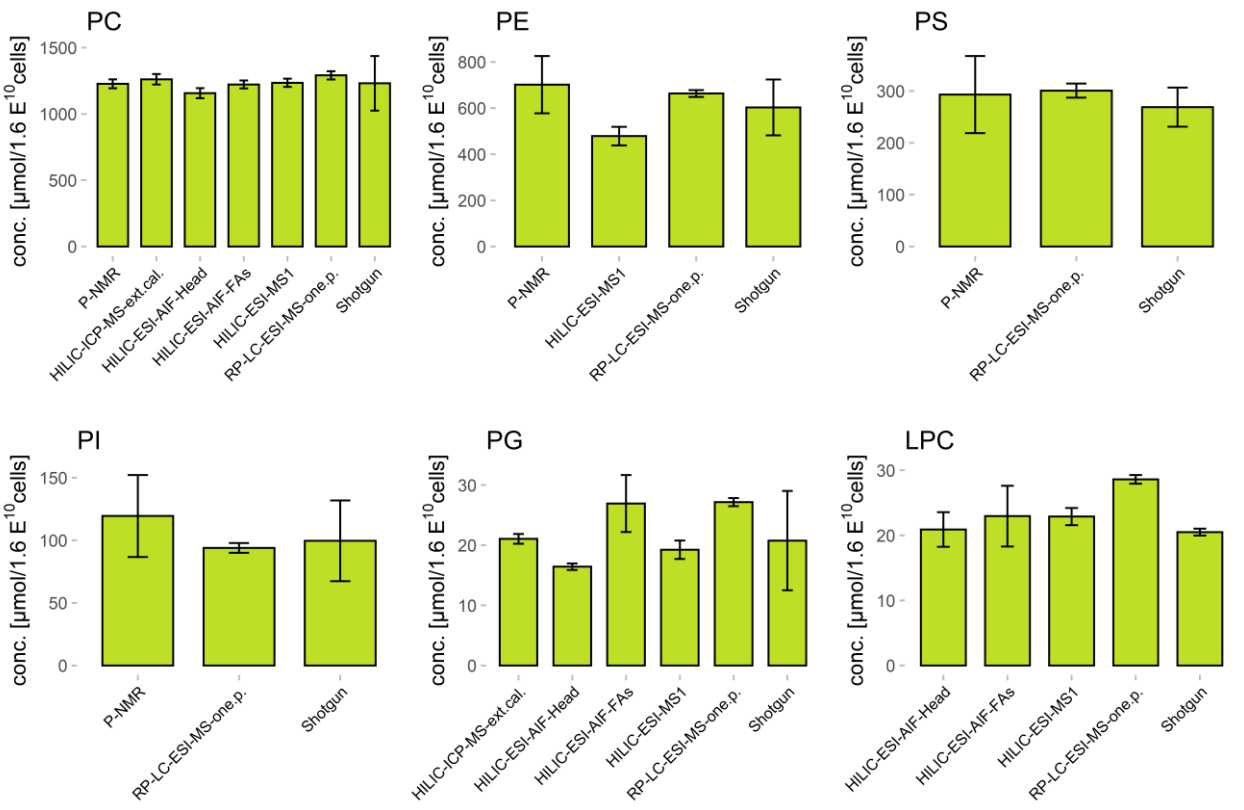

Figure 2 Absolute concentration values of different lipid classes in µmol/ 1.6 10^{10} yeast cells (equals 1 g wet weight of yeast cells). Error bars indicate technical repeatability (n=3). Values can be found in Supplementary Table 2. Classes with less than 7 bars were limited by sensitivity and selectivity. In detail, PE overlap with PA in HILIC methods, but as PA is relatively low concentrated, values still can be obtained shown in Supplementary Figure 6. Acidic lipids (PS, PI) were limited by their peak shape in HILIC separations reducing the sensitivity. While PG and LPC methods are only limited by the relatively low abundance in yeast.

Mass balancing lipid species methods

The limitations and critical points to consider in lipid species quantification are comprehensively described elsewhere.[10,52–54] It is well accepted that RP-LC using only one ISTD per class is not the method of choice for accurate absolute quantification. This holds true especially when highly unsaturated lipids[50] are analyzed. However, in the case of yeast lipidomics, this aspect is less important due to the lower complexity and lower degree of occurring unsaturated fatty acids if compared to the well-investigated matrix of human plasma.[8,55] Overall, co-ionization, achieved by minimal retention time differences between analytes and ISTD, is the key for accurate quantification.[10] HILIC-MS and shotgun lipidomics, both approaches ensuring co-ionization, in turn, are compromised by isomeric overlaps e.g. the classes PC and PE. In this work, the problem was solved as shotgun quantification was based on negative mode

measurements of the fatty acyl chain fragments and in HILIC-MS selectivity was provided by the chromatographic separation. Mass balances between the different methods showed overall good agreement. Excellent CoVs calculating the uncertainty-weighted mean[47] were calculated considering all methods delivering lipid class quantities and the sum of all lipids quantified on the species level (see Figure 2 and Supplementary Table 3). In total, a mean total concentration of 1920±60 µmol/ 1.6 10^{10}cells with a CoV of 3.2% across all methods was achieved(see Figure 3B). It must be kept in mind, that the observation cannot be generalized, as the investigated lipids are high-abundant, and the lipid species profile of yeast is not too complex. Correlation plots between the lipid species quantification methods (shotgun, HILIC, and RP-LC) are shown in supplementary Figure 7. A higher correlation of higher values in HILIC vs. RP was found than for low concentration values. In shotgun analysis concentrations < 1 µmol/ 1.6 10^{10}cells fall below the limit of quantification. The obtained correlations graphs are in accordance to other studies[11,50] reporting discrepancies to a certain extent between these methods.

Finally, the relative lipid class distribution across all quantified PLs enabled benchmarking with published data for *K. phaffii* (see Figure 3B). The lipidome of *K. phaffii* is interesting as it is well-known in biotechnological industries, can grow on methanol or glucose as sole carbon source, and has an important role as host for recombinant protein production.[42] The use as labeled biomass for internal standardization[41] profits from the possible high enrichment degree due to the use of sole carbon sources and the fact that its membrane composition is more similar to multicellular eukaryotes than in *S. cerevisiae*.[42] In detail, *K. phaffii* contains polyunsaturated lipids (FA 18:3) and it produces both (mannosyl) inositol phosphorylceramides ((M)IPC) and GlcCer, in which the latter is also present in mammalians but not in *S. cerevisiae*. Hydrolyzed FAs and PL have been studied by gas chromatography (GC)-MS and TLC, respectively,[43–45] and only our previous publication[46] applied shotgun lipidomics as lipid species method after a pre-fractionation via prep-SFC. In summary, the data revealed a consistent picture. PC values range around 50%, followed by PE with appr. 30%. The relatively high LOD of TLC (see Table 1), the high standard deviation, and the varying values for PI, PA, and LPC between the published TLC methods also highlight the limitation of this time-consuming lipid class quantification method.

The better characterization of the *K. phaffii* yeast lipidome can now further help to establish a benchmarking tool for MS-based lipidomics method development.[56] The applied traceable lipid class quantification methods in combination with the simple and reproducible production of *K. phaffii* fermentation pave the way for a cost-effective and accessible material, which is suitable as system QC for long-term as well as large-scale studies. The proposed workflows pave the way for quantitative

lipidomics studies including (1) species-unspecific standardization even for new PL classes by ^{31}P-NMR and ICP-MS and (2) support the development of lipid reference materials.

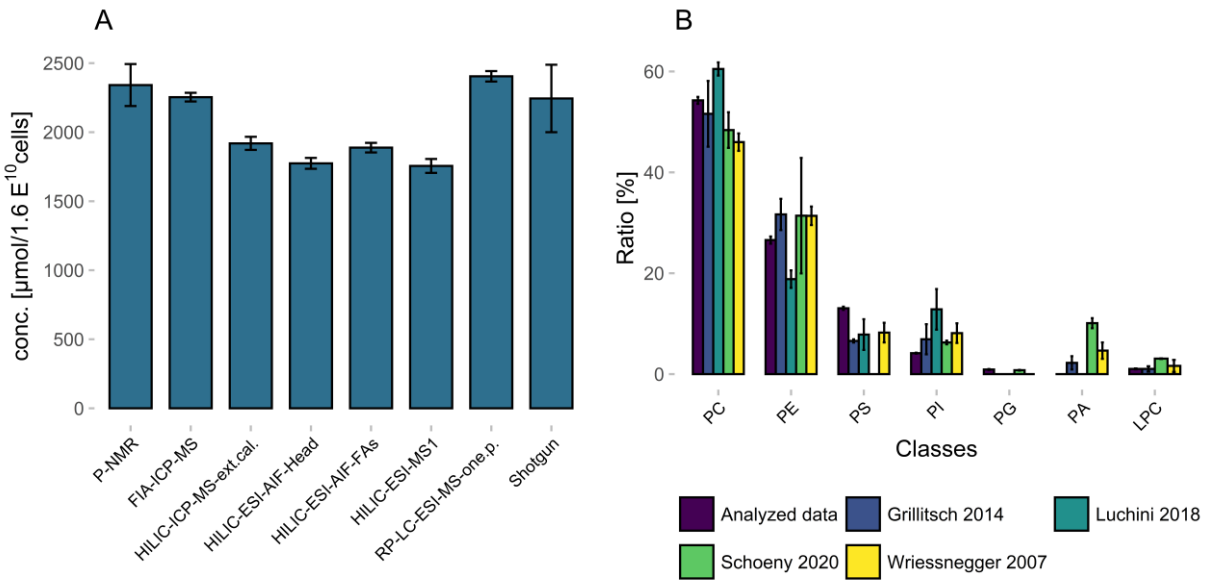

Figure 3 (A) Absolute concentration values for the total PL conc. in µmol/ 1.6 10^{10} yeast cells. (equals 1 g wet weight of yeast cells). An average value of 1920±60 µmol/ 1.6 10^{10}cells (CoV of 3.2%) for total PL conc. was achieved. **(B) Data comparison with literature values.** Analyzed data represent the mean value of the present study. Data in Grillitsch 2014[45] Luchini 2018[44] and Wriessnegger 2007[43] have been obtained by quantitative TLC and data from Schoeny 2020[46] by prep-SFC fractionation with subsequent shotgun analysis. If no bar is visible, values have not been determined in this study.

CONCLUSIONS

In conclusion, values from nine different methods on the lipid species, lipid class, or total PL content level were obtained. ^{31}P-NMR -as a fully traceable method- can be recommended for quantitative assessment of unknown samples or sample pools in bigger cohort studies. However, the high sample need, and the long acquisition time make it unpractical for direct sample comparison in bigger cohort studies. Also, overlaps of lipid signals are still possible and need further improvement. ICP-MS is a useful alternative where species-unspecific quantification is also possible. Special care must be taken about the selected HILIC method to avoid overlaps and improve peak shape and lipid class recovery. The use of FI-ICP-MS is a simple version for the traceable quantification of less complex samples and shows great potential for the degradation assessment of phosphorous-containing standards. We further introduced a novel lipid class quantification based on HILIC and AIF. Depending on the applied HILIC conditions, this was valuable for the lipid classes of PC, PE, PG, and LPC. If available, universal lipid detectors e.g. CAD and ELSD are interesting alternatives with competitive LODs. Whatever lipid class quantification method is finally

chosen, the benefit of cross-validated lipid concentrations was highlighted and can help to bring lipidomics further to a standardized and harmonized field.

ASSOCIATED CONTENT

Supporting information

PDF File

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Notes

The authors declare no competing financial interest.

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Hunting absolute molar lipid concentrations: A phospholipidomics cross-validation study

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EXPERIMENTAL SECTION

Sample and materials

The yeast sample extraction was conducted as described in Neubauer *et al.*[1] and Schoeny *et al.*[2] A final lipid extract from 16 billion cells (~10 mg lipid dry weight) was obtained and, if not other stated, dissolved in 1 mL chloroform and diluted 1:10, 1:100 and 1:1000 with the starting conditions. PC 36:2 was used for recovery experiments and was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). For ^{13}P-NMR analysis, sodium cholate hydrate (99%, purchased from Sigma-Aldrich, Vienna, Austria), EDTA (99.995%, trace metals basis, Sigma-Aldrich), deuterium oxide (D_2O, 99.9%, Sigma-Aldrich), trizmaBase (99.7%, Sigma-Aldrich) and NMR test tubes with a 5-mm outer diameter (Sigma-Aldrich) were used. O-phospho-L-serine (CRM, TraceCert, Sigma-Aldrich) was used as ISTD. For chromatographic methods, acetonitrile (ACN) and water were of LC-MS grade and ordered at Honeywell (Vienna, Austria). Chloroform, isopropanol (IPA), and methanol (MeOH) were also of LC-MS grade and ordered at Fisher Scientific (Vienna, Austria). Ammonium acetate and ammonium formate were ordered as eluent additives for LC-MS at Sigma Aldrich. SPLASH® Lipidomix® Mass Spec Standards were purchased from Avanti Polar Lipids, Inc.. Tributyl phosphate (CRM, Supelco, Sigma-Aldrich) was used as ESTD in FI-ICP-MS.

Methods

Nine different quantification methods using different platforms were used (see Table 1 in the manuscript) and are described in the following sections according to the applied separation

technique and the analyzer. The different modes and quantification strategies using the same separation technique and analyzer are combined in the same section.

^{31}P-NMR analysis (lipid class quantification with ISTD)

The protocol followed Kato *et al.* 2018.[3] Following solutions were prepared: surfactant solution (20%, 1 g sodium cholate in 5 mL D_2O), Tris HCl Buffer (1 M, pH 7), EDTA solution (100 mg mL^{-1} in Tris HCl Buffer), an ISTD solution (3.8 mM, 1 mg mL^{-1}, 20 mg phosphoserine in 2 mL EDTA solution and 18 mL Tris HCl Buffer) and a PC 36:2 standard solution (1 mM in chloroform). The dried lipid extract from 16 billion cells and 1 mL of the PC 36:2 standard solution, which was dried down before, were dissolved in a 1 mL surfactant solution. To each of them, 1 mL ISTD solution was added and shaken for 1 h at 50°C before it was centrifuged at 15.000 rpm. The obtained supernatant was pH adjusted to 6.9±0.04 using 1 M NaOH or 1 M HCl solutions. Following conditions were used for ^{31}P-NMR measurements on the Avance III 600 MHz (Bruker, Billerica, MA, USA): irradiation frequency (242 MHz), acquisition time (5.4 s), probe temperature (30°C), spectral width (80 ppm), FID data points (210934), spinning (15 Hz), dummy scans (4), 1H decoupling (Waltz16), pulse angle (90°) pulse width (14.8 µs). The peaks of the obtained spectra were integrated manually, the integral of the ISTD was normalized to 1, and the final lipid class concentration was calculated as in formula 1:

(1) $$conc\left[\frac{\mu mol}{1\ mL \approx 1.6\ 10^{10}\ cells}\right] = Norm.integral_{\ Analyte} \cdot conc\left[{}^{\mu mol}/_{mL}\right] \cdot Dil.factor$$

Limit of detection (LOD) was calculated as the conc. that corresponds to a signal-to-noise ratio (S/N) of 3. The noise value was obtained by integrating a region without peaks with peak width similar to analyte peaks in the spectrum (5.793-5.291 ppm).

