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Holistic Lipidomics of the Human Gut Phenotype Using Validated Ultra-High-Performance Liquid Chromatography Coupled to Hybrid Orbitrap Mass Spectrometry

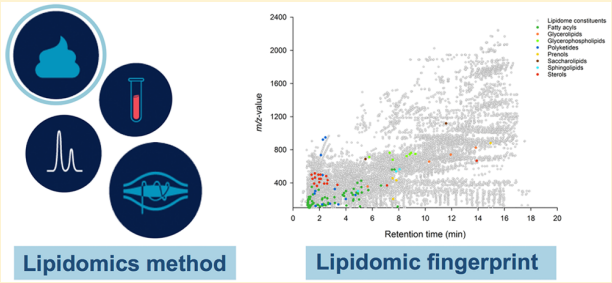
Lieven Van Meulebroek,[†] Ellen De Paepe,[†] Vicky Vercruysse,[†] Beata Pomian,[†] Simon Bos,[‡] Bruno Lapauw,[§] and Lynn Vanhaecke*[,†]

[†]Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[‡]Inflammatory Bowel Disease Research Unit, Department of Internal Medicine, and [§]Department of Endocrinology, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium

Supporting Information

ABSTRACT: As lipids are assigned a plethora of biological functions, it is evident that dysregulated lipid metabolism signifies a key element in many pathological conditions. With this rationale, this study presents a validated lipidomics platform to map the fecal lipidome, which integrates unique information about host−gut microbiome interactions, gastrointestinal functionality, and dietary patterns. This particular method accomplished coverage across all eight lipid categories: fatty acyls, glycerolipids, phosphoglycerolipids, polyketides, prenols, saccharolipids, sphingolipids, and sterols. Generic extraction of freeze-dried feces was achieved by solid−liquid extraction using methanol and methyl *tert*-butyl ether. Extracted components were separated by liquid chromatography, whereby the selected ethylene-bridged hybrid phenyl ultra-high-performance liquid chromatography stationary phase allowed fast separation of both individual lipid species and categories. Detection was achieved by high-resolution full-scan Q-Exactive Orbitrap mass spectrometry and covered a broad m/z scan range (67−2300 Da). Method validation was performed in a targeted fashion to evaluate the analytical performance across all lipid categories, revealing excellent linearity ($R^2 \geq 0.9921$), acceptable repeatability (coefficients of variance ≤15.6%), and stable recovery (coefficients of variance ≤11.9%). Method suitability for untargeted fingerprinting was verified, demonstrating adequate linearity ($R^2 \geq 0.90$) for 75.3% and acceptable repeatability (coefficients of variance ≤30%) for 84.5% of about 9000 endogenous fecal compounds. Eventually, the potential of fecal lipidomics was exemplified within a clinical context of type 2 diabetes, thereby revealing significant perturbations [orthogonal partial least-squares discriminant analysis $Q^2(Y)$ of 0.728] in the fecal lipidome between participants with normal blood glucose levels (n = 26) and those with type 2 diabetes (n = 17).



The chemical complexity among lipids was a major incentive for the LIPID MAPS consortium to establish a classification system based on eight lipid categories: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), polyketides (PK), prenol lipids (PL), saccharolipids (SL), sphingolipids (SP), and sterol lipids (ST). Each category is characterized by a profound hierarchy of classes and subclasses, by which a total of about 40 000 biologically relevant species have been incorporated to date.[1] Following this complexity, lipids are involved in a plethora of biological processes, including energy homeostasis, immune response, membrane architecture, enzyme activity, inflammation, cellular signaling, and transduction of cellular events.[2−5] Evidently, a dysregulated lipid metabolism has been implicated in a variety of pathological conditions such as diabetes mellitus, obesity, Alzheimer's disease, schizophrenia, cancer, atherosclerosis, and multiple sclerosis.[4,6−8] In-depth assessment of the biological relevance of lipid alterations in biological systems may thus yield valuable insights on metabolic homeostasis in relation to human health and disease.[9]

On this basis, the lipidomics domain has recently emerged, whereby the aim is to map the complete assortment of molecular lipid species together with associated metabolic fluxes.[10] Although any biological matrix can be selected, the most frequently used specimens to explore lipid alterations are serum,[11,12] plasma,[13,14] and urine.[15,16] Feces also represents an interesting choice because of the unique link with gastrointestinal functionality, encompassing gut integrity and digestive and absorptive processes.[17] Moreover, this biological matrix strongly reflects dietary intake and captures the tight

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interactions between the host and gut-residing microbiota, for which a causal role in various pathologies has been evidenced.[18−20] For these reasons and because of the noninvasive nature of sample collection, the fecal metabolome has recently been studied in colon cancer, ulcerative colitis, irritable bowel syndrome, and autism.[21−23] However, these studies tend to focus only on the polar subfraction of the metabolome, typically comprising small polar molecules such as amino acids, carbohydrates, bile acids, organic acids, etc.

To date, the 2013 study of Gregory et al.[17] is the sole one to report on fecal lipidomics, whereby the presented analytical methodology was demonstrated to cover six out of eight lipid categories and characterized up to 300 lipophilic species in fecal matter from premature infants. Lipid profiles were established by high-performance liquid chromatography (HPLC) coupled to high-resolution mass spectrometry (MS), which has indeed emerged as the most expedient strategy to perform global quantitative measurements of lipids in complex mixtures. Nevertheless, implementation of LC in lipidomics is not straightforward due to the wide polarity range across the lipid population, which hampers the efficient separation of lipid categories and molecular species therein. In this regard, complex LC methodologies with extended elution times, ternary or quaternary gradient programs, or the use of two-dimensional (2D) chromatography have been proposed.[24] Also at the level of extraction, the heterogeneity of the lipidome in terms of polarity and concentration range brings about difficulties in achieving adequate extraction of lipids across all categories. For example, in order to align with the varied lipid properties, the workflow of Gregory et al.[17] applied two separate and complementary extraction chemistries, dichloromethane and methyl *tert*-butyl ether/hexafluoro-2-propanol. The complexity of the lipidome thus is associated with some major analytical challenges but is also considered one of the reasons that lipidomics connotes a promising research field with lots of unexploited potential.

The objective of this study was to establish an analytical methodology for robust fingerprinting of the fecal lipidome. The specific intentions of this work were to (i) develop a simple extraction protocol based on a single extraction chemistry, (ii) optimize a short ultra-high-performance liquid chromatography (UHPLC) strategy to achieve separation of lipid categories as well as individual lipid species within the categories, (iii) establish full-scan detection on a hybrid system that also offers possibilities for selective fragmentation, and (iv) achieve coverage across all eight lipid categories. In addition, the methodology was validated in a targeted and untargeted fashion to assess the method of being fit-for-purpose for all eight lipid categories and in an omics context, respectively. Finally, lipidomic fingerprinting of fecal samples from type 2 diabetes patients and healthy controls was performed to demonstrate the discriminative power of fecal lipidomics in health and disease. Type 2 diabetes mellitus was particularly interesting due to the associated dyslipidemia,[25] acknowledged role of microbiota,[26] and strong impact of dietary pattern,[27] presumed to be integrated by the fecal lipidome.

