Large-Scale Lipid Profiling of a Human Serum Lipidome Using a High-Resolution, Accurate-Mass LC/MS/MS Approach

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Key Words
C30 column, Q Exactive HF, high resolution, accurate mass, Orbitrap mass detector, lipids, lipidomics profiling, relative quantification, human serum, human plasma, LipidSearch software, triglycerides, isomers, efficiency

Goal
Develop an optimal high-resolution, accurate-mass HPLC-MS platform to achieve improved lipid identification, quantification, and throughput from complex biological samples.

Introduction
Lipids play a key role in cell, tissue, and organ physiology. Many diseases, such as cancer and diabetes, involve disruption of their metabolic enzymes and pathways. Identification of unique lipid biomarkers to distinguish healthy humans compared to those with a disease can have an impact on the early detection of diseases and personalized medicine. Because of the complexity of a lipidome, which includes eight major categories of lipids, over 80 major classes, 300 sub-classes, and thousands of lipid species,[1] HPLC MS/MS methods are often used to separate many overlapping isomeric or isobaric molecular ions. It is critical that the adapted LC-MS platform offers the capability to separate and identify as many of the isobars and isomers from the biological lipid extracts using both chromatographic separation and the resolution of the MS detector. C30 reversed-phase HPLC columns uniquely offer high shape selectivity, which denotes a chromatographic quality exhibited by certain stationary phases for which enhanced separations of geometric isomers result based on their molecular structure, rather than other physical or chemical differences of the solutes.[2] This selectivity provides separation of structurally related isomers and improved lipid isomer separation efficiency compared to C18 columns.

The high resolving power offered by the quadrupole Orbitrap mass detector, in combination with accurate-mass capabilities, enables large lipid identification coverage and precise quantitation for complex biological samples.[3] Here we report that approximately one thousand lipid molecules from a human serum sample were simultaneously identified and quantified using a newly developed UHPLC Thermo Scientific™ Acclaim™ C30 column (2.1 x 250 mm, 1.9 µm) and a quadrupole Orbitrap high-resolution, accurate-mass (HRAM) MS instrument.

Experimental
Samples
Three human serum samples from donors with different diets and one human plasma sample from equal numbers of male and female donors were provided by the National Institute of Standards and Technology (NIST). Table 1 shows the detail description of the four samples. Chloroform, methanol, and water were used for the lipid extraction. Table 2 shows the extraction procedure used.

Table 1. Human serum and plasma sample description.

ID	Type	Description
2378-1	Human serum	Donors did not take fish or flaxseed oil
2378-2	Human serum	Donors took flaxseed oil supplements
2378-3	Human serum	Donors took fish oil supplements
SRM 1950	Plasma pool	Equal number of men and women

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Table 2. Lipid extraction procedure.

1	Take 80 µL of the sample aliquot into 4 mL glass tube.
2	Add internal standards purchased from Avanti (17:1 LPA, 17:1 LPC, 17:0-14:1 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, 19:0 Cholesterol ester, Sphingosine (d17:1), C17 Ceramide (d18:1/17:0), Lyso SM (d17:1), 17:0 SM (d18:1/17:0), Cholesterol (D7), Cardiolipin Mix I, Deuterated TG Mixture I, and Deuterated DG Mixture I).
3	Add 600 µL of methanol, vortex.
4	Add 1000 µL of chloroform, vortex.
5	Add 500 µL of water, vortex.
6	Centrifuge at 3000 R for 10 min.
7	Collect the lower (chloroform) phase.
8	Add additional 600 µL of chloroform, repeat step 6 to step 7.
9	Evaporate the combined organic phases to dryness in a vacuum centrifuge.
10	Reconstitute extracted lipids in 100 µL of IPA/methanol (50:50) for storage.

UHPLC Method

A Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system performed UHPLC separations using the gradient conditions shown in Table 3. Mobile phase A was 60:40 acetonitrile / water and mobile phase B was 90:10 IPA / acetonitrile; both A and B contained 10 mM ammonium formate and 0.1% formic acid. The column was a C30 prototype column (2.1 x 250 mm, 1.9 µm) operated at 45 °C. The flow rate was 200 µL/min and the injection volume was 2 µL.

Table 3. HPLC gradient conditions.

Time	% A	% B
0	60	40
7	45	55
8	35	65
12	35	65
30	30	70
31	12	88
51	5	95
53	0	100
60	0	100
60.1	60	40
70	60	40

Mass Spectrometer Method

A Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer equipped with a HESI-II probe was employed. The Q Exactive HF mass spectrometer features an ultra-high field Orbitrap analyzer, which almost doubles its speed and resolution compared to a first-generation Orbitrap analyzer. The ultra-high resolution (up to 240,000, FWHM at *m/z* 200) of the Q Exactive HF MS allows accurate mass measurement with less than 3 ppm accuracy with external calibration. The faster scan speed (up to 18 Hz) of the Q Exactive HF MS results in a higher number of precursor ions triggered for MS/MS. As a result, more lipid identifications in a single run can be achieved with improved sensitivity, accuracy, and productivity.[3]

The instrument was operated using a data-dependent LC-MS/MS method (top15 MS^2) in both positive mode and negative mode, respectively. The instrument method and operating conditions are shown in Table 4. The cycle time was 1.8 seconds, providing sufficient scans across the chromatographic peak profile for accurate relative quantification using the HRAM precursor ion while simultaneously acquiring dd-MS^2 spectra for lipid identification.