ICP-MS analysis (total phospholipid content with FI and lipid class quantification with HILIC)

For HILIC-ICP-MS measurements an Agilent 1260 Infinity Bio-Inert HPLC system was coupled with an Agilent 8800 Triple Quadrupole ICP-MS (both Agilent Technologies, Santa Clara, CA, USA). An iHILIC P column (2.1 mm x 150 mm, 5 µm, HILICON, Upsala, Sweden) connected to a PEEK -inlet filter was used to obtain class separation of polar lipids. A mixture of ACN/H_2O (50:50, v/v) with 10 mM ammonium acetate at pH 8.0 was used as eluent A and a mixture of ACN/H_2O (95:5, v/v) with 10 mM ammonium acetate at pH 8.0 was used as eluent B. The applied gradient was as follows: 0-2 min 100% B, 2-12 min gradient to 80% B, hold from 12-16 min, at 16 min switched back to 100%, and hold for column equilibration from 16-22 min. The flow rate was kept constant at 250 µL min^{-1} and the column oven at 40°C. The injector needle was washed with 75% IPA, 24.9% H_2O, 0.1% formic acid before each injection. SPLASH® Lipidomix® Mass Spec Standard (Avanti Polar Lipids) was used for both external calibration and standard addition via HILIC-ICP-MS. FI (Flow injection) experiments have been applied without the column, an injection volume of 10 µL, a flow of 250 mL $min^{-1,}$ and a

constant eluent mixture of 50% A/ 50% B. Tributyl phosphate was used as the reference standard for external calibration via FI. The sample introduction system consisted of a MicroMist nebulizer (AHF Analysentechnik AG, Tuebingen, Germany) and a quartz double-pass spray chamber (Agilent Technologies). The instrument was tuned on a daily basis in order to achieve maximum sensitivity while keeping the oxide formation ($^{140}Ce^{16}O^{+}/^{140}Ce^{+}$) and the doubly charged ratio ($^{140}Ce^{2+}/^{140}Ce^{+}$) below 2%. Phospholipids were detected via phosphorus in the oxygen gas mode using mass shift modality ($^{31}P^{+} \rightarrow {}^{31}P^{16}O^{+}$). Identification of phospholipid in the samples was performed via retention time on the LC column and the phosphorus tracer in ICP-MS.

For the ICP setting the following optimized conditions were used: Plasma gas 15 L min^{-1}, auxiliary gas 0.8 L min^{-1}, carrier gas 0.80 L min^{-1}, optional gas flow rate 35% (of carrier gas flow rate), rf power 1550 W (reflected power 10 W), ORC gas (oxygen) 30%, spray chamber temperature 2°C and sample depth (torch-interface distance) 7.8 mm. All chromatograms were smoothed before integration by using MassHunter software (Version 4.6, Agilent Technologies).

Results from external calibration were obtained via linear regression models of ESTDs and multiplying the results with the applied dilution factor (1:10 and 1:100). LOD was calculated by multiplying the standard deviation of 5 repetitive injections of a low concentrated recovery standard with 3. The external calibration for FI and HILIC-ICP-MS respectively was used to calculate the LOD concentration. Additionally, standard addition was applied for validation, and concentration values were obtained by multiplying the x-intercept value of a linear regression model (sample and sample + SPLASH® at different conc. levels) with the applied dilution factor (1:100).

HILIC-ESI-MS analysis (lipid class quantification with ESTD in AIF mode and lipid species quantification with ISTD in MS1 mode)

For the organic MS method, the same HILIC method (column, eluents, gradient, temperature, and flowrate) was applied as in the HILIC-ICP-MS analysis. However, a Vanquish Horizon HPLC was used instead and coupled to a high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (both Thermo Fisher Scientific, Waltham, MA, USA).

With the same chromatographic method, two modes can be applied to obtain either (1) lipid class concentrations, if AIF is used and samples are quantified with ESTDs (only Splash at different dilution levels), or (2) lipid species concentration if samples are measured in MS1 with Splash as ISTD. In both methods, 5 µL per sample was injected and source parameters were kept the same: spray voltage 3.5 kV (pos)/ -2.8 kV (neg), capillary temperature 280°C, sheath gas flow rate of 38, auxiliary gas flow rate of 3, sweep gas was switched off, auxiliary gas heater temperature of 320°C and S-lens radio frequency level of 30. In MS1 mode the mass range in both polarities was set to *m/z* 400-1000 and in

AIF to *m/z* 100-1000. Both had a maximum injection time (IT) of 200 ms, a resolution of 120000, and an automatic gain control (AGC) target of 3e6. In AIF, the normalized collision energy (NCE) was set to 25 (pos)/ 28 (neg). Skyline (version 20.2) was used for MS1 data processing and AIF fatty acyl chain fragments whereas head fragments in the AIF files were integrated manually with Qual Browser Thermo Xcalibur (version 4.0.27.19).

Concentrations of the lipid species in MS1 mode were calculated as in formula 2.

(2) $$conc\left[\frac{\mu mol}{1\ mL \approx 1.6\ 10^{10}\ cells}\right] = \frac{Area_{\ Analyte}}{Area_{ISTD}} \cdot conc_{ISTD}\left[\mu mol/_{mL}\right] \cdot Dilution\ Factor$$

Depending on the lipid class, different fragmentation led to different possibilities summarized in Supplementary Table 1 and exemplarily shown in Figure 1 (manuscript). A headgroup fragment is either present as a product ion (e.g. m/z 184 for PC) or neutral loss (e.g. m/z 141 for PE). For neutral losses, different fatty acyl chain compositions lead to different product ions and a mass range for integration is necessary. In negative mode, the FAs present in yeast were selectively integrated with Skyline, and the sum area was used for quantification.

Sample concentrations were calculated via linear regression models of ESTDs and the results were multiplied with the dilution factor (1:1000). LOD was calculated by multiplying the standard deviation of 3 repetitive injections of a low concentrated Splash ESTD with 3.

Shotgun analysis (lipid species quantification with ISTD)

Shotgun analysis was conducted as previously described at Schoeny *et al.*[2] Briefly, a high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) was connected with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca NY, USA) and a nanoelectrospray ionization (nanoESI) chip with spraying nozzles of 5 µm nominal internal diameter. IPA/MeOH/$CHCl_3$ (4:2:1, v/v/v) containing 7.5 mM ammonium formate was used for sample dissolving and dilution. SPLASH® Lipidomix® ISTD was added in a 1:500 dilution to a 1:1000 diluted yeast sample and a final volume of 200µL. Three replicates of 30 µL were placed in a 96 twin.tec® well plate (Eppendorf, Hamburg, Germany). Following settings were applied in the Chipsoft 8.3.1 software (Advion BioSciences): ionization voltage 1.25 kV (pos)/ -1.25 kV (neg); backpressure 0.9 psi and in the MS source parameters: capillary temperature 250 °C, S-Lens radio frequency level 50. A 9 min polarity switching method with data independent acquisition (DIA) and MS1 scans was used with the following settings: resolution 240,000 (MS1), 60,000 (MS2), AGC target 1e6 (MS1), 2e5 (MS2), maximum IT 150 ms (MS1), 130 (MS2), scan range in MS1 *m/z* 350-1050 (pos)/ *m/z* 200-1200 (neg), NCE for MS2 of 21 (pos)/ 26 (neg) and a fixed first mass of *m/z* 80 (pos)/ *m/z* 150 (neg). LipidXplorer 1.2.8 settings were the following: mass tolerance 5 ppm, min. occupation of 0, intensity

threshold 10000 (MS1)/ 5000 (MS2), resolution 260000 (MS1)/ 65000 (MS2), resolution gradient -102 (MS1)/ -60 (MS2). Identified Lipids were removed if the absolute mass error was above 3, if isobaric compounds were present and if the concentration was below LOQ. LOQ values were calculated by multiplying the standard deviation of 5 repetitive injections of a low concentrated Splash ISTD with 10.

RP-LC-MS analysis (lipid species quantification with ISTD)

A Vanquish Horizon HPLC (Thermo Fisher Scientific) with an Acquity HSS T3 (2.1 mm × 150 mm, 1.8 µm, Waters, Milford, MA, USA) with a VanGuard Pre-column (2.1 mm × 5 mm, 100 Å, 1.8 µm) was used for reversed-phase liquid chromatography-high resolution mass spectrometry (RP-LC-HRMS). The flow rate was set to 250 µL min^{-1} and the column temperature to 40°C. Solvent A was ACN/H_2O (3:2, v/v), and solvent B was IPA/ACN (9:1, v/v). Both solvents contained 0.1% formic acid and 10 mM ammonium formate. The following gradient was used: 0−2 min 30% B, 2−15 min ramp to 75% B, 15−17 min ramp to 100% B, 17−22 min 100% B and 22-27 min 30% B as equilibration step. The injection volume was 5 µL and the injector needle was washed with 75% IPA, 24.9% H_2O, and 0.1% formic acid before each injection.

A high field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) was used as MS. The following source parameters were applied: capillary temperature of 220°C (pos)/ 250°C (neg), sheath gas flow rate of 48 (pos)/ 35 (neg), an auxiliary flow rate of 10, sweep gas of 2, S-lens RF level of 40 and auxiliary gas heater temperature of 300 °C applying a spray voltage of 3.5 kV in positive mode and 2.8 kV in negative mode. In MS1 mode the mass range in both polarities was set to *m/z* 200-2000. A maximum IT of 200 ms, a resolution of 120000, and an AGC target of 1e6 was applied. Skyline (version 20.2) was used for peak integration and R/ R studio for final data processing.

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Table 1 Overview of detected lipid classes in HILIC-AIF. LOD was calculated by multiplying the standard deviation of a low standard by 3, LLOQ and ULOQ correspond to the borders of the linear range (the lowest and the highest standard in this range). *indicate lipids with double peaks, the RT of the highest is shown.

Lipid class	Type	Fragment	Formula	Mass (range)	RT [min]	[µmol L^{-1}] LOD	LLOQ	ULOQ	R^2
PC	HG	$[HG]^+$	$[C_5H_{15}O_4PN]^+$	184.0733	4.04	0.017	0.2	4	0.9973
LPC	HG	$[HG]^+$	$[C_5H_{15}O_4PN]^+$	184.0733	7.48*	0.010	0.05	9	0.998
SM	HG	$[HG]^+$	$[C_5H_{15}O_4PN]^+$	184.0733	6.58	-	0.04	8	0.9972
PI	HG	$[HG]^-$	$[C_6H_{11}O_8P]^-$	241.0119	8.86	0.133	0.1	2	0.9956
PE	HG	$[M\text{-}HG]^+$	$[M\text{-}C_2H_6O_4PN]^+$	570-605	6.0	0.001	0.007	7	0.9996
PG	HG	$[M\text{-}HG]^+$	$[M\text{-}C_2H_6O_4PN]^+$	570-605	2.9	0.003	0.035	0.7	0.9981
PC	FAs	$[FA+O]^-$	$[C_XH_YO_2]^-$	241-283	4.04	0.082	0.2	4	0.9971
LPC	FAs	$[FA+O]^-$	$[C_XH_YO_2]^-$	241-283	7.48*	0.011	0.045	9	0.9996
LPE	FAs	$[FA+O]^-$	$[C_XH_YO_2]^-$	241-283	9.47*	0.112	0.04	20.1	0.999
PE	FAs	$[FA+O]^-$	$[C_XH_YO_2]^-$	241-283	6.0	-	0.007	1.5	0.9993
PG	FAs	$[FA+O]^-$	$[C_XH_YO_2]^-$	241-283	2.9	0.013	0.03	0.7	0.9956

Table 2 lipid class concentration of *K. phaffii.* Data obtained by lipid class quantification methods (^{31}P-NMR, HILIC-ICP-MS, HILIC-ESI-AIF headgroup, and FA) only. Uncertainty is shown for classes quantified with more than one method.

Lipid class	Nr. Methods	Conc. [µmol/ 1.6 10^{10}cells]
PC	4	1218±17
PE	4	594±8
PS	1	293
PI	1	119
LPC	2	21±2.3
PG	3	18±1.6

Table 3 Absolute concentration values of different lipid classes and their overall value across all platforms.

