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Article

High Throughput LC-MS Platform for Large Scale Screening of Bioactive Polar Lipids in Human Plasma and Serum

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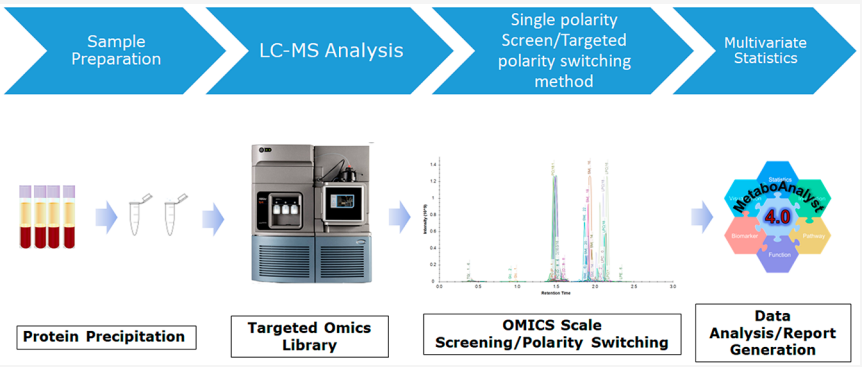

ABSTRACT: Lipids play a key role in many biological processes, and their accurate measurement is critical to unraveling the biology of diseases and human health. A high throughput HILIC-based (LC-MS) method for the semiquantitative screening of over 2000 lipids, based on over 4000 MRM transitions, was devised to produce an accessible and robust lipidomic screen for phospholipids in human plasma/serum. This methodology integrates many of the advantages of global lipid analysis with those of targeted approaches. Having used the method as an initial "wide class" screen, it can then be easily adapted for a more targeted analysis and quantification of key, dysregulated lipids. Robustness was assessed using 1550 continuous injections of plasma extracts onto a single column and via the evaluation of columns from 5 different batches of stationary phase. Initial screens in positive (239 lipids, 431 MRM transitions) and negative electrospray ionization (ESI) mode (232 lipids, 446 MRM transitions) were assessed for reproducibility, sensitivity, and dynamic range using analysis times of 8 min. The total number of lipids monitored using these screening methods was 433 with an overlap of 38 lipids in both modes. A polarity switching method for accurate quantification, using the same LC conditions, was assessed for intra- and interday reproducibility, accuracy, dynamic range, stability, carryover, dilution integrity, and matrix interferences and found to be acceptable. This polarity switching method was then applied to lipids important in the stratification of human prostate cancer samples.

KEYWORDS: *lipidomics, quantification, serum, plasma, high throughput, prostate cancer*

INTRODUCTION

Lipids are integral to human biology and represent approximately one-third of all known metabolites.[1] They play a critical role in energy storage, cell membrane architecture, cellular signaling, and cell−cell interactions and are thus important to cellular survival, growth, proliferation, interaction, and cell death.[2] Lipids are present in samples such as plasma and serum over a very wide range of concentrations from millimolar to attomolar. Thus, fatty acids, triacylglycerols, and cholesterol esters are found in millimolar to nanomolar amounts while the less abundant eicosanoid lipid mediators are present in the picomolar to attomolar concentration range.[3,4] It is widely acknowledged that the lipid composition of samples is dynamic, strongly influenced by endogenous and exogenous factors, and subject to tight regulation. A large number of diseases and disorders, including metabolic syndrome disorders, cardiovascular disease, cancer, and neurodegenerative diseases, can result in changes in the lipidome.[5,6] Whilst correlation is not causation, nevertheless associating alterations in the composition of the lipidome with these pathophysiological states may provide many benefits. Such benefits include the potential for a deeper understanding of disease mechanisms, diagnosis, patient stratification, treat-

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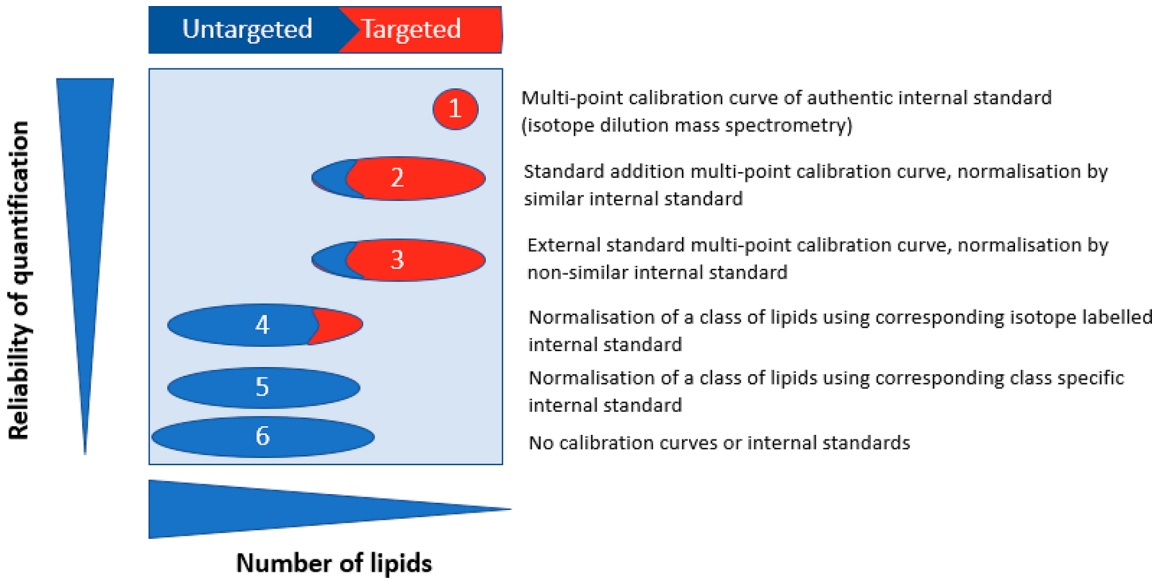

Figure 1. Untargeted and targeted lipidomics methods in relation to the number of detected lipids and reliability of quantitative results. Adapted with permission from ref 8. Copyright (2016) American Chemical Society.

ment evaluation, and monitoring clinical conditions. For lipidomics to provide these benefits it is therefore important to be able to accurately, rapidly, and reliably measure changes within the lipidome.

Currently, lipidomic analysis has two general approaches: (i) targeted analysis, often used to study signal processing and hypothesis-driven pathway analysis, or (ii) global analysis (untargeted) approaches[7] used to obtain a qualitative view of changes in the lipidome. The strategies/methodologies employed are dependent on the aims and scope of the underlying research question and have their advantages and disadvantages. As described by Cajka and Fiehn[8] (Figure 1), these approaches can be characterized based on the number of detected lipids and the reliability of quantification.

Various analytical methodologies can be utilized in lipidomics; however, mass spectrometry (MS) configured for direct infusion (DI) or chromatographic separation using gas chromatography (GC), reversed-phase liquid chromatography (RPLC), hydrophilic interaction chromatography (HILIC), or supercritical fluid chromatography (SFC) are the most commonly employed.[8−10]

DI-MS is used extensively for lipidomic analyses, due to its potential for high throughput, reproducibility, and ease of automation. However, as there is no separation prior to ionization, discriminating between isomers and isobaric species is challenging. GC-MS is typically used for fatty acid (FA) analysis but requires derivatization of the sample prior to analysis (e.g., see ref 9). For LC-MS-based lipidomics, the most widely used separation methods are RPLC and HILIC.[8,11] RPLC separates lipids based mainly on fatty acyl/alkyl chain hydrophobicity and is ideally suited for the analysis of isomers, whereas HILIC separates lipids based on their headgroup,[10] with lipids of the same class eluting together. HILIC based methods are therefore able to minimize elution dependent matrix effects due to coelution of internal standards and lipids of the same class.[11] However, ion suppression favoring detection of the most abundant lipids can also occur.[11] Although not as widely established, SFC shows a great potential as the comprehensive and high-throughput screening method for a large number of samples per data set in different 'omic fields including lipidomics.[11]

A major challenge correlating lipidomes at the onset and during the progression of a disease is the large disparity in available methodologies and technologies. This has resulted in discrepancies in published data and broader issues of irreproducibility, spurring the lipidomics community to address these inconsistencies.[12] To date, clinical lipid measurements have consisted primarily of cholesterol (total, LDL and HDL, etc.) or triglycerides, as a measure for cardiovascular risk and response to lipid-lowering drugs.[1,13]

Currently, "omics" studies often suffer from the inadequate or poor annotation of lipid species.[4,12,14,15] The bulk of published lipidomic data are reported as the sum composition(s) of lipids, e.g., (PC (34:1)) rather than as individual molecular species, i.e., (PC (16:0_18:1)), hindering the ability to describe pathways comprehensively.[16] Misidentification and over-reporting tend to be a result of the incorrect mapping of spectral features to potential lipid molecules.[12] Furthermore, the data are often reported in arbitrary units (ion counts of peak intensity or area) even though quantification (moles) is necessary for the calculation of the fraction of the total for each of the lipid classes and vital for the detailed interpretation and comparison of large data sets in multilaboratory studies.[12,13,17] Several large interlaboratory studies have shown the need for standardized performance verification parameters and quality control measures for the determination of data quality since batch-to-batch variations are unavoidable in high-throughput analytical settings.[18−20]

Here, we describe a validated, robust, high throughput, sensitive, and selective HILIC-based UHPLC-ESI-MS/MS "omics scale" and targeted profiling assay for the semi-quantitative analysis using a library of over 2000 lipids (comprised of more than 4000 measured and theoretical MRM transitions), for lipids commonly found in human plasma and serum.

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MATERIALS AND METHODS

Materials

LC-MS grade water and 2-propanol (IPA) were purchased from Honeywell (Birkenhead, UK); LC-MS grade acetonitrile (ACN) were purchased from Biosolve Chimie (Birkenhead, UK), and ammonium acetate was purchased from Fisher Scientific (Loughborough, UK). All lipid standards were obtained from Avanti (Alabama, USA). Avanti Odd-Chained LIPIDOMIX Mass Spec Standard was used to generate calibration curves and to spike QC samples at known concentrations. Stable isotope labeled (SIL) premixed standards (deuterated ceramide LIPIDOMIX and SPLASH LIPIDOMIX Mass Spec Standards) were used as internal standards (IS).

