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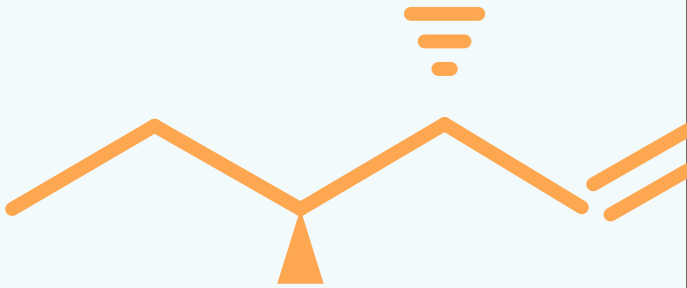

A Technical Guide to Metabolon's Complex Lipids Targeted Panel

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Table 1. Acronyms and abbreviations. See Table 2 for class abbreviations.

CoV	Compensation voltage
DMS	Differential mobility spectrometry
FIA	Flow injection analysis
IS	Internal standard
ISLE	Intact sample lipid extraction
MRM	Multiple reaction monitoring
MS	Mass spectrometry
QC	Quality control
RSD	Relative standard deviation (standard deviation/average of measurements)

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Introduction to Lipidomics

Structure and Nomenclature of Complex Lipids

Complex lipids are immensely diverse and numerous. The Lipid Maps Structure Database, a standard and continuously updated reference in the field,[1] currently contains over 40,000 unique lipid structures. Within mammalian biology, most biologically relevant lipids fall into roughly 10 to 20 chemical classes, defined by a portion of the lipid called the headgroup. In addition to the headgroup, each complex lipid may contain as many as four fatty acid side chains, each drawn from a pool of approximately 40 distinct fatty acid structures. For example, Figure 1A depicts a representative complex lipid that can be classified as a phosphatidylcholine (PC) due to the presence of a phosphocholine headgroup on the glycerol backbone. This headgroup is decorated by two fatty acid side chains, one containing 16 carbon atoms (represented as black spheres) and no double bonds, and the other containing 18 carbon atoms and a single double bond (shown as a bend in the chain of carbon atoms). This structure is designated PC(16:0/18:1). A change in either the headgroup or side chain(s) would produce a distinct lipid structure that may differ from PC(16:0/18:1) in molecular weight, chemical properties, and biological functions. For example, changing the headgroup to a phosphatidylethanolamine would produce PE(16:0/18:1), whereas introducing a second double bond to either side chain would result in either PC(16:1/18:1) or PC(16:0/18:2). In summary, one can imagine the complex lipidome as a matrix containing combinations of headgroups and fatty acids (Figure 1B), accounting for the large number of possible lipids.

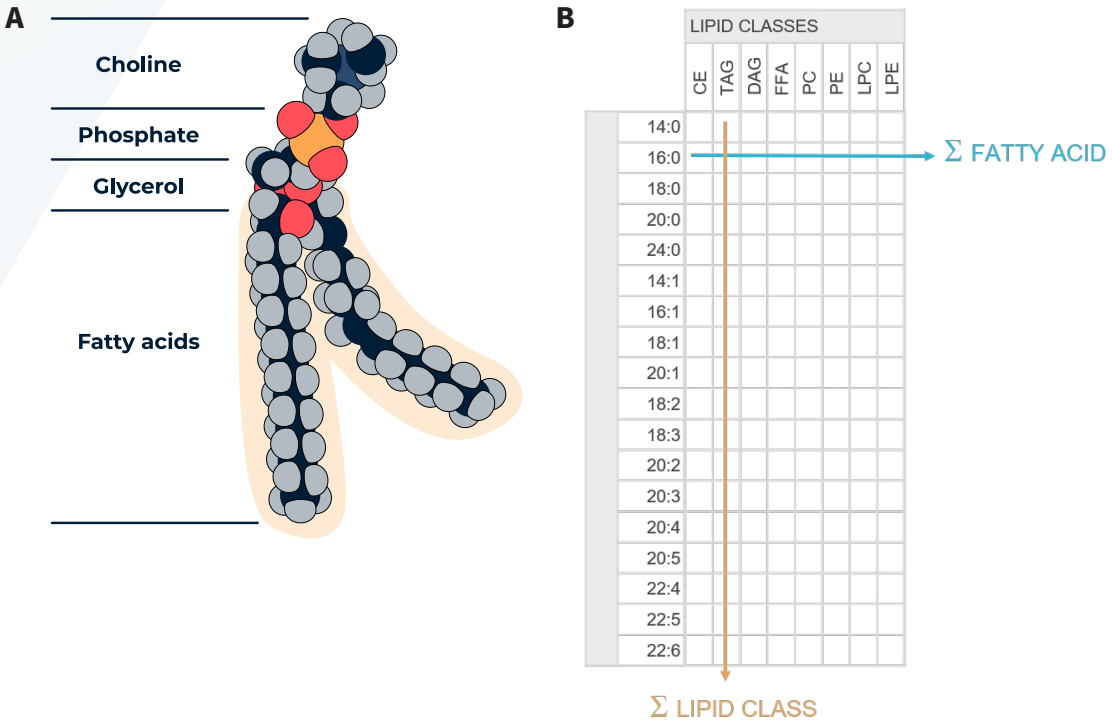

Figure 1. Representative complex lipids: A, Structural representation of PC(16:0/18:1) and B, matrix representation of complex lipids.