Method [µmol/ 1.6 10^{10}cells]	PC	PE	PI	PS	PG	LPC
^{31}P-NMR	1227±34	702±124	119±33	293±74	-	-
HILIC-ICP-MS	1261±39	637±26	-	-	21±0.8	-
HILIC-ESI-AIF-FAs	1222±30	616±16	-	-	27±5	23±5
HILIC-ESI-AIF-Head	1157±38	580±9	-	-	16±0.5	21±3
RP-LC-ESI-MS	1291±31	663±15	94±4	301±14	27±0.7	29±0.7
Shotgun	1231±206	603±121	100±32	269±38	21±8	21±0.5
HILIC-ESI-MS1	1235±30	479±40	-	-	19±1.5	23±1.3
Mean± uncertainty (CoV)	1234±14 (1.1%)	605±6 (1.0%)	94±2 (2.1%)	300±13 (4.3%)	21±2 (9.5%)	23.5±0.4 (1.7%)

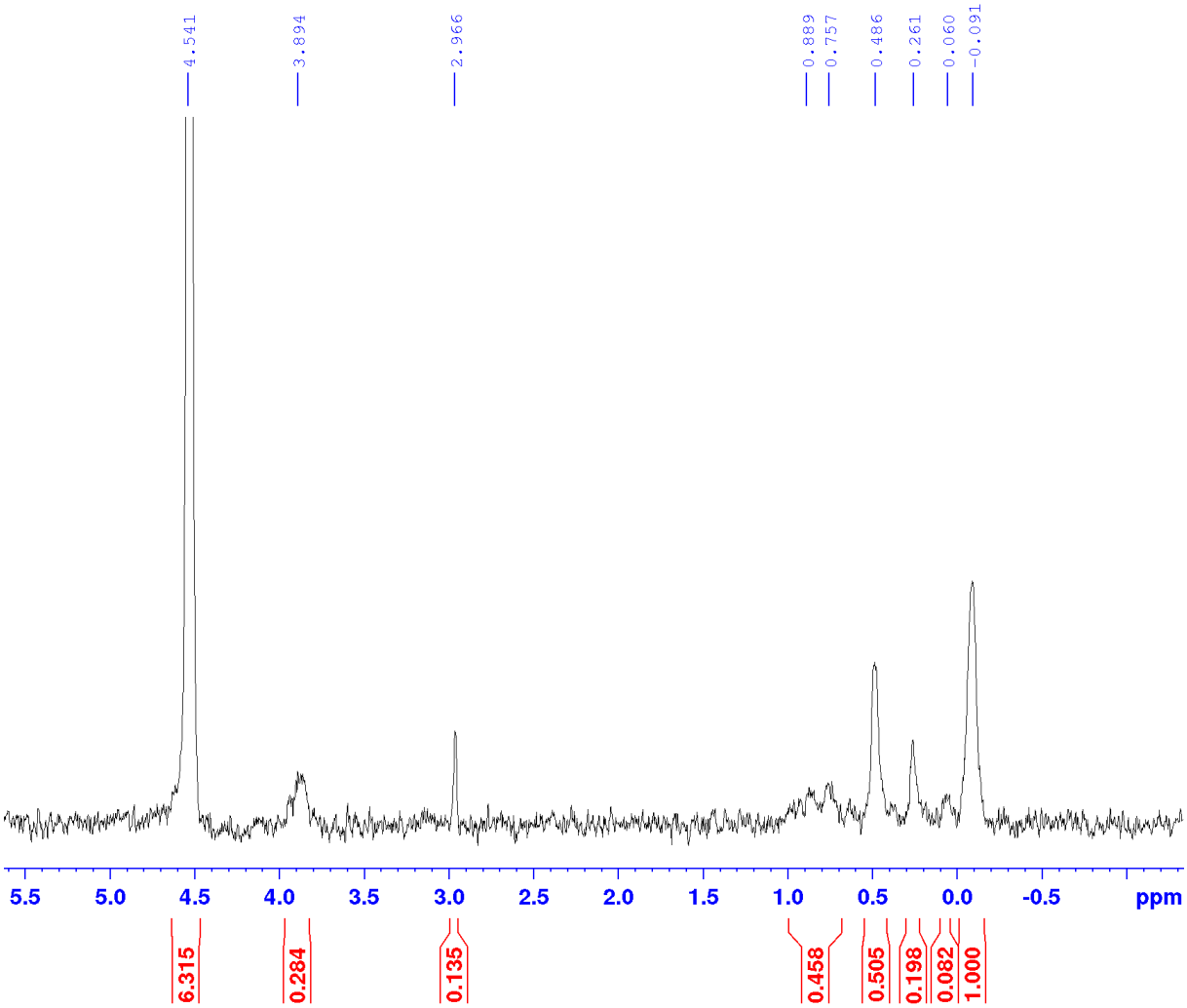

Figure 1 ^{31}P-NMR spectrum of 1.6 10^{10} yeast cells in 2 mL solution. Peak identification: PC -0.091; PI 0.06; PS 0.261; PE+LPC 0.486; PG+LPE 0.757+0.889; phosphoserine -ISTD 4.541; Impurity 2.966; PA 3.894; noise value was selected at 5.793-5.291.

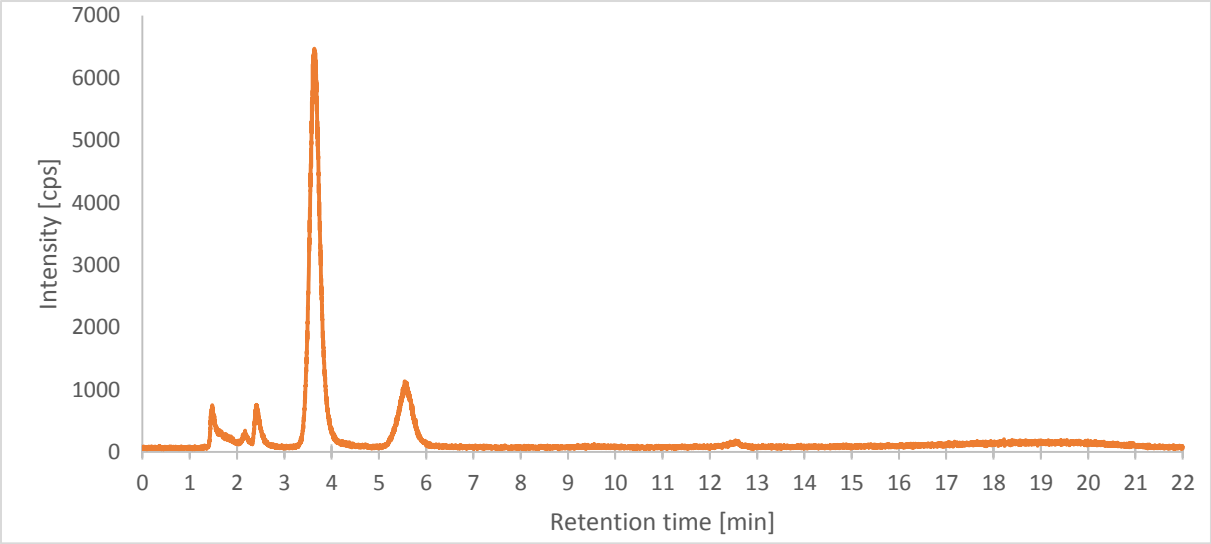

Figure 2 HILIC-ICP-MS chromatogram of yeast extract. Peak identification: Void volume 1.6 min; PG 2.5 min; PC 3.7 min; PE+PA 5.6 min.

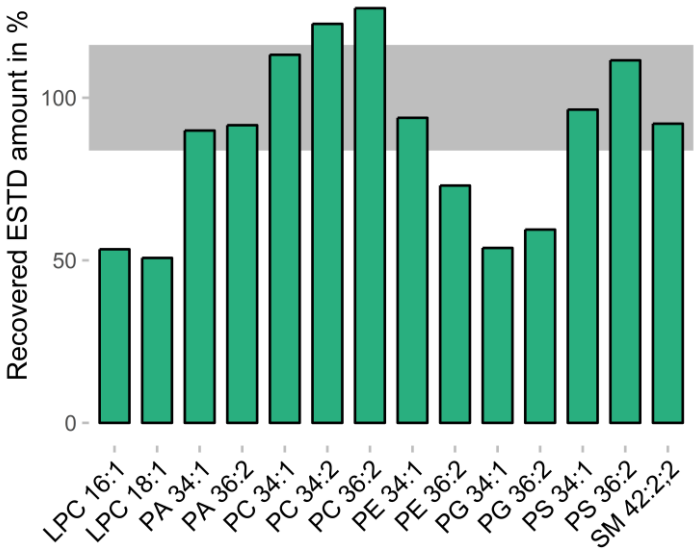

Figure 3 Recovery of two-year-old ESTDs analyzed via FI-ICP-MS and external calibration. ESTD had been stored at -80°C in chloroform/methanol solution. Grey area corresponds to ±9% precision via ICP measurements. Lower values indicate degradation, higher values can be explained by solvent evaporation.

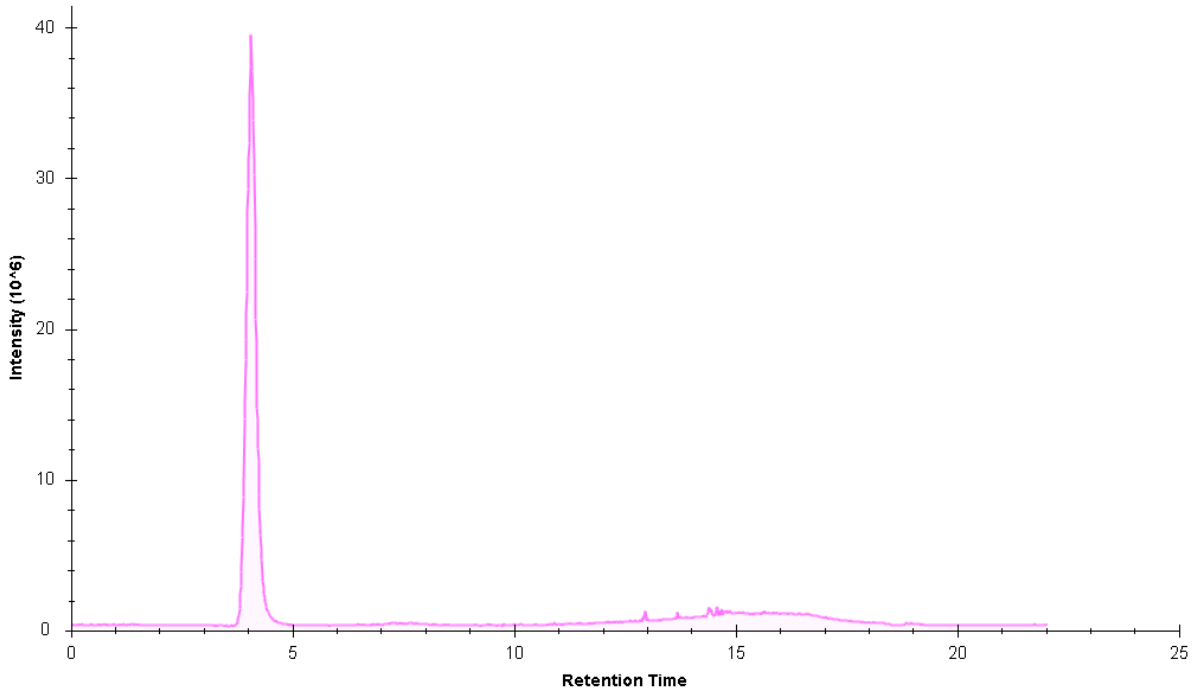

Figure 4 HILIC-ESI-AIF chromatogram of yeast extract in positive mode showing the mass trace of m/z 184.

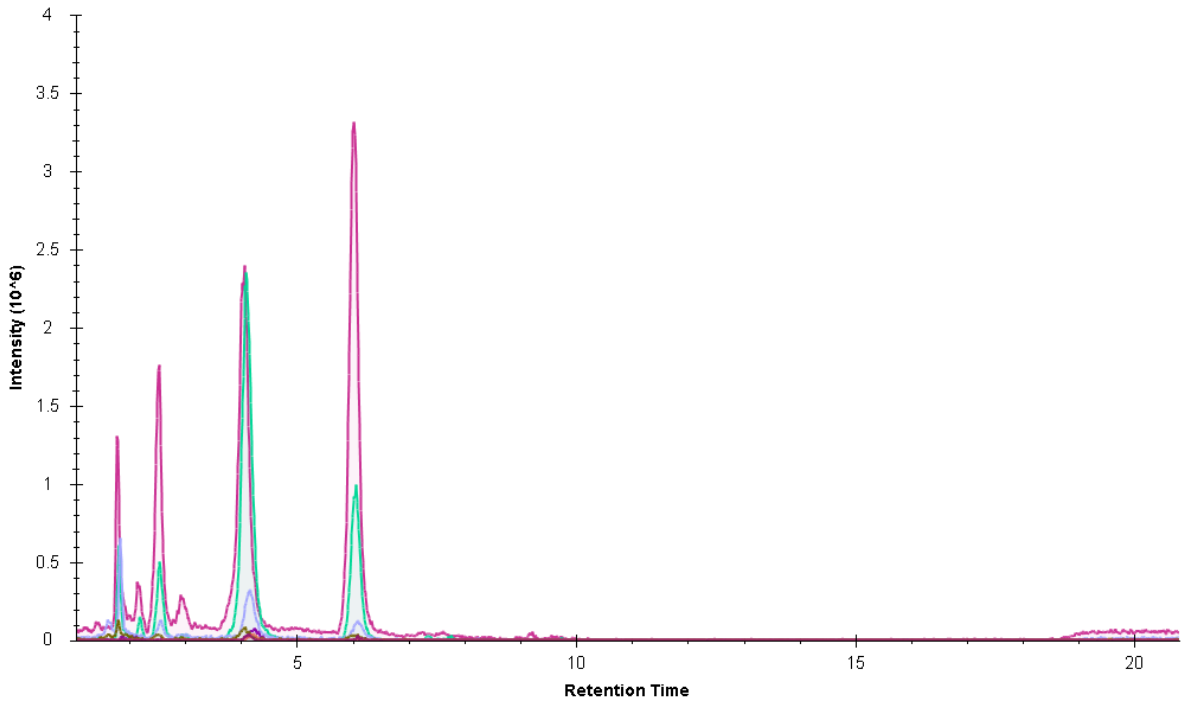

Figure 5 HILIC-ESI-AIF chromatogram of yeast extract in negative mode showing the mass traces of the different fatty acyl chain fragments.

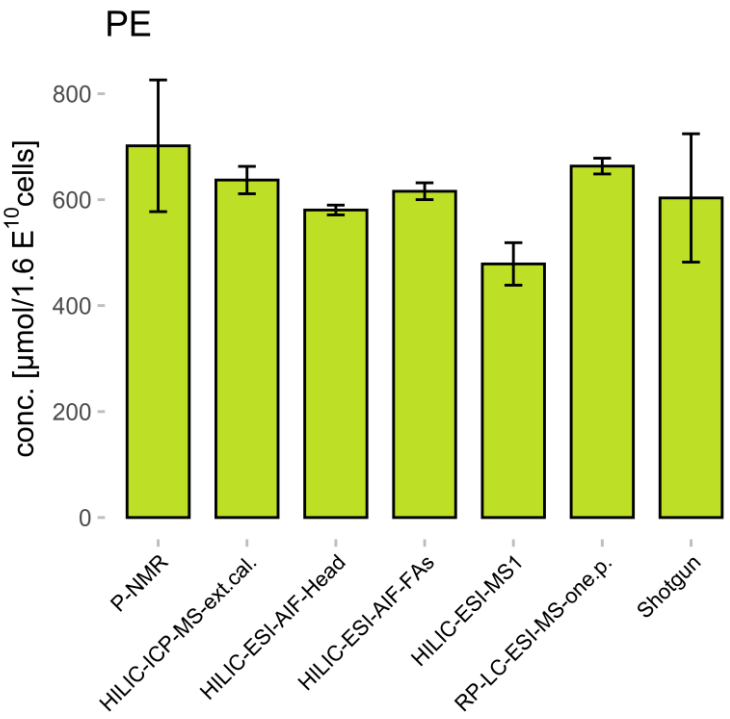

Figure 6 Absolute concentration values of PE in µmol/ 1.6 10^{10} yeast cells (equals 1 g wet weight of yeast cells). Error bars indicate technical repeatability (n=3).

