Title

Plasma and serum oxylipin, endocannabinoid, bile acid, steroid, fatty acid and nonsteroidal anti-inflammatory drug quantification in a 96-well plate format

Authors

Pedersen, Theresa L

Gray, Ira J

Newman, John W

Publication Date

2021

DOI

10.1016/j.aca.2020.11.019

Analytica Chimica Acta 1143 (2021) 189–200

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Plasma and serum oxylipin, endocannabinoid, bile acid, steroid, fatty acid and nonsteroidal anti-inflammatory drug quantification in a 96-well plate format

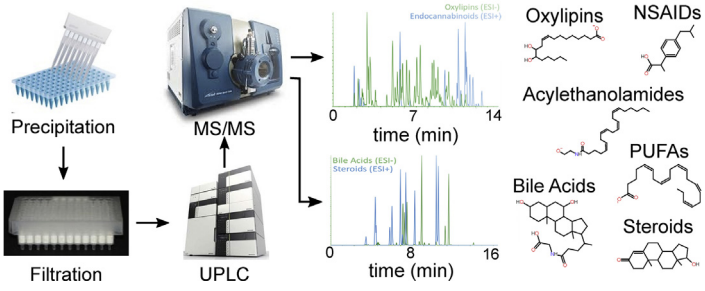
Theresa L. Pedersen [a], Ira J. Gray [b, c], John W. Newman [b, c, d, *]

[a] Dept of Food Science and Technology, University of California at Davis, Davis, CA, USA
[b] Obesity and Metabolism Research Unit, United States Department of Agriculture – Agricultural Research Service – Western Human Nutrition Research Center, Davis, CA, USA
[c] West Coast Metabolomics Center, UC Davis Genome Center, University of California Davis, Davis, CA, USA
[d] Dept of Nutrition, University of California Davis, Davis, CA, USA

HIGHLIGHTS

- Validation of 96-well plate quantitative metabolic profiling method.
- Quantification of 109 lipid mediators, bile acids and steroids in 50 μL of blood.
- First report of endocannabinoid and NSAID concentrations in NIST SRM 1950.
- Expanded oxylipin and glucocorticoid coverage in NIST SRM 1950.
- Fasting state influences endocannabinoids, oxylipins and bile acids.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 August 2020
Received in revised form
10 November 2020
Accepted 17 November 2020
Available online 23 November 2020

Keywords:
Lipid mediators
High-throughput
Metabolic profiling
NIST SRM 1950

ABSTRACT

The goal of this research was to develop a high-throughput, cost-effective method for metabolic profiling of lipid mediators and hormones involved in the regulation of inflammation and energy metabolism, along with polyunsaturated fatty acids and common over-the-counter non-steroidal anti-inflammatory drugs (NSAIDs). We describe a 96-well plate protein precipitation and filtration procedure for 50 μL of plasma or serum in the presence of 37 deuterated analogs and 2 instrument internal standards. Data is acquired in two back-to-back UPLC-MS/MS analyses using electrospray ionization with positive/negative switching and scheduled multiple reaction monitoring for the determination of 145 compounds, including oxylipins, endocannabinoids and like compounds, bile acids, glucocorticoids, sex steroids, polyunsaturated fatty acids, and 3 NSAIDs. Intra- and inter-batch variability was <25% for >70% of metabolites above the LOQ in both matrices, but higher inter-batch variability was observed for serum oxylipins and some bile acids. Results for NIST Standard Reference Material 1950, compared favorably with the 20 certified metabolite values covered by this assay, and we provide new data for oxylipins, N-acylethanolamides, glucocorticoids, and 17-hydroxy-progesterone in this material. Application to two independent cohorts of elderly men and women showed the routine detection of 86 metabolites, identified fasting state influences on essential fatty acid-derived oxylipins, N-acylethanolamides and conjugated bile acids, identified rare presence of high and low testosterone levels and the presence of

* Corresponding author. USDA-ARS-Western Human Nutrition Research Center, 430 West Health Sciences Dr, Davis, CA, 96595, USA.
E-mail address: John.newman2@usda.gov (J.W. Newman).

https://doi.org/10.1016/j.aca.2020.11.019

NSAIDs in ~10% of these populations. The described method appears valuable for investigations in large cohort studies to provide insight into metabolic cross-talk between the array of mediators assessed here.

Abbreviations

1+2-MAGs	the sum of the 1-acyl and 2-acyl monoacylglycerols
AA	arachidonic acid
ALA	alpha-linolenic acid
CUDA	1-cyclohexyluredio, 3-dodecanoic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ISTD	internal standards
LA	linoleic acid
LEA	linoleoyelethanolamide;
MAG	monoacylglycerol;
ROS/MAP	Religious Orders Study/Rush Memory and Aging Project
MEDMs	medians of laboratory means
MRM	multiple reaction monitoring
NE-PUFAs	non-esterified polyunsaturated fatty acids
NAEs	N-acylethanolamides
NAGs	N-acylglycines
NIST 1950	National Institutes of Standards and Technologies Standard Reference Material 1950, Metabolites in Human Plasma
NSAIDs	non-steroidal anti-inflammatory drugs
PUHA	1-phenylureido, 3-hexanoic acid
SSTDs	isotopically labeled analytical surrogate standards
UTAK	UTAK Laboratories defibrinated plasma

1. Introduction[1]

The simultaneous quantitation of regulatory metabolites in bio fluids has the potential to provide insight into the complex interactions between regulatory domains, how they are influenced by environmental (e.g. diet) and genetic factors, and their modulation by health and disease. The measurement of metabolite suites from single metabolic pathways, but derived from alternate substrates is one aspect that can provide data for pathway enrichment analyses that reinforce observed changes and can identify shifts in substrate availability and utilization [1–3]. For instance, modulation of omega-6 oxylipins by omega-3 fatty acid feeding may underlie portions of the anti-inflammatory benefits of such dietary interventions in diseases associated with chronic inflammation [4–6]. However, regulatory lipids are numerous, being derived through multiple routes including the metabolism of free and esterified acyl lipids (e.g. oxylipins and endocannabinoids), and the isoprenoid-derived cholesterol backbone (e.g. bile salts and steroids), to name a few. Notably, regulatory cross-talk within and among these pathways are well known and are modulated by disease processes. For instance, interactions between the oxylipin and endocannabinoid pathways are numerous and occur at levels of synthesis, receptor activation, and response modulation and these interactions may be altered during disease processes [7]. Similarly, bile acids are regulators of glucocorticoid metabolism, with important implications regarding energy metabolism, going far beyond their classic roles in lipid digestion [8,9]. Other well-established cross-pathway interactions include endocannabinoid-glucocorticoid interactions modulating hypothalamic-pituitary axis tone, glucocorticoid-dependent inhibition of pro-inflammatory oxylipin production, and glucocorticoid – sex steroid interactions influencing energy metabolism and stress responses [10–14]. Therefore, analytical tools that allow the efficient and simultaneous quantification of these and other interacting regulatory domains offer powerful new tools for biomedical research.