MATERIALS AND METHODS

Reagents and Chemicals. Analytical reference standards were obtained from diverse suppliers (Table S1) and carefully chosen in order to attain adequate representation of the LIPID MAPS[1] classification structure. Hereby, as a minimum condition, at least one reference standard for each of the eight main lipid categories was selected. Internal standards were obtained for most of these categories and included deuterium-labeled and non-naturally-occurring compounds (Table S2). Being aware of the fact that several classes or subclasses may not be covered by the constructed database of analytical standards, we considered it critically important to implement a truly untargeted evaluation approach during both method development and validation to ensure holistic coverage.

Stock solutions were made at a concentration of 1 mg·mL^{-1}, whereby appropriate solvents were selected on the basis of the compounds' polarity index (Tables S1 and S2). These solutions and dilutions thereof were stored in amber glass bottles at −20 °C. Solvents were of analytical grade for extraction purposes and LC/MS grade for UHPLC/MS applications and were purchased from VWR International (Merck) and Thermo Fisher Scientific. Ultrapure water (0.055 μS·cm^{-1}) was obtained via a purified water system (VWR International, Merck).

Biological Samples. For development and validation of the method, pools of lyophilized fecal material were used, containing equal shares from at least six healthy adults. These persons were not subjected to any antibiotic treatment during the 3 months before sample donation, and no dietary restrictions were imposed. Lyophilization of fresh samples was performed for approximately 48 h, leading to the removal of 69.3% ± 3.8% water. Lyophilized material was ground, sieved, and stored at −80 °C until use.

Optimization of Lipid Extraction. The extraction protocol was optimized on the basis of a fractional factorial screening design (Modde 5.0, Umetrics) by which six factors were assessed: amount of feces (design values of 100 and 250 mg dry weight), volume extraction solvent (5% and 10% w/v), vortex time (20 and 60 s), incubation time (0 and 60 min, at 4 °C and 0 rpm), type of extraction solvent [methanol/methyl *tert*-butyl ether (1/3 v/v), methanol/dichloromethane (1/2 v/v), and methanol/chloroform (1/2 v/v)], and incorporation of a rotation step (yes or no). This rendered a D-optimal design with 27 experiments (24 design runs and three center points), whereby the main effect of each variable was statistically evaluated (Modde 5.0, Umetrics) based on the metabolome coverage and intensity of 53 targeted lipid species, specifically representing all eight lipid categories.

Following the screening phase, response surface modeling (RSM) was applied to optimize significant quantitative factors, that is, amount of starting material (RSM values of 100, 200, and 300 mg), volume of extraction solvent (5.0%, 1.7%, and 10% w/v), vortex time (20, 40, and 60 s), and methanol/methyl *tert*-butyl ether solvent ratio (1/3, 1/6, and 1/9 v/v). Modeling was effectuated by considering the absolute metabolome coverage and intensity of the 53 selected lipid species. Along with the modeling-based optimizations, the following factors were assessed by full factorial designs with at least three technical replicates: addition of an antioxidant to the extraction solvents [0.01% (w/v) butylhydroxytoluene, BHT],[28] acidification of the water phase [5% (w/v) trichloroacetic acid],[9] shaking (200 rpm) instead of rotation, number of solid−liquid extraction cycles (one or two), and use of alternative nonpolar solvents (ethyl acetate and diethyl ether).[29] The final step concerned optimization of the extract dilution factor.

Final Extraction Protocol. The final protocol for generic extraction of lipids started with the addition of 1200 μL of methanol containing 0.01% BHT (w/v) to 200 ± 0.50 mg of lyophilized and homogenized fecal material. After this mixture

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was vortexed for 30 s, a total volume of 5.4 mL of methyl *tert*-butyl ether with 0.01% BHT (w/v) was added, after which a new vortex step of 30 s was applied. Subsequently, the sample was shaken for 20 min at 200 rpm at 20 °C in an incubator (New Brunswick Innova 42, Eppendorf). Thereafter, 3 mL of ultrapure water with 2.5% trichloroacetic acid (w/v) was added to induce phase separation, which was enforced by centrifugation for 5 min at 3000 rpm at 20 °C. Next, 1 mL of the upper layer, consisting of methyl *tert*-butyl ether, was collected and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue was sequentially suspended in 250 μL of chloroform and 650 μL of methanol, after which a 50-μL subfraction was transferred to an amber glass vial and diluted 1/2 by the addition of 50 μL of methanol internal standard mixture (internal standard concentrations between 2 and 100 ng·μL^{-1}). An aliquot (5 μL) of sample was injected into the chromatographic system.

Liquid Chromatographic Separation and Mass Spectrometric Detection. Liquid chromatography was achieved on a Dionex Ultimate 3000 XRS UHPLC system (Thermo Fisher Scientific), which was equipped with an Acquity ethylene-bridged hybrid (BEH) phenyl column (2.1 × 150 mm, 1.7 μm). Hereby, a binary solvent system consisting of ultrapure water (solvent A) and methanol (solvent B), both acidified with ammonium acetate (3.5 mM), was used to establish a gradient elution program. The following proportions (v/v) of solvent B were used: 0−1 min at 75%, 1−2 min from 75% to 90%, 2−6 min from 90% to 98%, 6−15 min from 98% to 100%, and 15−17 min at 100%, followed by 3 min of equilibration at initial conditions. A constant flow rate of 300 μL·min^{-1} and a column oven temperature of 40 °C were set.

MS analysis was carried out by high-resolution hybrid quadrupole Q-Exactive Orbitrap MS (Thermo Fisher Scientific), preceded by heated electrospray ionization (HESI-II) that operated in polarity switching mode. Analysis was realized through full-scan events with following instrumental settings; sheath gas flow of 40 arbitrary units (au), auxiliary gas flow of 10 au, sweep gas flow of 2 au, heater and capillary temperature both 325 °C, S-lens RF level of 80 au, and spray voltage for positive and negative ionization 3.5 and 3.0 kV, respectively. Furthermore, mass resolution of 70 000 full width at half-maximum (fwhm; 1 Hz), AGC (automatic gain control) setting of 2×10^5 ions, and maximum injection time of 50 ms were selected. The m/z scan range was set from 67 to 1000 Da for both positive and negative ionization modes. After 4.5 min, additional scan events with a scan range of 1000−2300 Da were initiated to cover high-mass lipid species.