Table 4. MS operating conditions.

ESI Probe	Q Exactive HF
Sheath Gas = 45	Pos. Ion (250–1200 amu)
	Neg. Ion (200–1200 amu)
Aux Gas = 8	MS Resolution R = 120K, FWHM at *m/z* 200
	Top15 dd-MS^2 R = 30K
Spray Voltage = 3.5 kV	FWHM at *m/z* 200
S-Lens = 50	MS^2 Isolation Width = 1 Da
Cap. Temp. = 320 °C	Stepped NCE Pos. Ion: 25, 30 Neg. Ion: 20, 24, 28
Heater Temp. = 350 °C	AGC target 1E+6 MS, 50 ms max. 1E+5 MS^2, 80 ms max.

Data Processing

Thermo Scientific™ LipidSearch™ software version 4.1 was used for lipid identification and quantitation. Key processing parameters are shown in Table 5. First, the individual data files were searched for product ion MS/MS spectra of lipid precursor ions. MS/MS fragment ions were predicted for all precursor adduct ions measured within ±5 ppm. The product ions that matched the predicted fragment ions within a ±5 ppm mass tolerance were used to calculate a match-score, and those candidates providing the highest quality match were determined. Next, the search results from the individual positive or negative ion files from each sample group were aligned within a retention time window (±0.1 min) and the data were merged for each annotated lipid.

Table 5. LipidSearch parameters.

Search Parameter	Settings
Precursor Ion Mass Tolerance	5 ppm
Product Ion Mass Tolerance	5 ppm
Lipid Sub-Classes	**Phospholipids:** LPC, PC, LPE, PE, LPS, PS, LPG, PG, LPI, PI, LPA, PA, CL
	Sphingolipids: SM, Cer, CerG1, CerG2, CerG3
	Glycerolipids: MG, DG, TG, DGDG, DGMG, MGDG, MGMG, SQMG, SQDG
	Neutral lipids: ChE, CoQ, SiE, StE, ZyE

The annotated lipids were then filtered to reduce false positives using criteria such as those listed in Table 6. For example, the main adduct ion observed for diglyceride (DG), triglyceride (TG), and cholesterol ester (ChE) lipid classes is M+NH_4. DG species that were identified as M+H or M+Na adduct ions are likely to be a false positive if there is no corresponding M+NH_4 adduct and were filtered out from the lipid ID results.

Table 6. Examples of criteria used for filtering lipid annotations.

Filter Criteria	Lipid Class
Main Ion = M+H	PC, LPC, Cer, CerG, So, SM
Main Ion = M+NH_4	DG, TG, ChE, PG
Main Ion = M+H or M-H	PE, LPE, PS
Main Ion = M+NH_4 or M-H	PI
Main Ion = M-H	PA, LPA
Main Ion = M-2H	CL
Main Grade = A, B	All except PC, SM (A, B, C)
Occupy > 30 and mScore > 20	TG

Results

Separation Efficiency of Lipid Isomers Using the Acclaim C30 Column

Due to the role of lipids in human diseases, such as Alzheimer's and diabetes and advances in mass spectrometry, the application of lipidomics to profiling disease phenotypes is emerging as a potential approach to hypothesis-free discovery. However, it remains challenging to identify and quantify thousands of lipid molecular species from a biologically complex sample due to inherent complexity. One of the main challenges is that lipids are diversified in structure, but many of them have similar physical/chemical properties. Within each particular lipid category, there can be additional great structural complexity. For example, although TGs are composed of one glycerol molecule and three fatty acid molecules, they have differences in fatty acid chain length, degree of saturation, position of double bonds, and position of particular fatty acid chain on the glycerol backbone, resulting in numerous isomeric and isobaric triglyceride molecular species. It is difficult to distinguish all these isomers solely relying on mass spectrometry.

Chromatographic separation is generally needed prior to MS analysis. C18 columns are commonly used for the lipid mixture separation. However, it is inefficient to use a C18 column to separate large numbers of isomeric lipid species.