To allow the broad application of such targeted metabolic profiling efforts, efficient and robust techniques are needed to support clinical research toward interpreting these interactions, and the roles these pathways play in health and disease. This work presents modifications and validaton of re recently reported protocols allowing the measurement of oxylipins, endocannabinoids and bile acids from a single 50 μL aliquot of human plasma or serum by UPLC-MS/MS[15]. The current method relies on protein precipitation and filtration in the presence of deuterated analytical surrogates in a 96-well plate-based format, followed by analysis over two LC-MS/MS acquisition. This method represents a significant increase in efficiency relative to other standardly used methods for oxylipins and endocannabinoids [2,16–20], and parallels modern methods for bile acid [21] and steroid quantification [22,23]. We expanded the base method of 72 oxylipins, 3 nitrolipids, 24 endocannabinoids and endocannabinoid like substances and 23 bile acids to include the determination of 4 additional oxylipins belonging to the specialized pro-resolving lipid mediator class [24], 9 high abundance endogenous steroids, 3 non-steroidal anti-inflammatory drugs (NSAIDs), and the semi-quantitative assessment of 5 non-esterified polyunsaturated fatty acids (NE-PUFAs). We apply this method to the analysis of plasma and serum, reporting estimated LODs, LOQs, and intra- and inter-assay variability for all observed analytes. We compare results for the National Institutes of Standards and Technologies Standard Reference Material 1950: Metabolites in Human Plasma (NIST 1950), and to the best of our knowledge provide the first report of endocannabinoids and NSAIDs in this material, while documenting 4 additional steroids and 16 additional oxylipins. Finally, we report metabolite value ranges observed in two cohorts of older individuals for plasma and serum.

2. Methods

2.1. Chemicals, reference materials and quality assurance/quality control measures

Calibration standards were constructed using 5-decimal place analytical balances, analytical syringes and volumetric flasks, with materials purchased from Cayman Chemical (Ann Arbor, MI),

Medical Isotopes (Pelham, NH), Avanti Polar Lipids Inc. (Alabaster, AL), Larodan Fine Lipids (Malmö, Sweden), Sigma-Aldrich (St. Louis, MO) and Steraloids (Newport, RI). A complete list of analytical targets listed by their common abbreviations including their estimated LODs and LOQs, international chemical identifier keys (InChIKeys), Pubchem IDs, and metabolic precursors can be found in Supplemental Table S1 and Table S2.

2.2. Quality assurance/quality control measures

To assess method performance, each experimental batch (96-well plate) of plasma and serum included two method blanks, two NIST standard reference material 1950s - Metabolites in Human Plasma (Sigma-Aldrich, St. Louis, MO) two UTAK Laboratories Inc (Valencia, CA) defibrinated plasma (UTAKs) samples and two study serum pools in serum batches. An additional set of 7 pooled plasma samples was processed in a single batch to assess intra-batch variability and reference material applicability to the included study populations. Inter-batch variability was calculated as the CV of the batch replicate averages, while intra-batch variability was calculated as the average within batch replicate CV. Method blanks were routinely negligible and are not discussed in this manuscript.

2.3. Plasma and serum sources, pool preparation and reference material sub-aliquoting

Plasma and serum samples were collected from control subjects in association with projects investigating biological changes in memory and aging. Plasma samples from men and women between 45 and 90 years of age (n = 281) were obtained from the Emory Goizueta Alzheimer's Disease Research Center at Emory University collected on EDTA as previously described [25]. Serum samples from men and women between 60 and 90 years of age (n = 230) were obtained from Rush University collected in association with the Religious Orders Study (ROS) of older nuns, priests, and brothers from across the United States and the Rush Memory and Aging Project (MAP) of older lay persons from the greater Chicago area [26]. Pooled samples were constructed for each matrix using a random selection of 10 samples. Samples were thawed on wet ice during the extraction procedure, and 100 μL aliquots were mixed, centrifuged for 10 min at 4500 rcf (g) and 4 °C prior to sub-aliquoting. For NIST 1950, 1 mL vials were thawed on wet ice prior to sub-aliquoting and freezing at -°80C. For UTAK defibrinated plasma, 500 mL was thawed over night at 4 °C, mixed gently, placed on wet ice with continuous gentle mixing prior to sub-aliquoting. All pools and reference materials were distributed as 125 μL sub-aliquots in methanol rinsed 500 μL polypropylene Eppendorf tubes and stored at -°80C until use.

2.4. Sample extraction

Plasma, serum and reference materials were randomized and thawed in racks on wet ice, covered in foil to avoid light and processed under amber lights. Aliquots (50 μL) of serum or plasma were added to methanol washed polypropylene 1 mL deep well plates containing: 10 μL methanolic solutions of 625 nM deuterated surrogates of oxylipins, endocannabinoids, PUFA and NSAIDs; 10 μL of 625 nM bile acids and steroids; 5 μL of 0.2 mg/mL of butylated hydroxytoluene and EDTA in 1:1 methanol/water (v/v); 5 μL of methanol solutions of 5 μM 1-cyclohexyluredio, 3-dodecanoic acid (CUDA; Sigma-Aldrich, St. Louis, MO) and 1-phenylureido, 3-hexanoic acid (PUHA; kind gift from Dr. B.D. Hammock, University of California-Davis, Davis CA) in methanol. Spike solutions were delivered with glass analytical syringes using a calibrated repeater. Samples were delivered with an adjustable tip-spacing VOYAGER 8-channel pipette (Integra Biosciences Crop, Hudson, NH). Samples were brought to a final volume of 250 μL with 170 μL methanol:acetonitrile (1:1), vortexed for 3 min to precipitate proteins and centrifuged for 10 min at 4500 rcf and 4 °C. The plate was capped with a silicone mat and chilled at −20 °C for 15 min. A 150 μL supernatant aliquot was pipetted into a 0.2 μm PVDF membrane filter plate (Agilent Technologies, Santa Clara, CA, USA) placed over a 450 μL polypropylene conical well plate. The plate stack was centrifuged for 3 min at 500 rcf at 4 °C. The plate was capped with a slit-top silicone mat and extracts were analyzed by LC-MS/MS as described below. In more recent projects, the 450 μL conical well plates and the silicone mats have been replaced with 450 μL microtiter plates with thermally sealed polypropylene-backed foil.