Precautions To Avoid Contamination during Analysis. To avoid the introduction of extraneous lipophilic substances from sources other than the fecal material, various precautionary measures were taken. First, all recipient vessels used during extraction consisted of Teflon (40 mL, VWR International, Germany), which is known to show high temperature and chemical resistance. These tubes were extensively cleaned by two cycles of chloroform and acetone, after which they were dried at 75 °C. Second, all other accessories (tips, beaker, pipet, Pasteur, Erlenmeyer) were made of glass and also cleaned by the protocol described. Third, a Hypersil Gold column (50 × 2.1 mm, 1.9 μm) (Thermo Scientific) was installed between the LC pump and injector valve. This column served as a delay column with the purpose of retarding any lipophilic compounds originating from the solvent system.[30]

Method Validation. In accord with the guidelines of Naz et al.,[31] the method's analytical performance was thoroughly assessed in an untargeted and targeted fashion. With respect to the latter, one representative per lipid category was considered: 12-tridecanoic acid (FA), 1-monoolein (GL), coenzyme Q10 (PR), chrysophanol (PK), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (GP), lithocholic acid (ST), lactonic sophorolipid (SL), and *N*-oleoyl-D-*erythro*-sphingosine (SP). Selection of these particular compounds was based on their endogenous absence or low abundance in the fecal material, simplifying any fortification experiments.

Linearity was assessed per lipid category by considering an eight-point calibration curve for each of the lipid target compounds. Associated concentration series were set up in the fecal material prior to extraction (Table 2). In case that the lipid target compound was endogenously absent in the fecal material, the lowest concentration was chosen so as to reach a minimum integrated peak area of about 1 000 000 au, whereas the upper level was 16 times higher. Usage of the peak area as a decision criterion instead of the signal-to noise ratio (S/N) was related to the features of the HRMS instrument, where S/N is often infinite. For those lipids that were endogenously present, selection of the upper calibration point was of course most important, whereby it was opted to add at least 2 times the endogenously expected amount of the compound to the fecal material. Linearity was also evaluated in an untargeted way through a nine-point dilution series (adjusted dilutions of fecal extract), covering factors from 1/50 to 8/1. Linearity performance was assessed each time on the basis of the determination coefficient. Recovery was evaluated in a targeted fashion only, whereby adjusted amounts of analytical standards were added to either fecal material or extract. The ratio of the recovered amounts in both cases was the measure for recovery. Hereby, three levels were considered with six replicates per level (Table 2). Repeatability was assessed by analysis of nine fecal samples by the same analyst under repeatable conditions, whereas an additional set of nine samples was analyzed by another analyst on a different day to allow evaluation of the intralaboratory reproducibility. Enriching these samples with some specific target compounds prior to extraction enabled the targeted assessment of precision across all categories (Table 2). In addition, the short-term instrumental precision was investigated by sequentially injecting three fecal extracts 10 times.

Data Analysis. XCalibur 3.0 software (Thermo Fisher) was used for targeted processing of full-scan data, including identification and quantification of lipid target compounds. Identification of a compound was realized by use of the m/z value of the molecular ion (mass deviation ≤3 ppm), the C isotope pattern ($^{13}C/^{12}C$ isotope ratio that is compliant with CD 2002/657/EC guidelines),[38] and the retention time relative to that of an internal standard (deviation ≤2.5%), all being determined from the corresponding analytical standard. Sieve 2.2 (Thermo Fisher Scientific) was applied for untargeted data interpretation, characterizing detected ions in terms of m/z value, retention time, and peak intensity. Parameters for automated peak alignment, noise removal, peak extraction, and deconvolution are presented in Table S3. This work did not intend to identify the individual species from the lipidomic fingerprints, with the exception of those that matched an analytical standard. Multivariate data analysis was performed with SIMCA 14.1 (Umetrics).

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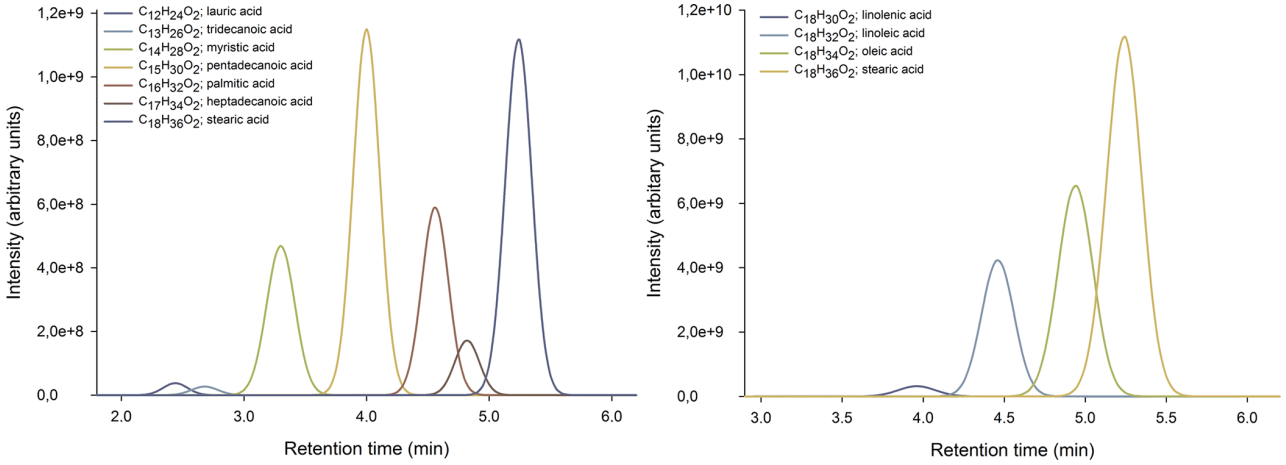

Figure 1. Interaction between fatty acids and the C18 BEH phenyl stationary phase with separation according to carbon chain length (left) and number of unsaturated bounds (right).

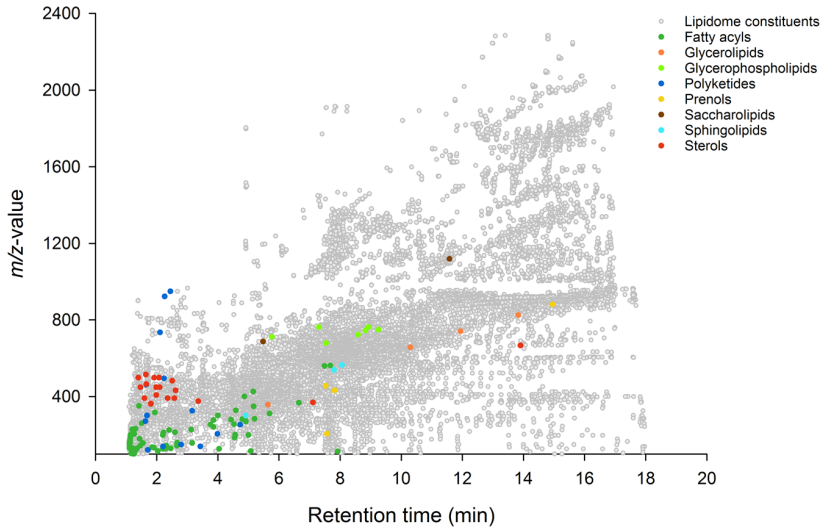

Figure 2. Fecal lipidomic fingerprint including both positively and negatively ionized constituents (total number of 13 153 ions). In total, 127 reference standards were located, demonstrating class-related time zones.