NIST SRM 1950—Metabolites in Frozen Human Plasma was sourced through Merck (Gillingham, UK). A full description of this material is provided in its certificate of analysis (COA) available from www.nist.gov/srm.

NIST SRM 971a—Hormones in Frozen Human Serum was obtained from NIST (Gaithersburg, USA). A full description of this material is provided in its certificate of analysis (COA) available from www.nist.gov/srm.

Pooled "normal human plasma" containing anticoagulant, K2 EDTA, was sourced from Innovative Research (Peary Court, Novi, MI, USA) and used to generate calibration curves and quality control (QC) samples. Prostate cancer (PCa) sera-based samples (University of Surrey, Guildford, UK) from 42 individuals were pooled to form phenotypic groups. The pool samples for this study were approved by Yorkshire and the Humber-Leeds East Research Ethics Committee, U.K., under Reference No. 08/H1306/115+5 and IRAS Project ID 3582. These pools comprised of healthy controls (n = 6), patient controls (individuals diagnosed with prostate cancer) (n = 6), patients undergoing active surveillance (AS) (n = 6), brachytherapy (n = 6), hormone therapy only (n = 6), combined radiotherapy and hormone therapy (n = 6), or prostatectomy (n = 6).

Preparation of Calibration and Quality Control (QC) Stock Solutions

Avanti Odd-Chained LIPIDOMIX Mass Spec Standard (Table S1) was used to prepare three spiking solutions at 10×, 20×, and 50× dilution in IPA. Each standard in the mixture was at a concentration designed to cover the expected concentration ranges of biological fluids[4] examined in this study. Both the neat standard mixture or spiking solutions were added directly into commercially available pooled "normal plasma" at less than 5% v/v (to minimize the impact of spiking on the matrix), generating a 10-point calibration curve (Table S2) and seven QC samples (Table S3). QCs were prepared with concentrations based on the maximum calibration curve range to be validated for each of the respective analytes as follows: ULOQC 1 = 100%, ULOQC 2 = 80%, HQC = 70%, MQC = 40%, LQC= 6.4%, LLOQC 1 = 5%, and LLOQC 2 = 2% (Table S3). QCs for NIST SRM plasma and serum cross validation were prepared at LQC, MQC, and HQC concentrations only.

Six dilution integrity QC samples were prepared by spiking plasma at ×2 ULOQ2 concentration (see Table S3) prior to extraction and diluted (1:5 v/v) to ensure that the final concentrations fell within the validated calibration ranges (see Table S2). Recovery QCs were prepared at LQC, MQC, and HQC concentrations by the addition of Avanti Odd Chain LIPIDOMIX Mass Spec Standard to the Internal Standard solution described below.

PCa pooled cancer study samples were prepared in LoBind Microtubes (Eppendorf Ltd., Stevenage, UK). Both a study reference (SR) pool of all 7 phenotypic pools, the PCa sera-based samples, and the NIST SRM 1950 human plasma were also used to help assess the methodology as described below.

Preparation of Internal Standard Solutions

An IPA/ACN (1:2 v/v) solution containing a 500-fold dilution of the deuterated ceramide LIPIDOMIX (Table S4A) and SPLASH LIPIDOMIX (Table S4A) Mass Spec Standards were used to prepare the calibration and QC samples via protein precipitation. The stable isotope labeled standards were used as internal standards throughout the study.

Sample Preparation

Sample preparation used the procedure described by Sarafian et al.[21] and was adopted for all calibrants, QCs, and samples. Aliquots of plasma (25 μL) were transferred to low protein binding Eppendorf tubes followed by 125 μL of IPA/ACN (1:2, v/v) to precipitate proteins. Samples were vortex mixed for 1 min prior to incubation at −20 °C for 10 min. The samples were then shaken at 500 rpm on a Thermo-Shaker PCMT (Grant-bio, Cambridge, UK) at 5 °C for 2 h to ensure complete protein precipitation. The extracted samples were then centrifuged at 10 300g for 10 min at 5 °C before transferring the supernatant to total recovery glass vials (Waters, Milford, MA, USA) for LC-MS/MS analysis.

UHPLC-MS Conditions

Analysis was performed on an ACQUITY I-Class Ultra Performance LC (Waters, Milford, MA, USA) comprising of a binary solvent manager, flow-through needle (FTN) sample manager, and column oven. Samples (1 μL polarity switching mode, 1 μL positive ESI mode, and 2 μL negative ESI mode) were loaded onto a 2.1 × 100 mm, 130 Å, 1.7 μm ACQUITY BEH Amide Column (Waters, Milford, MA, USA) which was maintained at 45 °C. The lipids were separated via multilinear gradient elution at a flow rate of 0.6 mL/min; mobile phase A was composed of 95% ACN, 5% 10 mM ammonium acetate (v/v), and mobile phase B was 50% ACN, 50% water, 10 mM ammonium acetate (v/v). The initial gradient conditions were 99.9% mobile phase A, reducing to 80% mobile phase A at 2 min and 20% at 5 min before returning to initial conditions at 5.1 min. Initial conditions were held from 5.1 to 8 min, allowing for re-equilibration prior to the next injection. The purge solvent (weak wash) was 95% ACN and 5% water, while the needle wash (strong wash) was 100% IPA.

MS detection was performed on a Xevo TQ-XS Mass Spectrometer (Waters, Wilmslow, UK) operating in multiple reaction monitoring (MRM) ESI Mode. The source parameters for each class of lipid were optimized using SPLASH LIPIDOMIX Mass Spec standards. The MS source capillary was maintained at 2.8 kV in positive ESI and 1.9 kV in negative ESI. The desolvation and source temperatures were set to 500 and 120 °C, respectively. The desolvation, cone, and collision gas were maintained at 1000 L/h, 150 L/h, and 0.13 mL/min, respectively, and the nebulizer gas was operated at 7.0 bar (see Table S5). The mass spectrometer was operated using either polarity switching, positive (ESI+), or negative (ESI−) ion modes. Both positive mode and negative targeted ESI plasma screen analyses were performed using the list of compound-specific parameters and MRM transitions detailed

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in the Supporting Information (Tables S6, S7). Conditions for the polarity switching method used for validation are detailed in Table S8, and the prostate cancer proof of concept application MRMs are listed in Table S9.

MRM Library Generation

The library used in this study was generated using authentic analytical standards to optimize the collision and cone voltages required to fragment lipids of specific classes. Once the appropriate energies required and the most intense fragment ions for each class were determined, these were used for lipids of the same class. Theoretical fragments for lipids of the class were searched against databases such as LIPIDMAPS and the Human Metabolite Database (HMDB),[22] added to the library, and assigned the appropriate collision energy and cone voltages.

Assay Robustness

Robustness Testing: Retention Time Reproducibility. Robustness testing to assess retention time reproducibility and the impact of different batches of stationary phase on retention times was performed as part of the method development process.

Column Life Testing. Three replicates of 100 μL of NIST SRM 1950—Metabolites in Frozen Human Plasma were protein precipitated using 400 μL SPLASH LIPIDOMIX:IPA (1:100, v/v) and shaken at 5 °C for 2 h followed by centrifugation (10 300*g*, 10 min, at 5 °C). Supernatants were then transferred to glass vials for LC-MS/MS analysis, and a total of 1550 sample injections were performed continuously to simulate the analysis of a large batch.

Column Batch Testing. In order to compare different batches of columns a UPLC vial containing a 100× dilution of SPLASH LIPIDOMIX Mass Spec Standard in IPA was prepared. After "conditioning" with 50 injections of IPA, each of the five columns evaluated received 150 injections of the diluted SPLASH LIPIDOMIX Mass Spec Standard sample. Each of the five columns were manufactured using different batches of the BEH Amide (130 Å, 2.1 × 100 mm, 1.7 μm) stationary phase. The serial numbers were Column 1 #0164371711, Column 2 #0166373411, Column 3 #0154360781, Column 4 #163371451, and Column 5 #016037003. Column 1 was also used to perform the column life testing.

Method Validation

Method validation was based as far as practicable on the FDA "Guidance for Industry" on bioanalytical methods.[23] However, as the assay contained in excess of 400 lipids, over a wide range of concentrations, less stringent acceptance criteria were applied than would be employed for the quantification of a single pharmaceutical. The following assessments were performed over three separate occasions on different days.

Linearity. The screening methods were not validated for quantification to the same extent as the polarity switching method, which is intended for biomarker confirmation. To be considered linear, the R^2 correlation coefficient determined from calibration standards was required to be >0.95 for the positive and negative mode plasma screen analyses (stage one: 'omics screen assay) and >0.99 using the polarity switching screening method (stage two: targeted method). Standards deviating from the nominal values by more than 25% were excluded. Per the FDA guidelines, for acceptance, at least 75% of the calibration standards had to be included and no more than two consecutive standard concentrations could be removed from the regression.

Intra- and Interassay Precision. To determine assay precision, seven replicate QC samples at seven concentrations (as described above) were prepared in a single batch using the methods described (Table S2 and Table S3).

The intra-assay variabilities of the three MRM methods (polarity switching, positive ion mode, and negative ion mode screen) were determined using the CV for replicate assays (n = 6) for each of the selected QC concentrations on a single occasion. Interassay variability was performed on three separate days (Day 1, Day 2 (48 h), and Day 3 (144 h)) using QC samples at three concentrations (n = 18) with the CVs obtained used to determine interday precision. The acceptance criteria employed in this study were those in the FDA "Guidance for Industry" on bioanalytical methods, where a minimum of 2/3 of the QC samples must have had a deviation of no more than 25% from their nominal concentration—and at least 50% of the QC injections at each concentration were required to meet these criteria. Each polarity switching run had a batch size of 103 injections with 42 QC injections for intra- and interday evaluation, while the positive and negative ion mode screens had batch sizes of 121 injections with 42 QC injections.

Stability. Stability of samples initially stored at −80 °C between analyses was evaluated using the response of standards in extracts by monitoring after 48 and 144 h. Samples were thawed at 5 °C for 2 h and vortexed for 30 s prior to reinjection. For analytes to be accepted as stable, concentrations had to be within ±10% of the original result.