2.5. Data acquisition and analysis

Oxylipins, N-acylethanolamides (NAEs), N-acylglycines (NAGs), monoacylglycerol (MAGs), nitrolipids, NE-PUFAs and NSAIDs (Oxy/Endo assay) were analyzed in a single UPLC chromatographic run using positive/negative switching with scheduled multiple reaction monitoring (MRM) on a tandem quadrupole mass spectrometer. Bile acids and steroids (BA/Sters assay) were quantified with a second injection on the same platform. Oxy/Endo assay analytes were separated with a Shimadzu Nexera X2 UPLC (Shimadzu, Kyoto, Japan) on a 2.1 × 150 mm, 1.7 μm Acquity BEH C18 column (Waters, Milford, MA, USA) [13]. All UPLC parameters for this assay are included in Supplemental Table S3. BA/Sters analytes were quantified in a second analysis, being separated on a 2.1 × 100 mm 1.7 μm Acquity BEH C18 column using modifications of previously reported procedures [15]. Important modifications to the chromatography gradient improved the method robustness. All UPLC parameters for this assay are included in Supplemental Table S4. Analytes were detected by electrospray ionization with positive/negative switching and scheduled MRM on an API 6500 QTRAP (AB Sciex, Framingham, MA, USA). The electrospray source parameters are described in Supplemental Table S5. For the Oxy/Endo assay, negative mode parameters for oxylipin, NSAIDs, PUFAs, along with retention times, MS/MS acquisition parameters, and quantitative analytical surrogate tags are provided as Supplemental Table S6 and positive mode parameters for NAEs, NAGs, and MAGs are in Supplemental Table S7. For the BA/Sters assay, Supplemental Table S8 includes the negative mode parameters for bile acids, and Supplemental Table S9 includes positive mode parameters for steroids.

Acquisition parameters for a number of metabolites identified as "Screens" are included. These compounds are not included in the calibration standards but have been identified based on either retention times compared to authentic standards (i.e.-NE-PUFAs), or coherent relative retention time matches and mass transitions from the literature. The NE-PUFAs are not included in the Oxy/Endo calibration solutions due to the potential for oxylipin formation in storage. These screened ions include Resolvin E1 and E2, eicosapentaenoylethanolamide (i.e. EPEA), palmitoleoylethanolamide (i.e. POEA), and the NE-PUFAs [i.e. linoleic acid (LA); alpha-linolenic acid (ALA); arachidonic acid (AA); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA)]. With the exception of Resolvin E1 and E2, each of these residues is routinely observed in samples and is reported as relative abundances across study samples after correction by their respective labeled surrogates, as a ratio response. The relative abundances of the NE-PUFAs correlate well with GC-MS based NEFA analyses of split samples (data not shown), supporting the reporting of relative abundances for this "screened" data. For simplicity, full validation for these residues are not reported here.

Data was processed with AB Sciex MultiQuant v 3.0.1. Auto-integrations were manually inspected and corrected as necessary to optimize integrations and confirm peak picking based on relative retention times to their analytical surrogates. Low abundance analytes often required manual integration. The majority of analytes were quantified against 6- to 10-point calibration curves by area ratio response using class-specific surrogate peak areas and 1/x curve calibration curve fits, with calibration point inclusion/exclusion at the low end being established by standard accuracy between 80 and 120%. See Supplemental Tables S6–S9 for surrogate associations to specific analytes. Surrogate recoveries were estimated by application of internal standards to labeled surrogates as an area ratio with either CUDU or PUHA area responses, depending on surrogate retention times. The use of the area ratio corrects for matrix effects at the instrument, thus correcting for sample-to-sample variance due to matrix associated ionization suppression/enhancement of the surrogate. Calibration standard concentration ranges for all analytes in the Oxy/Endo assay and BA/Sters assay are found in Supplemental Table S10 and Table S11, respectively.

2.6. Limits of detection and limits of quantification

LODs and LOQs were estimated according to the Environmental Protection Agency method Procedure 1c [27]. Specifically, 1-tailed t-tests were run between successive concentrations of calibration standard replicates to determine the region of the calibration where a significant change in sensitivity occurred ($p < 0.05$), 'i.e., a break in the slope of the calibration'. The standard deviation (σ) of the first replicated standard solution with calculated concentrations significantly different than the preceding calibration level was used to estimate the instrumental LOD. These values were then transformed into sample nM concentrations by multiplying the calculated concentration by the final sample volume (i.e. 250 μL) and dividing by the volume of sample extracted (i.e. 50 μL). Using the Students t-Distribution, the t-value was determined at a 95% 1-tail confidence level to define the LOD. Specifically, the sample LOD = (t-value) σ x (250 μL/50 μL) and the LOQ = 3 x LOD.

3. Results and discussion

3.1. Surrogate recovery precision and accuracy

Isotopically labeled analytical surrogates standards (SSTDs) are introduced into samples such that 100% recoveries would result in concentrations equivalent to those within the calibration standards. However, as sample matrix components can influence ionization behavior of both analytes and SSTDs, direct comparisons of sample to calibration solution SSTD area counts result in high sample-to-sample variance. To correct for sample specific perturbations in analyte ionization, independent internal standards (ISTDs) can be used to normalize the precision of surrogate recovery estimations, and provide insight into ion suppression/enhancement effects commonly encountered with electrospray ionization. As shown in Fig. 1, by comparing SSTD/ISTD area ratios to SSTD raw area response, the precision of SSTD recoveries is greatly enhanced by normalization with an internal standard. As SSTD standard additions were not performed, we cannot distinguish sample preparation losses from matrix effects including influences on metabolite ionization.

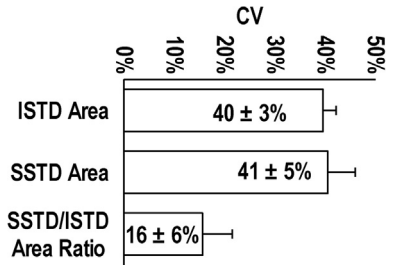


Fig. 1. The use of internal standards increases the precision of analytical surrogate recovery estimates. The average area count and area count ratio coefficient of variation (CV) is shown for the internal standards (ISTD) CUDA and PUHA and for the analytical surrogates (SSTDs) across both positive and negative mode assays in the Oxylipin/Endo assays. The CV of the SSTD/ISTD ratio is significantly reduced ($p < 0.0001$).