It has been demonstrated that an Acclaim C30 column provides higher resolution for separation of hydrophobic structurally related isomers compared to C18 columns.[2] Additionally, it is known that a smaller particle size and longer column length will increase the column efficiency for improved isomer separation.[4]

Aimed to provide better column resolution for separating lipid isomers from complex biological samples, a new UHPLC version of the Acclaim C30 column (2.1 x 250 mm, 1.9 µm), which uses a C30 stationary phase with smaller particle size and longer column length, was developed. To prove the concept, a bovine heart lipid extract (Avanti®, 2.5 µg on column) was analyzed using the C30 column coupled to a Q Exactive HF mass spectrometer. The same bovine heart lipid extract also was analyzed using three commercially available C18 columns (column A: 2.1 x 150 mm, 2.7 µm; column B: 2.1 x 100 mm, 1.7 µm; column C: 2.1 x 150 mm, 2.6 µm). A 60-minute non-linear gradient condition (Table 3) provided the best lipid separation profile using the C30 column. The gradient conditions of three C18 columns followed the published methods.[5,6,7] The lipid isomer separation efficiency and lipid ID results observed using the C30 column were compared with that from the C18 columns. Figure 1 shows extracted elution profiles of a TG (55:3) species observed from the C30 column and one of the C18 columns, respectively. The isomer separation efficiency was improved significantly using the C30 column: fourteen TG (55:3) isomer peaks were detected, while only five TG (55:3) isomer peaks were detected using the 150 mm C18 column. As a result, significantly more lipid species including all isomers from a bovine heart lipid extract were identified using the C30 column compared to using the conventional C18 columns (Figure 2).

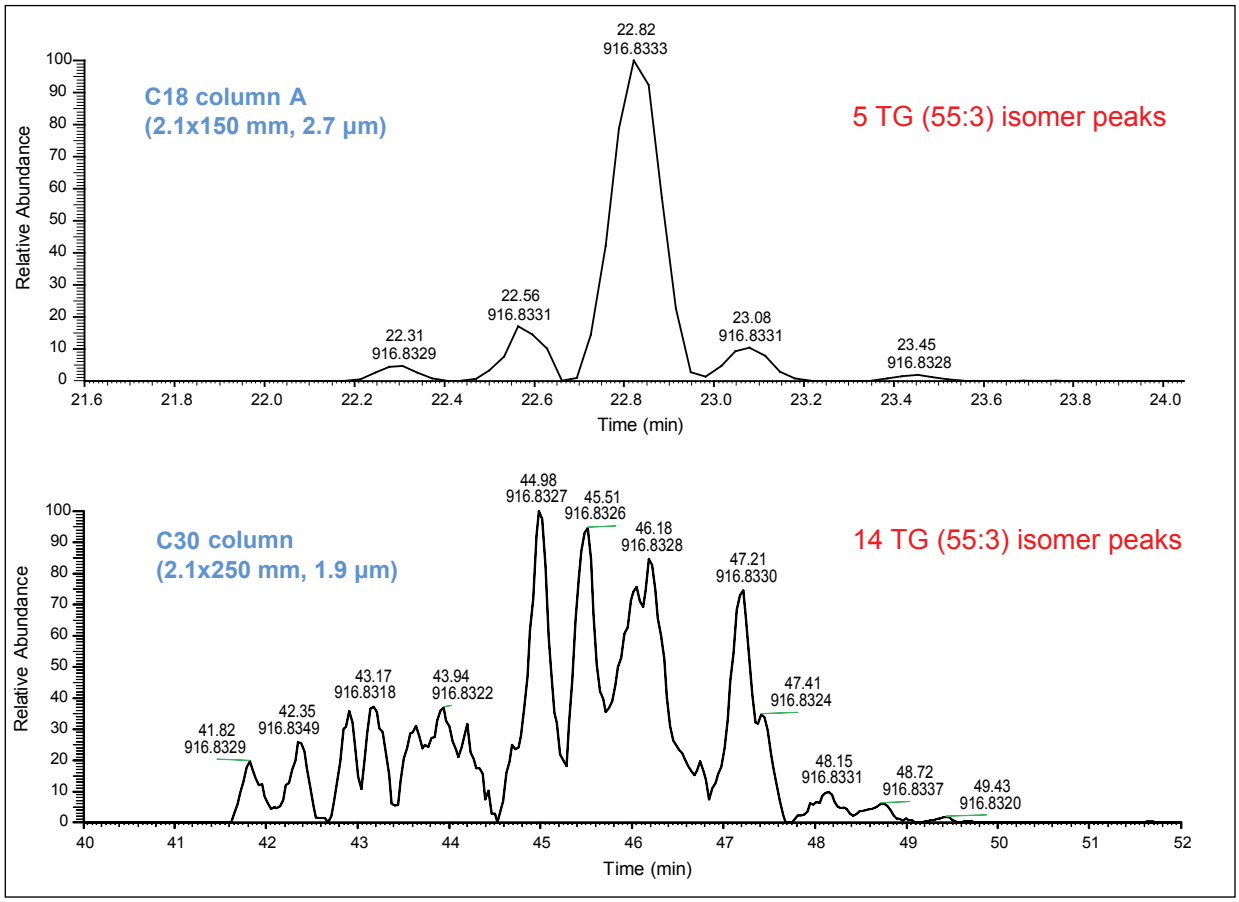

Figure 1. TG (55:3) isomer peak detection from a serum sample with the new Acclaim C30 column and a C18 column.