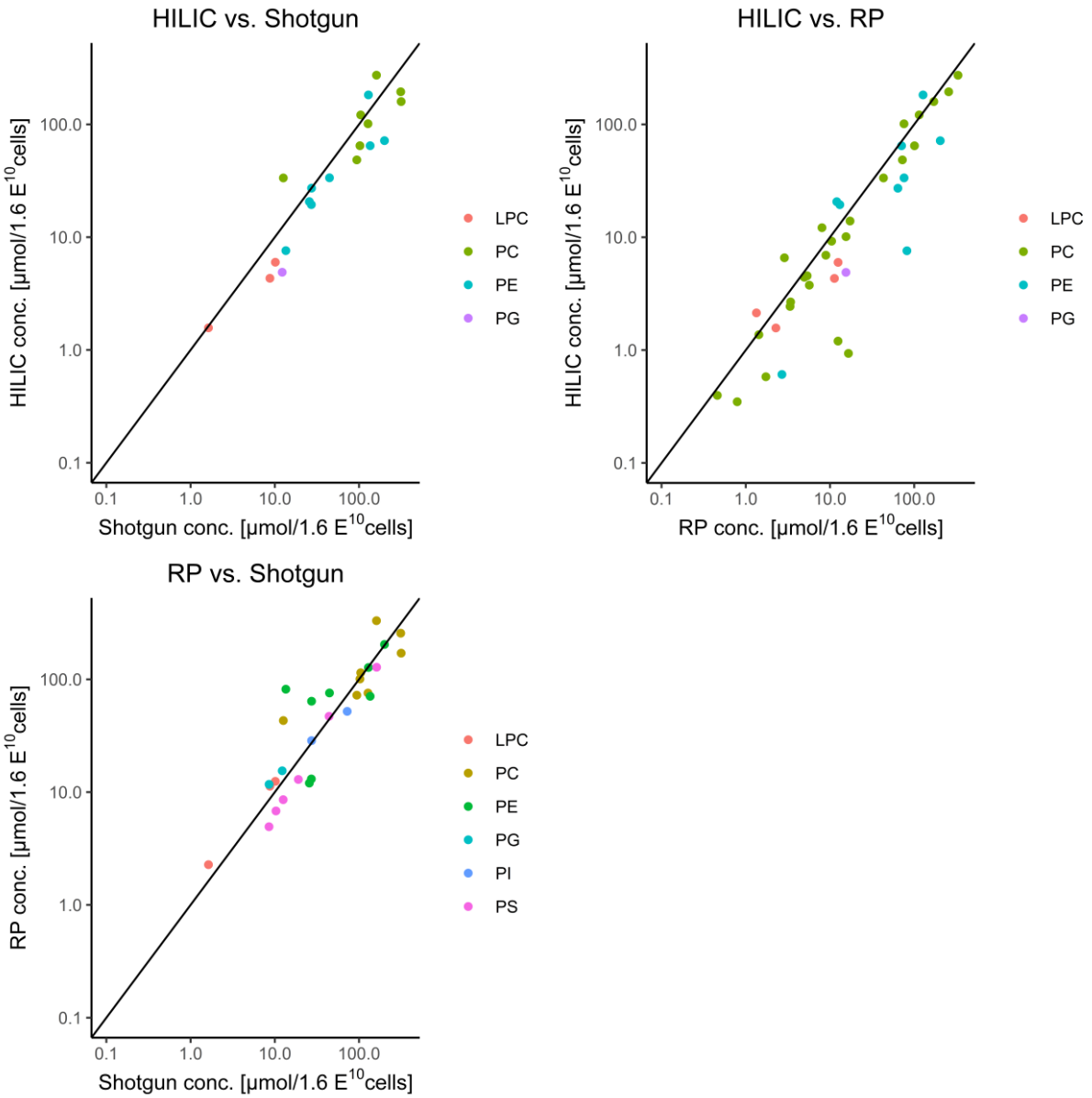

Figure 7 Correlation plot between the absolute concentrations obtained from the three lipid species quantification methods. Quantified yeast lipids are shown if present in the two compared methods. The line indicates y=x. Colors indicate the lipid class of the different analytes.

3.3. Publication III

Preparative supercritical fluid chromatography for lipid class fractionation—a novel strategy in high-resolution mass spectrometry-based lipidomics.

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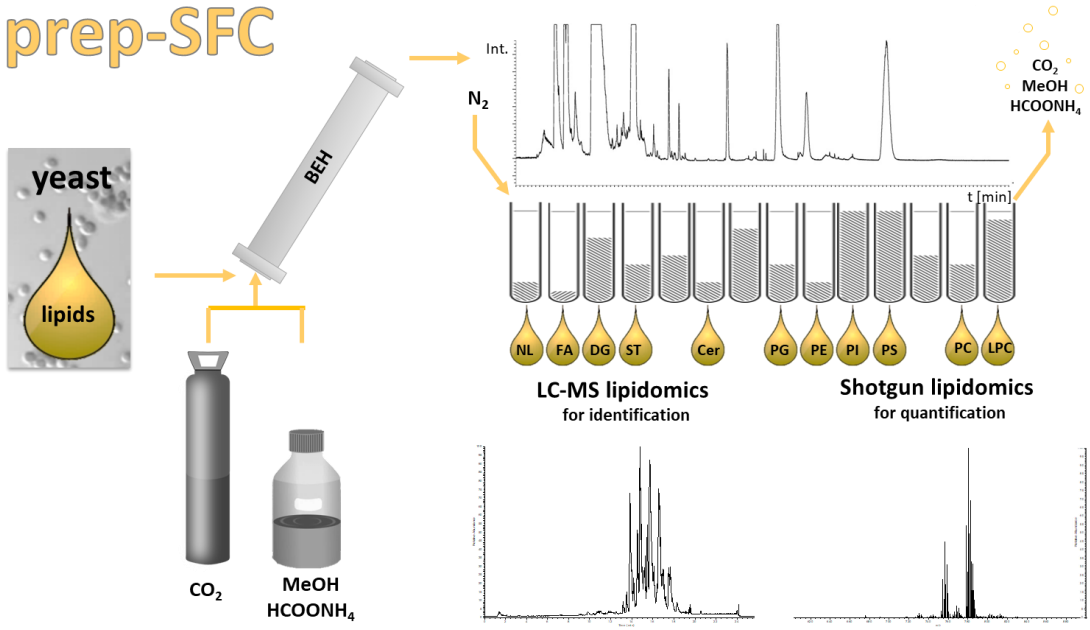

An offline semi-preparative lipid class-specific fractionation by supercritical fluid chromatography (SFC) has been implemented, which can be followed by high-resolution mass spectrometry methods such as shotgun lipidomics and RP-LC-MS-based lipidomics for in-depth characterization. The powerful SFC approach offers separation of a wide polarity range for lipids, enables enrichment (up to 3 orders of magnitude) of lipids, selective fractionation of 14 lipid classes/subclasses, and increases dynamic range. A significantly increased coverage of low abundant lipids, improving lipid identification by numbers and degree (species and molecular level), has been obtained in *Pichia pastoris* when comparing high-resolution mass spectrometry based lipidomics with and without prior fractionation. In total, 400 lipids have been detected in the *Pichia pastoris* yeast lipidome which enables now a more specific application than a global lipidomics approach and paves the way to a biotechnological production of single ^{13}C-labeled molecules.

I have developed the method including the upscaling of the sample preparation and the optimization of the SFC run. I have been responsible for the data evaluation and the writing of the manuscript.

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RESEARCH PAPER

Preparative supercritical fluid chromatography for lipid class fractionation—a novel strategy in high-resolution mass spectrometry based lipidomics

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Abstract

In this work, a lipidomics workflow based on offline semi-preparative lipid class-specific fractionation by supercritical fluid chromatography (SFC) followed by high-resolution mass spectrometry was introduced. The powerful SFC approach offered separation of a wide polarity range for lipids, enabled enrichment (up to 3 orders of magnitude) of lipids, selective fractionation of 14 lipid classes/subclasses, and increased dynamic range enabling in-depth characterization. A significantly increased coverage of low abundant lipids improving lipid identification by numbers and degree (species and molecular level) was obtained in *Pichia pastoris* when comparing high-resolution mass spectrometry based lipidomics with and without prior fractionation. Proof-of-principle experiments using a standard reference material (SRM 1950, NIST) for human plasma showed that the proposed strategy enabled quantitative lipidomics. Indeed, for 70 lipids, the consensus values available for this sample could be met. Thus, the novel workflow is ideally suited for lipid class-specific purification/isolation from milligram amounts of sample while not compromising on omics type of analysis (identification and quantification). Finally, compared with established fractionation/pre-concentration approaches, semi-preparative SFC is superior in terms of versatility, as it involved only volatile modifiers and salt additives facilitating any follow-up use such as qualitative or quantitate analysis or further purification down to the single lipid species level.

Keywords Lipidomics · Lipid fractionation · Preparative supercritical fluid chromatography · *Pichia pastoris* · Human plasma · SRM 1950

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Introduction

High-resolution mass spectrometry (HRMS) has evolved as key technology in the field of lipidomics showing an unrivaled potential to pursue quantification and lipid species identification in parallel [1]. Shotgun lipidomics, i.e., direct infusion HRMS (DI-HRMS) [2] enables accurate quantification of hundreds of lipids in complex samples. Evidently, the combination of HRMS and liquid chromatography (LC) is powerful when aiming at in-depth characterization of the lipidome, as increased dynamic range improves the lipidome coverage, which in turn enables higher numbers of identified species within one analytical run [3, 4]. In this work, for the first time, both HRMS and LC-HRMS have been combined to lipid class-specific fractionation as obtained by upscaling analytical supercritical fluid chromatography (SFC). The aim has been to create a workflow enabling purification/fractionation of as many

lipid classes as possible at semi-preparative scale together with in-depth characterization of the lipidome. At analytical scale, SFC-based lipid analysis has been introduced in the late 1980s [5–7] but the number of studies had remained low, until significant technological improvements (e.g., the introduction as sub 2 μm particles, backpressure regulation and improved injection systems) have led to a renaissance of SFC in lipidomics [8]. Above all, the facilitated combination with mass spectrometry (MS) accelerated these developments. Bamba et al. [9] has pioneered the field. Studying different column chemistries, separation of phospholipids, glycolipids, neutral lipids, and sphingolipids in relatively short time (15 min) could be accomplished. Depending on stationary phase selection, SFC separation is either governed by head group or by fatty acid chain length, degree of saturation and double bond position. A reversed-phase-like separation has been proposed when using non-polar stationary phases [10–13], while HILIC type of separation is enabled when using polar stationary phases. The current state-of-the-art method has been introduced by Lísa et al. [14] who have developed a powerful high-throughput SFC high-resolution mass spectrometry (HRMS) method to separate non-polar and polar lipids within one run. More specifically, by ultra-high performance SFC (UHPSFC) on an ethylene-bridged hybrid stationary phase with 1.7 μm particles, a separation of 30 lipid classes has been achieved. The optimized chromatographic gradient starting with pure non-polar CO_2, which is then followed by the addition of up to 51% methanol/water (99:1, v/v) containing 30 mM of ammonium acetate, is key to separate both polar and non-polar lipids. Further applications of analytical SFC in lipidomics are comprehensively summarized elsewhere [15–18]. However, the lipid class-specific SFC separation introduced by Lísa et al. remains unrivaled in both separation speed and coverage up to date.

Based on the SFC studies on analytical scale, in this work, we have addressed the development of a short, semi-preparative SFC method for both polar and non-polar lipids within one run to separate milligram amounts of dry lipid extract. Most well-established preparative lipid separations resort to techniques developed already decades ago, such as thin layer chromatography (TLC) [19, 20], column chromatography (CC) [21, 22], preparative high-performance liquid chromatography (prep-HPLC) [23, 24], or solid-phase extraction (SPE) [25–28]. As a major drawback, not all of them allow for lipid class fractionation. On top of that, the techniques, regardless whether implemented offline or online, are time and solvent consuming, often requiring multiple steps. For example, a combined SPE protocol addressing fractionation of 11 different lipid classes [26, 28] has implied the use of 12 different solvent mixtures, two cartridges per sample, and several hours of subsequent drying steps. While preparative SFC (prep-SFC) is widely used in pharmaceutical sciences [29, 30], its application is less common in the realm of lipids and rather focused on purification of selected classes [20, 21] (e.g., non-polar lipid classes [31–34], polyunsaturated fatty acids (PUFAs) [35], or phospholipids (PL) [31]). A comprehensive fractionation attempt covering the whole lipidome is still lacking. However, due to the key advances of (1) easy removal of supercritical CO_2 by evaporation upon fractionation, (2) avoidance of complex buffer systems, which complicate the further use of the obtained fractions, and (3) relatively short run time compared with other chromatographic mechanisms, prep-SFC shows great potential for a global lipidome fractionation.

The novel workflow has been applied to the lipidome characterization of *Pichia pastoris* (Guillierm.) Phaff 1956 (*Komagataella phaffii* Kurtzman) [36]. The yeast strain *Pichia pastoris* is a well-known cell factory related to lipidomics but not as well studied as the yeast *Saccharomyces cerevisiae* [37–39]. The existing lipid studies have served as a reference [40–43] for this work.

Materials and methods

In Fig. 1, a simplified workflow is shown. The different working steps are described below and in the Electronic Supplementary Material (ESM).

Sample preparation

A detailed sample preparation procedure can be found in the ESM. Briefly, the yeast was fermented according to a previously established protocol [44] and the cells were extracted following the procedure of Folch [45]. Human plasma was purchased from the National Institute of Standards and Technology (NIST), USA, as standard reference material 1950 (SRM 1950) and also extracted to an adopted Folch extraction.

Semi-preparative supercritical fluid chromatography for lipid class fractionation

For semi-preparative SFC, the Waters Prep-15 SFC system was used. The system comprised a fluid delivery module (connected to an Accel 500 LC chiller by Thermo Fisher Scientific), a Waters 2767 sample manager, a ten-port column oven, a back pressure regulator, a heat exchanger, a make-up pump, a Waters 2998 Photo Diode Array (PDA, at 210 nm), and a Waters 2424 Evaporative Light Scattering Detector (ELSD). The software MassLynx V4.1 was used for controlling the chromatography.