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Requirements for Lipid Analysis

Quantifying both lipid classes and fatty acid side chains is critical to understanding lipid biology. Lipids with different headgroups may have drastically different biological functions: for example, PCs and PEs are building blocks of cell membranes; PIs play a role in cell signaling; and TAGs are used for energy storage. The same is true for the fatty acids: for example, saturated fatty acids (ie, fatty acids with no double bonds, such as 16:0) are used primarily for energy storage, whereas the polyunsaturated fatty acids (PUFAs, eg, 20:4 and 22:6), which include the well-known omega-3 fatty acids, play key roles in signaling. Different headgroups and fatty acids also derive from different sources, including numerous metabolic reactions as well as diet, and can have distinct effects on cell membrane properties. Therefore, a successful lipidomic profiling method must measure the abundance of both lipid headgroups and fatty acids, leading directly to three key requirements:

- **Coverage:** A lipidomic method should measure as many relevant lipids as possible in order to provide the most complete understanding of the lipidome. A method lacking broad coverage may not only fail to reveal changes in important individual lipids, but also will not accurately represent the abundance of lipid classes and fatty acids across the lipidome.

- **Quantification:** Untargeted metabolomics, such as Metabolon's Global Discovery Panel, report metabolite levels in terms of relative abundance, which allows the level of a single metabolite to be compared across samples, for example, to determine the fold change in an experimental vs. a control group. In lipidomics, by contrast, an important goal is to measure the contribution of each lipid class and fatty acid to the lipidome; therefore, it is necessary to compare and sum the quantity of lipids across different classes. Because the lipid classes differ dramatically in their chemical properties, such comparisons and calculations can only be done if each lipid's abundance is measured in units of concentration (eg, µM).

- **Specificity:** To understand the contribution of each fatty acid to the lipidome, the fatty acid composition of each complex lipid must be resolved. For example, the fatty acid composition of the lipid in Figure 1A is PC(16:0/18:1). Many existing lipidomic profiling methods are capable of determining only the sum composition of the lipid (ie, the total number of carbons and double bonds across all of its side chains). For example, the sum composition corresponding to PC(16:0/18:1) would be PC(34:1). However, this sum composition could also correspond to PC(16:1/18:0) or a variety of other possible structures. Thus, methods that identify lipids only to the level of sum composition are blinded to the changes in abundance of individual fatty acids in the lipidome. Metabolon's complex lipid methodology characterizes every lipid reported to the individual fatty acid level. In this way, Metabolon's methodology can reveal biologically relevant changes in lipid metabolism, such as, for example, increased incorporation of 22:6 fatty acid as opposed to 16:0 fatty acid, within a specific lipid class or across the entire lipidome in a given study.

The next section explains how the technology behind Metabolon's Complex Lipids Targeted Panel fulfills these three requirements.

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Metabolon's Complex Lipids Targeted Panel

FIA-DMS-MRM Technology

The Complex Lipids Targeted Panel utilizes three key technologies to achieve broad coverage, quantification, and specificity in lipidomic analysis. These are flow injection analysis (FIA), differential mobility spectrometry (DMS), and multiple reaction monitoring (MRM; Figure 2).

Flow injection analysis refers to the fact that the lipid sample solution is continuously infused into the mass spectrometer without the use of any chromatographic column. FIA is used because chromatographic separation of lipids is extremely challenging and can actually hinder quantification if a lipid elutes from the column at a different time, and in the presence of different interferences, than its internal standard (see Quantification section below). With FIA, a uniform sample is analyzed throughout the infusion (typically ~6 minutes in the case of the Complex Lipids Targeted Panel), allowing multiple replicate measurements for more robust and reproducible results.

The DMS technology takes advantage of the fact that a molecule's trajectory through an electric field is affected by its size, shape, and dipole moment. After ionization in the source of the mass spectrometer, lipids are introduced into the SelexION DMS cell. The DMS cell acts as a lipid filter or gate that permits a specific lipid class to pass into the mass spectrometer (for example, the PC class, denoted by blue circles in Figure 2), while the other lipid classes are filtered out. The DMS cell dramatically limits the complexity of the lipid mixture entering the mass spectrometer at a given time. This technology removes the isomeric lipid interferences between lipid classes, permitting more specific identification of all detected lipid species. The DMS cell cycles through the different lipid classes over the course of a single sample infusion, sequentially passing each lipid class into the mass spectrometer for analysis.

During the development of the Complex Lipids Targeted Panel, it became clear that certain lipid classes, particularly CEs, are unstable and cannot be subjected to the DMS process. Thus, the Complex Lipids Targeted Panel analysis was split into two parts: Method 1, comprising lipids that require DMS separation because they cannot be fully distinguished by MRM alone, and Method 2, comprising CEs and other lipids that do not require DMS separation because they have unique precursor/fragment ion pairs (Table 2). Separate injections of each lipid sample are performed for each of the two analyses.

After exiting the DMS cell, the lipids from the selected lipid class enter the mass spectrometer for MRM analysis. MRM analysis requires a triple quadrupole mass spectrometer, in which the first and third quadrupole can filter the individual lipid species on the basis of mass-to-charge ratio (m/z). In an MRM analysis, the first quadrupole (Q1) filters on the basis of the m/z of the intact lipid species, while the third quadrupole (Q3) filters on the basis of the m/z of a characteristic fragment of that same lipid species, such as one of the fatty acid side chains. MRM analysis is the standard methodology used for mass spectrometry-based clinical assays, as it is the most quantitatively accurate and provides a high degree of analyte specificity. Together, the DMS cell and the MRM analysis ensure that the signal that is finally recorded by the detector specifically and accurately measures an individual lipid species (Figure 2).