Table 1
Surrogate inter-assay and intra-assay percent variability by assay [a].

	Oxy/Endo Assay		BA/Sters Assay	
	-ESI	+ESI	-ESI	+ESI
Inter-batch Variability Geometric Mean [Range] (%)				
Plasma (n = 4)	9.6, [2.8, 41]	15, [9.3, 22]	14, [3.7, 55]	6.9, [4.8, 10]
Serum (n = 3)	3.1, [0.56, 10]	7.2, [5.3, 9.5]	9.1, [4.5, 42]	12, [7.9, 25]
Intra-batch Variability Geometric Mean [Range] (%)				
Plasma (n = 4)	13 [10,18],	20 [12,28],	13 [9,22],	10, [7.3, 12]
Serum (n = 3)	21 [16,28],	24 [21,27],	20 [16,27],	21 [20,23],

[a] . Results are calculated from the analysis of 298 plasma samples analyzed in four batches and 228 serum samples analyzed in three batches.

The estimated surrogate recovery precision across analyte class was stable across the 3–4 plates analyzed for both plasma and serum, with serum showing greater intra-assay variability (Table 1). Across all analyte profiles, the average inter-assay variance for plasma surrogate recoveries ranged from 6.9% to 15% and for serum 3.1%–12%. The average intra-assay variance for plasma surrogate recoveries ranged from 10% to 20%, and for serum 20%–24%.

The artifactual migration of the 1- and 2- acylchains in monoacylglycerols is a particularly difficult problem associated with the measurement of these compounds. Numerous methods have been reported which attempt to minimize this isomerization during sample processing and analysis [28,29]. In the current methods, deuterated 2-arachidonoylglycerol (d8-2-AG) revealed substantial isomerization (>80%) during this simple protein precipitation approach consistent with previous reports of severe isomerization with acetonitrile precipitation [29]. However, quantifying the two isomers as a single peak suggest ~50% recoveries (Fig. 2.). Since these analytes are present at high concentrations this apparent recovery level is acceptable to correct for loss/suppression during the analysis. Therefore, while isomerization was substantial, the sum of the quantified isomers (i.e. 1+2-MAGs) can be used as a measure of the total MAG levels in the sample. It should be stressed, however, that the measure of individual MAG isomers with these methods are not appropriate, and alternative methods should be considered if these targets are critical to the research question [30].

The estimated recoveries for individual surrogates across both assays are displayed in Fig. 2. As reflected in the intra- and inter-assay variability, performance also appeared more stable in plasma than in serum for the Oxy/Endo assay, but equivalent in the BA/Sters assay. In addition, in each assay SSTDs eluting in the first 30% of the chromatographic run show inflated recovery estimates. This high variability likely reflects poor chromatographic resolution of these early eluting surrogates from matrix components influencing the ionization of the analytical surrogates which are not fully corrected by the use of the early eluting internal standard, PUHA. Together these findings demonstrate the importance of an increased density of analytical surrogates in early eluting chromatographic regions, to allow for accurate concentration corrections for analytical targets.

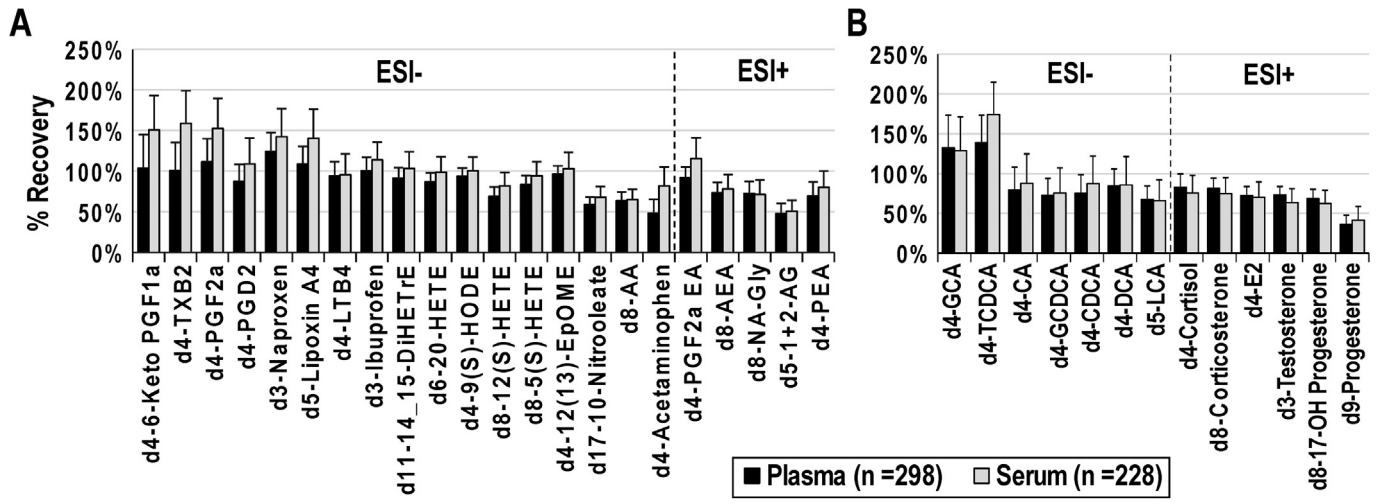


Fig. 2. Estimated analytical surrogate recoveries arranged by increasing retention time in two study cohorts of plasma and serum. A) Oxy/Endo assay. B) BA/Sters assay.

Estimated recoveries are also generally reduced for compounds eluting in the last 10% of the chromatographic run regardless of class. Notably, the stability of these estimated surrogate recoveries across the entire chromatographic run are substantially greater than observed in our previous efforts using solid phase extraction cleanups of 100–200 μL plasma samples [31]. Moreover, optimization of sample volumes in this procedure indicated that greater suppression was observed if 100 μL sample aliquots were used, while reducing sample sizes to 10 μL provided no improvement in SSTD precision or estimated recoveries, but reduced the number of detectable analytes. Together, these findings support the contention that dilution of matrix interferences, rather than extraction and concentration of larger sample sizes provides considerable analytical benefits when using electrospray ionization techniques to evaluate such a diverse array of compounds.