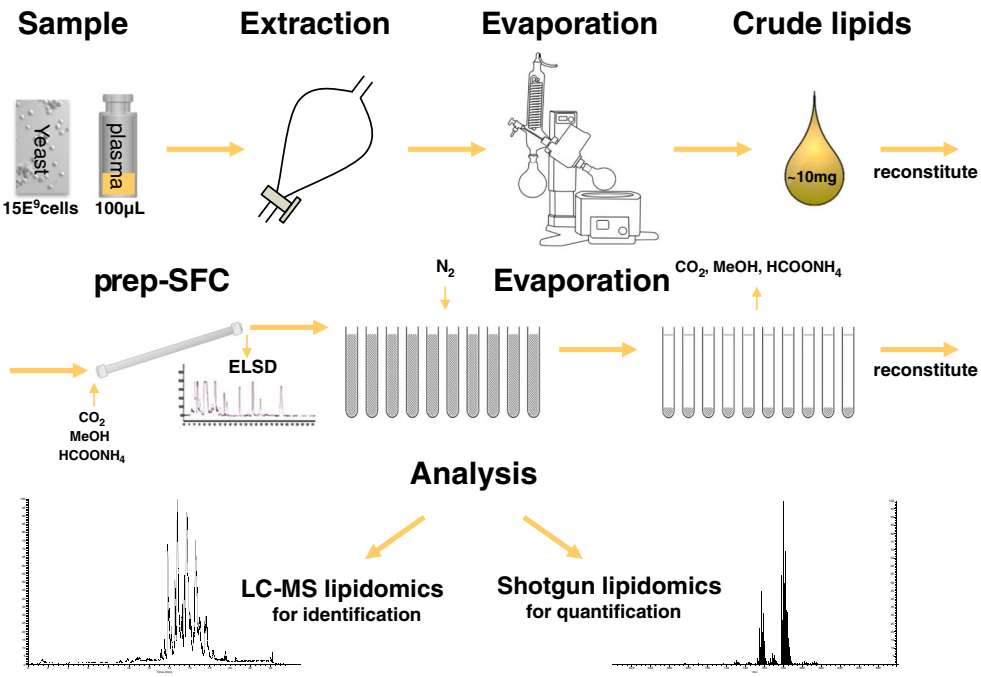

Fig. 1 Overview of the applied workflow including sample preparation, SFC separation, and MS analysis

A Viridis BEH column (250 × 10 mm, 5 µm, Waters Corporation) was used at 60 °C column temperature. The following gradient was used with pure CO_2 as solvent A and methanol (MeOH) with 30 mM ammonium formate (1.892 g dissolved prior in 5 mL water for 1 L MeOH) as modifier (solvent B): 0–4.0 min 5% B, 4.0–17.0 min ramp to 55% B, 17.0–25.0 min 55% B, 25.0–25.5 min ramp down to 5% B, and 25.5–27.0 min 5% B as equilibration step.

The injection volume was 300 µL and the injector needle was washed with methanol for 5 s prior to each injection. Samples were dissolved in chloroform. The flow rate was 15 mL min^{-1}, the backpressure was set to 120 bar, and after the column, the eluents were combined with a methanol make-up flow of 3 mL min^{-1} to facilitate the detection and the fraction collection. The fractionation time events can be found in the ESM (Table S1). The retention time stability was checked each time prior fraction collection. The fractions were collected, dried under nitrogen, and stored at − 80 °C prior analysis.

High-resolution mass spectrometry shotgun lipidomics

For the direct infusion analysis of the fractions, a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY, USA) was coupled to a high-field Q Exactive HF™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The dried samples were diluted in isopropanol (IPA)/MeOH/$CHCl_3$ 4:2:1 (v/v/v) containing 7.5 mM ammonium formate and 30 µL was placed in a 96-well twin.tec® plate (Eppendorf, Hamburg, Germany). Nano-electrospray ionization (nano-ESI) chips with spraying nozzles of 5 µm nominal internal diameter were used and the whole NanoMate was controlled by the Chipsoft 8.3.1 software (both Advion BioSciences). The following settings were applied: ionization voltage 1.25 kV(+)/− 1.25 kV (−); backpressure 0.9 psi; capillary temperature 250 °C; S-Lens radio frequency (RF) level 50.

Each sample was measured for 17 min and polarity switching was triggered after 8 min (afterwards 1 min for equilibration) via contact closure signal by the mass spectrometer as described previously [46]. For each polarity, only MS1 spectra were acquired at the beginning for 30 s before 200 data independent acquisition (DIA) scans alternated with a MS1 scan for quantification. In MS1, the resolution was set to 240,000, the automatic gain control (AGC) target to 1e6, and the maximum injection time (IT) to 150 ms. For the DIA scans, a resolution of 60,000 was applied and the AGC target and the max IT was set to 2e5 and 130 ms, respectively. Normalized collision energy (NCE) of 25 was used in positive mode and 28 in negative mode. The scan range was set to *m/z* 200–1600 in both modes.

Data evaluation for identification and quantification was performed with LipidXplorer 1.2.7 software [47]. The spectra were imported into a Master Scan database using the following settings: mass tolerance 5 ppm, min.; occupation of 0; intensity threshold 10,000 (MS1)/5000 (MS2); resolution 260,000 (MS1)/65,000 (MS2); resolution gradient − 102 (MS1)/− 60 (MS2). The molecular fragmentation query language (MFQL) files used for identification can be found in the ESM. Limits of quantification (LOQ) were calculated

according to EURACHEM, The Fitness for Purpose of Analytical Methods, 2nd edition (2014), by repetitive injections of a low concentrated internal standard.

Reversed-phase chromatography high-resolution mass spectrometry

For reversed-phase (RP) chromatography of lipids, an Acquity HSS T3 (2.1 mm × 150 mm, 1.8 μm, Waters) with a VanGuard Pre-column (2.1 × 5 mm, 100 Å, 1.8 μm) was used. The column temperature was set to 40 °C and the flow rate to 250 μL min^{-1}. Acetonitrile (ACN)/H_2O (3:2, v/v) was used as solvent A and IPA/ACN (9:1, v/v) as solvent B, both containing 0.1% formic acid and 10 mM ammonium formate. The following gradient was applied: 0–2.0 min 30% B, 2.0–17.0 min ramp to 75% B, 15.0–17.0 min ramp to 100% B, 17.0–22.0 min 100% B, 22.0 min fast switch to 30% B, and equilibrated at the starting conditions for 5 min (22.0–27.0 min 30% B). The injector needle was washed with 75% isopropanol, 25% H_2O, and 0.1% formic acid for 5 s prior to each injection. The temperature of the autosampler was set to 10 °C. The same samples as for shotgun lipidomics were used (IPA/MeOH/$CHCl_3$ 4:2:1 (v/v/v) containing 7.5 mM ammonium formate) and the injection volume was 2 μL. A high-field Thermo Scientific™ Q Exactive HF™ quadrupole-Orbitrap mass spectrometer equipped with an electrospray source was used for HRMS. The ESI source parameters were the following: sheath gas 35, auxiliary gas 5, spray voltage 2.8 kV in negative and 3.5 kV in positive mode, capillary temperature 220 °C, S-Lens RF level 30, and auxiliary gas heater 300 °C. Spectral data were acquired in profile mode.

The full MS runs in positive mode were acquired in the range of *m/z* 200–2000 at a resolution of 120,000, an AGC target at 1e6, and a maximum IT of 200 ms. Data-dependent MS2 (ddMS2) fragmentation spectra were acquired for identification in positive and negative mode. For both, a Top8 method with a NCE of 25(+)/28(−) and an isolation window of *m/z* 1 was applied. The resolution in the MS2 was set to 30,000, the AGC target to 2e5 (minimum 8e3), and the max IT to 60 ms. The dynamic exclusion of triggered *m/z* was set to 15 s. Both an inclusion and an exclusion list were used for the possible lipids in yeast and the background compounds identified in a blank run, respectively.

Lipid identification was performed with Lipid Data Analyzer (LDA) 2.6 [48] and LipidSearch 4.2 from Thermo Scientific, in which following filters were applied: RT tolerance 0.25 min, m-score threshold 5, ID quality filter A,B,C (D- only for free fatty acids and cardiolipins), calculate unassigned peak area TRUE, and top rank filter TRUE. The identifications were curated manually following the criteria in Table S2 (see ESM).

Results and discussion

Semi-preparative supercritical fluid chromatography method development

The developed semi-preparative class-specific separation was based on the work of Lísa et al. [14]. This rigorously optimized analytical SFC utilizing sub 2 μm particle stationary phases enabled the separation of polar and non-polar lipid classes within 6 min. Accordingly, the selection of column chemistry involving ethylene-bridged hybrid (BEH) material and the optimized parameter (such as column temperature, water-additive composition, modifier gradient) served as a starting point of the upscaling process. As a drawback, the 400 bar pressure limit of the prep-SFC system (suggested operating range, 100–200 bar) only permitted the use of stationary phases with 5 μm particle size instead of sub 2 μm. A 10-mm column operated at flow rates of 15 mL min^{-1} was implemented. Additionally, the different injection modes between prep-SFC and analytical SFC needed to be considered. Indeed, the former system provides a so-called modifier-stream injection mode, in which the sample is injected into the modifier (solvent B) before it is mixed with CO_2. This mode benefits from reduced impact of the injection solvent compared with a mixed-stream injection [49, 50] established in analytical SFC. However, as the modifier-stream injection mode only works while using a modifier, a minimum of 5% modifier is required. The method developed by Lísa et al. [14] involved 100% CO_2 (solvent A) as starting condition and column temperatures of 60 °C, a prerequisite for the achieved excellent separation of non-polar lipids. The starting conditions of 100% CO_2 (A) could not be accomplished by the applied prep-SFC system (Waters Prep-15 SFC). Hence, the separation power regarding non-polar lipid classes was compromised. On the other hand, using the modifier-stream injection mode, the impact of sample matrix on the separation power was reduced. The final SFC method was based on a CO_2 (A) methanol-ammonium formate (B) gradient. The addition of volatile salts such as ammonium formate to the modifier was crucial with regard to peak shape of polar lipids. Omitting the salt additives was reported to hamper class-specific separation for these lipids. As a major advantage, the follow-up characterization or simply the use of the lipid class fractions obtained by prep-SFC was straightforward requiring no additional sample preparation steps as only volatile modifiers and salt additives were involved. Compared with other preparative lipid fractionation methods such as TLC, CC, prep-HPLC, and SPE, prep-SFC is fast, cost-efficient, less laborious, environmentally friendly [51], and has additional upscaling potential. In this work, up to 300 μL volumes could be injected containing 10 mg mL^{-1} dry lipid extract, which is 3 mg dry lipid extract compared with 0.5 μg lipid dry mass sample intake in analytical SFC (0.5 mg mL^{-1}, 1 μL) [14].

Figure 2 shows the optimized prep-SFC separation monitored by evaporative light scattering detector (ELSD) for lipid

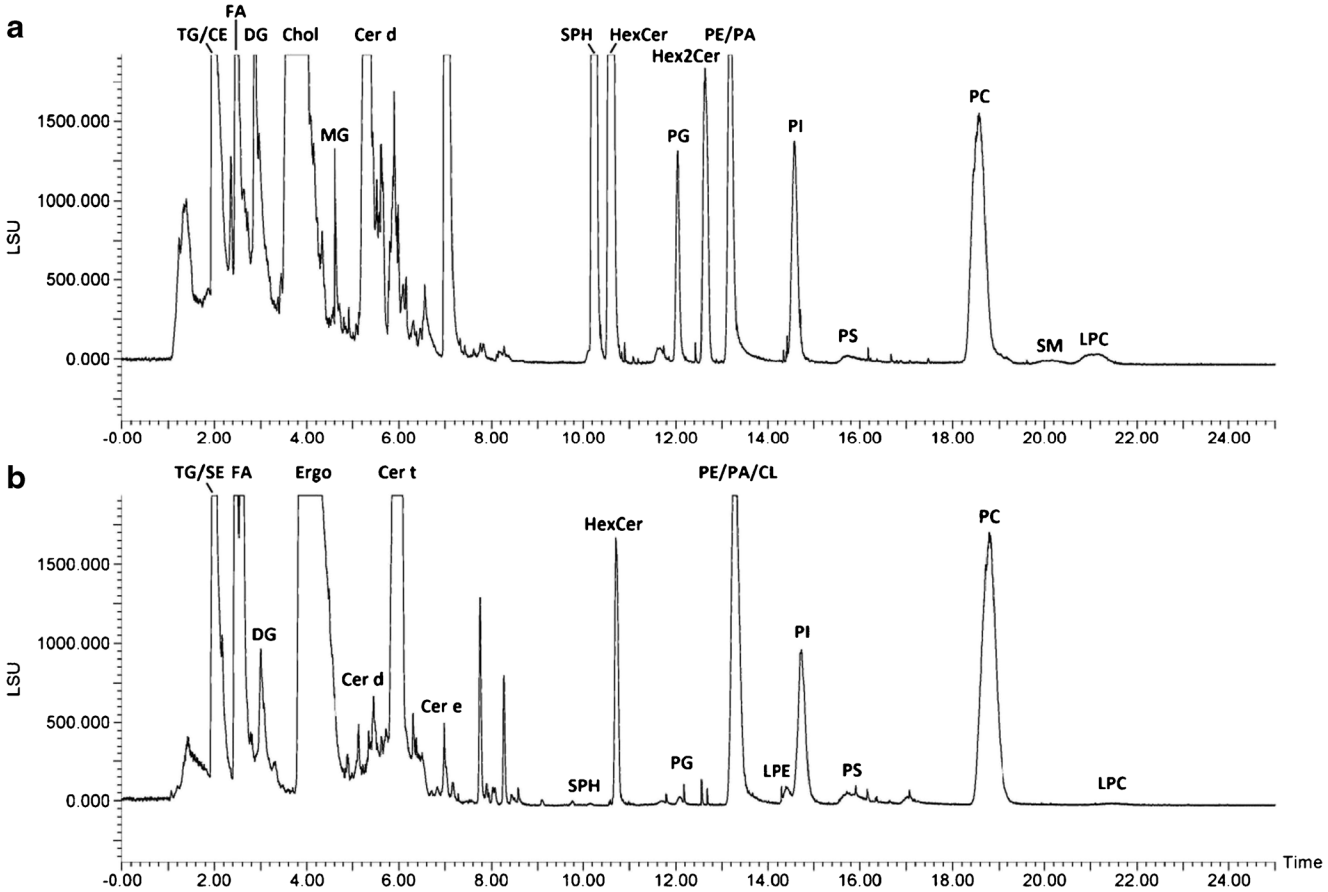

Fig. 2 Supercritical fluid chromatogram of a **a** multi-lipid mix and **b** yeast (*Pichia pastoris*) detected with ELSD. Peak annotation: TG, triacylglycerols; CE, cholesteryl esters; SE, steryl esters; FA, fatty acids; DG, diacylglycerols; Chol, cholesterol; Ergo, ergosterol; MG, monoacylglycerols; Cer, ceramides (d,t,e, di,tri,tetra hydroxylated); SPH, sphingosine bases, HexCer, hexosyl ceramides; PG, phosphatidylglycerols; Hex2Cer, dihexosyl ceramides; PE, phosphatidylethanolamines; PA, phosphatidic acids; CL, cardiolipins; PI, phosphatidylinositols; LPE, lysophosphatidylethanolamines; PS, phosphatidylserines; PC, phosphatidylcholines; SM, sphingomyelins; LPC, lysophosphatidylcholines. "/" indicates co-eluting lipid classes

standards and yeast samples. As can be readily observed, a broad lipid polarity range was covered (from non-polar lipids such as triacylglycerols (TG) to polar lipids such as lysophosphatidylcholines (LPC)). In analogy to analytical SFC, neutral lipids showed lower retention compared with polar lipids. The elution orders were also comparable, with the exception of the lipid classes phosphatidylserines (PS), phosphatidic acids (PA), and cardiolipins (CL). The final prep-SFC method featured a separation of 22 fractions within a runtime of 27 min. The collection of the lipid fractions was performed after the backpressure regulator, which was responsible of the density control along the column to maintain the solubility and the retention behavior of the analytes. Additional building blocks (heater, gas-liquid separator, make-up solvent) improved the recovery of the eluting fractions. Pure methanol was used as make-up solvent and the lipids were collected in defined time windows. First, the fraction collection timing was based on the detection of non-volatile compounds by ELSD only. This timing was fine-tuned by an iterative approach involving offline HRMS-based lipidomics screening of the fractions. The exact timing is given in Table S1 (see ESM).