Fecal Lipidomics in a Clinical Context. The lipidomics methodology was employed to assess any perturbations in the fecal lipidome in relation to blood glycemic state. Hereby, individuals without (n = 26) and with type 2 diabetes (n = 17) were engaged to participate and deliver one fecal sample. Classification of individuals was based on the glycated hemoglobin (Hb A1c) level, measured in venous whole blood by HPLC (Tosoh). Hereby, a Hb A1c level of 60 mmol·mol^{-1} was taken as the criterion for type 2 diabetes. This particular study was approved by the UZ Ghent Ethical Committee (EC 2016/0673). Other factors such as age, weight, body mass index, gender, and diet were verified as nonconfounding, as the factor-specific orthogonal partial least-squares discriminant analysis (OPLS-DA) models were not validable.

RESULTS AND DISCUSSION

Ultra-High-Performance Liquid Chromatography Optimization. Although direct infusion ESI-based shotgun lipidomics represents a powerful approach for rapid analysis of lipids, there are some serious limitations in terms of ion suppression and separation of isobaric and isomeric compounds. These limitations may be addressed by liquid chromatography, which offers high resolution and better reproducibility.[2,6] In the case of reversed-phase LC, separation mainly depends on the characteristics of the fatty acid chain, which allows one to distinguish between species within a lipid class or subclass. Conversely, normal-phase chromatography relies on the physical properties of the lipid headgroup and generally achieves separation between lipid (sub)classes.[3] Only recently, it was accomplished by Olsson et al.[32] to combine both separation principles, by using 5-μm particles of phenyl-coated silica (i.e., Reprosil-Pur 120 phenyl). Accordingly, in this study, an Acquity BEH phenyl C18 column (2.1 × 150 mm, 1.7 μm) was tested and proven to show an analogous separation performance while being compatible with UHPLC. Indeed, as exemplified by the elution profile of some saturated and unsaturated fatty acids (Figure 1), interaction with the stationary phase appeared to be a function of the length of the carbon chain and its degree of saturation. Chromatographic separation of individual lipid species was thus dependent on the

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arrangement of the fatty acid groups, providing opportunities for retention time prediction.[32]

In addition, the use of retention time windows (Figure 2) may also assist in structural elucidation of unknowns, as information on the polar headgroup and thus lipid category could be acquired as well. In conclusion, the BEH phenyl stationary phase was found highly suitable for high-throughput lipidomics chromatography.

Mobile-phase composition was optimized by testing various organic solvents (methanol, acetonitrile, and 2-propanol) in combination with adapted complements of ultrapure water. Although the speed of elution could be increased for various lipids by using 2-propanol, this solvent was disadvantageous for the chromatographic peak shape of several compounds, in particular FA (average A_s of 1.26 ± 0.13). In this respect, methanol and acetonitrile performed much better (average A_s ≤ 1.12 ± 0.14 for FA and ≤1.14 ± 0.19 for all lipids), whereby better sensitivity was generally achieved in case of methanol. Indeed, when methanol was used instead of acetonitrile, peak intensities were increased by a factor between 1.5 (for FA) and 15 (for prenol lipids). Additionally, various mobile-phase additives were evaluated: ammonium acetate, ammonium formate, acetic acid, formic acid, and combinations thereof. The addition of 3.5 mM ammonium acetate was most suitable, as the sensitivity was significantly improved for almost all lipid species, resulting in an increase in class-based average sensitivity by a factor of about 4.

Eventually, a column oven temperature of 40 °C was set, as higher temperatures were accompanied by a significant degradation of numerous lipid species. In cases where temperatures were ≥45 °C, a strong reduction in peak intensity (28.1% on average, ranging between 5.4% and 79.6%) was noted for compounds from all lipid classes (with the exception of FA, which were found to be rather inert up to 50 °C). However, 35 °C was observed to result in decreased signal intensities (on average 1.8−17.6%) for most lipid classes with the exception of ST and PR (increased by 1.7% and 7.1%, respectively). Moreover, lower temperatures were also not feasible, as these caused the column back pressure to exceed its maximum limit (mainly due to combination with the delay column). Also for this reason, the mobile-phase flow rate was kept at 300 $\mu L \cdot min^{-1}$.

Optimization of Heated Electrospray Ionization and Full-Scan Orbitrap Mass Spectrometry. Prior to determining the optimal MS conditions, lipid analytical and internal standards were directly infused into the HESI source (50 $ng \cdot min^{-1}$) or separately injected (up to 100 ng) to determine the most abundant adducts and their accurate masses (m/z values). Hereby, $[M - H]^-$, $[M + H]^+$, and $[M + NH_4]^+$ adducts were most common. In this context, it should be noted that the most intense signal for SL Kdo2-lipid A was obtained for the double-charged molecular ion in negative ionization mode. From this, it may be expected that other SL species, also characterized by high molecular weights, may be retrieved as double-charged ions as well. Another finding concerned the analysis of cholesteryl linoleate; it was found that this particular cholesterol ester releases cholesterol through in-source fragmentation, as observed upon analysis of the analytical standard (≥98% purity). As the associated cleavage process takes place after LC separation, this process does not interfere with the semi-quantitation of cholesterol (directly originating from the fecal extract), which elutes earlier than cholesteryl linoleate (Table S1). Optimization of parameters was mainly based on the peak intensity of the individual lipids as well as the overall sensitivity per lipid class.

Full-scan MS parameters were optimized for fecal extracts and mainly concerned the mass resolution and AGC target. Evaluation of resolution was based on the achieved mass accuracies and number of data points across the chromatographic peaks. Increased mass resolution is accompanied by improved mass accuracy and selectivity, due to superior exclusion of isobaric matrix interferences. However, this also implies prolonged scan times with fewer data points across a peak, which can be detrimental for repeatability and sensitivity. Accordingly, a mass resolution of 70 000 fwhm was found to deliver good quantitative performance, as low mass deviations were noted (<3 ppm) while the number of data points was still satisfactory (≥7).[33] The optimal AGC target was 2×10^5 ions, as this was associated with the highest S/N ratios and low mass deviations (<3 ppm).