3.2. Analytical precision

Analytical precision was assessed based on the intra-batch variability for each analyte detected in the NIST 1950, pooled plasma, UTAK defibrinated plasma, and pooled serum samples. Inter-batch variability was assessed in all but the pooled plasma. Aggregate intra-batch variability and ranges for each metabolite class for compounds detected above the LOQ are assembled in Table 2. A list of LODs and LOQs for all analytes in these assays can be found in Supplemental Table S1 and Table S2. Metabolite specific CVs ranged from 5 to 93%, with variance increasing as concentrations decreased. Across all matrices, >77% of metabolites detected above the LOQ had intra-batch variability of <25%, decreasing to >67% of metabolites detected above the LOD. For the NIST 1950, the pooled plasma and the UTAK defibrinated plasma73%, 83% and 83% of metabolites detected > LOQ had inter-assay variability <25%, respectively, while in pooled serum, 68% of metabolites achieved this level of performance.

Aggregated data for inter-batch variability for metabolites detected above the LOQ are shown in Table 3. As with the intra-batch variability, inter-batch variability was also influenced by proximity to the LOQ and serum was found to have higher variance than the other matrices. Serum specific issues are discussed below. In the NIST 1950 and the UTAK defibrinated plasma, the average inter-batch variability was <25% across all metabolite classes at concentrations greater than the LOQ. Pooled serum on the other hand showed aggregate inter-batch variability ranging from 16 to 38% for the endogenous metabolites with oxylipins showing the highest variance. Moreover, extremely high variability was seen in 2 of 3 detected NSAIDs. Acetaminophen showed high but variable levels in the three serum analysis batches, while Naproxen was only detected above the detection limit in 2 of 3 batches, with an inter-batch CV >100%. In contrast, NSAID precision was <10% in the NIST 1950 analyses conducted across 7 batches, however Naproxen was not detected in this sample. Notably, ibuprofen was detected in all matrices and performed extremely well.

Across all metabolite classes. 81% and 74% of metabolites in plasma and defibrinated plasma, respectively had inter-assay variability <25%, at concentrations > LOQ. In serum, only 47% of metabolites achieved this level of precision, with particularly high variance in a number of high abundance oxylipins and the detected NSAIDs. The inter-batch variability for individual metabolites are shown in Fig. 3. These figures clearly demonstrate that inter-batch precision increases with metabolite concentrations and highlight matrix specific differences. In NIST 1950, only a handful of detected metabolites showed higher variance than metabolites observed at

Table 2
Intra-batch % variability for aggregate metabolites > LOQ by class [a].

Sample	n[b]	Oxylipins	NAEs/NAGs	1 + 2 MAGs	NSAIDs	BAs	Steroids
NIST 1950	8	16 [5,43],	24 [19,37],	13 [10,23],	5 [5,7],	16 [8,44],	16 [6,43],
Plasma Pool	1	12 [3,43],	16 [9,34],	10 [5,20],	4 [4,5],	10, [3, 80]	9 [5,13],
Serum Pool	3	19 [6,41],	26, [7, 60]	25 [17,33],	19 [14,29],	12, [5, 56]	13 [9,15],
UTAK	6	11 [4,36],	16, [6, 61]	16 [12,23],	22 [13,36],	21, [10, 93]	16 [8,38],

[a] . Reported values are geometric means [range] of the mean coefficients of variation (CV) for all metabolites within a given class detected above the LOQ in replicates measured within analytical batches (i.e. intra-batch variability).
[b] . Number of analytical batches. NIST 1950, Serum Pools and UTAK defibrinated plasmas were run in duplicate in each batch. A total of 7 plasma pools were processed in a single batch.

Table 3
Inter-batch variability (%) for aggregate metabolites > LOQ by class [a].

Sample	n[b]	Oxylipins	NAE/NAG	1 + 2 MAGs	NSAIDs	BAs	Steroids
NIST 1950	7	17, [8, 79]	16 [6,34],	15 [12,21],	7 [6,8],	17, [9, 53]	12 [7,20],
Serum Pool	3	38, [7, 108]	22 [6,41],	16 [12,22],	58, [26, 144]	26, [11, 60]	19 [11,44],
UTAK	6	18, [4, 50]	15 [10,33],	19 [16,20],	24 [15,37],	24, [13, 64]	23 [18,32],

[a] . Reported values are geometric means [range] of coefficients of variation (CV) in mean concentrations measured across all batches (i.e. inter-batch variability) for all metabolites within a given class detected above the LOQ in replicates measured within analytical batches.
[b] Number of analytical batches. NIST 1950, Serum Pools and UTAK defibrinated plasmas were run in duplicate in each batch. A total of 7 plasma pools were processed in a single batch.