Carryover. Carryover was assessed via the analysis of a double blank, which was run at the beginning of the run and immediately after a ULOQ calibration standard, and carryover was deemed acceptable if the response obtained was ≤20% of the average response of the LLOQ standards. For the internal standards (ISs), carryover of ≤5% of the average response from the calibration standards (including the single blank) was acceptable.

Matrix Interferences. Matrix-to-analyte interferences were investigated via the analysis of six double blanks for signals at the retention times of the analytes. Such responses were compared to the mean analyte responses determined in the LLOQ calibration standards. To be acceptable a minimum of five of the six double blanks had to have responses that were ≤20% of that seen in the LLOQ calibration standard. To meet the acceptance criteria, the SIL IS responses had to be less than 5% of the average IS response of the standards in the calibration curve in a minimum of five of the six double blanks.

Selectivity. IS to analyte selectivity was investigated with three aliquots of the same lot of blank matrix prepared using the IS solution, single blank (SB). Any response for any of the IS's at the retention time(s) of the analyte(s) was compared to the mean of the analyte(s) response(s) in the LLOQ calibration standards. To achieve the validation criteria, such responses had to be less than 20% of the LLOQ response.

Dilution Integrity. Six dilution integrity QC samples were prepared by spiking plasma at 2× ULOQ2 concentration prior to extraction and diluted (1:5, v/v) to ensure final concentrations fell within validated calibration ranges. A minimum of 2/3 of these dilution integrity QC samples must have had a deviation of no more than 25% from their nominal concentration—and to meet the validation criteria, at

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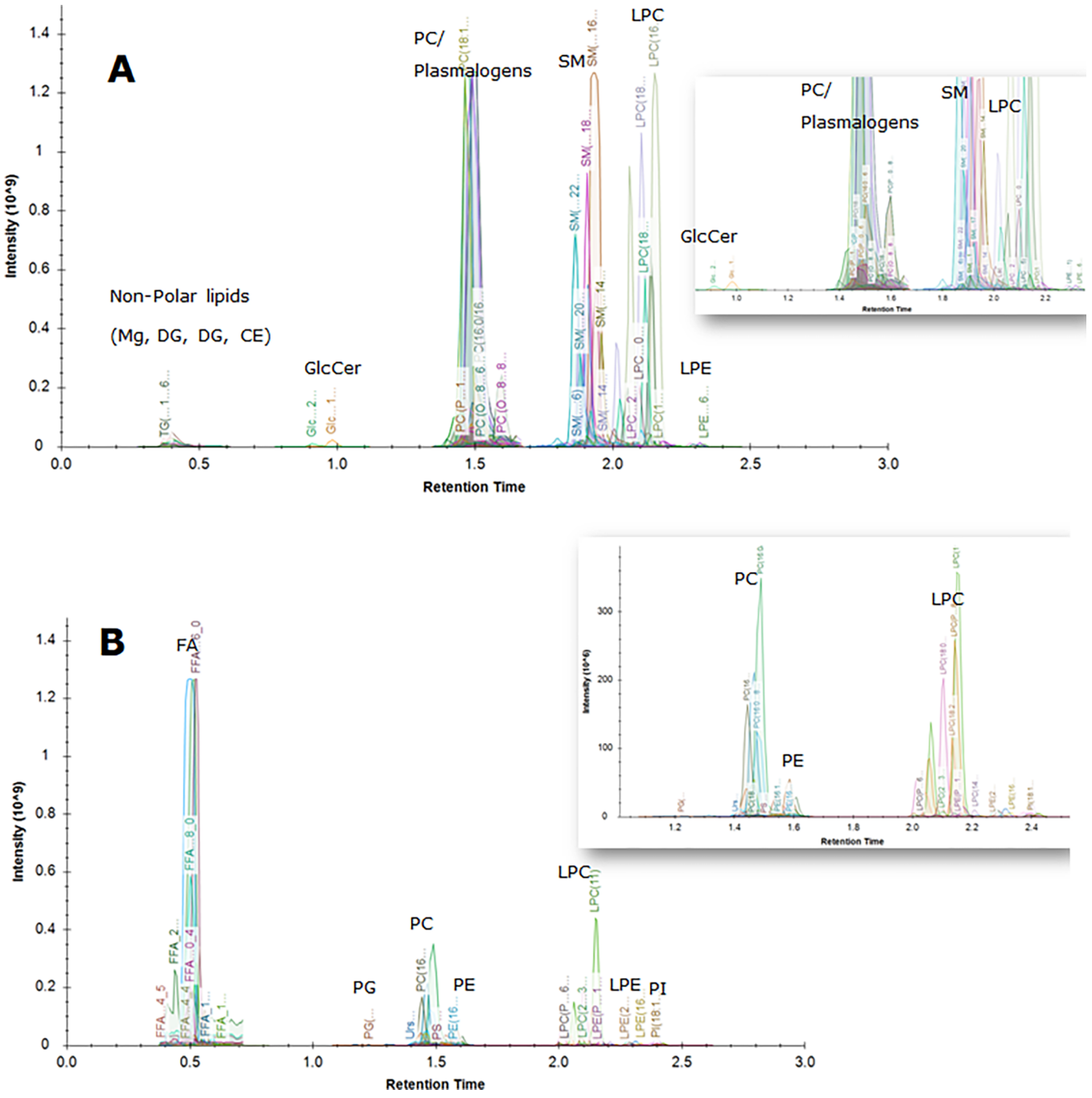

Figure 2. Chromatograms representing the NIST SRM 1950 plasma for the ESI+ screen (A) and ESI− screen (B).

least 50% of the QCs at each concentration had to meet these criteria.

Recovery. Experiments to assess recovery were performed using plasma samples spiked with Odd Chain LIPIDOMIX Mass Spec Standard at LQC, MQC, and HQC concentrations before protein precipitation versus extracts of blank plasma with Odd Chain LIPIDOMIX Mass Spec Standard postextraction (at LQC, MQC, and HQC). The % recovery was assessed by comparing the results of extracted samples with corresponding extracts of blanks spiked postextraction.

Plasma and Serum Cross validation. Cross validation experiments to determine assay accuracy and precision when analyzing serum rather than plasma were performed using the NIST SRM serum. For this both NIST SRM plasma and serum were prepared at the LQC, MQC, and HQC concentrations described above (Table S2 and Table S3). The intra-assay variabilities were determined using the CV for replicate assays ($n = 6$) for each of the selected QC concentrations on a single occasion. The % mean bias of the back-calculated concentrations were also assessed. In order to pass the QC samples must have had a deviation of no more than 25% from their nominal concentration—and for validation at least 50% of the QCs at each concentration had to meet these criteria.

Data Analysis

Data processed using TargetLynx XS software vs4.2 (Waters, Wilmslow, UK) were used to generate all quantitative outputs. Skyline (MacCoss Lab Software, University of Washington) was used only for data visualization and the generation of

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graphics. Quantification was based on the relative responses of the analytes measured against the standard curves for the SIL. The responses for the calibration standards (Response Cal), QCs, and the endogenous lipids were calculated by

$$\text{Response Cal} = \text{Area} \times (\text{IS Conc}/\text{IS Area})$$

- Area = Peak area.
- IS Conc = Concentration of selected IS for the lipid class.
- IS Area = Area of selected IS for the lipid class.

The Avanti Odd-Chained LIPIDOMIX Mass Spec Standard signal responses were used for the construction of the calibration curves for the various lipid classes and to generate a linear regression curve with a 1/X weighting. Concentrations of endogenous lipids and back calculated spiked standards in the QCs were determined by their response compared to those of these calibration curves.

Ceramides were quantified using the known concentration of the deuterated ceramide LIPIDOMIX Mass Spec Standard internal standard responses via a single point calibration approach.

$$(\text{Analyte Area}/\text{IS Area}) \times \text{IS concentration}$$

The MS lipidomic data relating to this manuscript have been uploaded to the EMBL-EBI MetaboLights database[24] with the data set identifier MTBLS4906.

RESULTS AND DISCUSSION

Method Development

There are multiple published methods for lipid quantification using either infusion-based MS or LC-MS/MS methods, which can detect 1000 lipids or more.[18,25] However, these methods are often time-consuming (30−60 min/sample), suffer from analyte coelution and poor resolution of isobaric species, and are typically focused on the quantification of high abundance lipids such as glycerolipids.[19,26] Although more rapid 2 min methodologies have been reported, these methods also suffer from ion suppression and lack of isomeric resolution.[27] Other rapid approaches using ion mobility have been used to quantify approximately 300 lipids in 4 min.[28]

From an "analytical chemist's" point of view, lipids are frequently considered to be either polar (mainly glycerophospholipids, sphingolipids, and saccharolipids) or nonpolar (glycerolipids, sterol lipids, and fatty acyls). Polar and nonpolar lipids require different procedures in their sample preparation, chromatographic separation, and mass spectrometric detection[29] for optimal analysis. HILIC methodologies are most suited to the separation of polar and ionic compounds; nonpolar compounds on the other hand are not retained and elute with the solvent front. The HILIC-MS/MS methodology presented here was specifically designed to rapidly measure the low abundant and bioactive polar lipids such as PA, PS, and LPE, which play key roles in cell signaling and cell recognition and are important biomarkers of diseases such as cancer and diabetes, etc.,[30] as well as monitoring the common, highly abundant lipids.