Optimization of Extraction. The extraction procedure was optimized by a D-optimal design (G-efficiency of 99.9%), whereby the significance of each factor was evaluated on the basis of metabolome coverage and absolute peak areas of 53 lipid species, representing all eight lipid categories. The results for this screening phase are summarized in Table 1.

Table 1. Impact of Investigated Factors on Extraction[a]

factor	no. of lipids affected positively	no. of lipids affected negatively	metabolome coverage
volume extraction solvent (upper value)	4	12	no effect
amount of feces (upper value)	12	4	no effect
vortex time (upper value)	2	1	no effect
incubation (upper value)	8	0	no effect
rotation step (yes)	5	1	no effect
type of extraction solvent			
methanol/chloroform	1	5	decrease
methanol/dichloromethane	1	8	no effect
methanol/methyl *tert*-butyl ether	20	2	increase

[a]Number of lipid species for which the recovery (expressed by absolute signal intensities) was significantly affected (p-value ≤0.05) or a significant change (p-value ≤0.05) in metabolome coverage was noted.

The type of extraction solvent was ascribed the highest impact (variable importance in the project of 1.30). Usage of methanol/methyl *tert*-butyl ether rendered the highest signal intensities for lipid species from FA, GP, SP, ST, and PK (Table S4). Moreover, this extraction solvent was accompanied by higher metabolome coverage. This is in accordance with Matyash et al.,[34] who found similar or better recoveries for most lipid categories when using methyl *tert*-butyl ether instead of applying the Folch or the Bligh and Dyer method. Here, the low density of methyl *tert*-butyl ether simplifies collection of the lipid-containing phase (which is the upper layer during liquid-phase extraction), allowing easy removal of nonextractable interferences such as denatured proteins by centrifugation.[9] The incorporation of methanol intended to disrupt the strong interactions between lipids and cell biopolymers such as proteins and polysaccharides,[9] presumed to manifest during the incubation period, as this factor affected the recovery of polar lipids, mainly short-chain FA (Table S4). Therefore, to

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Table 2. Method Validation Results for Representatives of Eight Lipid Categories

compd	lipid class	endogenous concn (ng·mg^{-1} feces)	added concn range (ng·mg^{-1} feces)	linearity (R^2)	fortification levels (ng·mg^{-1} feces)	recovery ± SD (%)	coefficient of variance ± SD[a] (%) repeatability	coefficient of variance ± SD[a] (%) within-lab reproducibility
chrysophanol	PK	0	0−128	0.9936	25, 50, 100	99.7 ± 12.0	13.6 ± 6.8	22.4 ± 3.3
12-tridecanoic acid	FA	0.5	0−64	0.9974	12.5, 25, 50	119.8 ± 4.7	2.8 ± 0.5	11.2 ± 3.5
1-palmitoyl-2-deoyl-*sn*-glycero-3-phospho-L-serine	PL	1.2	0−128	0.9945	25, 50, 100	100.8 ± 11.9	5.1 ± 3.7	9.5 ± 3.0
N-oleoyl-D-*erythro*-sphingosine	SP	128.6	0−320	0.9949	25, 50, 100	113.1 ± 2.4	7.0 ± 4.2	8.5 ± 1.7
coenzyme Q10	PR	130.4	0−384	0.9941	50, 100, 200	101.4 ± 5.4	7.0 ± 6.2	8.9 ± 2.50
monoolein	GL	357.1	0−1000	0.9985	250, 500, 1000	127.8 ± 10.7	7.2 ± 3.3	10.1 ± 2.0
lactonic sophorolipid	SL	0	0−292	0.9921	25, 50, 100	114.2 ± 1.8	15.6 ± 2.8	16.1 ± 2.4
litocholic acid	ST	6.1	0−195	0.9936	25, 50, 100	110.0 ± 4.6	7.0 ± 3.7	13.4 ± 3.8

[a]SD, standard deviation.

overcome hydrophobic as well as polar interactions between lipids and biopolymers, a balanced solvent ratio between methanol and methyl *tert*-butyl ether was optimized during the subsequent RSM phase, together with other important quantitative factors (amount of feces, volume of extraction solvent, and vortex and incubation times).

During RSM modeling, the optimal factor settings were ascertained on the basis of absolute peak areas of the 53 targeted lipid species only, as differences in metabolome coverage were almost not present. Results were visualized by surface response plots. With respect to the solvent ratio, the optimum was generally found at a methyl *tert*-butyl ether/methanol ratio of 9/1 (v/v). Although this value coincided with the upper limit of the tested range, the use of pure methyl *tert*-butyl ether would not be feasible, as the extraction for some species belonging to TGL and PL was strongly improved in the presence of higher amounts of methanol. Therefore, the 9/1 ratio was concluded to be adequate for holistic, class-representative extraction of lipids. In addition, low volumes of extraction solvent (5.4 mL) and high amounts of feces (300 mg) were determined to be optimal. However, as instrument saturation was noted for some lipid species, the amount of feces was lowered to 200 mg. The final volume of solvent for dissolving the dried extract was also optimized in order to avoid this kind of saturation as well as to minimize matrix effects by checking linearity performance (R^2) together with metabolome coverage (both as a function of a dilution series). Despite these preventive measures, matrix effects can never be excluded completely.[35,36]

Method Validation. The methodology was validated for various criteria, which were evaluated in a targeted and/or untargeted fashion. Identification of compounds was based on the relative retention time, m/z value of the molecular ion, and presence of the corresponding ^{13}C isotope.[37]

Linearity. Targeted evaluation revealed good linearity for the eight class representatives, as determination coefficients R^2 were all ≥0.9921 (Table 2). Additionally, a dilution series was established to obtain linearity data for those compounds that were endogenously present in the fecal material. However, it should be noted that associated findings are merely indicative for the linearity performance, as altered extract dilutions are accompanied by differences in matrix density, which may strongly affect possible matrix ionization effects.[31] For the identifiable species (i.e., those that matched one of the 53 compounds that were targeted during method development), good linearity was concluded for the majority of compounds (about 90%) (Table S5). Although a higher dilution could be designated to anticipate biological variation and avoid saturation in the case of some compounds, reduced metabolome coverage (at least 2%) would be the case. Moreover, evaluation of the linearity based on the calibration curves (with no differences in matrix density) did not reveal any saturation effects. Finally, a truly untargeted five-point linearity evaluation was performed for the known unknowns (i.e., nonidentified compounds), whereby the established R^2 histogram indicated acceptable linearity ($R^2 \geq 0.90$) for 75.3% of 9126 compounds.