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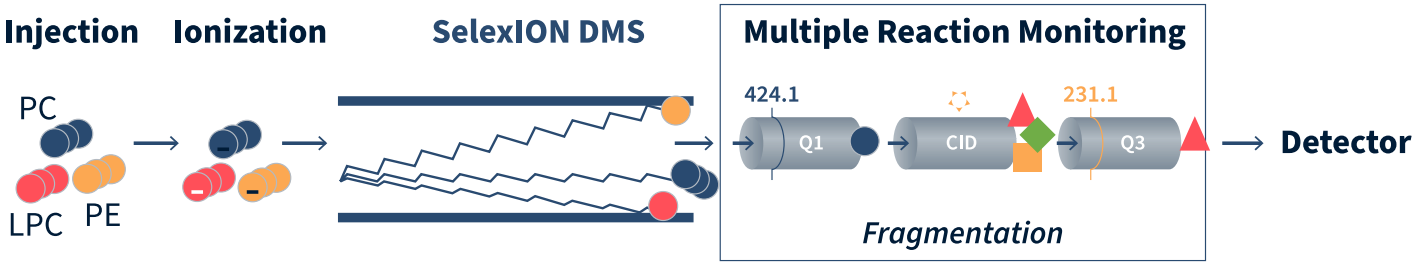

Figure 2. FIA-DMS-MRM technology underlying the Complex Lipids Targeted Panel.

Together, these technologies accomplish the three aims set out in the previous section:

- **Coverage:** Because the MRM analysis takes only 20 milliseconds to measure each lipid, the Complex Lipids Targeted Panel monitors over 1,100 lipid species in a single sample, a breadth of coverage that compares very favorably to other lipidomic platforms. Moreover, the instruments cycle through the entire MRM table 17 times over the course of each sample infusion, obtaining 17 replicate measurements for each lipid. Only lipids that are detected in at least 12 out of the 17 cycles are reported, while all others are considered undetectable. This enables increased confidence in the method's reproducibility and robustness, particularly in comparison to "shotgun" methods that collect only a single measurement per lipid.

- **Quantification:** The Complex Lipids Targeted Panel quantifies the absolute concentration of each of the >1,100 monitored lipids using isotopically labeled internal standards, which are included at known concentration during the preparation of each sample. The MRM technology utilized in this methodology is the industry standard for mass spectrometry-based clinical quantitative measurements. See the Quantification section below for more information on this process.

- **Specificity:** The Complex Lipids Targeted Panel includes several layers of specificity that ensure correct lipid species identification to the individual fatty acid level. In the MRM analysis, the combination of precursor and fragment (Q1 and Q3) ions provides extensive information about each lipid, including its overall molecular weight, its fragmentation pattern (which is often diagnostic of lipid class), and one of its fatty acids. In addition, DMS resolves those lipids, such as certain PCs and PEs, that cannot be adequately distinguished by the MRM analysis alone and can often lead to interferences in MRM-only analyses.

Target Analytes

The Complex Lipids Targeted Panel is a targeted methodology, which monitors a pre-defined list of over 1,100 analytes, specified in the MRM table, in every sample (Table 2). These analytes were selected based on pre-existing knowledge of the most abundant lipids in human plasma, and in fact, over 700 of them are routinely detected in a reference plasma sample (see Assurance and Quality Control section below). The Complex Lipids Targeted Panel is optimized for studies of mammalian lipid biology and currently focuses on lipid classes present in plasma. As a result, in samples from organisms whose lipid content differs dramatically from that of mammalian plasma, such as plants and bacteria, the Complex Lipids Targeted Panel may not capture the complete picture of the lipidome. There is no technical obstacle to analyzing A variety of sample types have been successfully analyzed with the Complex Lipids Targeted Panel, however some are generally low in lipid content (Table 3), but researchers should be aware of the target analyte list and of the possibility that the analysis may exclude relevant lipids to their sample type. A variety of sample types have been successfully analyzed with the Global Discovery Panel; however, some are generally low in lipid content (Table 3). Analysis of low lipid content sample types on the Complex Lipids Targeted Panel is generally not recommended unless elevated lipids are strongly expected due to the specific model system or perturbation under study (eg, media from cultured cells known to secrete high levels of lipids).

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Table 2. Lipid classes monitored by the Complex Lipids Targeted Panel.

METHOD 1			METHOD 2		
Class	**Abbrev.**	**Species**	**Class**	**Abbrev.**	**Species**
Lysophosphatidylcholine	LPC	26	Cholesteryl ester	CE	26
Lysophosphatidylethanolamine	LPE	26	Ceramide	CER	12
Phosphatidylcholine	PC	140	Diacylglycerol	DAG	59
Phosphatidylethanolamine	PE	216	Dihydroceramide	DCER	13
Phosphatidylinositol	PI	28	Free fatty acid*	FFA	26
Sphingomyelin	SM	12	Hexosylceramide	HCER	12
			Lactosylceramide	LCER	12
			Monoacylglycerol	MAG	26
			Triacylglycerol	TAG	519
Total (Method 1)		**448**	**Total (Method 2)**		**705**
TOTAL SPECIES MONITORED: 1,153					

**Free fatty acid levels can be heavily influenced by environmental and process contamination including contact with plastics. For this reason Metabolon has paused the return of data from this class.*

Table 3. Selected sample types that have been analyzed on the Complex Lipids Targeted Panel.