Analysis of lipids in serum and plasma requires the quantitative extraction of the lipids and, critically for robustness, the removal of endogenous proteins to prevent their precipitation during LC-MS analysis and subsequent fouling of the LC system. The extraction of lipids from plasma has been extensively reviewed elsewhere;[31] in this study we chose to employ a simple isopropanol/acetonitrile solvent mixture to precipitate the proteins and extract the lipids, as previously described by Sarafian et al.[21] The advantage of this methodology is that it is rapid, does not require the separation of immiscible solvent layers, and lends itself to automation. This extraction procedure also has the benefit that the resulting solvent-based solution can also be used for the analysis of polar small molecule metabolites, thus allowing a comprehensive view of changes in both the lipidome and metabolome. The lipid extract was separated by a HILIC column using a 5 min acetonitrile/aqueous formic acid gradient separation, with an overall analysis time of 8 min. The lipid elution order was based on the polarity of the lipid headgroup with the neutral lipids such as mono-, di-, triglycerides, FFA's, and cholesterol esters eluting earliest, at or just after the solvent front, followed by the ceramides, hexosyl ceramides, PG, PC, SM, LPC, LPE, PS, PA, PI, LPA, and LPI. The average peak width was 6 s at the base giving a peak capacity of ca. 50 for the 5 min separation (overall analysis time 8 min). Whilst this peak width can be considered quite wide for a UHPLC separation, it is important to note that the gradient is quite shallow and the lipids elute by class. These wider peaks facilitate the use of several simultaneous MRM transitions for the simultaneous accurate quantification of multiple lipids. Example chromatograms representing the NIST SRM 1950 plasma extract for positive and negative ESI are shown in Figure 2. Additional chromatograms corresponding to the odd chain mix and SPLASH LIPIDOMIX Mass Spec standards are provided in Figure S1 and Figure S2, respectively.

Analytical Strategy/Deployment

For the assay described here a two-stage approach was employed. Stage one offers a semiquantitative assay providing a screen for approximately 500 preselected bioactive lipids. This is performed using the single polarity methods in positive and/or negative ESI mode for broad profiling in epidemiological studies or when prior biological knowledge is limited. Stage two represents a more focused approach building on the knowledge derived from stage one, from which the bioactive lipids considered as biologically important as potential "biomarkers" can be determined. Similarly, this method can be used immediately where an initial hypothesis exists without the need for the stage 1 screening. Thus, with this more targeted assay, the number of lipids that require analysis is greatly reduced and the assay platform can be limited to their validation. Here an ion polarity switching mode method was employed to improve throughput.

It has been broadly demonstrated that the ionization efficiencies of polar lipid species predominantly depend on the electrical properties of their head groups, and the effects of acyl chains are negligible at lower concentrations (i.e., concentrations at which lipid aggregates begin to form) in the ESI-MS data acquisition.[32] Therefore, one species in a polar lipid class can be employed as an internal standard to quantify multiple individual species within the same class. Reasonable accuracy (>95%) in the MS survey scan mode can be achieved providing the measurement is made in the low concentration region.[32]

The stable labeled isotope (SIL) used as internal standards, and odd chain standards used as calibrants, are eluted within the LC peak envelope of each endogenous lipid class and experienced the same ionization conditions. This allowed for significantly fewer standards to be employed, thereby

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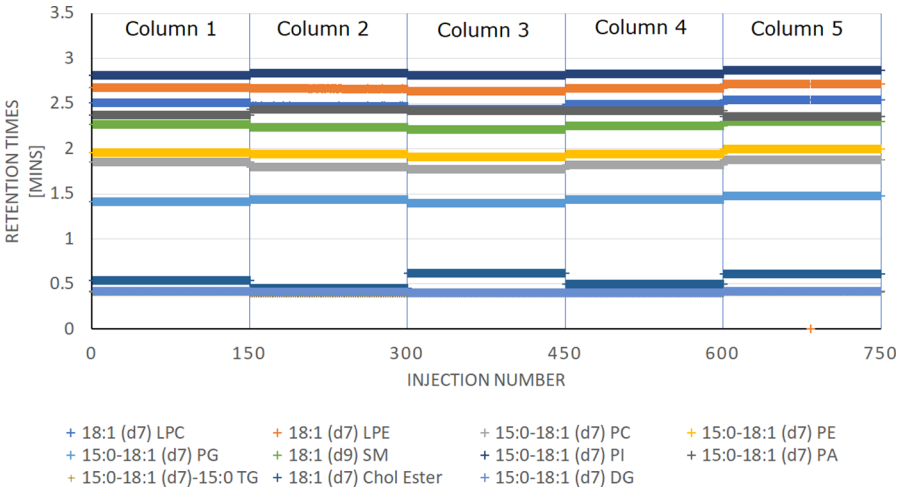

Figure 3. Evaluation of column to column t_R variability. 150 injections on each of the five different batches of stationary phases were performed.

Table 1. Average t_R for 5 Columns Originating from Different Lot Numbers (n = 750; i.e., 150 ×5)

	18:1 (d7) LPC	18:1 (d7) LPE	15:0−18:1 (d7) PC	15:0−18:1 (d7) PE	15:0−18:1 (d7) PG	18:1 (d9) SM	15:0−18:1 (d7) PI	15:0−18:1 (d7) PS	15:0−18:1 (d7) PA	15:0−18:1 (d7)-15:0 TG	18:1 (d7) Chol Ester	18:1 (d7) MG	15:0−18:1 (d7) DG
average t_R (min)	2.49	2.68	1.82	1.95	1.43	2.25	2.83	2.27	2.40	0.40	0.54	0.50	0.41
std. dev.	0.03	0.03	0.04	0.03	0.03	0.03	0.02	0.03	0.03	0.01	0.06	0.04	0.01
CV (%)	1.36	0.98	2.10	1.52	1.95	1.33	0.79	1.21	1.30	2.46	11.90	8.01	2.18

simplifying the assay and reducing operational costs. As these standards were commercially available, the process of calibration line generation was significantly simplified, as no accurate weighing of authentic standards was required (and thus assay transfer between laboratories should be simplified).

The reproducibility and ruggedness of the methodology was evaluated via the analysis of a large batch of injections of the NIST SRM 1950 control human plasma (n = 1550). The coefficients of variation (CV) of the lipid retention times ranged from CV = 0.40% for the LPC to CV = 1.96% for PG with the majority of CVs ≤ 1.0%, Figure S3. These results suggest that the assay had good reproducibility and is thus well suited to the analysis of samples from large cohort studies.

The method reproducibility was also evaluated between columns to assess transferability of the assay. The lipid standard retention times (t_R) were determined using five separate HILIC columns containing different batches of stationary phase (ACQUITY BEH Amide column, 1.7 μm). A total of 150 injections were performed on each of the five columns, and the retention time CVs varied from 0.40% for MG to 2.83% for the PI (Figure 3; Table 1).

As can be seen from these data, there were small differences between the mean t_R's obtained for the different batches of 1.7 μm ACQUITY BEH Amide stationary phase (though there was good reproducibility for the data for each column). Based on the t_R of the standards, batch correction to account for the use of different columns, in, e.g., different laboratories, or if a column needed to be changed part way through a long clinical study, could be performed.

Lipid Screening Platform

A plasma or serum lipid screening method was developed based on literature findings of the most biologically important lipids and the most recent harmonized studies using NIST SRM 1950 plasma.[11,18,19] Recent studies suggest lipid species in membranes act not as single molecules but as a collective and therefore would benefit from being analyzed both quantitatively and comprehensively to better understand their biological function.[13,33] An example of an investigation showing bioactive lipid species which act in concert was the FINRISK 2002 Cohort large-scale study, which identified circulating ceramides as risk markers for cardiovascular disease,[34] fatty-acid-derived pro-inflammatory mediators (for example, prostaglandins and leukotrienes) and anti-inflammatory mediators (for example, resolvins, protectins, and maresins).[35] Such promising findings have driven interest in lipidomics across related research communities. Therefore, it is important that any methodology employed be comprehensive and quantitative, or at least semiquantitative.

In this study the lipid library containing MRM transitions for over 2000 lipids covering "markers" for various classes was used to develop a "targeted discovery" screen. Analysis was then performed on samples using a predefined set of endogenous lipid species from this library. The majority of the library consisted of phospholipids with around 390 PC and plasmalogens, 280 PE, 300 PA, 220 PG, 280 PS, and 90 PI. At least two MRM transitions were assigned to each lipid with one MRM transition used for quantification and the second for confirmation. The library also contains around 25 LPC, 25 LPE, 25 LPI, 50 Cer, 25 GlcCer, 30 SM, and these were monitored using individual SRMs. The library contains a further 300 nonpolar lipids such as CE, MG, DG, and TG that can be included in future screening methods, if needed.

The FA chains used for this screen were predominantly those most abundant FAs in plasma. Oleic acid (18:1) followed by palmitic acid (16:0) and stearic acid (18:0) make up 78% of all free FA in plasma.[36] Some FA species within the unsaturated family are long-chain polyunsaturated FAs (LC-PUFAs) which are taken up in the diet. These are

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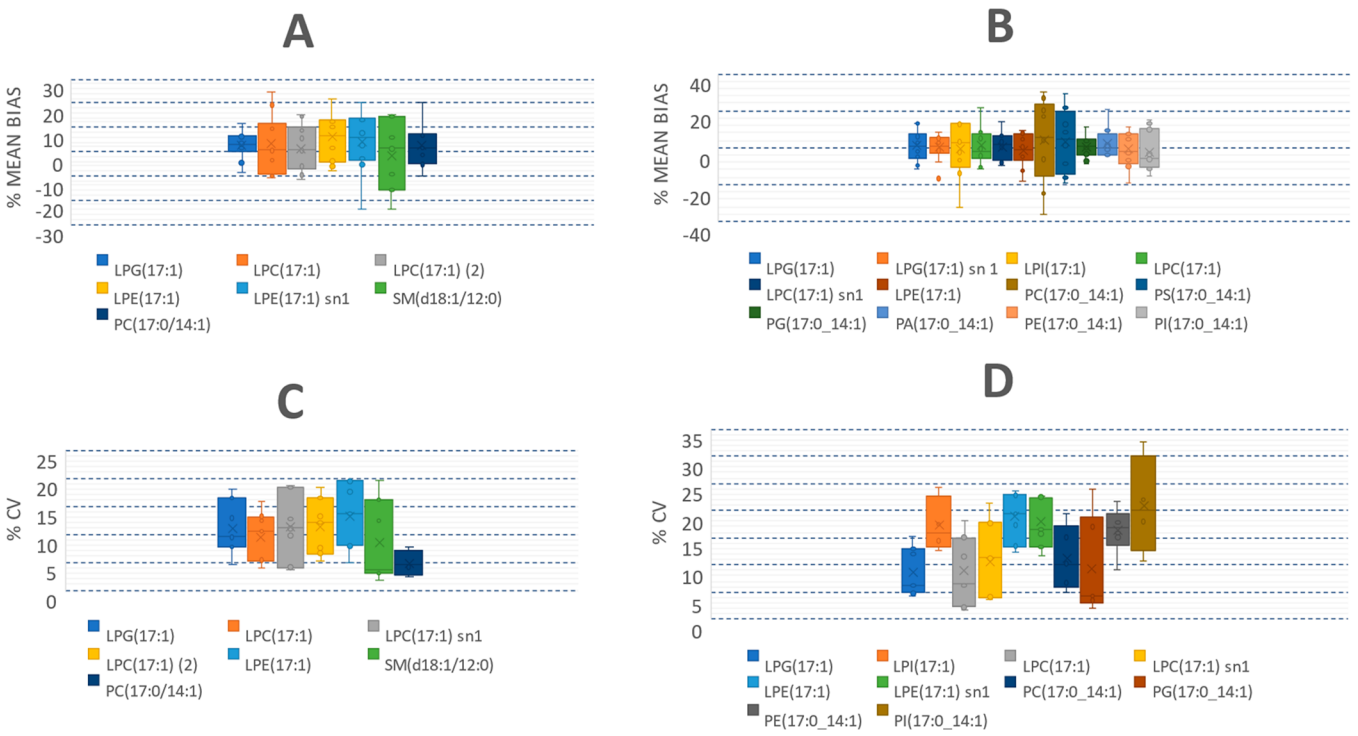