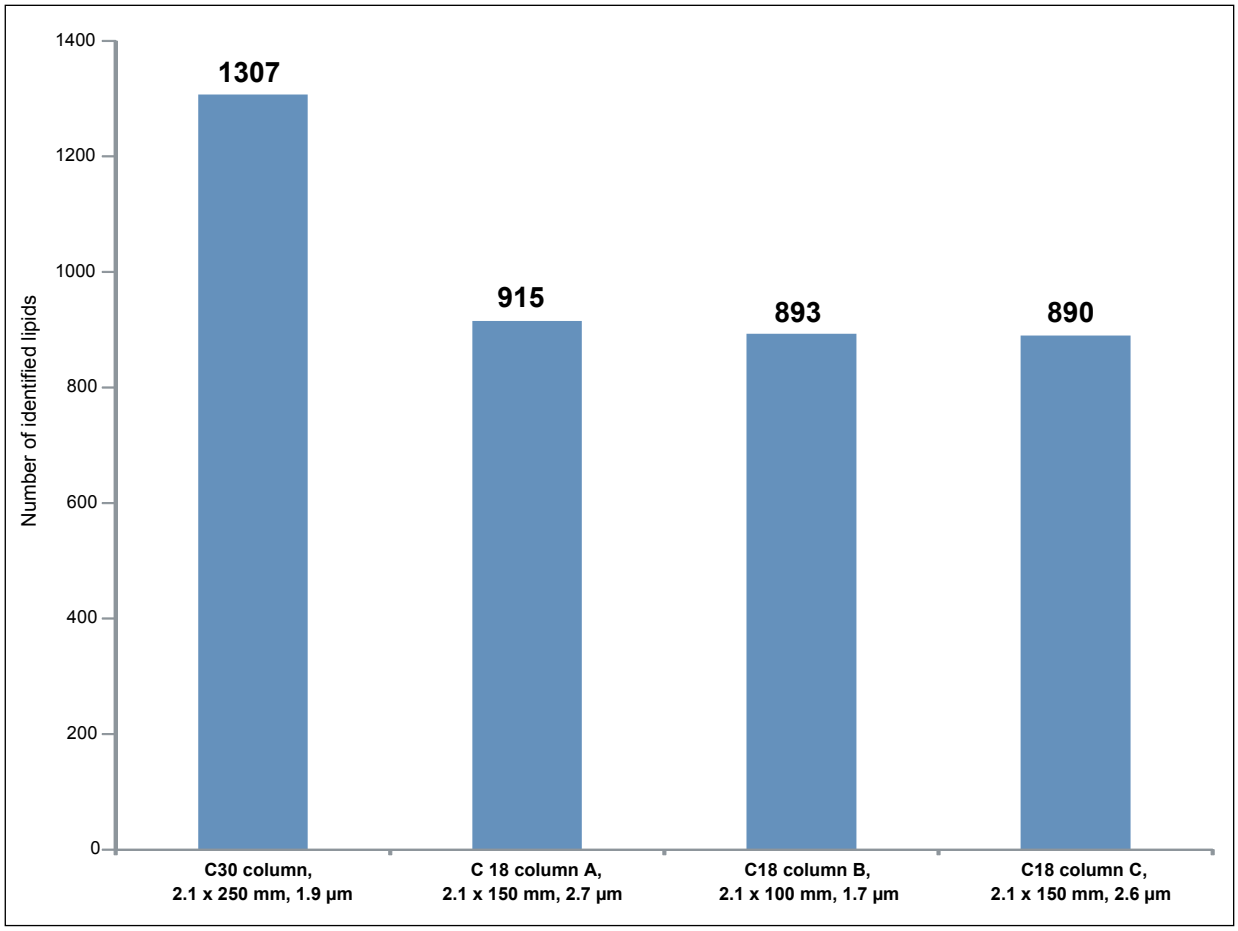

Figure 2. Summary of lipid IDs (including all isomers) comparing the new Acclaim C30 column and three types of conventional C18 columns.