Using standards and yeast samples, it could be shown that lipid class-specific fractionation was successfully accomplished for 14 different lipid classes or subclasses (FA, DG, ST, MG, SPH, HexCer, PG, Hex2Cer, PC, SM, LPC, Cer d, Cer t, Cer e) paving the way to further purification, in-depth profiling, or facilitating a detailed fatty acyl chain composition determination via gas chromatography-mass spectrometry (GC-MS) as previously addressed by TLC or SPE [52, 53]. Figure 3 gives a detailed overview of the fractionated lipid classes in yeast; the retention time of cholesterol, MG, Hex2Cer, AcCa, and SM was determined by the multi-lipid mix only. Early eluting non-polar lipid classes such as TG, SE, and ubiquinones (coenzymes, Co) could not be separated due to the compulsory minimum amount of modifier upon injection. Additionally, class-specific fractionation was only partly successful for PLs which could be to some extent attributed to peak tailing. While PG, PC, and LPC were clearly separated,

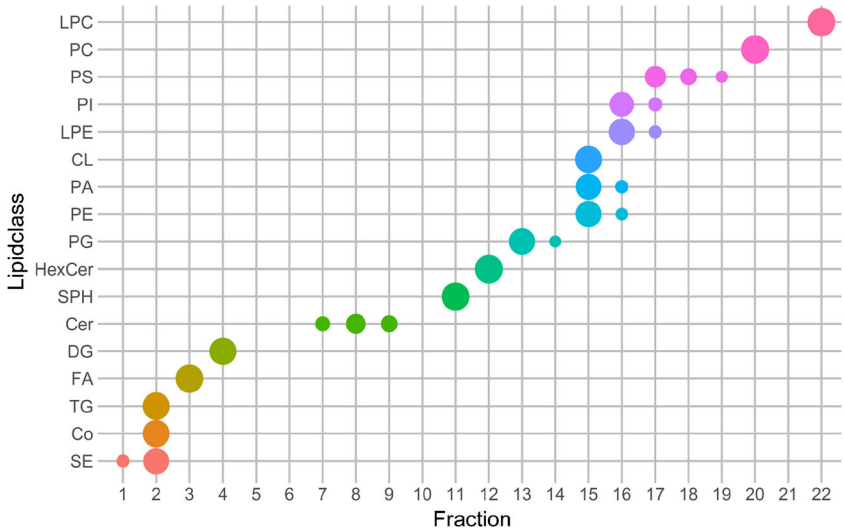

Fig. 3 Distribution of each yeast lipid class over the fractionated SFC run. Values are calculated by the summarized area values of the lipid species of each lipid class obtained by LipidSearch. The size of the points accounts for the relative area of each lipid class over all fractions (smaller points indicate a distribution over several fractions). Colors emphasize the different lipid classes on the y-axis

others were only partially fractionated (e.g., fraction 15 contained PE/PA/CL; fraction 16 contained PI/LPE).

However, the fractionation strategy allowed overcoming several selectivity challenges of MS analysis, e.g., isomeric classes PC and PE (also LPC and LPE) were separated. PC, SM, and LPC, all producing the head group fragment *m/z* 184 in positive mode due to their choline-containing head group, were separated. Finally, important classes showing corresponding in-source fragments or degradation products were successfully fractionated into different lipid classes such as sphingolipids (SPH, Cer, HexCer, and Hex2Cer) sterol/sterol esters or lysophospholipids and their corresponding phospholipids.

Interestingly, for ceramides, differences in hydrophilicity due to long-chain bases (LCB) as well as hydroxylated and non-hydroxylated fatty amid chains [54] led to SFC separation within the class. Using *P. pastoris*, ceramides were recovered in three fractions (7–9). Ceramides containing two hydroxyl groups eluted in a separate fraction as well as ceramides with three respectively four hydroxyl groups (see Fig. 4).

Identification, purification, and quantification of lipids from *Pichia pastoris*

In a next step, the workflow was evaluated for in-depth characterization of a yeast lipidome. *Pichia pastoris* selected as comprehensive lipidomics is still behind [40–43], when compared with the well-known yeast *Saccharomyces cerevisiae.* The collected SFC fractions were analyzed by RP-LC-HRMS using gradient elution of 22 min for identification and shotgun HRMS for quantification (see supplementary Excel table "Yeast_quant_shotgun_results" in the ESM). In parallel, non-fractionated yeast extracts were analyzed by RP-LC-HRMS to compare the number of identifications. Overall, a tremendous increase (by 170%) of lipid identification was achieved by the novel workflow (see Fig. 5). In total, 404 lipid species of 18 different lipid classes from six different lipid categories were identified either on the lipid or molecular species level in the fractions (corresponds to the whole bar per class). This number exceeded common workflows such as shotgun lipidomics (250 in *S. cer.* [38]) and HPLC analysis (ca. 200 in *P. pastoris* [40, 55]) Without SFC prefractionation, only 150 lipids (purple and white bar) were identified in *Pichia pastoris* using RP-HRMS in this work. The novel strategy proved to be advantageous in the case of low abundant lipid classes (Cer, FA, SPH) and lipids with low ionization efficiency (SE, ST). Moreover, due to the reduced complexity, the number of PLs, both in the isolated classes, e.g., PC and PG, as well as in the mixed fractions, e.g., PE/PA/CL, was increased. Finally, not only the number of identified lipids could be enhanced, also the degree of identification was improved (blue bar). An example is the lipid class PC. This class ionize highly efficiently in positive mode but the corresponding MS2 spectra by higher-energy collisional dissociation (HCD) only delivers head group-specific fragments that cannot help to identify the fatty acyl chain composition (compare lipid species level (e.g., PC 32:1) and molecular species level (e.g., PC 16:0_16:1)). However, in negative mode, the main fragments are the fatty acyl chain fragments, which make this mode preferable for identification. Unfortunately, in this mode, the ionization efficiency is less and higher concentrations, as obtained with SFC fractionation, can double the number of identified lipids on the molecular species level (see Fig. 5, also for PE, PS, PI). A detailed list of identified lipids (including identification level) is given in the supplementary Excel table (see ESM). The list was obtained after thorough corroboration of first screening data obtained by the application of commercial software tools (LipidSearch 4.2 from Thermo Scientific). Orthogonal open-source software tools based on fragmentation rules (Lipid Data analyzer 2.6 [48] and LipidXplorer 1.2.7 [47] for LC and shotgun data, respectively) were used to confirm the identifications based

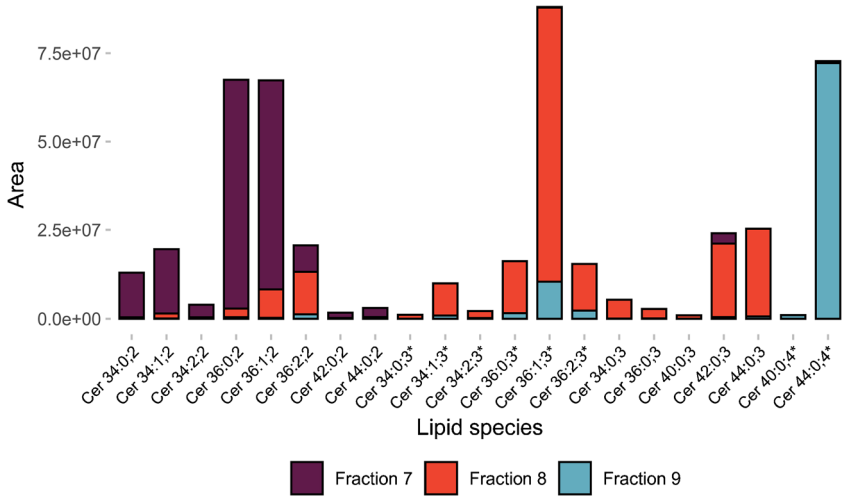

Fig. 4 Distribution of ceramides in the ceramide-containing fractions. Ceramides ordered after the number of hydroxyl groups in the three fractions, where ceramides were identified (fraction 7–9). Lipids marked with asterisk have a hydroxyl group on the fatty amid chain

on in silico-generated database search. As a drawback, this strategy is restricted to classes, where reliable fragmentation rules are available. Therefore, for some lipids, the validation of lipid identification resorted to a probability check through literature data [37, 38, 40–43]. Only a limited number of lipids were confirmed by authentic standards. It has to be mentioned that following the definition according to Schymanski et al. [56], only a standard matched identification corresponds to level 1 identification. Thus, all other lipids were identified on level 3 as the exact structure (position of double bonds, stereochemistry) cannot be determined with the applied HCD MS2 fragmentation. The total list can be found in the supplementary Excel tab "Yeast_ident_LC" (see ESM). Overall, 317 out of 404 lipid species could be validated by orthogonal approaches.

Validation of prep-SFC for offline quantification

In a next step, the SFC fractionation was combined to shotgun HRMS and was validated with regard to quantification accuracy. Proof-of-principle experiments were performed using the established internal standardization strategy (one-point calibration with one deuterated internal standard per lipid class) and a standard reference material from NIST, USA (SRM 1950), of human plasma. A recent international interlaboratory comparison provided consensus values for a large number of lipids [57]. An online tool denoted as LipidQC [58] facilitated cross-validation. The concentration was calculated according the following equation:

$$\mathrm{conc}_{\mathrm{Analyte}}\left[\mu\mathrm{mol\ L}^{-1}\right] = \mathrm{conc}_{\mathrm{ISTD}} \cdot \frac{\mathrm{Intensity}_{\mathrm{Analyte}}}{\mathrm{Intensity}_{\mathrm{ISTD}}}$$

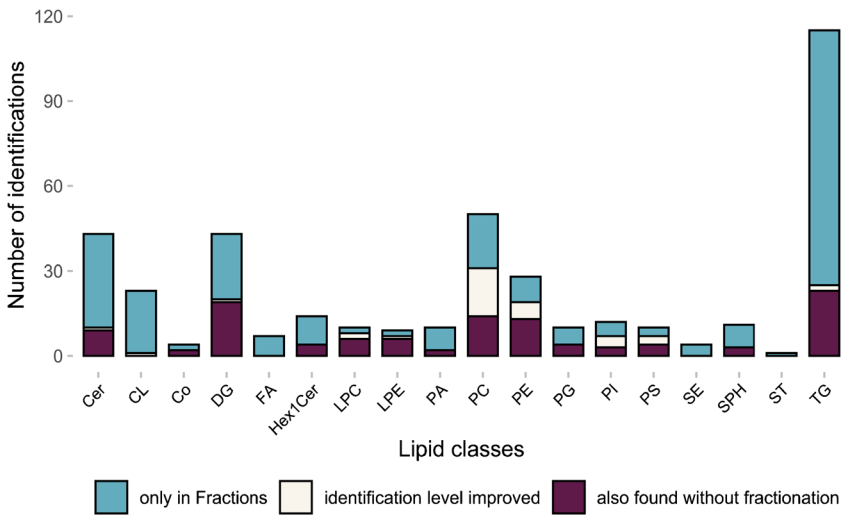

Fig. 5 Improved lipid identification in yeast with SFC fractionation. The total number of identifications over all fractions of the fractionated yeast (whole bar) compared with identifications in the non-fractionated full extract (purple and white bar) was more than doubled (404 compared with 150). The white bar corresponds to the lipids identified in both fractionated and non-fractionated full extract, but the level of identification was improved in the fractionated yeast (molecular species level, e.g., PC 16:0_16:1 instead of lipid species level, e.g., PC 32:1). The blue bar shows the number of lipids only identified in the fractionated yeast

As the internal standard was added prior extraction and therefore also before SFC separation, any losses during the process influence the internal standard and the analytes equally. Even a lower recovery rate can still lead to a decreased complexity in the following analysis, thus enabling quantification of low abundant lipid species. Figure 6 gives an overview of the accuracy assessment for both fractionated and non-fractionated plasma. The figure compares quantitative values (> LOQ by both approaches) for the lipid classes DG, TG, LPE, PC, PE, and SM. Overall, 70 out of 79 lipids were absolutely quantified within the 99% confidence interval by both approaches, which in turn confirmed the quantitative capability of SFC fractionation at semi-preparative scale. Finally, the quantitative semi-preparative SFC/shotgun lipidomics workflow was applied to *P. pastoris*. The results of this study are summarized in the supplementary Excel table (see ESM). It can be readily seen that starting from 1 g yeast resulting in 10 mg/mL lipid extract following sample preparation, high microgram amounts of lipid classes can be obtained.