Figure 4. Intraday % mean biases of the calibration standards for the positive ion (n = 3) (A) and negative ion (n = 3) (B) methods are provided. Intraday QC CVs for positive mode CVs (n = 6 for each point on the bar) (C) and negative ESI (D) are also shown (for full results, see Table S12). Failed ULOQ and LLOQC CVs in negative mode were excluded.

essential because they cannot be synthesized de novo. Linoleic acid (18:2) and arachidonic acid (20:4) are the main PUFAs (accounting for 8% of the total free FA), while essential PUFAs such as ω-linolenic acid (18:3 ω-3), eicosapentaenoic acid (20:5, EPA), and docosahexaenoic acid make up about 1% of the total free FA.[36] The screening method was generated largely based on the assumed FA chain distribution described here. The final screens for validation were optimized based on literature searches, particularly those discussing lipid coverage for interlaboratory studies[4,11,18−20] as well as in-house method development testing. This set of 433 lipids was used to evaluate the reproducibility and accuracy of the methodology.

In contrast to the areas of clinical and candidate drug evaluation where there are rigorous guidelines for method validation, there is less guidance, or agreement, on the criteria for assay acceptance for biomarkers. Therefore, for this assay a fit-for-purpose approach has been adopted here, following the FDA "Guidance for Industry" on bioanalytical methods where possible.

Following the development of a suitably robust UPLC-MS/MS methodology, intraday assessments of the method were performed for various lipid classes using the polarity switching method as well as positive and negative ion ESI screens. Interday validation experiments were also performed using the polarity switching method only. A range of analytical attributes were investigated, including linearity, intra- and interday accuracy and precision, lower and upper limits of quantification (LLOQ, ULOQ) specificity, carryover, matrix, and other interferences.

Validation of Single Polarity Assays (Stage One). The lipid screening method was developed using ESI mass spectrometry operated in either positive or negative modes, allowing for the detection of over 433 lipids. A total of 431 MRM positive ion (239 individual lipids) and 446 MRM negative ion (232 individual lipids) ESI transitions were employed for the quantification of endogenous plasma lipids. The total number of lipids monitored using these screening methods was 433 with an overlap of 38 lipids in both modes. Representative chromatograms from these screening methods are shown in Figure 2. Excluding the standards used for quantification, a total of 13 acyl carnitines, 23 bile acids, 36 FFA, 6 cholesterol esters, 14 ceramides, 23 GlcCer, 10 LacCer, 6 LPA, 24 LPC, 23 LPE, 23 LPI, 2 LPS, 5 PA, 38 PC diacyls, 39 plasmalogens, 24 PE, 22 PG, 23 PI, 12 PS, 23 SM, 3 sphingolipids, 5 mono-, 11 di-, and 16 triglycerides were used for the two screening methods. In addition, 17 LPC, 10 LPE, and 17 PC species were monitored in both modes.

Although these single polarity screening methods were not intended to be validated for the quantification of lipids, it was felt to be important that the reproducibility, sensitivity, and dynamic ranges of the methods be determined, and these parameters were measured via an abbreviated validation study.

The intraday accuracy was assessed using the percentage mean biases of back-calculated concentrations for the calibration standards using the three methods, polarity switching and positive and negative ESI screens. The results showed that the back-calculated concentrations for the nine lipid standards were within the ±20% CV of the nominal value, as shown in Figure 4. The full results, including CVs, are shown in Table S10 and Table S11.

The intraday precision of the positive and negative mode assays was determined by examining the CVs of replicate QC samples. The intraday precision for the positive and negative mode screening methods are displayed in Figure 4C and 4D. The full results, including CVs, are provided in Table S12A,B. All of the CV values calculated using the positive ESI data were below 20% across all 42 QC samples at seven concentrations except for the HQC and ULOQC for PC (17:0/14:1) due to

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detector saturation as discussed later. The CVs corresponding to the negative ESI screen were slightly higher; however, the LQC, MQC, and HQC samples were all below 25% except for PI (17:0/14:1) at the HQC level. These classes of lipids including PG are referred to as anionic lipids and have been shown to benefit from pH modification of the mobile phase to improve responses and peak shape.[37] Nevertheless, the results obtained here were considered to be acceptable for the level of quantification necessary for this type of screening analysis.

Polarity Switching Analysis (Stage Two). To demonstrate the extension of the methodology to the provision of accurate measurements of a number of specifically targeted lipids, the assay performance was evaluated for a smaller subset of lipids. For the quantitative assay, a total of 16 odd chain lipid standards were monitored using a combination of 33 positive and negative ion mode transitions. The PL standards available in the mix were LPG (17:1), LPA (17:1), LPI (17:1), LPC (17:1), LPE (17:1), PA (17:0/14:1), PC (17:0/14:1), PE (17:0/14:1), PG (17:0/14:1), PI (17:0/14:1), PS (17:0/14:1), and SM(d18:1/12:0). The nonpolar lipids DG (17:0/17:0), TG (17:0/17:0/17:0), and CE (17:0) were also included but were not found to be suitable for quantification using these conditions and were included for monitoring purposes only. Commercially sourced SIL lipids (Avanti SPLASH and Deuterated Ceramide LIPIDOMIX Mass Spec Standards) were employed as internal standards.

Of the 33 odd-chain transitions monitored (nonpolar lipids included) by the polarity switching method, 26 gave a satisfactory linear response, generating correlation coefficients (R^2) of 0.97 or better with (1/X) weightings (Table S13). A total of 26 of the 29 phospholipid signals met the above-stated criteria, the remaining 3 were suitable for monitoring fold changes. Example calibration curves for key phospholipid standards are shown in Figure S4. Using the available premixed standards, the PC calibration curve required truncation in positive mode at a concentration of 52 μM (37 750 ng/mL) due to detector signal saturation, likewise for LPC (17:1) (508.3 > 184.1) saturation was observed at 56.7 μM (Figure 5A). PC plasmalogen species which are present in plasma are of low abundance, and hence were quantified against the truncated calibration curve of PC (17:0/14:1) ES+ (718.5 > 184.1) over the range of 2 to 52 μM.

However, the PC calibration in negative mode covered the range (2.1 to 263 μM or 1510 to 18 8750 ng/mL) Figure 5B. Saturation of the detector signal was also observed for SM (d18:1/12:0) (647.5 > 184.1) between 40.2 and 50.3 μM.

The IS responses from the SIL standards were used whenever a lipid of the same class was quantified. However, it was interesting to note that a good linear response was observed in both positive and negative ESI for lipids such as LPG (17:1) even without employing an internal standard correction (Figure S4E for LPG (17:1) in positive ESI).

The intra- and interday % mean biases of the back-calculated concentrations using the polarity switching method were less than ±20% of the nominal concentrations as shown in Figure S5A and S5B. The back calculated concentrations of the LQC, MQC, and HQC samples within the calibration range showed that intraday precision for the polarity switching method ranged from 1.57 to 22.52% for the lipids PE (17:0/14:1) and PI (17:0/14:1), respectively (n = 6) (Table S14), and 2.96–23.96% for the interday comparison (n = 18) (Table S15). The majority of the interday CVs for the concentrations assessed showed a CV < 10%. Lipid standards corresponding to the

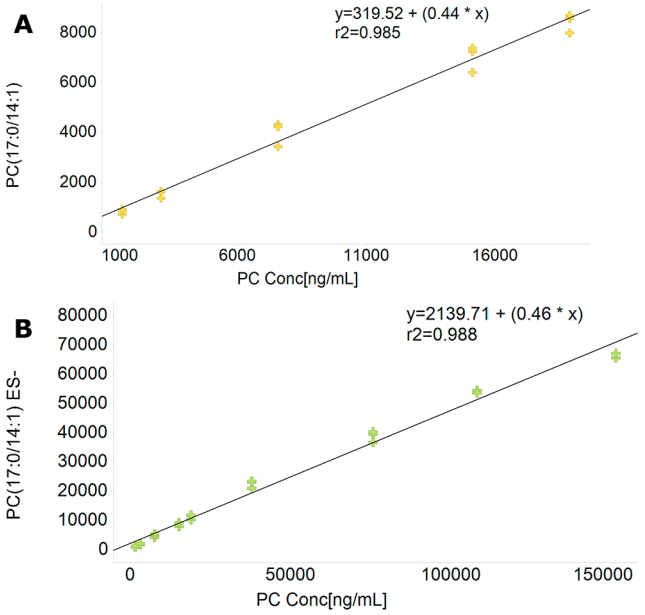

Figure 5. (A) Truncated calibration curve for PC (17:0/14:1) in ESI + mode. (B) Calibration curve for PC (17:0/14:1) in ESI− mode showing good linearity up to 18 8750 ng/mL.

concentrations of the HQC and ULOQ had mean biases >25%. Although most of the analytes measured were within the acceptable range of <20%, intraday CVs were found to be less than 25% for PE and PI standards at secondary lower limit (LLOQQC2) and HQC levels (Table S14). The SM standard, however, failed to meet the 25% CV limit when using the choline headgroup transition (647.5 > 184) at the HQC and can only be used in monitoring mode at these concentrations.