Large Scale Lipid Profiling of the Human Serum/ Plasma Samples Using the UHPLC C30 Column and Q Exactive HF MS

The developed UHPLC MS workflow was applied to NIST human serum samples for evaluating its capability to carry out large scale lipidomics profiling on highly complex biological fluids samples. Each human serum/ plasma lipid extract was separated with the C30 column using the optimized 60 minute gradient prior to MS analysis. Each sample was analyzed in triplicate using the positive ionization mode and the negative ionization mode, respectively. Figure 3 shows both positive and negative basepeak chromatograms observed from one serum sample (S2). High lipid separation efficiency of major lipid classes was achieved by using the UHPLC Acclaim C30 column. Ultra-high resolution used for full-scan MS enabled high mass accuracy over wide inter-scan dynamic range. Sub-ppm mass accuracy was observed even for very low abundant TG molecular species (Figure3, insert). Plus, the high sensitivity and faster scan speed of the Q Exactive HF mass spectrometer[3] allowed MS/MS data being collected on both high and low abundant lipid species with clean and enriched fragment ion information, yielding high lipid identification coverage at molecular composition levels. Figure 4 shows that the fragment ion information observed from the low abundant precursor ion of *m/z* 822.7545 was clear and enriched enough to determine the molecular composition of this TG lipid species. The lipid identification and peak integration results from all 24 raw files were combined together for lipid profiling. Figure 5 shows the parameter setup for the alignment of the total 24 raw files using LipidSearch software.

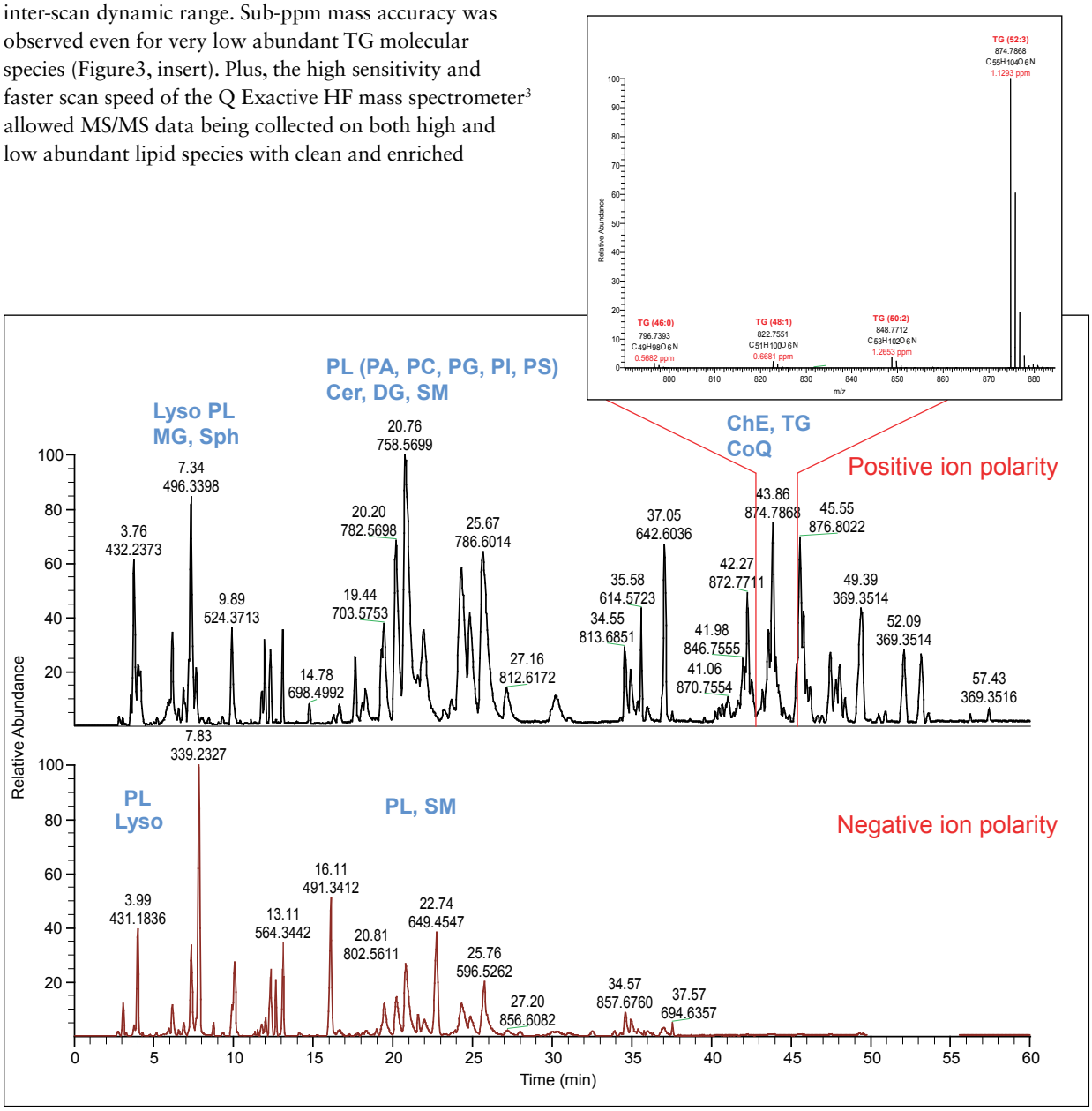

Figure 3. Basepeak chromatograms of a serum sample and one example of MS spectrum of TG lipids (insert).