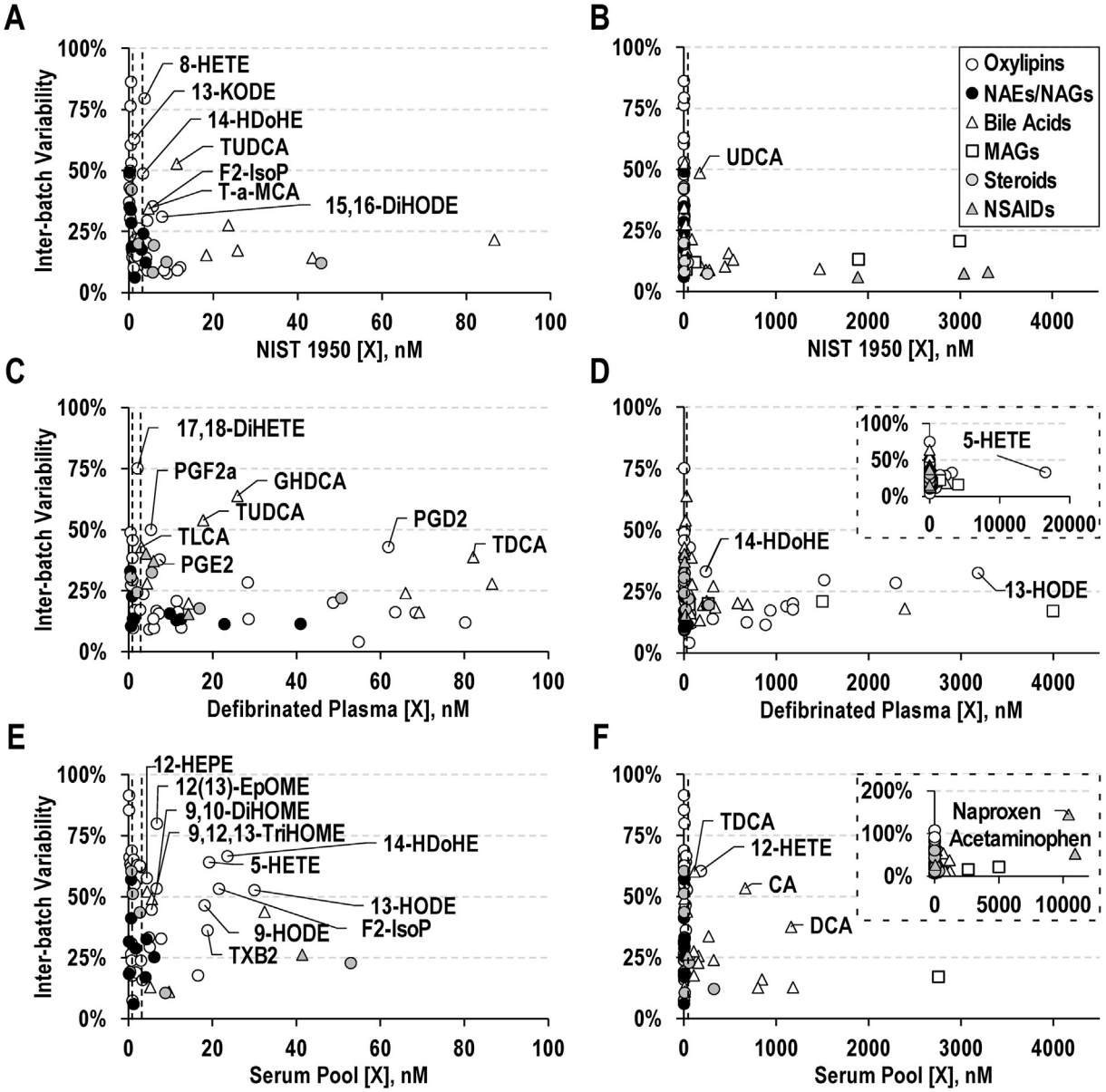


Fig. 3. Inter-batch variability as a function of concentration in each analyzed matrix. Data are the CV of batch averages for NIST 1950 (n = 7) A) low and B) high abundance metabolites, defibrinated plasma (n = 7) C) low and D) high abundance metabolites, and pooled Serum (n = 3) E) low and F) high abundance metabolites. For NIST and defibrinated plasma, labeled residues have >30% inter-batch variability at concentrations >1 nM. For serum, labeled residues have >35% variance at concentrations >3 nM. Vertical dashed lines indicate the average LOD and LOQ across all assays.

similar concentrations. In the UTAK defibrinated plasma, the conjugated bile acids, prostaglandins, and the 5-lipoxygenase metabolite 5-HETE appeared uniquely variable. In contrast, serum displayed high variance in the bile acids cholic acid (i.e. CA) and deoxycholic acid (i.e. DCA), an array of oxylipins, and two of the detected NSAIDs as described above. Assessing the relationships between highly variant serum oxylipins showed two highly correlated sets of metabolites (See Supplemental Fig. S1). In the

first, 12-HETE was found at the highest concentration, and was strongly correlated with other 12-lipoxygenase products (i.e. 12-HEPE, 14-HDoHE and 15-HETE), the LA- and ALA-derived alcohols (i.e. HODEs and HOTEs), the LA-derived-epoxides (i.e. EpOMEs) and the AA cyclooxygenase products TXB2 and PGE2. The second group was dominated by 5-HETE, with strong correlations to other 5-lipoxygenase products (5-HEPE and 4-HDoHE) and the auto-oxidation products 9-HETE and F2-isoprostanes. Notably, these two groups were not correlated with each other. It is interesting to note that the enzymes responsible for producing many of these products are known to be involved in coagulation and thrombosis, and that handling time can influence their levels [32,33]. These results suggest that activity of these enzymes may be preserved in archived serum samples, and suggest that post thaw extraction times should be closely controlled to limit such activity if there is interest in investigating serum levels of oxylipins [34].

3.3. NIST 1950 values, estimated LODs and LOQs, and analytical accuracy

Using the described assays, a total of 86 compounds were quantified in NIST SRM 1950 including 20 certified compounds, 25 previously estimated compounds, and 37 previously unreported residues. The novel measures include 3 bile acids, 4 steroids, and 16 oxylipins, along with the first reports of endocannabinoids and endocannabinoid-like substances in this reference material, including 9 NAEs and NAGs, 3 1+2-MAGs, along with levels of the NSAIDs ibuprofen, acetaminophen, and naproxen. The

Table 4
Thromboxane, prostaglandin and fatty acid diols in NIST SRM 1950 [a].