Matrix Interference. The lipid odd chain mix was spiked into calibrant solvent to provide a comparison solution and the MS responses compared to the standards spiked into the plasma matrix at the same concentration in order to assess matrix effects at the LLOQ. No interferences were observed at the retention times associated with the evaluated lipid standards or SIL standards. For the matrix-to-analyte and matrix-to-internal standard, acceptance criteria were set at ≤20% and ≤5% CV, respectively. Based on the odd chain mix, 21 of the 29 monitored transitions (matrix-to-analyte) and 15 of the 21 transitions (matrix-to-internal) were within acceptable levels (Table S16 and S17). Of the polar lipids only LPS (17:1) and PS (17:0/14:1) gave values of ≥20% CV (Table S16). The majority of the lipid species that failed to meet these criteria were the nonpolar lipids eluting at the beginning of the chromatogram where there was only limited resolution. (As previously noted, the aim of this methodology was only to monitor these lipids for gross changes, and thus this level of interference was deemed acceptable).

Selectivity: Internal Standard to Analyte Interference. Three single blank plasma sample spiked only with internal standards were used to assess internal standard to analyte interference. With the exception of PS (d7) and Cer (d18:1/24:1) (d7), all the internal standards gave values of ≤5% CV (Table S17).

Sample Dilution Integrity. It is often the case that the reanalysis of samples which have concentrations which exceed the validation range of the assay is required, and thus the dilution accuracy of the methodology was evaluated. Six QC

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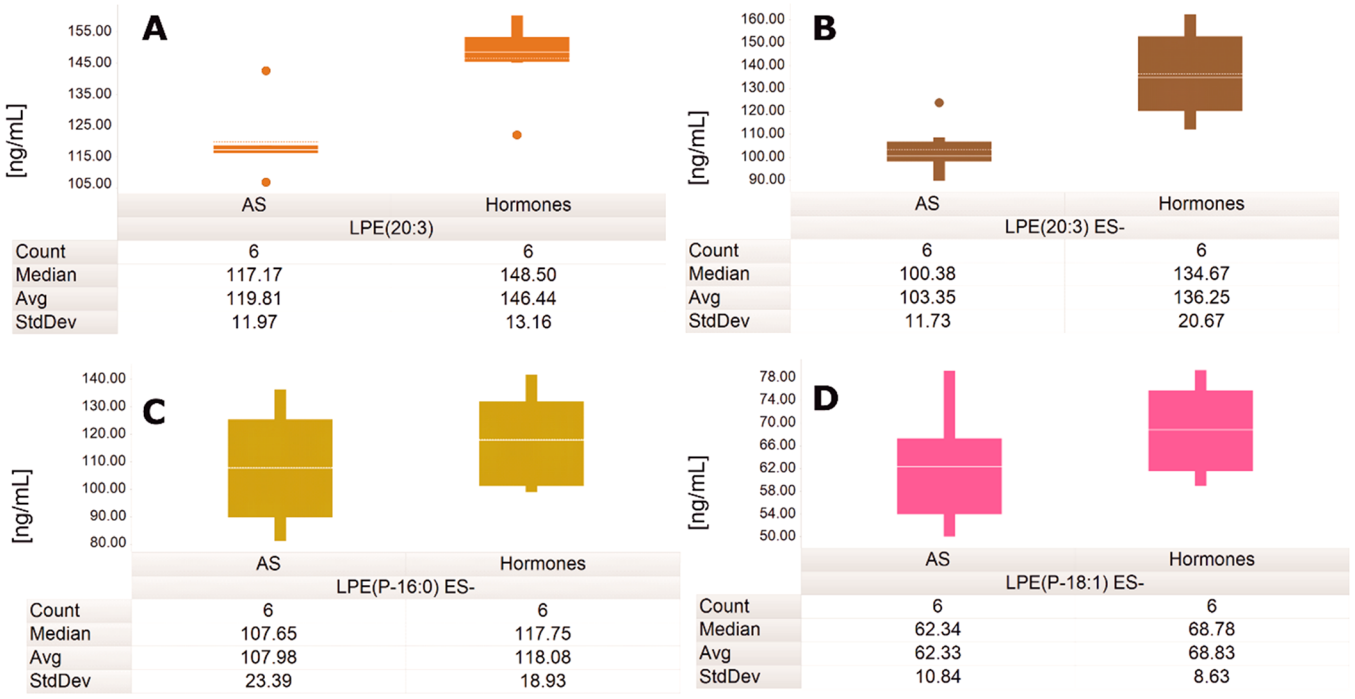

Figure 6. Box plots representing key LPE lipids, which show differential expression between active surveillance (AS) and hormone therapy treated individuals. LPE (20:3) was analyzed in both positive (A) and negative ESI (B). LPE (P-16:0) (C) and LPE (P-18:1) (D) were analyzed in negative mode only. Quantification was performed using LPE (17:1) calibration curves.

samples were prepared by spiking with the odd chain mix at twice the ULOQ, and these samples were then diluted (1:5 v/v) prior to analysis. The resulting data demonstrated that the samples can be diluted 5-fold to bring them within the linear dynamic range of the assay. For example, after dilution the LPC and SM lipids had % mean biases of 1.62 and 1.59%, respectively.

Carryover. The Odd Chain LIPIDOMIX Mass Spec Standard calibration standards were employed to assess assay carryover. The results were considered to be acceptable if the response for the lipid signals in the double blank sample were ≤20% of the average response from the acceptable LLOQ standards in the batch (Table S18). Using the SIL internal standards, the carryover limit of ≤5% was achieved, with the maximum carryover being 3.1% for the PI (d7), as shown in Table S19.

Recovery. The Odd Chain LIPIDOMIX calibration Mass Spec standards spiked in plasma prior to protein precipitation and during extraction were employed to assess recovery using samples prepared at the LQC, MQC, and HQC concentrations. On analysis the majority of those spiked pre-extraction had and recoveries of 95−105% with CV's of <10% when compared to those spiked post-extraction. A few, particularly at the LQC level, had higher CV's, but all were less than 20% (Table S20a). The % mean biases of the back-calculated concentrations obtained using the polarity switching method were also acceptable with values of between 95-105 ±25% of those of the post-extraction spiked samples. A total of 47 out of the 51 mean values for the pre-spiked QCs and 43 out of 51 recovery QCs mean values were within ±25% of the nominal concentration (Table S20b).

Plasma vs Serum Cross Validation. NIST 1950 SRM plasma and serum samples were prepared at LQC, MQC, and HQC concentrations as part of a cross validation experiment to assess accuracy and precision using these different matrices against calibration curves and QCs prepared using control plasma. The majority of the odd chain calibration standards spiked into either plasma or serum had CV's < 10% (Table S21a). The % mean biases of back-calculated concentrations using the polarity switching method were between ±25% of their nominal concentrations and were therefore considered to be acceptable for this application. In total, 50 out of 54 mean values met these criteria for the control QCs, 49 out of 54 NIST SRM 1950 plasma QCs and 48 out of 54 NIST SRM 971a serum QCs mean values met these criteria (Table S21b). The failures were the PC (17:0/14:1) and SM (d18:1/12:0) species in all three matrices as well as LPC (17:1) for the serum.

Application of the Targeted LipidQuan Methodology for the Analysis of Serum from a Prostate Cancer Study

Prostate cancer (PCa) accounts for approximately 40% of all cancers worldwide, but nevertheless both its diagnosis and prognosis remains challenging.[38] Circulating prostate-specific antigen (PSA) concentrations are the most common of the few blood-based protein biomarkers currently available in clinical practice. However, PSA by itself is not accurate especially as there is no reliable PSA range that is an explicit signifier for the presence of prostate cancer.[39] To predict the risk of relapse, and to reduce the need for active monitoring using PSA, the discovery of early biomarkers for prostate cancer progression has gained urgency.[40] Combinations of multiple levels of molecular information have the potential to improve biomarker panels and help gain better understanding into the biology underpinning a given disease, such as pancreatic and ovarian cancer, inflammatory bowel disease, and Alzheimer's disease.[41−44] Studies on potential biomarkers and measurable signatures for PCa thus remain a key area of translational research. Appropriate assay accuracy, precision, and sensitivity

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still need to be attained to make these a reality in clinical settings.

Previous studies have identified LPC, PC, LPE, PE, and SM as providing potential diagnostic signatures to characterize PCa.[45,46] Numerous studies have highlighted the intricate relationship between oncogenic signaling and lipid metabolism regulation to promote cancer cell growth and survival. Lipids also regulate the processes that initiate cell dissemination and metastasis formation, and to control the communication between cancer and immune cells within the cancer microenvironment.[47]

The lipid LC-MS workflow described within this paper was therefore applied to the analysis of serum samples from a PCa study. Phenotypic pools were created as described in the Materials and Methods by pooling sera from 42 patients comprised of 6 patient groups (PCa diagnosed, active surveillance (AS), brachytherapy, hormone therapy, combined radiotherapy with hormone therapy, or prostatectomy). For brevity here we discuss the use of the selected lipid targeted assay, with the lipid selection based upon information obtained from a previous multiomic study which utilized Stage 1 of the LipidQuan Methodology. The targeted polarity switching LC-MS method was used here to selectively monitor 39 endogenous lipids across the Cer, LPE, LPI, PG, PI, and SM classes, which appear to be key differentiators of sample cohorts (Figure S6A). An example chromatogram is shown in Figure S6B.

The samples were randomized and analyzed by the LC-MS platform described above, along with the QC samples at 3 concentrations, SR and NIST 1950 SRM serum samples. Calibration curves were analyzed at the beginning and at the end of the analytical batch. A representative chromatogram of the study samples is displayed in Figure S6C.