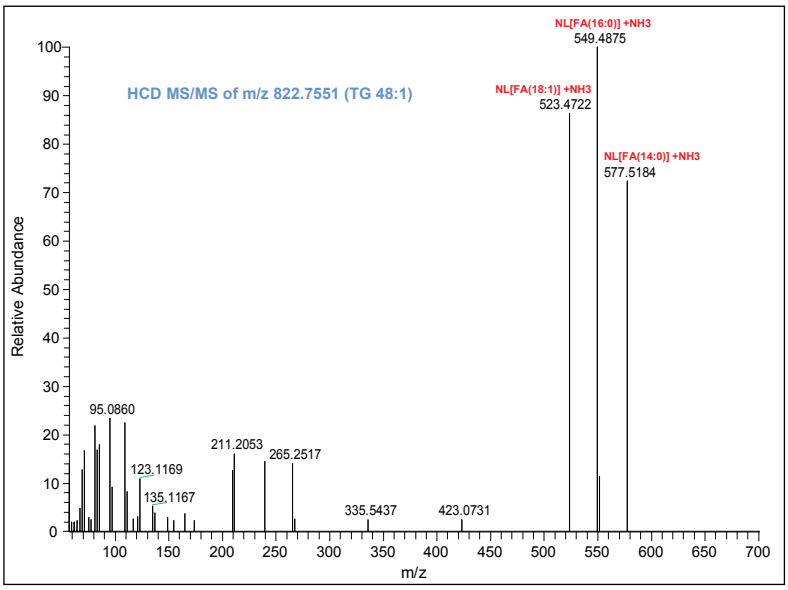

Figure 4. Confident TG molecular composition determination using MS/MS of low abundant TG (48:1) species.

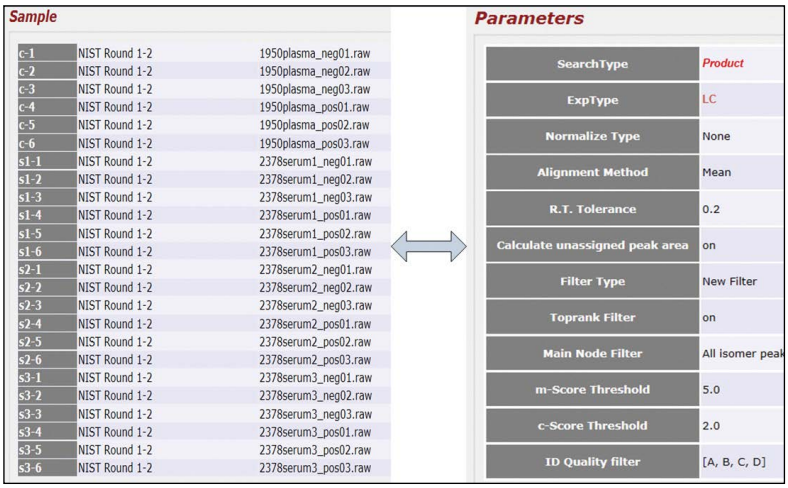

Figure 5. Parameters to merge 24 raw files for lipid profiling.

The C30 column coupled with the Q Exactive HF mass spectrometer successfully identified and quantified a large range of lipid molecules from the four human serum/plasma samples. After filtering and manual validation,[8] 981 lipid species were identified with high confidence and quantified with great analytical precision from the human serum/plasma samples (Table 7). Figure 6 shows the observed lipid profile for each lipid class. The triglycerols (TG) and phosphatidylcoline (PC) showed significant abundance differences within three human serum and plasma samples. The increased lipid isomer separation efficiency offered by the C30 column allowed the clear separation of the human donor serum groups by PCA analysis at the molecular lipid species level (Figure 7) and helped interpretation of the biological rules for each lipid class. Figure 8 shows one separation example of TG 54:6 isomers. The good separation of three lipid isomers enabled clear MS/MS spectra to be collected on each isomer peak, yielding confident identification of each molecular lipid species. In addition, the high-resolution, accurate-mass capability offered by the Q Exactive HF MS further enabled great analytical precision for lipid quantification. Most lipids showed less than 15% CVs. Table 8 shows the calculated amount of TG 54:6 isomers using deuterated TG internal standard. All of the molecular TG species were quantified with CVs less than 7%.

Table 7. Number of lipid species Identified from the human serum/plasma samples after filtering using criteria listed in Table 6.