Repeatability. Repeatability was assessed in a targeted manner by considering the samples from the recovery experiment, thereby calculating the coefficients of variance for the eight lipid class representatives and for each fortification level. The method was found repeatable as the average coefficients of variance were ≤15.6% (Table 2). Repeatability was also assessed for the endogenously present compounds, thereby first focusing on those lipid species that were considered during method optimization. As coefficients of variance were ≤30%,[31] good repeatability was concluded for all compounds (Table S5). Even more, with the exceptions of valeric acid, anandamide, trimyristin, and tripalmitin, coefficients of variance were ≤16.2%. This finding was strengthened by the complete set of detected lipid species, whereby an acceptable coefficient of variance (i.e., ≤ 30%)[31] was retrieved for 84.5% of 9349 compounds (compounds that were not present in at least one sample of the considered batch were pre-excluded).

Reproducibility. On the basis of samples from the recovery experiment, the within-laboratory reproducibility was also evaluated in a targeted fashion, focusing on the eight lipid class representatives. As coefficients of variance were ≤22.4%, acceptable reproducibility was concluded (Table 2). Additionally, this parameter was evaluated by considering the compounds that were endogenously present in the fecal material. When focusing on the lipid species that were targeted during method optimization, acceptable reproducibility was concluded, as coefficients of variance were generally ≤30.0%[31] (Table S5) with the exception of anandamide and the short-chain fatty acids propionic acid, butyric acid, and valeric acid. With respect to the short-chain fatty acids, their relative high volatility forms a possible explanation, whereby routine execution of the extraction (as performed by the expert analyst) seems sufficient to warrant reproducible results (coefficients of variance for repeatability were ≤20.0%).

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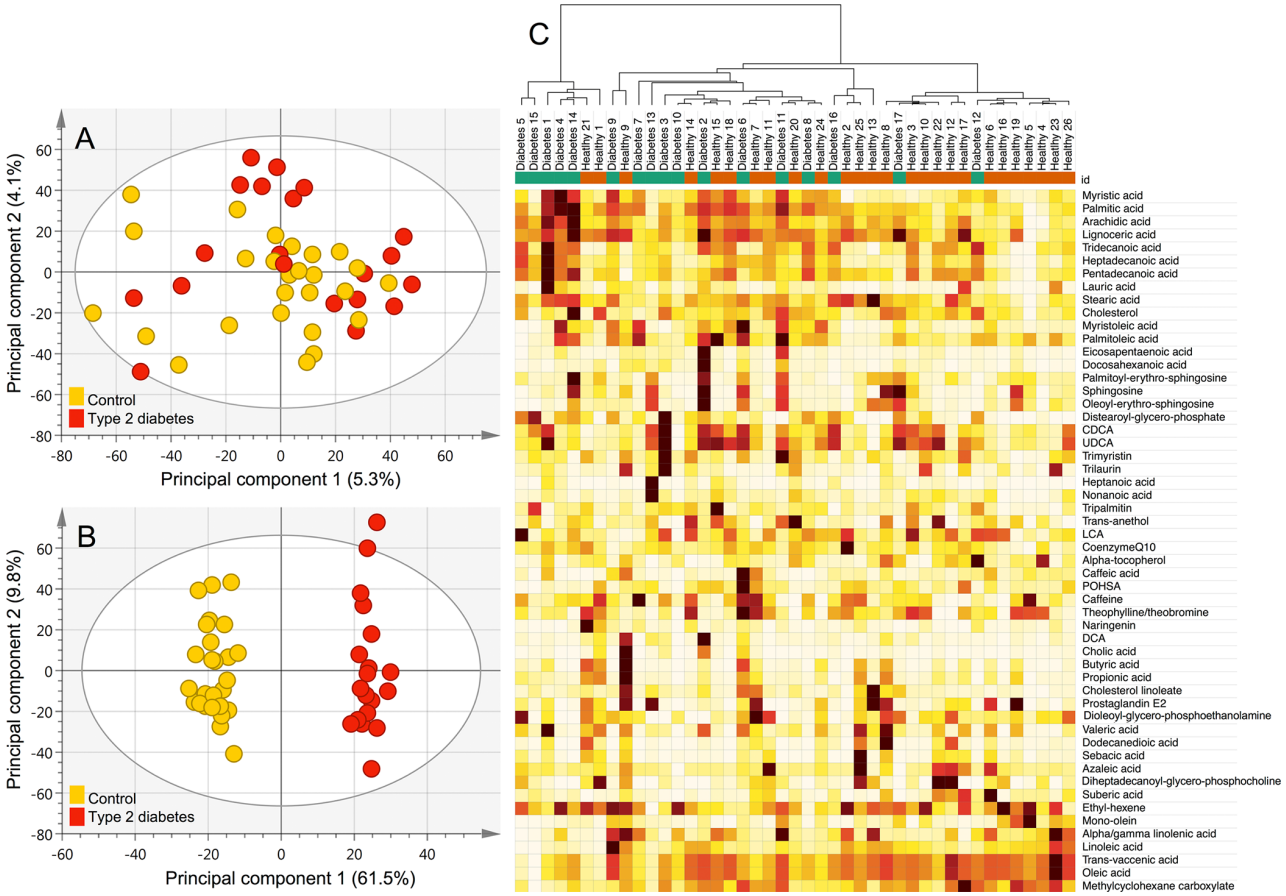

Figure 3. (A) Multivariate analytical workflow with PCA-X score plot, (B) OPLS-DA score plot, and (C) heat map with hierarchical clustering to determine any fecal lipidome discrepancies between control and type 2 diabetes patients.

When the complete set of endogenous lipids was considered, coefficients were ≤30% for 72.6% of 6663 compounds (compounds that were not present in at least one sample of the considered batches were pre-excluded).

Instrumental Precision. As a crucial parameter within metabolomics analyses, instrumental precision was evaluated in an untargeted manner by considering the repeatability of three samples analyzed 10 times within a time frame of about 10 h. It was determined that 71.96% of all 8963 compounds had a coefficient of variance ≤15%. In this regard, to minimize the impact of instrumental drift on the metabolomics data, internal quality control samples[38] are usually introduced. Therefore, the repeatability performance was also assessed for a shorter time span (3 h), corresponding to a sample batch size that would normally be normalized based on the same internal quality control samples. As such, 79.31% and 48.28% of all compounds had a coefficient of variance ≤15% and ≤10%, respectively. Based on these findings, a large part of the variations that were determined for precision (i.e. repeatability and within-laboratory reproducibility) can be linked to prevailing instrumental performance.

Recovery. Adequate trueness was concluded for the target lipid species, as recovery values ranged from 100.8% to 127.8%, thereby noting a low maximum variation of 12.0% across the different fortification levels. This is a fundamental finding within the omics context, as untargeted approaches seek for differences in metabolite abundance, whereby unstable recovery could mask these often-subtle differences.