Typical R^2 values for the calibration curves ranged from 0.985 to 0.996 (Table S22), with examples shown in Figure S7. QC samples spiked with odd-chain lipid standards at 3 concentrations were used to monitor method precision. Box and whisker plots for these QC samples are represented in Figure S8. The results in Table S23 show that the CVs were all below 15% across the acquisition.

The analysis of the PCa-derived samples highlighted several significant lipid classes including the SM, Cer, and LPEs, which could be used to differentiate between the subject groups. For example, LPE (20:3), LPE (P-16:0), and LPE (P-18:1) were key lipids for differentiating between patient groups categorized as active surveillance (i.e., individuals diagnosed with prostate cancer and those undergoing hormone therapy). Box and whisker plots comparing the calculated concentrations of these LPEs are displayed in Figure 6. The concentration of LPE (20:3) was determined in both positive and negative ESI (Figure 6A and 6B) with the concentration relating to active surveillance individuals being quantified as 119.81 ng/mL and 103.35 ng/mL in positive and negative ESI, respectively. Comparatively, the concentration of LPE (20:3) in the hormone therapy patient pool sample was reported as 146.5 ng/mL in positive and 136.3 ng/mL for negative ESI. LPE (P-16:0) and LPE (P-18:1) were only measured in negative ESI but were also shown to be elevated for the hormone-treated group.

CONCLUSIONS

A rapid HILIC based LC-MS/MS method for the targeted quantification of over 400 polar lipids (selectable from a library of 2000 lipids) in 8 min, requiring only 25 μL of human plasma/serum, has been developed and subjected to a fit for purpose validation. The use of HILIC chromatography greatly simplified analysis since the lipids were eluted from the LC column according to the polarity of the headgroup. It was therefore only necessary to employ a small number of lipid standards to quantify lipids of the same class. The use of odd-chain lipid standards provided a cost-effective approach to the quantification of these lipids. The assay was found to be sensitive, robust, reproducible, and specific for the quantification of lipids in plasma and serum. The short analysis time makes the methodology ideal for the analysis of the large cohorts typically observed in population "omics" studies. The LC methodology was demonstrated as being robust for at least 1500+ injections and was transferrable across columns prepared from different batches of the same stationary phase. The methodology was applied to a human prostate cancer study and shown to differentiate between the individual treatment therapies and identify the lipids responsible for the statistical separation of the patient groups.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.2c00297.

Figures and tables of the concentrations of various types of standards used for calibration and QC purposes, optimized MRM transitions and mass spectrometer parameters, calibration regression, LLOQ, and ULOQ, intra- and interday accuracy and precision, matrix and standard interference, recovery performance, validation performance of analytes in plasma and serum, and prostate cancer proof of concept data (PDF)

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) O'Donnell, V. B.; Ekroos, K.; Liebisch, G.; Wakelam, M. Lipidomics: Current state of the Art in a Fast Moving Field. *WIREs: Syst. Biol. Med.* **2020**, *12*, e1466.

(2) Perrotti, F.; Rosa, C.; Cicalini, I.; Sacchetta, P.; Del Boccio, P.; Genovesi, D.; Pieragostino, D. Advances in Lipidomics for Cancer Biomarkers Discovery. *Intnl J. Mol. Sciences* **2016**, *17*, 1992.

(3) Murphy, R. C.; Gaskell, S. J. New Applications of Mass Spectrometry in Lipid Analysis. *J. Biol. Chem.* **2011**, *286*, 25427−25433.

(4) Burla, B.; Arita, M.; Arita, M.; Bendt, A. K.; Cazenave-Gassiot, A.; Dennis, E. A.; Ekroos, K.; Han, X.; Ikeda, K.; Liebisch, G.; Lin, M. K.; Loh, T. P.; Meikle, P. J.; Orešič, M.; Quehenberger, O.; Shevchenko, A.; Torta, F.; Wakelam, M. J. O.; Wheelock, C. E.; Wenk, M. R. MS-based Lipidomics of Human Blood Plasma - a Community-initiated Position Paper to Develop Accepted Guidelines. *J. Lipid Res.* **2018**, *59*, 2001−2017.

(5) Wenk, M. R. The Emerging Field of Lipidomics. *Nat. Rev. Drug Discovery* **2005**, *4*, 594−610.

(6) Shevchenko, A.; Simons, K. Lipidomics: Coming to Grips with Lipid Diversity. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 593−598.

(7) Yang, K.; Han, X. Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends Biochem. Sci.* **2016**, *41*, 954−969.

(8) Cajka, T.; Fiehn, O. Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics. *Anal. Chem.* **2016**, *88*, 524−545.

(9) Kopf, T.; Schmitz, G. Analysis of Non-esterified Fatty Acids in Human Samples by Solid-phase-extraction and Gas Chromatography/mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2013**, *938*, 22−26.

(10) Lísa, M.; Holčapek, M. High-Throughput and Comprehensive Lipidomic Analysis Using Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry. *Anal. Chem.* **2015**, *87*, 7187−7195.

(11) Lange, M.; Fedorova, M. Evaluation of Lipid Quantification Accuracy Using HILIC and RPLC MS on the Example of NIST® SRM® 1950 Metabolites in Human Plasma. *Anal. Bioanal. Chem.* **2020**, *412*, 3573−3584.

(12) Lipidomics Standards Initiative Consortium. Lipidomics Needs More Standardization. *Nat. Metab.* **2019**, *1*, 745−747.

(13) Vvedenskaya, O.; Wang, Y.; Ackerman, J. M.; Knittelfelder, O.; Shevchenko, A. Analytical challenges in human plasma lipidomics: A winding path towards the truth. *Trends Anal. Chem.* **2019**, *120*, 115277.

(14) Liebisch, G.; Ekroos, K.; Hermansson, M.; Ejsing, C. S. Reporting of lipidomics data should be standardized. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2017**, *1862*, 747−751.

(15) Koelmel, J. P.; Ulmer, C. Z.; Jones, C. M.; Yost, R. A.; Bowden, J. A. Common cases of improper lipid annotation using high-resolution tandem mass spectrometry data and corresponding limitations in biological interpretation. *Biochim. Biophys. Acta* **2017**, *1862*, 766−770.

(16) Ekroos, K. Lipidomics Perspective: From Molecular Lipidomics to Validated Clinical Diagnostics. In *Lipidomics: Technologies and Applications*, 1st ed.; Wiley Online Library, 2012; pp 1−20.

(17) Pinu, F. R.; Beale, D. J.; Paten, A. M.; Kouremenos, K.; Swarup, S.; Schirra, H. J.; Wishart, D. Systems Biology and Multi-omics Integration: Viewpoints From the Metabolomics Research Community. *Metabolites* **2019**, *9*, 76.

(18) Bowden, J. A.; Heckert, A.; Ulmer, C. Z.; Jones, C. M.; Koelmel, J. P.; Abdullah, L.; Ahonen, L.; Alnouti, Y.; Armando, A. M.; Asara, J. M.; Barr, J. R.; Bergquist, J.; Borchers, C. H.; Brandsma, J.; Breitkopf, S. B.; Cajka, T.; Cazenave-gassiot, A.; Checa, A.; Cinel, M. A.; Colas, R. A.; Cremers, S.; Dennis, E. A.; Evans, J. E.; Fauland, A.; Fiehn, O.; Gardner, M. S.; Garrett, T. J.; Gotlinger, K. H.; Han, J.; Huang, Y.; Neo, A. H.; Hyötyläinen, T.; Izumi, Y.; Jiang, H.; Jiang, H.; Jiang, J.; Kachman, M.; Kiyonami, R.; Klavins, K.; Klose, C.; Köfeler, H. C.; Kolmert, J.; Koal, T.; Koster, G.; Kuklenyik, Z.; Kurland, I. J.; Leadley, M.; Lin, K.; Maddipati, K. R.; McDougall, D.; Meikle, P. J.; Mellett, N. A.; Monnin, C.; Moseley, M. A.; Nandakumar, R.; Oresic, M.; Patterson, R.; Peake, D.; Pierce, J. S.; Post, M.; Postle, A. D.; Pugh, R.; Qiu, Y.; Quehenberger, O.; Ramrup, P.; Rees, J.; Rembiesa, B.; Reynaud, D.; Roth, M. R.; Sales, S.; Schuhmann, K.; Schwartzman, M. L.; Serhan, C. N.; Shevchenko, A.; Somerville, S. E.; St John-Williams, L.; Surma, M. A.; Takeda, H.; Thakare, R.; Thompson, J. W.; Torta, F.; Triebl, A.; Trötzmüller, M.; Ubhayasekera, S. J. K.; Vuckovic, D.; Weir, J. M.; Welti, R.; Wenk, M. R.; Wheelock, C. E.; Yao, L.; Yuan, M.; Zhao, X. H.; Zhou, S. Harmonizing lipidomics : NIST Interlaboratory Comparison Exercise for Lipidomics Using SRM 1950 - Metabolites in Frozen Human Plasma. *J. Lipid Res.* **2017**, *58*, 2275−2288.

(19) Thompson, J. W.; Adams, K. J.; Adamski, J.; Asad, Y.; Borts, D.; Bowden, J. A.; Byram, G.; Dang, V. D.; Dunn, W. B.; Fernandez, F. M.; Fiehn, O.; Gaul, D. A.; Huhmer, A.; Kalli, A.; Koal, T.; Koeniger, S.; Mandal, R.; Meier, F.; Naser, J.; O'Neil, D.; Pal, A.; Patti, G. J.; Pham-Tuan, H.; Prehn, C.; Raynaud, F. I.; Shen, T.; Southam, A. D.; St. John-Williams, L.; Sulek, K.; Vasilopoulou, C. G.; Viant, M.; Winder, C. L.; Wishart, D.; Zhang, L.; Zheng, J.; Moseley, M. A. International Ring Trial of a High Resolution Targeted Metabolomics and Lipidomics Platform for Serum and Plasma Analysis. *Anal. Chem.* **2019**, *91*, 14407−14416.