Lipid Class	Number of Species After Filtering
ChE	18
DG	45
TG	452
PC	220
LPC	57
PE/LPE	32
PI	24
Cer	21
CerG	13
SM	91
Total	**973**

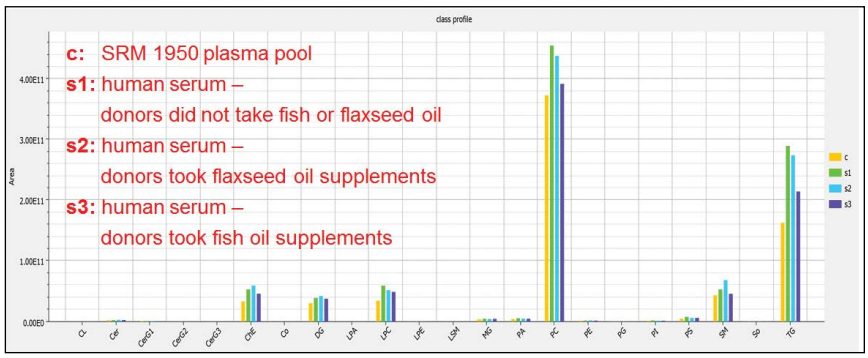

Figure 6. Lipid class profile. The 973 molecular lipid species, including all isomers, were identified from the merged search results of the 24 human serum/plasma runs after filtering using criteria of Table 7.

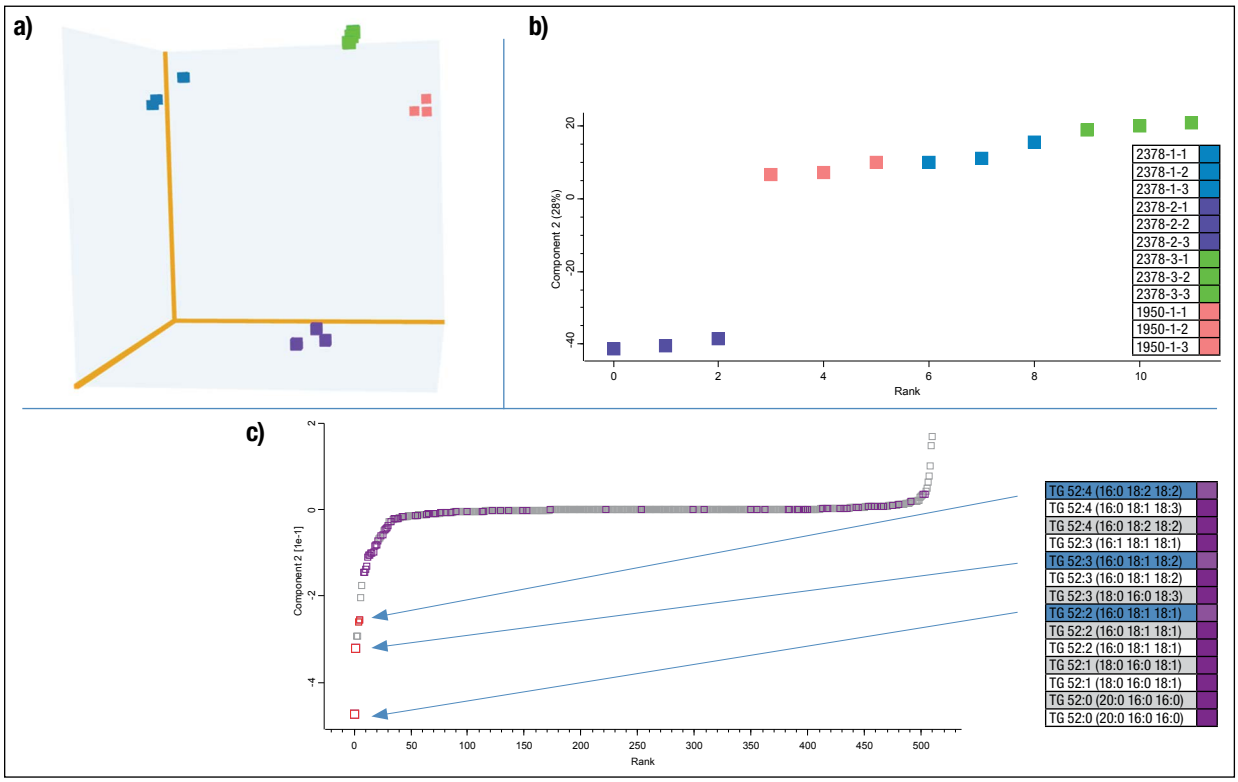

Figure 7. PCA of all samples and all lipid classes except cholesterol esters*. First 3 components shown in 3D plot (a); second component alone in 1D plot above (b), with loadings below (c). Loadings (weights applied to intensity values per lipid, to map into the principal component space) show TG class lipids in purple and select TG lipids in red. [Perseus software for PCA is developed by the Max Planck Institute Computational Systems Biochemistry Lab under Prof. Jürgen Cox.].
* ChE class was removed to enable lower abundance lipid observations.