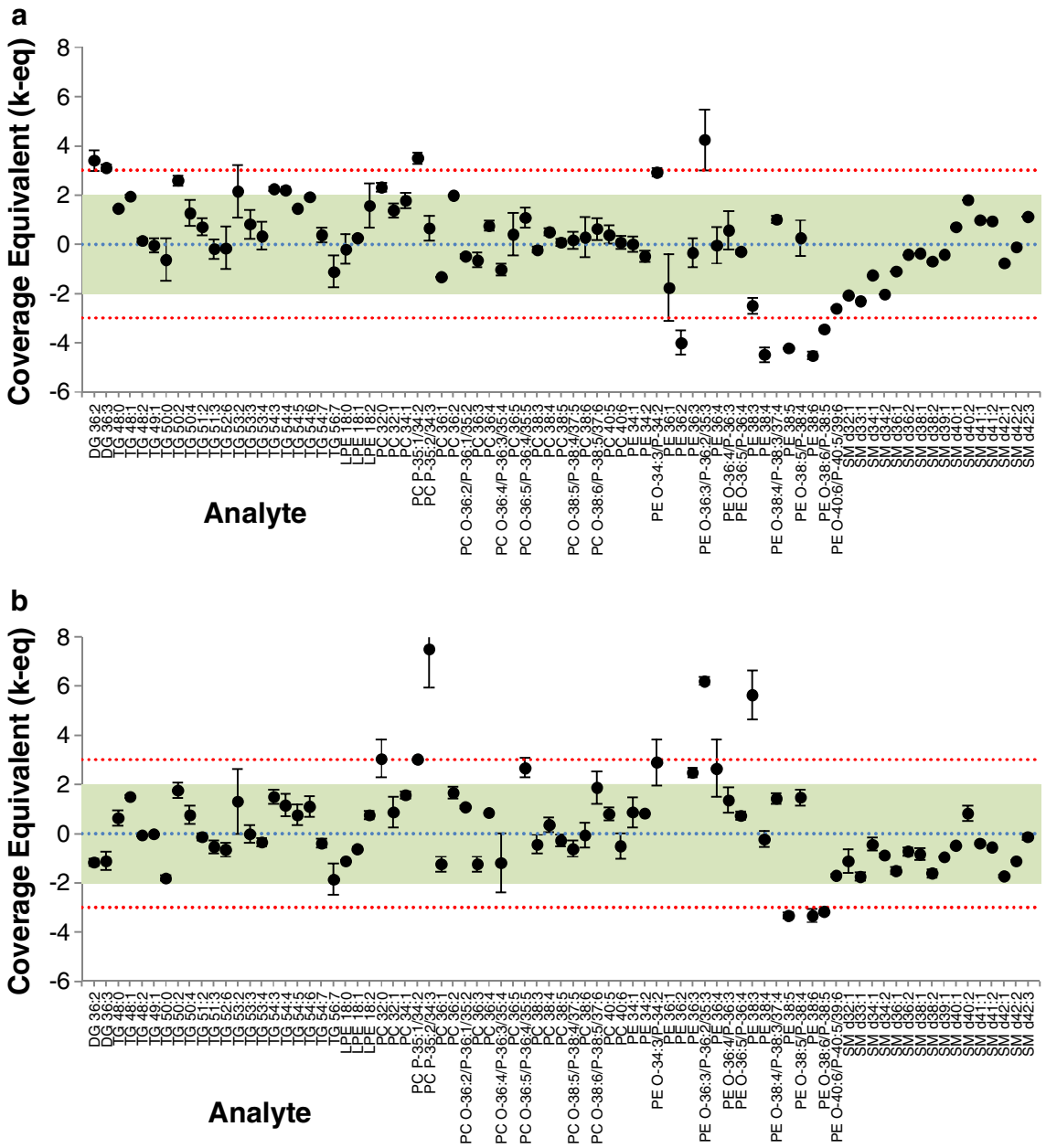

Fig. 6 Accuracy assessment for SRM 1950 - "Metabolites in Frozen Human Plasma." Values are presented as normalized coverage equivalents at the mean (dots) and stdev (error bars, $N = 2$) of measurements, overlaid onto the consensus mean value (blue line) and uncertainty (95% coverage, green region; 99% coverage, red region). **a** Fractionated with SFC. **b** Non-fractionated full extract. See ESM Tables S3 and S4 for detailed results. Graphic is produced with LipidQC [58]

Conclusions

The presented novel lipidomics workflow proved to be very versatile. The key advantages can be summarized as follows: (1) The class-specific fractionation by SFC offered reduced complexity and enrichment of low abundant lipids and lipid classes, respectively, thus overall enhancing the number of identified lipids (compare shotgun lipidomics (250 in *S. cer.* [38]) and HPLC analysis (ca. 200 in *P. pastoris* [40, 55]); (2) the workflow enabled absolute quantification as shown in a validation study using SRM1950; and (3) SFC commonly accepted as a green method provided "ready-to-use" lipid class fractions. Thus, follow-up lipid analysis is not limited to LC-MS-based assays. Any other additional lipid characterization method can be applied such as GC-MS analysis following hydrolysis and methylation (fatty acid methyl ester (FAME) analysis to determine the fatty acyl chain composition of each class) or structural elucidation via NMR. Additionally, in the future, isotopically labeled and non-labeled lipid standards/fractions could be produced from any organism of interest. The relatively short run time of 27 min, the automatization, and the use of CO_2 makes this fractionation method attractive for many applications.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Preparative supercritical fluid chromatography for lipid class fractionation – a novel strategy in high-resolution mass spectrometry based lipidomics

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Standards and solvents

Acetonitrile (ACN), isopropanol (IPA), methanol (MeOH), chloroform ($CHCl_3$) and water were of LC-MS grade and ordered at Fisher Scientific (Vienna, Austria) or Sigma Aldrich (Vienna, Austria). Ammonium formate was ordered as eluent additive for LC-MS at Sigma Aldrich. Formic acid was also of LC-MS grade and ordered at VWR International (Vienna, Austria).

Lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabama, USA). All standards were weighed and dissolved in an appropriate solvent. A multi-lipid mix was prepared in the used sample solvent. SPLASH® Lipidomix® Mass Spec Standard was purchased from Avanti Polar Lipids, Inc. (Alabama, USA).

Fermentation of *Pichia pastoris*

Yeast fermentation was conducted according to a previously established protocol [1]. Shortly, a cell culture of *Pichia pastoris* was grown on glucose as the only carbon source. The whole lipidome of the yeast can be uniformly ^{13}C labeled by using $U^{13}C$-glucose, whereas the labeling efficiency was improved by cultivating yeast precultures in shaker flasks prior to the actual inoculation of the fermenter. A BioFlo 310 Fermenter (New Brunswick™) was used to perform the fed-batch fermentation for 72 h. The fermentation broth was transferred into 50 mL falcon tubes and centrifuged for 5 min at 4000 rcf at 4 °C. The supernatant was discarded and the cell pellet was stored at −80 °C until further processing.

Lipid extraction of yeast cells

The extraction followed the procedure of Folch [2]. A dried cell suspension of *Pichia pastoris* (1 g ±0.2 g equals to 1.5 E10 cells) was washed with 20 mL ammonium bicarbonate (ABC) buffer (150 mM) to remove salts from the matrix. It was centrifuged for 10 min at 1000 rcf and the supernatant was descanted and discarded. The pellet was again dissolved in 15 mL ABC buffer and mixed vigorously. During all steps the sample was stored at ice if possible.

The cell suspension was aliquoted 16 times into 1 mL fractions into a 2 mL polypropylene tube, which is suitable for mechanical disruption device, in which glass beads had already been inserted with an amount corresponding to a liquid equivalent of 400 µL. It had to be aliquoted because the used *Peqlab Minilys* for tissue homogenization was only suitable for this 2 mL tubes. Each tube was shaken three times for 10 s at full speed respectively and between each lysis step the sample was cooled on ice for one minute.

Afterwards approximately 750 µL lysate of each tube was collected in a 1000 mL glass flask with a teflon lid. 500 mL (745 g) of $CHCl_3$ and 250 mL (198 g) methanol were added and the flask was shaken on ice for 1.5 h at 200 rpm. Afterwards it was removed from ice and 250 mL ABC buffer (150 mM) was added and shaken to initiate phase separation. After the two phases were clear the upper phase was put into Falcon tubes by an adjustable 10 mL pipette. The interphase which contained the insoluble proteins was collected separately. After the lower phase was almost totally free of any upper layer, it was put into a 500 mL round bottom flask by a DLAB Levo ME electric pipette with 50 mL glass tips. The organic lower phase was evaporated to dryness in a rotary evaporator, transferred into a 2 mL glass vial with $CHCl_3$, dried again and stored at -20 °C until further treatment. The lipids were dissolved in 1 mL $CHCl_3$ to obtain a concentration of 1.5 billion cells mL^{-1}.

Lipid extraction of human plasma

To a 100 µL plasma sample aliquot (standard reference material 1950, NIST, USA), which was placed on ice in a glass tube with a Teflon-lined cap, 1.253 mL Methanol, 80 µL SPLASH® Lipidomix® Mass Spec standard and 2.667 mL Chloroform were added. After each addition the tube was shaken vigorously. The mixture was shaken (400 rpm) for 1 h at 4 °C (ThermoMixer C, Eppendorf). After that, 1 mL ABC Buffer (150 mM) was added and the phase separation was done at room temperature. The aqueous phase was collected and the organic lower phase was put in a glass vial. The aqueous phase was re-extracted with 667 µL $CHCl_3$ and 333 µL MeOH. The combined phases were dried under nitrogen and stored at -20 °C until SFC measurement. Before analysis, the dried lipids were dissolved in 800 µL $CHCl_3$ and transferred into a 2 mL glass vial.

Table S1 Collection time ranges and fraction number for the SFC run

Fr.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
t [min]	1-2	2-2.5	2.5-3	3-3.5	3.5-5	5-6.4	6.4-7.2	7.2-7.9	7.9-8.5	8.5-10	10-10.6
Fr.	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
t [min]	10.6-11.3	11.3-12.2	12.2-12.9	12.9-14.2	14.2-15.5	15.5-16.8	16.8-17.6	17.6-18.2	18.2-19.2	19.2-20.5	20.5-23

Table S2 LipidSearch filter criteria

Class	Criteria	Class	Criteria
AEA	+H, ethanolamine and M+H frag	LPE	+H, NL, isomer separation→ 2 peaks
Cer	+H, SPH frag, A or B grade	PC	+H, 184 frag
CL	+H/-H/-2H, DG frag in pos, FA in neg	PE	+H/+Na, NL
Co	$+NH_4$, M-Q frag, alkyl-pattern	PG	$+NH_4$, NL
DG	$+NH_4$, NL (FA), alkyl-pattern, A or B grade	PI	$+NH_4$, NL
FA	-H	PS	+H/-H/+Na, NL in pos, FA in neg
HexCer	+H, SPH frag and NL, A grade	SPH	+H, waterloss
LPC	+H, 184 frag, isomer separation→ 2 peaks	SE	$+NH_4$, sterol frag, alkyl-pattern
TG	$+NH_4$, M-Q frag, alkyl-pattern	ST	$+H-H_2O$, alkyl-pattern

Table S3 Measurement accuracy summary compared against SRM 1950 – Metabolites in Frozen Human. Results of the fractionated human plasma

Lipid Species	Measurement* [nmol/mL]			Consensus Value** [nmol/mL]			No. of labs	Notes
DG 36:2	3.6	±	0.3	6.2	±	2.2	16	
DG 36:3	4.8	±	1.2	8.4	±	3.3	15	
TG 48:0	5.3	±	0.4	4.5	±	1.2	10	
TG 48:1	17.8	±	0.2	13	±	3.2	16	
TG 48:2	15.8	±	0.2	16	±	2.8	15	
TG 49:1	2.0	±	0.0	2.0	±	0.42	9	
TG 50:0	2.3	±	0.1	3.8	±	0.83	11	
TG 50:2	68.1	±	3.8	47	±	12	15	
TG 50:4	10.9	±	1.1	8.7	±	2.9	15	
TG 51:2	4.7	±	0.2	4.8	±	1.1	8	
TG 51:3	3.8	±	0.5	4.8	±	1.9	5	
TG 52:6	3.1	±	0.4	4.0	±	1.4	8	
TG 53:2	2.4	±	0.5	1.9	±	0.41	9	
TG 53:3	3.7	±	0.4	3.7	±	1.1	6	
TG 53:4	2.1	±	0.1	2.4	±	0.76	6	
TG 54:3	40.7	±	2.8	26	±	9.8	15	
TG 54:4	51.0	±	6.0	36	±	13	15	
TG 54:5	35.4	±	4.5	27	±	11	15	
TG 54:6	19.6	±	2.2	14	±	5.1	16	
TG 54:7	5.0	±	0.3	5.6	±	1.5	7	
TG 56:7	8.0	±	1.7	13	±	2.7	8	
LPE 18:0	1.0	±	0.0	1.6	±	0.55	15	
LPE 18:1	1.1	±	0.0	1.4	±	0.47	14	
LPE 18:2	2.3	±	0.1	1.9	±	0.56	16	
PC 32:0	10.3	±	0.8	7.2	±	1.0	18	
PC 32:1	14.7	±	1.2	13	±	1.9	18	
PC 34:1	153.0	±	3.1	120	±	21	19	
PC P-35:1/34:2	382.0	±	1.8	240	±	47	18	Includes only PC 34:2 results.
PC P-35:2/34:3	24.8	±	2.7	12	±	1.7	18	Includes only PC 34:3 results.
PC 36:1	20.3	±	1.4	26	±	4.6	17	
PC 36:2	181.5	±	6.2	140	±	25	18	
PC O-36:2/P-36:1/35:2	9.2	±	0.1	7.4	±	1.7	17	Includes only PC 35:2 results.
PC 36:3	82.7	±	4.4	100	±	14	17	
PC 36:4	173.7	±	1.2	150	±	28	19	
PC O-36:4/P-36:3/35:4	10.4	±	1.7	12	±	1.4	17	Includes only PC O-36:4 results.
PC 36:5	30.1	±	1.9	11	±	1.8	16	
PC O-36:5/P-36:4/35:5	11.2	±	0.6	6.9	±	1.6	11	Includes only PC O-36:5 results.
PC 38:3	23.8	±	2.0	26	±	5.2	14	
PC 38:4	88.9	±	4.1	84	±	14	18	
PC 38:5	39.9	±	1.9	42	±	7.9	18	
PC O-38:5/P-38:4/37:5	10.0	±	0.5	11	±	1.6	16	Includes only PC O-38:5 results.
PC 38:6	40.7	±	2.2	41	±	4.4	18	
PC O-38:6/P-38:5/37:6	5.5	±	0.7	3.6	±	1.0	12	Includes only PC O-38:6 results.
PC 40:5	7.6	±	0.3	6.7	±	1.1	18	
PC 40:6	12.7	±	1.3	14	±	2.6	17	
PE 34:1	1.3	±	0.1	1.2	±	0.17	14	
PE 34:2	2.4	±	0.0	2.2	±	0.26	16	
PE O-34:3/P-34:2	2.7	±	0.4	1.5	±	0.41	11	Includes only PE O-34:3 results.
PE 36:1	7.6	±	0.4	1.3	±	0.26	14	
PE 36:2	19.9	±	0.1	6.7	±	0.79	16	
PE 36:3	3.3	±	0.1	2.4	±	0.38	16	
PE O-36:3/P-36:2/35:3	7.9	±	0.1	3.2	±	0.76	15	Includes only PE O-36:3 results.
PE 36:4	4.1	±	0.5	3.1	±	0.39	16	
PE O-36:4/P-36:3	2.0	±	0.1	1.6	±	0.29	14	Includes only PE O-36:4 results.