Analyte	Reported NISTs[b]	Measured NIST	LOD	LOQ
TXB2 [c]		0.406 ± 0.292	0.233	0.699
PGE2	0.4	0.140 ± 0.086	0.198	0.595
PGD2	0.17	0.473 ± 0.463	0.267	0.802
PGF2α [c]		0.608 ± 0.397	0.299	0.897
F2-IsoP [c]		5.63 ± 2.11	0.243	0.729
5,15-DiHETE	0.25	0.476 ± 0.236	0.352	1.06
9,12,13-TriHOME [c]		2.29 ± 0.38	0.276	0.828
9,10-e-DiHO [c]		4.35 ± 1.49	1.15	3.45
12,13-DiHOME	5	3.64 ± 0.45	0.212	0.635
9,10-DiHOME	7	4.39 ± 0.49	0.206	0.617
15,16-DiHODE [c]		7.88 ± 2.51	0.311	0.934
12,13-DiHODE [c]		0.342 ± 0.395	0.187	0.561
9,10-DiHODE [c]		0.264 ± 0.097	0.32	0.961
14,15-DiHETrE	1.1	0.876 ± 0.167	0.172	0.515
8,9-DiHETrE	0.65	0.392 ± 0.254	0.298	0.895
5,6-DiHETE	1.5	1.35 ± 0.289	0.136	0.407
17,18-DiHETE [c]		4.15 ± 1.58	1.08	3.24
19,20-DiHDoPE [c]		1.30 ± 0.203	0.211	0.632
13-HODE	13	12.1 ± 1.3	1.18	3.55
9-HODE	9.7	9.00 ± 0.77	0.645	1.94
13-HOTrE	0.56	0.500 ± 0.248	0.405	1.22
9-HOTrE	0.8	0.288 ± 0.103	0.207	0.621
15-HETE	2.4 ± 0.64	2.34 ± 0.39	0.208	0.625
12-HETE	6.8 ± 1.5	8.31 ± 0.95	0.173	0.518
11-HETE	1.5	1.14 ± 0.16	0.25	0.749
9-HETE	0.85	0.811 ± 0.206	0.63	1.89
8-HETE	1.1	1.29 ± 0.35	0.651	1.95
5-HETE	10 ± 1.3	11.5 ± 1.4	0.242	0.726
15-HEPE	0.42	0.251 ± 0.125	0.191	0.574
12-HEPE	0.98	0.674 ± 0.136	0.312	0.935
9-HEPE	0.5	0.159 ± 0.075	0.386	1.16
5-HEPE	0.86	0.952 ± 0.294	0.706	2.12
14-HDoHE	1.3	3.44 ± 1.43	0.545	1.64
4-HDoHE [c]		2.39 ± 0.71	0.288	0.865
13-OxoODE [c]		1.27 ± 1.05	1.27	3.81
12,13-EpOME	7.8	4.08 ± 0.71	0.303	0.91
9(10)-EpOME	4.2	1.57 ± 0.43	0.133	0.399
15(16)-EpODE [c]		1.80 ± 0.39	0.498	1.49
9(10)-EpODE [c]		0.195 ± 0.126	0.443	1.33
14(15)-EpETrE [c]		0.204 ± 0.136	0.19	0.57
11(12)-EpETrE [c]		0.0653 ± 0.0344	0.2	0.599

[a] Values are in nM (i.e. pmol/mL). Measured values are means ± SD (n = 14). Measured values < LOD have >3:1 signal to noise.
[b] Reference values are medians of laboratory means MEDMs. Variance is included if 5 or more laboratories are included in the MEDM.
[c] Reference values for detected residues have not been reported.

Table 5
Bile acids and Steroids in NIST SRM 1950[a].

Analyte	Reported NISTs [b]	Measured NIST	LOD	LOQ
Bile Acids				
CA	120 ± 34	163 ± 30	1.41	4.23
CDCA	300 ± 110	452 ± 60	2.03	6.10
UDCA	110 ± 24	172 ± 79	1.15	3.46
DCA	350 ± 83	491 ± 76	1.36	4.08
LCA	14 ± 3.6	19.0 ± 5.3	9.43	28.0
w-MCA	5.7	1.22 ± 0.83	4.97	14.9
a-MCA	12		5.52	16.6
b-MCA	3.3	1.06 ± 0.675	2.64	7.91
TCA	26 ± 5.6	26.1 ± 5.2	0.659	1.98
TCDCA	84 ± 5	106 ± 13	0.283	0.846
TUDCA [b]		11.9 ± 6.3	0.047	0.143
TDCA	40 ± 6.4	44.7 ± 7.9	0.143	0.430
TLCA	2.7 ± 0.69	2.03 ± 0.62	1.55	4.66
T-a-MCA [b]		4.7 ± 1.77	0.505	1.52
GCA	240 ± 69	279 ± 30	1.38	4.17
GCDCA	1100 ± 180	1480 ± 160	0.532	1.6
GUDCA	150 ± 24	242 ± 27	0.621	1.86
GDCA	430 ± 69	543 ± 77	0.356	1.07
GHDCA [b]		23.3 ± 8.6	0.297	0.891
GLCA	25 ± 1.8	88.7 ± 19.6	0.522	1.57
Steroids				
Cortisol	231 ± 4.69	258 ± 31	0.875	2.63
Cortisone [c]		46.3 ± 6.1	0.283	0.848
Corticosterone [c]		6.01 ± 1.41	1.52	4.56
11-Deoxy-Cortisol [c]		0.609 ± 0.321	0.506	1.52
Testosterone	7.68 ± 0.163	8.94 ± 1.28	0.430	1.29
17OH-Progesterone [c]		2.26 ± 0.70	0.357	1.07
Progesterone	4.71 ± 0.121	5.70 ± 0.57	0.379	1.14

[a] Values are in nM (i.e. pmol/mL). Measured values are means ± SD (n = 14). Measured values < LOD have >3:1 signal to noise.
[b] Reference values are medians of laboratory means (MEDMs). Variance is included if 5 or more laboratories are included in the MEDM.
[c] Reference values for detected residues have not been reported.

Table 6
NAEs, NAGs, 1+2-MAGs, and NSAIDs in NIST SRM 1950[a].

Analyte	Measured NIST	LOD	LOQ
N-acyl ethanolamides (NAEs)/N-acyl glycines (NAGs)[b]			
OEA	3.97 ± 0.66	0.193	0.58
LEA	3.4 ± 1.0	0.157	0.472
aLEA	0.121 ± 0.074	0.0796	0.239
DGLEA	0.144 ± 0.063	0.149	0.447
AEA	1.36 ± 0.19	0.133	0.399
DEA	0.402 ± 0.168	0.398	1.19
DHEA	0.643 ± 0.143	0.0755	0.227
NO-Gly	2.97 ± 0.65	0.156	0.469
NA-Gly	0.574 ± 0.186	0.164	0.491
Monoacyl glycerols (MAGs)			
1+2-LG	1890 ± 305	1.2	6.31
1+2-AG	124 ± 18.1	0.565	1.69
1+2-OG	3000 ± 770	1.06	3.19
Nonsteroidal anti-inflammatory drugs (NSAIDs)			
Ibuprofen	3300 ± 289	2.79	8.38
Naproxen	3040 ± 249	9.07	27.2
Acetaminophen	1890 ± 120	2.47	7.4

[a] Values are in nM (i.e. pmol/mL). Measured values are means ± SD (n = 14). Measured values < LOD have >3:1 signal to noise.
[b] Palmitoylethanolamide (PEA) and stearoylethanolamide (SEA) are routinely observed in these assays. However, calibration solution contamination artificially elevated LODs during this study preventing their accurate reporting.