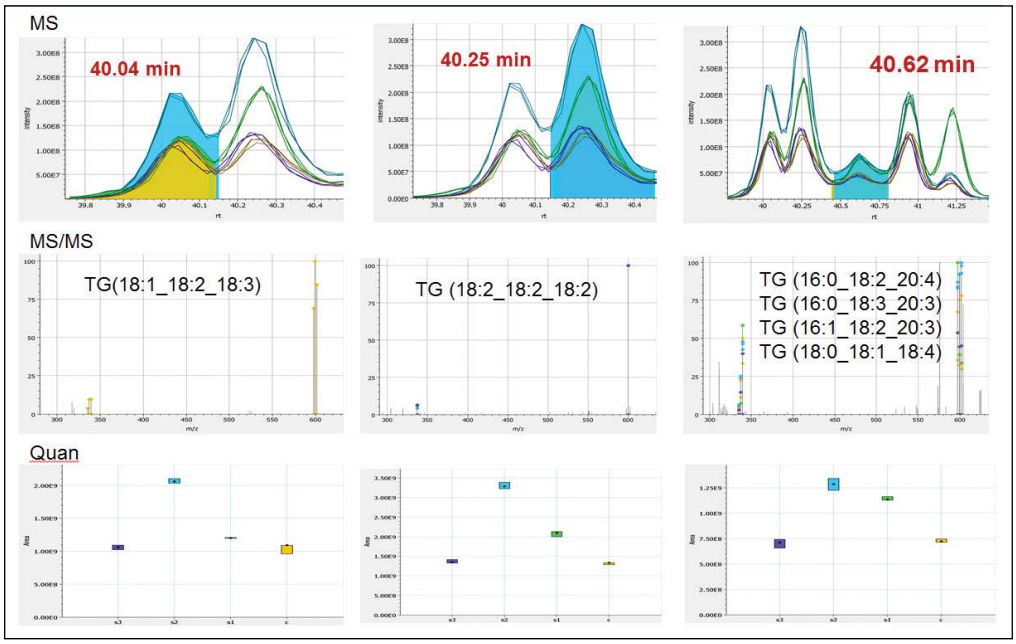

Figure 8. Simultaneous identification and quantitation of 54:6 TG isomers from human serum/plasma. The good separation of three lipid isomers enabled clear MS/MS spectra to be collected on each isomer peak, which can be used for confident identification of each molecular lipid species. Plus, the high-resolution, accurate-mass MS data were used for precise relative quantification of each identified lipid species within four sample groups.

Table 8. Calculated amount of TG 54:6 using deuterated TG internal standard.

TG Species	Rt, min	Concentration, nmol/mL				CV, %			
		2378 S-1	2378 S-2	2378 S-3	1950 P-1	S-1	S-2	S-3	P-1
TG(18:1/**18:2/18:3**)	40.04	3.22	**5.31**	2.26	3.01	3.1	2.6	2.4	5.1
TG(**18:2/18:2/18:2**)	40.25	5.57	**8.49**	2.91	3.79	4.1	3.5	1.7	3
TG(54:6)	40.62	3.06	**3.31**	1.5	2.1	3.2	5.5	7.3	6
TG(54:6)	40.94	4.79	**4.84**	2.43	3.37	4.4	4.1	6.6	4.1
TG(16:0/18:1/**20:5**)	41.21	**4.38**	1.28	0.82	0.95	1.6	5.1	2.7	4.7
TG(16:0/16:0/**22:6**)	41.9	**2.76**	0.73	0.55	0.34	2.2	2.1	6.1	1.1

- Serum-2 (flaxseed oil) significantly higher amount TG containing 18:2/18:3 FA
- Serum-1 (no supp.) had the highest levels of 22:5 and 22:6 FA
- Serum-3 (fish oil) had the lowest levels of TG, similar to the pooled plasma

Conclusions

- We developed an optimal LC-MS workflow for lipid profiling on a 2.1 x 250 mm, 1.9 µm UHPLC C30 column for efficient lipid isomer separation, coupled to a new generation Orbitrap detector for increased lipid identification coverage and quantitative accuracy, and LipidSearch software for high-throughput lipid identification and quantification.
- The increased lipid isomer separation efficiency of the C30 column allows clear and high-quality MS/MS data of individual lipid species obtained with the Q Exactive HF instrument, yielding confident lipid species identification from the highly complex human serum and plasma extracts. Almost a thousand lipid species can be identified routinely and quantified from a single LC-MS run.
- The coefficients of variation of most quantified lipids were less than 15%, showing the speed of analysis using the Q Exactive HF leading to excellent quantitative reproducibility.
- Orbitrap data combined with LipidSearch software allows the simultaneous lipid identification at high coverage and quantitation. Each lipid identification was obtained with a single, high-quality Orbitrap MS^2 scan over four orders of concentration dynamic range.
- This workflow can be applied to any lipidome, including serum/plasma, cell, plants, and food samples to improve the molecular resolution of complex lipid mixtures.

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