SAMPLE TYPE	NOTES
Body fluids	
Serum, plasma, whole blood	Favorable
Milk	Favorable
CSF	Low Lipid Content
Amniotic fluid	Low Lipid Content
Urine	Low Lipid Content
Solids	
Tissue (incl. liver, muscle, neural, skin, tumor)	Favorable
Dog food	Favorable
Feces	Low Complex Lipid Content
Pollen	Lipids not well-represented by Complex Lipids Targeted Panel
Other	
Cultured mammalian cells	Favorable
Cell culture media	Low Lipid Content
Sebum*	See Sebum Targeted Panel at Metabolon.com

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Quantification

Absolute quantification is the measurement of a metabolite's abundance in molar units, which is accomplished by comparing an unknown sample with a reference standard(s) of known concentration. Most quantitative assays monitor no more than 30 analytes, so to obtain the absolute concentration of over 1,100 analytes in a single assay is remarkable. The Complex Lipids Targeted Panel accomplishes this through the use of 54 isotopically labeled internal standards (IS), a series of lipid molecules that are structurally identical to representative Complex Lipids Targeted Panel analytes, except that between 5 and 9 of the hydrogen atoms in each molecule have been replaced with deuterium atoms. Isotopic labeling ensures that the IS molecules behave identically to their corresponding analytes throughout sample extraction and FIA-DMS-MS analysis; at the same time, the mass spectrometer can distinguish the IS molecules from the analytes due to the mass difference between deuterium and hydrogen. Known quantities of each IS are introduced into each sample at an early stage of extraction. Any experimental factors that may affect the signal detected for each analyte (eg, variation in extraction efficiency or instrument performance, sample evaporation, signal suppression by contaminants) are assumed to equally affect the signal detected for the corresponding IS as well, and thus the ratio between analyte intensity and IS intensity should depend only on the concentration of the analyte. Based on this assumption and the known concentration of IS, the analyte concentration is calculated according to the formula in Figure 3.

$$\frac{\text{Analyte intensity}}{\text{IS intensity}} \times \text{IS concentration (nmol/mL)} \times \frac{\text{IS volume Added (mL)}}{\text{Sample Weight (g)}}$$

Figure 3. Formula for calculating the concentration of an analyte based on the MS signal intensities of the analyte and corresponding internal standard (IS).

The number and diversity of internal standards is a key distinguishing hallmark of the Complex Lipids Targeted Panel. Quite unusually for a broad lipidomic profiling method, the Complex Lipids Targeted Panel includes ISs across 14 classes, with most classes comprising multiple ISs to maximize the similarity between each analyte and its internal standard. As a result, every lipid in the Complex Lipids Targeted Panel is quantified using an IS from the same lipid class, except for PIs, which are quantified using the PE internal standards. The validity of the Complex Lipids Targeted Panel's quantification methodology is confirmed by its quantitative precision and accuracy, which have been verified through a rigorous validation process. In particular, the accuracy of the lipid concentration measurements has been validated by comparison to a benchmark technology (a combination of thin-layer chromatography and gas chromatography / mass spectrometry). Moreover, sensitivity, intra- and inter-day precision, and accuracy are continually monitored as part of the Assurance and Quality Control process; for example, a typical plasma dataset would be expected to contain at least 700 lipid species with a median relative standard deviation (RSD) of <10% (see Quality Assurance and Quality Control section below for more details). Taken together, the quantification methodology, breadth of lipid coverage, precision, and accuracy of the Complex Lipids Targeted Panel provides a powerful dataset for understanding lipid biology.

Although the Complex Lipids Targeted Panel provides a quantitative measure of concentration for each lipid, it differs in several important ways from smaller targeted assays that use calibration curves. A calibration curve consists of at least five calibrator samples that contain a range of known concentrations of each analyte, as well as isotopically labeled internal standards; the calibrators and the unknown samples are subjected to the same analysis, and the intensity ratios between analytes and internal standards measured in each unknown sample are compared to those in the calibrator samples to calculate the analyte concentrations in the unknown samples. Clearly, this approach would be impractical if not impossible for quantifying the >1,100 analytes included in the Complex Lipids Targeted Panel, many of which lack commercially available authentic standards and isotopically labeled internal standards. Instead, as described above, the Complex Lipids Targeted Panel relies on a single known concentration of each isotopically labeled IS, in what is known as a single-point calibration. Therefore, the Complex Lipids Targeted Panel does not provide lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for each analyte, which are typically reported for smaller targeted assays. Moreover, because it is physically impractical and cost-prohibitive to include >1,100 isotopically labeled internal standards in every sample, the Complex Lipids Targeted Panel includes many more analytes than ISs, and therefore many analytes are not structurally identical to their ISs. A mitigating factor is that, in the FIA paradigm, all analytes co-elute with their ISs, and therefore encounter the same interferences despite any structural differences, which eliminates a major potential source of discrepancy in response between ISs and analytes. Also, the number and variety represented by the 54 ISs within the current Complex Lipids Targeted Panel does enable the methodology to account for as much lipid structural diversity as possible, considering the tradeoffs of cost as

well as ease of synthesis and handling. Therefore, despite these practical limitations, the Complex Lipids Targeted Panel provides the highest level of quantification currently available in a broad-based lipidomic profiling method.