Lipid Species	Measurement* [nmol/mL]			Consensus Value** [nmol/mL]			No. of labs	Notes
PE O-36:5/P-36:4	6.3	±	0.3	4.9	±	1.9	15	Includes only PE O-36:5 results.
PE 38:3	2.1	±	0.2	0.95	±	0.20	14	
PE 38:4	7.8	±	0.4	8.1	±	1.2	16	
PE O-38:4/P-38:3/37:4	1.2	±	0.0	0.94	±	0.18	9	Includes only PE O-38:4 results.
PE 38:5	1.1	±	0.1	2.7	±	0.47	12	
PE O-38:5/P-38:4	8.6	±	0.6	5.8	±	1.9	17	Includes only PE O-38:5 results.
PE 38:6	1.2	±	0.2	3.2	±	0.59	15	
PE O-38:6/P-38:5	1.1	±	0.2	4.9	±	1.2	16	Includes only PE O-38:6 results.
PE O-40:6/P-40:5/39:6	0.8	±	0.0	1.3	±	0.31	14	Includes only PE O-40:6 results.
SM d32:1	6.8	±	0.7	8.4	±	1.4	14	
SM d33:1	3.6	±	0.1	4.7	±	0.64	14	
SM d34:1	93.6	±	3.8	100	±	15	21	
SM d34:2	14.1	±	0.2	16	±	2.2	17	
SM d36:1	14.5	±	0.5	20	±	3.7	22	
SM d36:2	8.5	±	0.2	9.6	±	1.5	22	
SM d38:1	8.4	±	0.8	11	±	3.1	17	
SM d38:2	3.1	±	0.2	5.2	±	1.3	17	
SM d39:1	2.7	±	0.0	3.6	±	1.0	14	
SM d40:1	17.6	±	0.1	20	±	5.1	17	
SM d40:2	14.3	±	0.9	12	±	2.8	15	
SM d41:1	6.9	±	0.1	7.7	±	2.1	14	
SM d41:2	5.0	±	0.1	5.8	±	1.4	14	
SM d42:1	10.7	±	0.3	20	±	5.4	21	
SM d42:2	31.8	±	0.3	44	±	11	18	
SM d42:3	16.4	±	0.6	17	±	4.7	12	

Table S4 Measurement accuracy summary compared against SRM 1950 – Metabolites in Frozen Human. Results of the non-fractionated human plasma

Lipid Species	Measurement* [nmol/mL]	Consensus Value** [nmol/mL]	No. of labs	Notes
DG 36:2	13.7 ± 0.9	6.2 ± 2.2	16	
DG 36:3	18.7 ± 0.4	8.4 ± 3.3	15	
TG 48:0	6.3 ± 0.0	4.5 ± 1.2	10	
TG 48:1	19.3 ± 0.2	13 ± 3.2	16	
TG 48:2	16.4 ± 0.3	16 ± 2.8	15	
TG 49:1	2.0 ± 0.1	2.0 ± 0.42	9	
TG 50:0	3.3 ± 0.7	3.8 ± 0.83	11	
TG 50:2	78.1 ± 2.5	47 ± 12	15	
TG 50:4	12.4 ± 1.6	8.7 ± 2.9	15	
TG 51:2	5.6 ± 0.4	4.8 ± 1.1	8	
TG 51:3	4.5 ± 0.8	4.8 ± 1.9	5	
TG 52:6	3.8 ± 1.2	4.0 ± 1.4	8	
TG 53:2	2.8 ± 0.4	1.9 ± 0.41	9	
TG 53:3	4.6 ± 0.6	3.7 ± 1.1	6	
TG 53:4	2.7 ± 0.4	2.4 ± 0.76	6	
TG 54:3	48.0 ± 0.8	26 ± 9.8	15	
TG 54:4	64.8 ± 1.6	36 ± 13	15	
TG 54:5	43.1 ± 0.4	27 ± 11	15	
TG 54:6	23.8 ± 0.4	14 ± 5.1	16	
TG 54:7	6.2 ± 0.5	5.6 ± 1.5	7	
TG 56:7	10.0 ± 1.8	13 ± 2.7	8	
LPE 18:0	1.5 ± 0.3	1.6 ± 0.55	15	
LPE 18:1	1.5 ± 0.0	1.4 ± 0.47	14	
LPE 18:2	2.8 ± 0.5	1.9 ± 0.56	16	
PC 32:0	9.5 ± 0.2	7.2 ± 1.0	18	
PC 32:1	15.6 ± 0.5	13 ± 1.9	18	
PC 34:1	157.5 ± 6.4	120 ± 21	19	
PC P-35:1/34:2	404.6 ± 10.8	240 ± 47	18	Includes only PC 34:2 results.
PC P-35:2/34:3	13.1 ± 0.9	12 ± 1.7	18	Includes only PC 34:3 results.
PC 36:1	19.9 ± 0.0	26 ± 4.6	17	
PC 36:2	189.6 ± 0.9	140 ± 25	18	
PC O-36:2/P-36:1/35:2	6.6 ± 0.1	7.4 ± 1.7	17	Includes only PC 35:2 results.
PC 36:3	91.0 ± 4.3	100 ± 14	17	
PC 36:4	171.2 ± 5.5	150 ± 28	19	
PC O-36:4/P-36:3/35:4	10.6 ± 0.3	12 ± 1.4	17	Includes only PC O-36:4 results.
PC 36:5	11.7 ± 1.6	11 ± 1.8	16	
PC O-36:5/P-36:4/35:5	8.6 ± 0.7	6.9 ± 1.6	11	Includes only PC O-36:5 results.
PC 38:3	24.9 ± 0.7	26 ± 5.2	14	
PC 38:4	91.1 ± 1.9	84 ± 14	18	
PC 38:5	42.6 ± 1.3	42 ± 7.9	18	
PC O-38:5/P-38:4/37:5	11.3 ± 0.6	11 ± 1.6	16	Includes only PC O-38:5 results.
PC 38:6	42.3 ± 3.6	41 ± 4.4	18	
PC O-38:6/P-38:5/37:6	4.2 ± 0.4	3.6 ± 1.0	12	Includes only PC O-38:6 results.
PC 40:5	7.1 ± 0.4	6.7 ± 1.1	18	
PC 40:6	14.2 ± 0.7	14 ± 2.6	17	
PE 34:1	1.2 ± 0.1	1.2 ± 0.17	14	
PE 34:2	2.1 ± 0.1	2.2 ± 0.26	16	
PE O-34:3/P-34:2	2.7 ± 0.1	1.5 ± 0.41	11	Includes only PE O-34:3 results.
PE 36:1	0.8 ± 0.4	1.3 ± 0.26	14	
PE 36:2	3.5 ± 0.4	6.7 ± 0.79	16	
PE 36:3	2.3 ± 0.2	2.4 ± 0.38	16	
PE O-36:3/P-36:2/35:3	6.4 ± 0.9	3.2 ± 0.76	15	Includes only PE O-36:3 results.
PE 36:4	3.1 ± 0.3	3.1 ± 0.39	16	
PE O-36:4/P-36:3	1.8 ± 0.2	1.6 ± 0.29	14	Includes only PE O-36:4 results.

Lipid Species	Measurement* [nmol/mL]			Consensus Value** [nmol/mL]			No. of labs	Notes
PE O-36:5/P-36:4	4.3	±	0.2	4.9	±	1.9	15	Includes only PE O-36:5 results.
PE 38:3	0.4	±	0.1	0.95	±	0.20	14	
PE 38:4	2.7	±	0.4	8.1	±	1.2	16	
PE O-38:4/P-38:3/37:4	1.1	±	0.0	0.94	±	0.18	9	Includes only PE O-38:4 results.
PE 38:5	0.7	±	0.0	2.7	±	0.47	12	
PE O-38:5/P-38:4	6.3	±	1.4	5.8	±	1.9	17	Includes only PE O-38:5 results.
PE 38:6	0.5	±	0.1	3.2	±	0.59	15	
PE O-38:6/P-38:5	0.8	±	0.1	4.9	±	1.2	16	Includes only PE O-38:6 results.
PE O-40:6/P-40:5/39:6	0.5	±	0.0	1.3	±	0.31	14	Includes only PE O-40:6 results.
SM d32:1	5.5	±	0.0	8.4	±	1.4	14	
SM d33:1	3.2	±	0.0	4.7	±	0.64	14	
SM d34:1	81.2	±	0.0	100	±	15	21	
SM d34:2	11.5	±	0.0	16	±	2.2	17	
SM d36:1	15.9	±	0.0	20	±	3.7	22	
SM d36:2	9.0	±	0.0	9.6	±	1.5	22	
SM d38:1	9.9	±	0.0	11	±	3.1	17	
SM d38:2	4.3	±	0.0	5.2	±	1.3	17	
SM d39:1	3.2	±	0.0	3.6	±	1.0	14	
SM d40:1	23.6	±	0.0	20	±	5.1	17	
SM d40:2	17.0	±	0.0	12	±	2.8	15	
SM d41:1	9.8	±	0.0	7.7	±	2.1	14	
SM d41:2	7.1	±	0.0	5.8	±	1.4	14	
SM d42:1	15.9	±	0.0	20	±	5.4	21	
SM d42:2	42.9	±	0.0	44	±	11	18	
SM d42:3	22.4	±	0.0	17	±	4.7	12	

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4. Outlook

As the lipidome is influenced by the most studied diseases of our time (cancer, cardiovascular disease, diabetes), the future of lipidomics as a diagnostic and therapeutic tool is promising. However, it should not be ignored that after twenty years of existence as a term, the outcome for clinical application is limited. For a successful and useful transition into regular heath controls interdisciplinary cooperation is necessary including, medical, biological, chemical, pharmaceutical, statistical, economic, and legal aspects. With this thesis and ongoing community efforts, important analytical considerations have been tackled as only trustworthy data can build the basis for further application. The search for lipid biomarkers will continue but it might be possible that only a precision or personal medicine approach will enable the often promised and highly desired breakthrough of lipidomics application. Additionally, the high complexity and diversity of the lipidome are still not fully understood and our workflows can also help to investigate the lipid pathways themselves. Standardization and harmonization are prerequisites for each further application and hopefully contribute to a prosperous healthy future.

Further contributions to peer-reviewed publications

El Abiead, Y., Milford, M., Schoeny, H., Rusz, M., Salek, R., Koellensperger, G. Benchmarking feature quality assurance strategies for nontargeted metabolomics. *Analytical Chemistry*, submitted.

Rampler, E., El Abiead, Y., Schoeny, H., Rusz, M., Hildebrand, F., Fitz, V., Koellensperger, G. Recurrent Topics in Mass Spectrometry-Based Metabolomics and Lipidomics—Standardization, Coverage, and Throughput. *Analytical Chemistry*, 2021, 93, 1, 519–545.

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Contributions to conferences

Talk: H. Schoeny, E. Rampler, Y. El Abiead, F. Hildebrand, G. Hermann & G. Köllensperger. *The other way! - Alternative lipid quantification via RP-LC and 13C-labeled internal standard.* 9th International Singapore Lipid Symposium, Online iSLS9, 2021, Singapore, Singapore

Talk: H. Schoeny, E. Rampler, Y. El Abiead, F. Hildebrand, G. Hermann & G. Köllensperger. *How accurate is my lipid quantification? Insights into internal standardization by uncertainty calculation.* Joined online 8th European Lipidomics Meeting (ELM) and 6th Lipidomics Forum, 2020, Regensburg, Germany

Poster: H. Schoeny, E. Rampler, F. Hildebrand, O. Zach, G. Hermann & G. Köllensperger. *A combined direct infusion/RP-LC-HRMS workflow for accurate absolute quantification with ^{13}C- internal standards for a high number of lipids.* Online ASMS – 68th ASMS Conference on Mass Spectrometry and Allied Topics, 2020, Houston, USA

Invited Talk: H. Schoeny. *^{13}C Internal standardization in lipidomics*. Institute seminar at the Analytical Chemistry Department, University Vienna, 2019, Vienna, Austria

Talk: H. Schoeny, E. Rampler, F. Hildebrand, O. Zach, G. Hermann & G. Köllensperger. *How to solve the limits in absolute quantification on reversed phase LC-MS-based lipidomics approaches? -A novel flow injection/ reversed phase LC-MS workflow with LILY as ISTD.* 5th Lipidomics Forum, 2019, Borstel, Germany

Talk: H. Schoeny, E. Rampler, G. Hermann, U. Grienke, J. M. Rollinger & G. Köllensperger. *^{13}C Internal standardization in lipidomics.* ANAKON, 2019, Münster, Germany

Talk: H. Schoeny, E. Rampler G. Hermann, & G. Köllensperger. *^{13}C Internal standardization in lipidomics.* Summer school LipoSysMed, 2019, Leipzig, Germany

Poster: H. Schoeny, E. Rampler G. Hermann, U. Grienke, J. M. Rollinger & G. Köllensperger. *Fractionation of the ^{13}C-labeled yeast lipidome by prep-SFC for a new scope of internal standardization. 4th* Lipidomics Forum, 2018, Dortmund, Germany

Poster: H. Schoeny, E. Rampler G. Hermann, & G. Köllensperger. *^{13}C-labeled Yeast Lipidome as Internal Standard- Challenges and Perspectives. 7th* European Lipidomics Meeting, 2018, Leipzig, Germany

Poster: H. Schoeny, E. Rampler, Y. El Abiead, M. Schwaiger-Haber, G. Hermann & G. Köllensperger. *^{13}C Internal Standardization in Lipidomics- a comparative study on different quantification methods.* ASMS – 66th ASMS Conference on Mass Spectrometry and Allied Topics, 2018, San Diego, USA

Short-term stay abroad during the PhD

6th September – 5th November 2021	San Pablo-CEU University, Madrid, Spain, Research stay at the CEMBIO Laboratory, Faculty of Pharmacy in the group of Dr. Coral Barbas (2 months)

Awards and scholarships obtained during the PhD

2021	Erasmus + Traineeship Mobility
2021	"Best short talk", 9th international Singapore Lipid Symposium, Online
2020	Full scholarship for the Entrepreneurial Leadership Program, Austrian Startups
2020	Club Alpbach Niederösterreich Scholarship for the Forum Alpbach
2019	Full scholarship for the Summer school LipoSysMed, Germany
2019	"Award for the best scientific talk", Lipidomics Forum, Germany
2019	Nomination for the Agnes and István Halász Young Scientist Award, Anakon, Germany
2019	Gesellschaft Deutscher Chemiker (GDCh) travel scholarship
2019	Deutsche Gesellschaft für Massenspektrometrie (DGMS) travel scholarship
2019	Gesellschaft Östereichischer Chemiker (GÖCh) travel scholarship
2018	TOP Stipendium 2018 des Landes Niederösterreich travel scholarship
2018	Austrian Society of Analytical Chemistry (ASAC) travel scholarship
2018	Austrian Proteomics and Metabolomics Association (APMA) travel scholarship
2018	Gesellschaft Östereichischer Chemiker (GÖCh) travel scholarship