Detection Capability. As a reflection of detection power, data on the dilution experiment (Table S5) offer a good basis. Focusing on the targeted approach, out of the 36 endogenously present compounds (i.e., those that could be detected at the foreseen dilution), the majority of compounds (30) were still detectable at the maximum dilution of 1/50, whereas the other compounds could still be detected at a dilution of at least 1/2.5. Given the fact that S/N ratios are often infinite, the main criterion to warrant detection and thus correct compound identification was the presence of the required number of diagnostic ions in HRMS measurements: that is, the presence of the monoisotopic peak as well as the ^{13}C isotope ion (isotope ratio according to CD 2002/657/EC).[37]

Fecal Lipidomics in a Clinical Context. To demonstrate the applicability of the validated lipidomics methodology, fecal samples from individuals without hyperglycemia and from type 2 diabetes patients were analyzed to reveal any health-related lipidome discrepancies. Fingerprints covered in total 13 153 monoisotopic ions, whereby the majority (70.8%) were obtained in positive ionization mode. The corresponding data matrix of ion intensities was subjected to multivariate statistics, whereby pareto scaling and log transformation were applied to standardize the range of peak intensities and induce normality, respectively. Unsupervised principal component analysis (PCA-X) indicated no outliers (95% Hoteling's T^2 statistics) and

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suggested acceptable instrument stability during sample analysis, as internal quality control samples were closely clustered. The PCA-X score plot (Figure 3A) revealed only limited clustering according to health state, indicating that other factors, such as diet, may have a more pronounced impact on the fecal lipidome. Nevertheless, supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) (Figure 3B) was able to uncover health-related lipidome discrepancies, as the corresponding model was compliant with all validation criteria [i.e., $Q^2(Y)$ of 0.728, analysis of variance of cross-validated residuals (CV-ANOVA) p-value of 1.17×10^{-9}, and good permutation testing]. This implied that a portion (20.4%) of the fecal lipidome significantly (p-value <0.05) differed according to whether or not type 2 diabetes was present, enclosing potential to unravel etiological and pathological pathways in type 2 diabetes and offering new opportunities for prediction, diagnosis, prognosis, treatment, and management. In addition, a targeted profiling approach was performed, thereby focusing on the 127 compounds defined in Table S1 and using the retention time, m/z value of the molecular ion, and ^{13}C isotope pattern of the analytical standards to confirm the endogenous presence of these compounds in the fecal material of the participants. As such, 54 out of 127 compounds were found to be frequently present in fecal material (Figure 3C). Some other compounds (n = 13) were only sporadically present across participants (prevalence <50% within a study cohort; not shown in Figure 3C). However, by use of the relative levels of these metabolites, full discrimination of the study groups was not achieved on the basis of hierarchical clustering (one minus Pearson correlation, Morpheus online software). This finding points toward the added value of true holistic untargeted lipidomic fingerprinting compared to targeted analytical strategies.

CONCLUSIONS

On the basis of the fact that lipids have been associated with various biological functions, the domain of lipidomics has emerged as a valuable complement of polar metabolomics. However, a validated method that covers the analysis of all eight lipid categories was missing for feces. Indeed, in recent years, feces has surfaced as an essential matrix for in-depth metabolomics studies because of its unique link to the gut microbiome and the interaction with diet. This shortcoming was tackled by the present work, establishing a lipidomics methodology with good analytical performance in terms of linearity and repeatability for a high percentage (i.e., about 75%) of about 9000 endogenous fecal compounds under consideration. Besides, as the analytical performance for all lipid categories was taken into account during both method development and validation, holistic coverage was warranted. Moreover, the possibility to fragment quadrupole-selected metabolites by the hybrid quadrupole Q-Exactive Orbitrap platform may aid in identification of unknowns, which is acknowledged as the major bottleneck in metabolomics. In this regard, the acquired LC elution profile offers potential for retention time prediction, thus also providing valuable input for the identification process. Eventually, analysis of fecal samples from euglycemic individuals and patients with type 2 diabetes revealed that our method was capable of detecting 13 153 monoisotopic ions and allowed differentiation according to glycemic state. These findings indicate the method's high lipidome coverage and robust semiquantitation of lipid concentration levels. As such, this lipidomics method is considered highly suited for holistic fingerprinting of the gut phenotype, which may open the door toward advanced progress in clinical and nutritional research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b03606.

Five tables listing authentic reference standards, authentic analytical internal standards, parameter settings for extraction of features from full-scan MS data by use of Sieve 2.2 software, statistical output to evaluate factors' significance, and validation results for targeted endogenous lipids (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail Lynn.Vanhaecke@UGent.be.

ORCID

Lieven Van Meulebroek: 0000-0002-5489-8915

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Fahy, E.; Subramaniam, S.; Murphy, R. C.; Nishijima, M.; Raetz, C. R. H.; Shimizu, T.; Spener, F.; van Meer, G.; Wakelam, M. J. O.; Dennis, E. A. *J. Lipid Res.* **2009**, *50* (April suppl.), S9−S14.

(2) Gao, X.; Zhang, Q.; Meng, D.; Isaac, G.; Zhao, R.; Fillmore, T. L.; Chu, R. K.; Zhou, J.; Tang, K.; Hu, Z.; Moore, R. J.; Smith, R. D.; Katze, M. G.; Metz, T. O. *Anal. Bioanal. Chem.* **2012**, *402* (9), 2923−2933.

(3) Li, M.; Yang, L.; Bai, Y.; Liu, H. *Anal. Chem.* **2014**, *86*, 161−175.

(4) Vaz, F. M.; Pras-Raves, M.; Bootsma, A. H.; van Kampen, A. H. C. *J. Inherited Metab. Dis.* **2015**, *38* (1), 41−52.

(5) Nzoughet, J. K.; Gallart-Ayala, H.; Biancotto, G.; Hennig, K.; Dervilly-Pinel, G.; Le Bizec, B. *Metabolomics* **2015**, *11* (6), 1884−1895.

(6) Hu, C.; van der Heijden, R.; Wang, M.; van der Greef, J.; Hankemeier, T.; Xu, G. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877* (26), 2836−2846.

(7) Sandra, K.; Pereira, A. D. S.; Vanhoenacker, G.; David, F.; Sandra, P. *J. Chromatogr. A* **2010**, *1217* (25), 4087−4099.

(8) Teo, C. C.; Chong, W. P. K.; Tan, E.; Basri, N. B.; Low, Z. J.; Ho, Y. S. *TrAC, Trends Anal. Chem.* **2015**, *66*, 1−18.

(9) Pati, S.; Nie, B.; Arnold, R. D.; Cummings, B. S. *Biomed. Chromatogr.* **2016**, *30* (5), 695−709.

(10) Orešič, M. *Nutr., Metab. Cardiovasc. Dis.* **2009**, *19* (11), 816−824.