Similarities and Differences with the Global Discovery Panel

Many important differences exist between the Complex Lipids Targeted Panel and the Global Discovery Panel (Table 4). Most obviously, the Complex Lipids Targeted Panel analytes are much more hydrophobic than most Global Discovery Panel analytes, necessitating the use of different sample extraction methods, including stronger organic solvents, as compared to the Global Discovery Panel. As described above, the Complex Lipids Targeted Panel is a targeted methodology, so only those lipids included in the pre-determined list of ~1,100 target analytes are measured (see Target Analytes section above). By contrast, the Global Discovery Panel does not utilize a targeted workflow but rather detects all ions produced from the LC/MS analysis of a sample, so that novel metabolic products can be detected. As a result, the Global Discovery Panel is more readily customizable (eg, for analysis of drugs and drug metabolites of interest to a specific investigator). Another difference is that the Complex Lipids Targeted Panel results are fully quantitative (ie, µM units), whereas the Global Discovery Panel provides relative quantification only. Both technologies have unique strengths and weaknesses that can make them more or less suitable for specific biological applications.

Approximately 300 molecules are measured by both the Complex Lipids Targeted Panel and Global Discovery Panel, including multiple members of the MAG, DAG, FFA, phospholipid (LPC, LPE, PC, PE, PI) and sphingolipid (SM and CER) classes. Note, currently, lipid nomenclature differs between Metabolon's Global Discovery Panel and the Complex Lipids Targeted Panel for those lipid species detected on both panels. Please reach out to your Metabolon representative if you have any questions.

Typically, for projects that include both the Complex Lipids Targeted Panel and Global Discovery Panel, lipids are reported from the Complex Lipids Targeted Panel analysis of the samples. This is because, as noted above, the Complex Lipids Targeted Panel provides more comprehensive and quantitative lipid coverage, meaning that lipid data from the Complex Lipids Targeted Panel can be interpreted in biologically meaningful ways (ie, at the level of lipid class and fatty acid composition) that are impossible to do with lipid data from the Global Discovery Panel.

Table 4. Major differences between the Complex Lipids Targeted Panel and the Global Discovery Panel.

	Complex Lipids Targeted Panel	Global Discovery Panel
Extraction method	Biphasic (dichloromethane/methanol/ water or methyl-tert-butyl ether/methanol/water)	Monophasic
Targeted or not?	Targeted • Measure only pre-determined list of analytes	Untargeted • Detect all ions (including unknowns)
Basis for metabolite ID	• Precursor/product ion pair (MRM) • Differential Mobility Spectrometry	• Exact mass • Retention time • MS/MS fragmentation
Chromatography	None (direct infusion into mass spec) • cannot resolve isomers	Multiple separations • can resolve many isomers
Curation	None	Required for correct ID
Typical approaches for interpretation	• lipid classes • fatty acid totals • composition and concentration	• individual metabolites • metabolic pathways • relative up- or downregulation

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Complex Lipids Targeted Panel Workflow

Sample Analysis

The Complex Lipids Targeted Panel sample extraction protocols are different from those of the Global Discovery Panel due to the much more hydrophobic nature of the Complex Lipids Targeted Panel analytes. Many lipids are naturally found in lipoprotein complexes, which must be disrupted with strong organic solvents in order to efficiently extract the analytes. Accordingly, the Complex Lipids Targeted Panel uses biphasic extraction methods in which two liquid layers (phases) are formed in each sample, with the lipids partitioning to the hydrophobic layer, which is then collected, dried, and reconstituted for analysis. The reconstituted lipids are infused directly into a SCIEX QTRAP 5500 mass spectrometer for both positive and negative ionization analysis in MRM mode (see Methods section).

Quality Assurance and Quality Control

Several quality assurance (QA) and quality control (QC) measures are utilized at every step, from sample receipt to data analysis. These quality checks and procedures allow for monitoring, documentation, and, where possible, correction of process variances. If the data do not meet Metabolon's established acceptance criteria, then the samples are reanalyzed either by reinjecting the extracts or re-extracting the samples. Metabolon's workflow and processes are also designed in such a way that they prevent or mitigate the occurrence of errors. The combination of these quality measures allows Metabolon to deliver, with confidence, high-quality data to researchers.

Pre-Analytical: Quality processes begin at sample receipt, when each sample is individually inventoried against a sample manifest received from the investigator and stored at -80C. Samples are logged into an internal Laboratory Information Management System (mLIMS), ensuring fidelity of metadata and allowing traceability throughout the life of the sample. From this point the LIMS system is used to guide and facilitate the remaining steps in the analytical process including sample extraction, sequence generation for analytical plates, and QC of results.

Balancing and Randomization: Within a study, samples are extracted and analysed in sets of up to 40 samples. Prior to any analysis samples are balanced across these sets on the basis of any known confounding variables (case vs control, gender, etc.) To further remove any potential systematic bias the injection order of the individual client samples within a set are randomized. Interspersed at regular intervals are QC samples which have been extracted in parallel with the client samples for that set. The samples dedicated to QC include CMTRX, MTRX and PRCS samples and are described in detail in the following sections. Figure 4 illustrates a typical injection order of a study highlighting the randomization of sample groups and interspersion of QC samples throughout sample sets. The interspersion of quality control samples, within and across the analysis, ensures that analytical variability will be monitored throughout the entire study. Note that for every set of up to 40 experimental samples analyzed, at least four technical replicates and three process blanks are also analyzed.