(20) Siskos, A. P.; Jain, P.; Römisch-margl, W.; Bennett, M.; Asad, Y.; Marney, L.; Richardson, L.; Koulman, A.; Julian, L.; Raynaud, F.; Scalbert, A.; Adamski, J.; Prehn, C.; Keun, H. C. Inter-laboratory Reproducibility of a Targeted Metabolomics Platform for Analysis of Human Serum and Plasma. *Anal. Chem.* **2017**, *89*, 656−665.

(21) Sarafian, M. H.; Gaudin, M.; Lewis, M. R.; Martin, F.-P.; Holmes, E.; Nicholson, J. K.; Dumas, M.-E. Objective Set of Criteria for Optimization of Sample Preparation Procedures for Ultra-High

Throughput Untargeted Blood Plasma Lipid Profiling by Ultra Performance Liquid Chromatography-Mass Spectrometry. *Anal. Chem.* **2014**, *86*, 5766−5774.
(22) Wishart, D. S.; Guo, A. C.; Oler, E.; Wang, F.; Anjum, A.; Peters, H.; Dizon, R.; Sayeeda, Z.; Tian, S.; Lee, B. L.; Berjanskii, M.; Mah, R.; Yamamoto, M.; Jovel, J.; Torres-Calzada, C.; Hiebert-Giesbrecht, M.; Lui, V. W.; Varshavi, D.; Varshavi, D.; Allen, D.; Arndt, D.; Khetarpal, N.; Sivakumaran, A.; Harford, K.; Sanford, S.; Yee, K.; Cao, X.; Budinski, Z.; Liigand, J.; Zhang, L.; Zheng, J.; Mandal, R.; Karu, N.; Dambrova, M.; Schiöth, H. B.; Greiner, R.; Gautam, V. HMDB 5.0: the Human Metabolome Database for 2022. *Nucleic Acids Res.* **2022**, *50*, D622−631.
(23) Food and Drug Administration. *Center for Drug Evaluation and Research Center for Veterinary Medicine, Guidance Document Bioanalytical Method Validation Guidance for Industry*. https://www.fda.gov/media/70858/download (accessed on January 28, 2022).
(24) Haug, K.; Cochrane, K.; Nainala, V. C.; Williams, M.; Chang, J.; Jayaseelan, K. V.; O'Donovan, C. MetaboLights: A Resource Evolving in Response to the Needs of its Scientific Community. *Nucleic Acids Res.* **2020**, *48* (D1), D440−D444.
(25) van Meer, G. Cellular lipidomics. *EMBO J.* **2005**, *24*, 3159−65.
(26) Ubhi, B. K. Direct Infusion-Tandem Mass Spectrometry (DI-MS/MS) Analysis of Complex Lipids in Human Plasma and Serum Using the Lipidyzer Platform. *Methods Mol. Biol.* **2018**, *1730*, 227−236.
(27) Weckwerth, W.; Fiehn, O. Can we Discover Novel Pathways Using Metabolomic Analysis? *Curr. Opin. Biotechnol.* **2002**, *13*, 156−160.
(28) Merciai, F.; Musella, S.; Sommella, E.; Bertamino, A.; D'Ursi, A.-M.; Campiglia, P. Development and Application of a Fast Ultra-high Performance Liquid Chromatography-trapped Ion Mobility Mass Spectrometry Method for Untargeted Lipidomics. *J. Chromatogr. A* **2022**, *1673*, 463124.
(29) Holčapek, M.; Cífková, E.; Červená, B.; Lísa, M.; Vostálová, J.; Galuszka, J. Determination of Nonpolar and Polar Lipid Classes in Human Plasma, Erythrocytes and Plasma Lipoprotein Fractions Using Ultrahigh-performance Liquid Chromatography-mass spectrometry. *J. Chromatgr. A* **2015**, *1377*, 85−91.
(30) Chandler, P. D.; Song, Y.; Lin, J.; Zhang, S.; Sesso, H. D.; Mora, S.; Giovannucci, E. L.; Rexrode, K. E.; Moorthy, M. V.; Li, C.; Ridker, P. M.; Lee, I.-M.; Manson, J. E.; Buring, J. E.; Wang, L. Lipid biomarkers and long-term risk of cancer in the Women's Health Study. *Am. J. Clin. Nutr.* **2016**, *103*, 1397−1407.
(31) Liakh, I.; Pakiet, A.; Sledzinski, T.; Mika, A. Modern Methods of Sample Preparation for the Analysis of Oxylipins in Biological Samples. *Molecules* **2019**, *24*, 1639.
(32) Wang, M.; Wang, C.; Han, X. Selection of Internal Standards for Accurate Quantification of Complex Lipid Species in Biological Extracts by Electrospray Ionization Mass Spectrometry-What, How and Why? *Mass Spectrom Rev.* **2017**, *36*, 693−714.
(33) Ernst, R.; Ejsing, C. S.; Antonny, B. Homeoviscous Adaptation and the Regulation of Membrane Lipids. *J. Mol. Biol.* **2016**, *428*, 4776−4791.
(34) Havulinna, A. S.; Sysi-Aho, M.; Hilvo, M.; Kauhanen, D.; Hurme, R.; Ekroos, K.; Salomaa, V.; Laaksonen, R. Circulating Ceramides Predict Cardiovascular Outcomes in the Population-Based FINRISK 2002 Cohort. *Arterioscler. Thromb. Vasc. Biol.* **2016**, *36*, 2424−2430.
(35) Serhan, C. N.; Levy, B. D. Resolvins in Inflammation: Emergence of the Pro-resolving Superfamily of Mediators. *J. Clin Invest.* **2018**, *128*, 2657−2669.
(36) Quehenberger, O.; Armando, A. M.; Brown, A. H.; Milne, S. B.; Myers, D. S.; Merrill, A. H.; Bandyopadhyay, S.; Jones, K. N.; Kelly, S.; Shaner, R. L.; Sullards, C. M.; Wang, E.; Murphy, R. C.; Barkley, R. M.; Leiker, T. J.; Raetz, C. R. H.; Guan, Z.; Laird, G. M.; Six, D. A.; Russell, D. W.; Mcdonald, J. G.; Subramaniam, S.; Fahy, E.; Dennis, E. A. Lipidomics Reveals a Remarkable Diversity of Lipids in Human Plasma. *J. Lipid Res.* **2010**, *51*, 3299−3305.
(37) Wang, C.; Wang, M.; Han, X. Applications of Mass Spectrometry for Cellular Lipid Analysis. *Mol. Biosyst.* **2015**, *11*, 698−713.
(38) Worldwide Cancer Statistics. *Cancer Research UK*. https://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer#heading-Zero (accessed on July 17, 2021).
(39) Drabovich, A. P.; Saraon, P.; Drabovich, M.; Karakosta, T. D.; Dimitromanolakis, A.; Hyndman, M. E.; Jarvi, K.; Diamandis, E. P. Multi-omics Biomarker Pipeline Reveals Elevated Levels of Protein-glutamine Gamma-glutamyltransferase 4 in Seminal Plasma of Prostate Cancer Patients. *Mol. Cell Proteomics.* **2019**, *18*, 1807−1823.
(40) Kiebish, M. A.; Cullen, J.; Mishra, P.; Ali, A.; Milliman, E.; Rodrigues, L. O.; Chen, E. Y.; Tolstikov, V.; Zhang, L.; Panagopoulos, K.; Shah, P.; Chen, Y.; Petrovics, G.; Rosner, I. L.; Sesterhenn, I. A.; McLeod, D. G.; Granger, E.; Sarangarajan, R.; Akmaev, V.; Srinivasan, A.; Srivastava, S.; Narain, N. R.; Dobi, A. Multi-omic Serum Biomarkers for Prognosis of Disease Progression in Prostate Cancer. *J. Transl. Med.* **2020**, *18*, 10.
(41) Di Carlo, C.; Sousa, B. C.; Manfredi, M.; Brandi, J.; Dalla Pozza, E.; Marengo, E.; Palmieri, M.; Dando, I.; Wakelam, M. J. O.; Lopez-Clavijo, A. F.; Cecconi, D. Integrated Lipidomics and Proteomics Reveal Cardiolipin Alterations, Upregulation of HADHA and Long Chain Fatty Acids in Pancreatic Cancer Stem Cells. *Sci. Rep.* **2021**, *11*, 13297.
(42) Cheng, L.; Zhang, K.; Qing, Y.; Li, D.; Cui, M.; Jin, P.; Xu, T. Proteomic and Lipidomic Analysis of Exosomes Derived From Ovarian Cancer Cells and Ovarian Surface Epithelial Cells. *J. Ovarian Res.* **2020**, *13* (1), 9.
(43) Titz, B.; Gadaleta, R. M.; Lo Sasso, G.; Elamin, A.; Ekroos, K.; Ivanov, N. V.; Peitsch, M. C.; Hoeng, J. Proteomics and Lipidomics in Inflammatory Bowel Disease Research: From Mechanistic Insights to Biomarker Identification. *Int. J. Mol. Sci.* **2018**, *19*, 2775.
(44) Xu, J.; Bankov, G.; Kim, M.; Wretlind, A.; Lord, J.; Green, R.; Hodges, A.; Hye, A.; Aarsland, D.; Velayudhan, L.; Dobson, R. J. B.; Proitsi, P.; Legido-Quigley, C. Integrated Lipidomics and Proteomics Network Analysis Highlights Lipid and Immunity Pathways Associated with Alzheimer's Disease. *Transl. Neurodegener.* **2020**, *9*, 1−15.
(45) Zhou, X.; Mao, J.; Ai, J.; Deng, Y.; Roth, M. R.; Pound, C.; Henegar, J.; Welti, R.; Bigler, S. A. Identification of Plasma Lipid Biomarkers for Prostate Cancer by Lipidomics and Bioinformatics. *PLoS One* **2012**, *7*, e48889.
(46) Perrotti, F.; Rosa, C.; Cicalini, I.; Sacchetta, P.; Del Boccio, P.; Genovesi, D.; Pieragostino, D. Advances in lipidomics for cancer biomarkers discovery. *Int. J. Mol. Sci.* **2016**, *17*, 1992.
(47) Snaebjornsson, M. T.; Janaki-Raman, S.; Schulze, A. Greasing the Wheels of the Cancer Machine: The Role of Lipid Metabolism in Cancer. *Cell Metab.* **2020**, *31*, 62−76.

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