(11) Floegel, A.; Stefan, N.; Yu, Z.; Mühlenbruch, K.; Drogan, D.; Joost, H. G.; Fritsche, A.; Häring, H. U.; De Angelis, M. H.; Peters, A.; Roden, M.; Prehn, C.; Wang-Sattler, R.; Illig, T.; Schulze, M. B.; Adamski, J.; Boeing, H.; Pischon, T. *Diabetes* **2013**, *62* (2), 639−648.

(12) Lu, J.; Xie, G.; Jia, W.; Jia, W. *Front. Med.* **2013**, *7* (1), 4−13.

(13) Fiehn, O. *Plant Mol. Biol.* **2002**, *48*, 155−171.

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Anal. Chem. XXXX, XXX, XXX−XXX

(14) Padberg, I.; Peter, E.; González-Maldonado, S.; Witt, H.; Mueller, M.; Weis, T.; Bethan, B.; Liebenberg, V.; Wiemer, J.; Katus, H. A.; Rein, D.; Schatz, P. *PLoS One* **2014**, *9* (1), No. e85082.
(15) Salek, R.; Maguire, M.; et al. *Physiol. Genomics* **2007**, *29* (2), 99−108.
(16) Wei, H.; Pasman, W.; Rubingh, C.; Wopereis, S.; Tienstra, M.; Schroen, J.; Wang, M.; Verheij, E.; van der Greef, J. *Mol. BioSyst.* **2012**, *8* (5), 1482.
(17) Gregory, K. E.; Bird, S. S.; Gross, V. S.; Marur, V. R.; Lazarev, A. V.; Walker, W. A.; Kristal, B. S. *Anal. Chem.* **2013**, *85*, 1114−1123.
(18) Vanden Bussche, J.; Marzorati, M.; Laukens, D.; Vanhaecke, L. *Anal. Chem.* **2015**, *87* (21), 10927−10934.
(19) Kootte, R. S.; Vrieze, A.; Holleman, F.; Dallinga-Thie, G. M.; Zoetendal, E. G.; de Vos, W. M.; Groen, A. K.; Hoekstra, J. B. L.; Stroes, E. S.; Nieuwdorp, M. *Diabetes, Obes. Metab.* **2012**, *14* (2), 112−120.
(20) Musso, G.; Gambino, R.; Cassader, M. *Annu. Rev. Med.* **2011**, *62* (1), 361−380.
(21) De Angelis, M.; Piccolo, M.; Vannini, L.; Siragusa, S.; De Giacomo, A.; Serrazzanetti, D. I.; Cristofori, F.; Guerzoni, M. E.; Gobbetti, M.; Francavilla, R. *PLoS One* **2013**, *8* (10), No. e76993.
(22) Bickston, S. J.; Comerford, L. W.; Cominelli, F. *Curr. Gastroenterol. Rep.* **2003**, *5* (6), 518−523.
(23) Raman, M.; Ahmed, I.; Gillevet, P. M.; Probert, C. S.; Ratcliffe, N. M.; Smith, S.; Greenwood, R.; Sikaroodi, M.; Lam, V.; Crotty, P.; Bailey, J.; Myers, R. P.; Rioux, K. P. *Clin. Gastroenterol. Hepatol.* **2013**, *11* (7), 868−875.
(24) Knittelfelder, O. L.; Weberhofer, B. P.; Eichmann, T. O.; Kohlwein, S. D.; Rechberger, G. N. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2014**, *951*−*952* (1), 119−128.
(25) Krauss, R. M. *Diabetes Care* **2004**, *27* (6), 1496−1504.
(26) Qin, J.; Li, Y.; Cai, Z.; Li, S.; Zhu, J.; Zhang, F.; Liang, S.; Zhang, W.; Guan, Y.; Shen, D.; Peng, Y.; Zhang, D.; Jie, Z.; Wu, W.; Qin, Y.; Xue, W.; Li, J.; Han, L.; Lu, D.; Wu, P.; Dai, Y.; Sun, X.; Li, Z.; Tang, A.; Zhong, S.; Li, X.; Chen, W.; Xu, R.; Wang, M.; Feng, Q.; Gong, M.; Yu, J.; Zhang, Y.; Zhang, M.; Hansen, T.; Sanchez, G.; Raes, J.; Falony, G.; Okuda, S.; Almeida, M.; LeChatelier, E.; Renault, P.; Pons, N.; Batto, J.-M.; Zhang, Z.; Chen, H.; Yang, R.; Zheng, W.; Li, S.; Yang, H.; Wang, J.; Ehrlich, S. D.; Nielsen, R.; Pedersen, O.; Kristiansen, K.; Wang, J. *Nature* **2012**, *490* (7418), 55−60.
(27) Khazrai, Y. M.; Defeudis, G.; Pozzilli, P. *Diabetes/Metab. Res. Rev.* **2014**, *30* (S1), 24−33.
(28) Abbott, S. K.; Jenner, A. M.; Mitchell, T. W.; Brown, S. H. J.; Halliday, G. M.; Garner, B. *Lipids* **2013**, *48* (3), 307−318.
(29) Lin, J.; Liu, L.; Yang, M.; Lee, M. *J. Agric. Food Chem.* **2004**, *52*, 4984−4986.
(30) Herrero, L.; Calvarro, S.; Fernández, M. A.; Quintanilla-López, J. E.; González, M. J.; Gómara, B. *Anal. Chim. Acta* **2015**, *853* (1), 625−636.
(31) Naz, S.; Vallejo, M.; García, A.; Barbas, C. *J. Chromatogr. A* **2014**, *1353*, 99−105.
(32) Olsson, P.; Holmbäck, J.; Herslöf, B. *J. Chromatogr. A* **2014**, *1369*, 105−115.
(33) Henry, H.; Sobhi, H. R.; Scheibner, O.; Bromirski, M.; Nimkar, S. B.; Rochat, B. *Rapid Commun. Mass Spectrom.* **2012**, *26* (5), 499−509.
(34) Matyash, V.; Liebisch, G.; Kurzchalia, T. V.; Shevchenko, A.; Schwudke, D. *J. Lipid Res.* **2008**, *49* (5), 1137−1146.
(35) Koal, T.; Deigner, H.-P. *Curr. Mol. Med.* **2010**, *10*, 216−226.
(36) Antignac, J. P.; Courant, F.; Pinel, G.; Bichon, E.; Monteau, F.; Elliott, C.; Le Bizec, B. *TrAC, Trends Anal. Chem.* **2011**, *30* (2), 292−301.
(37) European Commission. *Off. J. Eur. Commun.* **2002**, *L221*, 8−36.
(38) Kamleh, M. A.; Ebbels, T. M. D.; Spagou, K.; Masson, P.; Want, E. J. *Anal. Chem.* **2012**, *84* (6), 2670−2677.

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