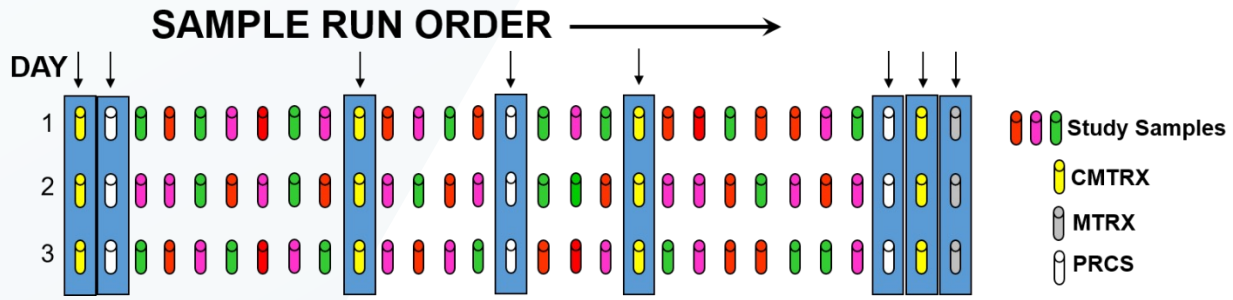

Figure 4. Example run order. Arrowheads indicate quality control samples as follows: CMTRX, client sample pool; MTRX, bulk human plasma pool; PRCS, process blank.

Process blanks: Process blanks are water samples extracted and analyzed in the same manner as client samples. These blanks help monitor for any potential contamination occurring during storage, sample prep, or analytical processes.

Technical replicate QC samples: Depending on the study sample matrix, and the volume received, technical replicate QC samples are extracted from either a bulk lot of commercially available human plasma (MTRX), or a pool of the client samples within that set (CMTRX). MTRX is used for all all plasma and serum studies, while CMTRX is used for all other matrices, unless insufficient client sample is available. The advantage of CMTRX is that, as a pool of the study samples, it perfectly mimics the study sample matrix, which allows run-day variability to be monitored in rare, complex, or highly variable matrices, such as liver tissue. Whether CMTRX or MTRX is used, the purpose of these technical replicates is to monitor run-day variability.

MTRX historical samples: Regardless of the type of technical replicate sample used in a study, each Complex Lipids Targeted Panel sample batch includes additional samples of MTRX (bulk pooled human plasma). Because the same lot of MTRX is analyzed extensively over the course of several years, the quantitative data from this sample can be compared to historical values previously obtained by analyzing many replicate MTRX samples. This provides an additional QC check to confirm that the concentrations obtained on each instrument are consistent over time. In addition to verifying the data from each run day, MTRX is also used in validating new reagents and new instruments.

Daily QC Procedure: Similar to the Global Discovery Panel, Complex Lipids Targeted Panel data is subjected to Platform Quality Control (QC) immediately after acquisition, typically the morning after an overnight instrument run. The platform analyst responsible for each instrument visually inspects the Total Ion Chromatogram for all samples to ensure that each one was infused in a uniform and consistent fashion and verifies that all internal standards were detected in each sample. Samples with low-quality injections may be re-injected at this time. Once all injections are complete, batch-level QC is performed using the technical replicate QC samples in each set as described above.

Several QC criteria are applied to verify adequate sensitivity, precision, and accuracy of the Complex Lipids Targeted Panel measurements. Sensitivity is measured as the number of lipid species detected in QC MTRX samples, which must be over 700. Precision refers to the reproducibility of replicate measurements, which is measured by comparing the five MTRX replicates (or four CMTRX replicates) in each sample set to each other. Specifically, the percent relative standard deviation (%RSD; defined as the standard deviation divided by the average) is calculated for each lipid across these four or five replicates, and the median of these %RSD values must be below 10%. Finally, accuracy is evaluated by comparing the MTRX concentrations of major plasma lipid classes (CE, FFA, PC, PE, and TAG) to historical values established over many weeks of instrument operation; the experimental data must be within 20% of these historical averages. This verification of accuracy is the reason for including additional samples of MTRX in each sample set and ensures that instrument performance remains stable over time and that data from different sample batches can be compared. If a batch fails to meet QC standards, instrument cleaning and other troubleshooting steps are performed, followed by reinjecting the same samples and/or performing a new extraction of the same biological starting material, if available. Together, these QC steps play a key role in ensuring the quality and reproducibility of the data that then proceeds to further analysis in the Statistics and Project Management groups.

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Data Post-processing and Interpretation

Normalization

The direct output of the Complex Lipids Targeted Panel analysis is a measurement of up to 1,100 individual lipid concentrations in units of µM (equivalent to nmol/mL). For liquid samples such as plasma (the sample type most analyzed with the Complex Lipids Targeted Panel), the results are easily and directly interpreted as the concentration in the original liquid sample. Similarly, for tissue and other solid sample types, the data is simply reported as nmol/g, using sample weights measured at the start of the sample preparation process. Notably, although the measurements for the lipid species monitored on the complex targeted panel are quantitative, the effects of background contamination and any potential batch effects are also addressed. Due to the near ubiquitous presence of lipids in various areas including labware, Metabolon uses the detected levels of lipids in the process blanks to deliver accurate measurements via background subtraction. Similarly any differences in the quantified measurements due to batch effects is removed via a median scaling run-day correction.

Normalization of cell data: For sample types such as cultured cells, the pellets are first homogenized in water and then a known volume of the homogenate is subjected to lipid extraction, while another aliquot of the same homogenate is utilized to measure protein or DNA content. For normalization purposes, the background subtracted and run-day normalized data are divided by the protein or DNA values. It is noted that if normalization to cell counts is desired, investigators should count the cells in their samples before shipping them to Metabolon.

Class, Fatty Acid, and Composition Calculations

A typical Complex Lipids Targeted Panel analysis measures the concentrations of up to 1,100 individual lipid species. However, because of the matrix-like structure of the complex lipidome, considering individual lipid measurements may not be sufficient to fully characterize a biological system (Table 4). For example, many lipids in a single class may coordinately increase or decrease due to the up- or downregulation of a single metabolic enzyme or process. Also, many lipid classes can be simultaneously affected by alterations in the supply of available fatty acids (eg, due to dietary effects or to changes in processes like fatty acid oxidation, desaturation, and elongation). To better capture these potentially crucial effects, the lipid species concentrations directly measured by the Complex Lipids Targeted Panel are used to calculate several additional metrics, which are reported to investigators and can assist in data interpretation:

- Lipid class concentration is calculated by summing the concentrations of each individual lipid in that class. Note, as a result of some TAG measurements having contributions from multiple triglyceride species, the Total TAG concentration incorporates a correction factor to account for this.
- Lipid species composition is calculated by representing each lipid species concentration as a percentage of its lipid class concentration. Thus, for example, all the DAG species compositions in a sample will total 100%.
- Fatty acid concentration is calculated by summing the concentrations of each individual lipid containing that fatty acid. Fatty acid concentrations are calculated both across individual lipid classes (eg, PC[FA16:0], which would represent the total concentration of fatty acid 16:0 within the phosphatidylcholine class) or across the entire lipidome (eg, Total[FA16:0]). Fatty acid composition is calculated by converting these fatty acid concentrations into percentages.
- All concentrations are reported in units of concentration (see section on Normalization above), whereas all compositions are reported in mol%.

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In one example of the utility of these calculations, a study of plasma from human volunteers subjected to vigorous exercise showed that the TAG class concentration decreased and the FFA class concentration increased, but there was no change in the fatty acid composition of either class, reflecting the utilization of TAGs for energy (which requires conversion to FFAs) without regard for their fatty acid composition. By contrast, consumption of a beverage rich in oleic acid (18:1) led to an increase in the mol% contribution of fatty acid 18:1 within the TAG class, demonstrating the storage of the consumed fatty acids in the form of TAGs. Analyzing the Complex Lipids Targeted Panel data at the level of lipid classes and fatty acids as well as individual lipid species, and at the level of composition as well as concentration, improves the chances of uncovering significant changes and fully understanding the biological phenomenon of interest.

Imputation

Imputation is a statistical procedure that facilitate certain analyses of large-scale datasets such as lipidomics and metabolomics studies. Imputation refers to the treatment of missing values (ie, analytes that were not detected). For the Complex Lipids Targeted Panel, missing concentration values (species, class, and fatty acid concentration data) are replaced with the minimum value detected for that analyte, which is also consistent with the procedure followed for Global Discovery Panel. For example, if Sample X has an undetectable FA(20:0) level, and the lowest FA(20:0) concentration detected in any sample in the dataset is 0.2 µM, then the imputed FA(20:0) concentration in Sample X will be 0.2 µM. By contrast, missing composition values (species, class, and fatty acid composition data) are replaced with 0; therefore, if Sample X has an undetectable FA(20:0) level, then the imputed percent composition of FA(20:0) in Sample X will be 0%. This procedure avoids recalculating the composition data based on imputed concentration values, which is less reliable. Together, these post-processing steps enable appropriate statistical analysis and reporting of the data.

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Data Delivery

To assist investigators in understanding and interpreting their lipidomic data, Complex Lipids Targeted Panel results are reported in several ways. One major deliverable is an Excel spreadsheet containing six tabs, with each tab corresponding to lipid class, species, or fatty acid data reported as concentration or composition (See Class, Fatty Acid, and Composition Calculations). This "six-tab file" is neither scaled nor imputed and represents the data in its rawest form. For combination Global Discovery Panel/Complex Lipids Targeted Panel projects, an investigator may also receive a second Excel file containing heat maps and box plots that visually depict the most statistically significant changes detected on the Complex Lipids Targeted Panel, in a manner consistent with the typical presentation of Global Discovery Panel data. These heat maps and box plots currently utilize only the species and class concentration data. For most projects, Complex Lipids Targeted Panel data (imputed but not scaled) is also uploaded to the Surveyor online visualization tool. This program enables investigators to dynamically explore their data (eg, by comparing fatty acid or class concentrations (or compositions) between their experimental groups). Surveyor also incorporates information about metabolic pathways that interconvert various lipid classes and fatty acids, including customizable "signatures" that can report on the activity of enzymes such as stearoyl-CoA desaturase. Significant changes in any of these metrics or pathways are easily visualized in heat maps, which can then be exported for reporting and publication (Figure 5). Finally, combination Global Discovery Panel/Complex Lipids Targeted Panel projects may include a narrative report, similar to standalone Global Discovery Panel projects. In general, Metabolon will work with each investigator on a project-by-project basis to identify the most appropriate deliverables to meet the investigator's research needs.

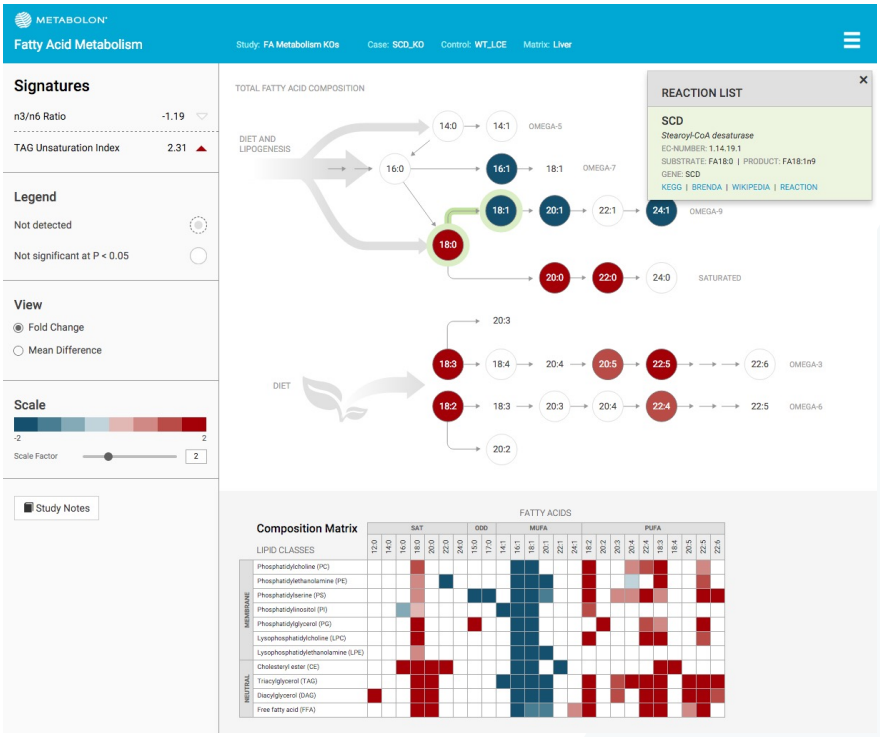

Figure 5. The Surveyor data visualization tool. Note the fatty acid pathway diagram at the top and the composition matrix at the bottom, both of which display differences in fatty acid composition between two experimental groups specified by the user.

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Methods

Sample Preparation

For plasma and serum, lipids are extracted from the sample in the presence of deuterated internal standards using an automated methyl-tert-butyl ether based extraction as previously described.[2] Other liquid samples are extracted in the presence of deuterated internal standards following a modified Bligh-Dyer procedure[3]. Solid samples (such as tissue, cultured cells) are either homogenized by ultrasonication or soaked overnight in dichloromethane/methanol, and the resulting solution is also subjected to a modified Bligh-Dyer extraction. Lipid extracts are dried under nitrogen, reconstituted in running solution consisting of dichloromethane/methanol supplemented with ammonium acetate, and subjected to flow injection and mass spectrometry (FIA-MS) as described below.

Flow Injection and Mass Spectrometry

FIA-MS is performed on a SCIEX QTRAP 5500 equipped with a SelexION DMS cell, and which is operated in MRM mode using both positive and negative mode electrospray in a Turbo V ion source. For analysis, a 50 µL sample is infused at a flow rate of 8µL/min.

Of note, for the 14 lipid classes in the Complex Lipids Targeted Panel that comprise only one or two fatty acid side chains, identifying the lipid class and one fatty acid side chain through the MRM analysis is sufficient to define the entire molecular structure. For example, for PC(16:0/18:1), the DMS and precursor (Q1) ion define the lipid's sum composition, PC(34:1), while the fragment (Q3) ion identifies one of the fatty acids as 16:0. Simple subtraction of the carbon and double-bond numbers then defines the remaining fatty acid side chain as 18:1, identifying the complete lipid structure as PC(16:0/18:1).

Unlike the other lipid classes, the TAGs contain three fatty acid side chains, only one of which can be identified by the MRM analysis. As a result, the Complex Lipids Targeted Panel cannot resolve the full fatty acid side chain composition of TAGs, such as TAG(16:0/18:0/18:1); instead, the TAGs are denoted as a combination of sum composition and the single identified fatty acid (eg, TAG50:1-FA18:1). Importantly, this approach still allows the fatty acid composition of the TAGs to be calculated.

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Summary and Conclusion

Metabolon's Complex Lipids Targeted Panel is a unique lipidomic profiling technology that offers accurate identification and quantification of over 1,000 lipid species, representing the most biologically relevant lipids in mammalian plasma. The fundamental and distinguishing features of the Complex Lipids Targeted Panel are the use of Differential Mobility Spectrometry and Multiple Reaction Monitoring for accurate identification, as well as the use of over 50 isotopically labeled internal standards for accurate quantification. This technology, combined with our rigorous quality assurance and control procedures and advanced capability for data analysis and interpretation, makes Metabolon the premier choice for investigators seeking a comprehensive lipidomic profiling solution.

References

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