concentrations of these compounds, their LODs, LOQs and comparisons to reported values for NIST SRM 1950 are provided in Tables 4–6. While LODs establish concentrations below which reported data may vary by greater than 100%, low precision data can be generated at lower concentrations indicating the presence of metabolites with an instrumental signal to noise intensity >3:1. It is advantageous to report and analyze all collected data, regardless of their proximity to the detection limit as this practice retains the true data structure and prevents left skewing that results from data removal and/or replacement with arbitrary values [35]. Based on the variance in low abundance calibration standards LODs were ~1 nM for the majority of metabolites. As a class oxylipins and NAEs/NAGs had lower average LODs of ~0.6 and ~0.1 nM, respectively, while bile acids and steroids were higher with average LODs between 1 and 2 nM. The described method is comparable to similar methods using larger sample volumes, more extensive sample cleanups, and/or more targeted assays [2,16–21,36,37]. It should be noted that derivatization can be used to improve the LC-MS/MS sensitivity of some analytes. For instance, employing N-(4-aminomethylphenyl) pyridinium, detection limits for eicosanoids may increase 10 to 20-fold using a Waters triple quadrupole instrument [38], but are more modest when using Sciex triple quadrupoles [39]. Similarly, derivatization of ketone containing steroid hormones with hydroxylamine can increase detection limits from 2 to 250-fold [40], while hydroxyl-containing steroid derivatization with isonicotinoyl chloride enhanced the LC-MS/MS quantification of a broader suite of steroids allowing the use of as little as 100 μL of plasma [41]. How such derivatization processes would influence the broad metabolite profiles described in the current method would need to be evaluated, but may offer advantages for specific research questions where greater sensitivity is required.

Assay accuracy was evaluated by comparing measured values from 14 independent analyses of NIST 1950 to certified and estimate values [42]. Certified values are calculated as the medians of laboratory means (MEDMs) from 5 or more laboratories, while estimated values are reported for results from fewer than five laboratories and do not have associated estimates of error. As seen in Fig. 4A, measured and certified values were highly correlated. Comparing the measurement averages to the certified MEDMs, 10 of 20 metabolites had accuracies between 75 and 125%, 18 of 20 were between 70 and 160%, and 14 of 20 compounds had measured ranges which overlapped the certified range. As seen in Fig. 4B, the bile acids were most often over estimated relative to the certified values, and the source of this inaccuracy is unclear. For metabolites with only

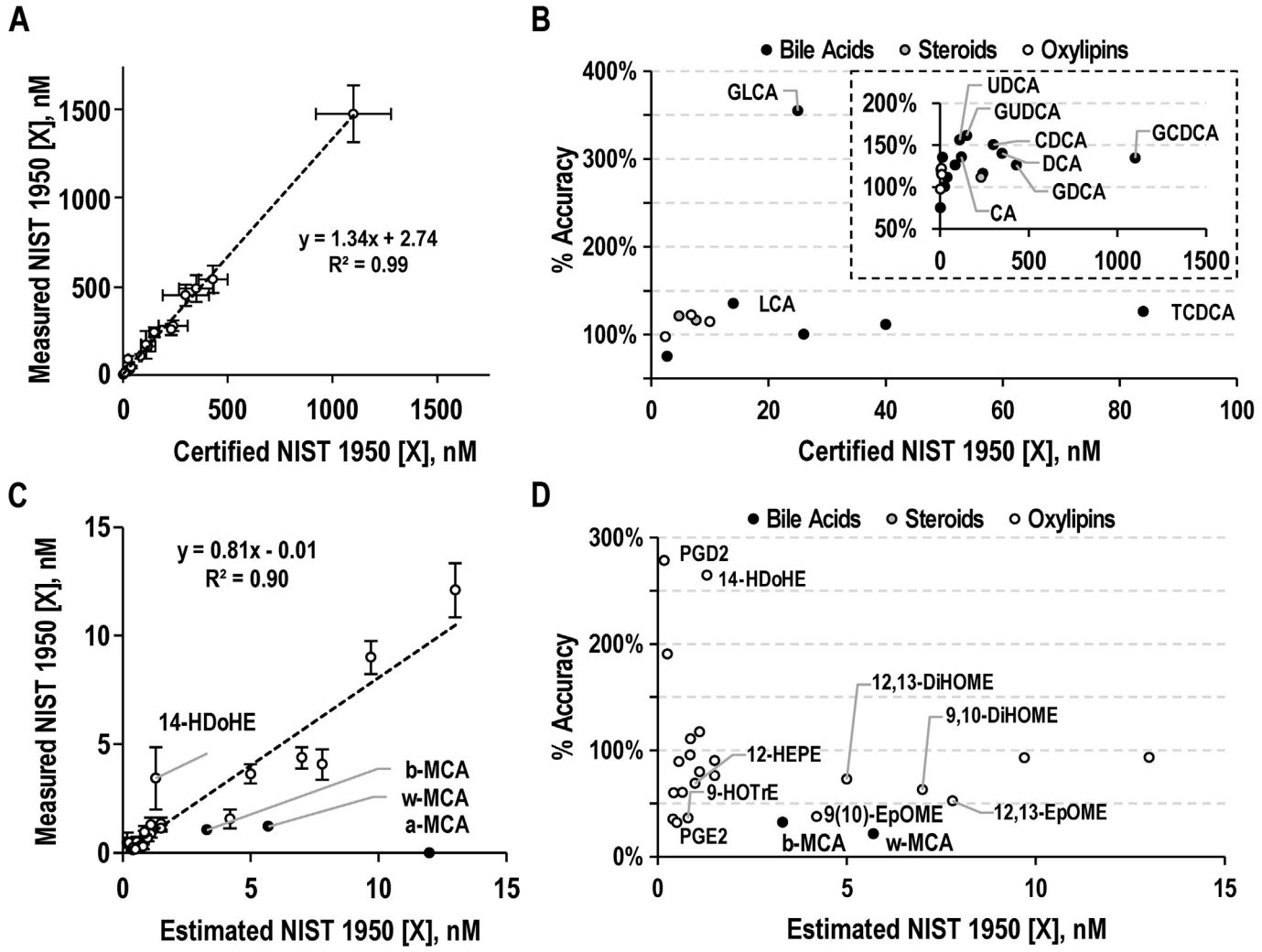


Fig. 4. Accuracy of metabolites concentrations measured in NIST SRM 1950 (n = 14). Measured and certified concentrations are compared by A) correlation, and B) calculated accuracy (i.e. measured/certified) x100% as a function of concentration. Measured and estimated concentrations are compared by C) correlation and D) calculated accuracy as a function of concentration, Certified values are calculated as the medians of laboratory means (MEDMs) from 5 or more laboratories, while estimated values are reported for results from fewer than five laboratories and do not have associated estimates of error [42].

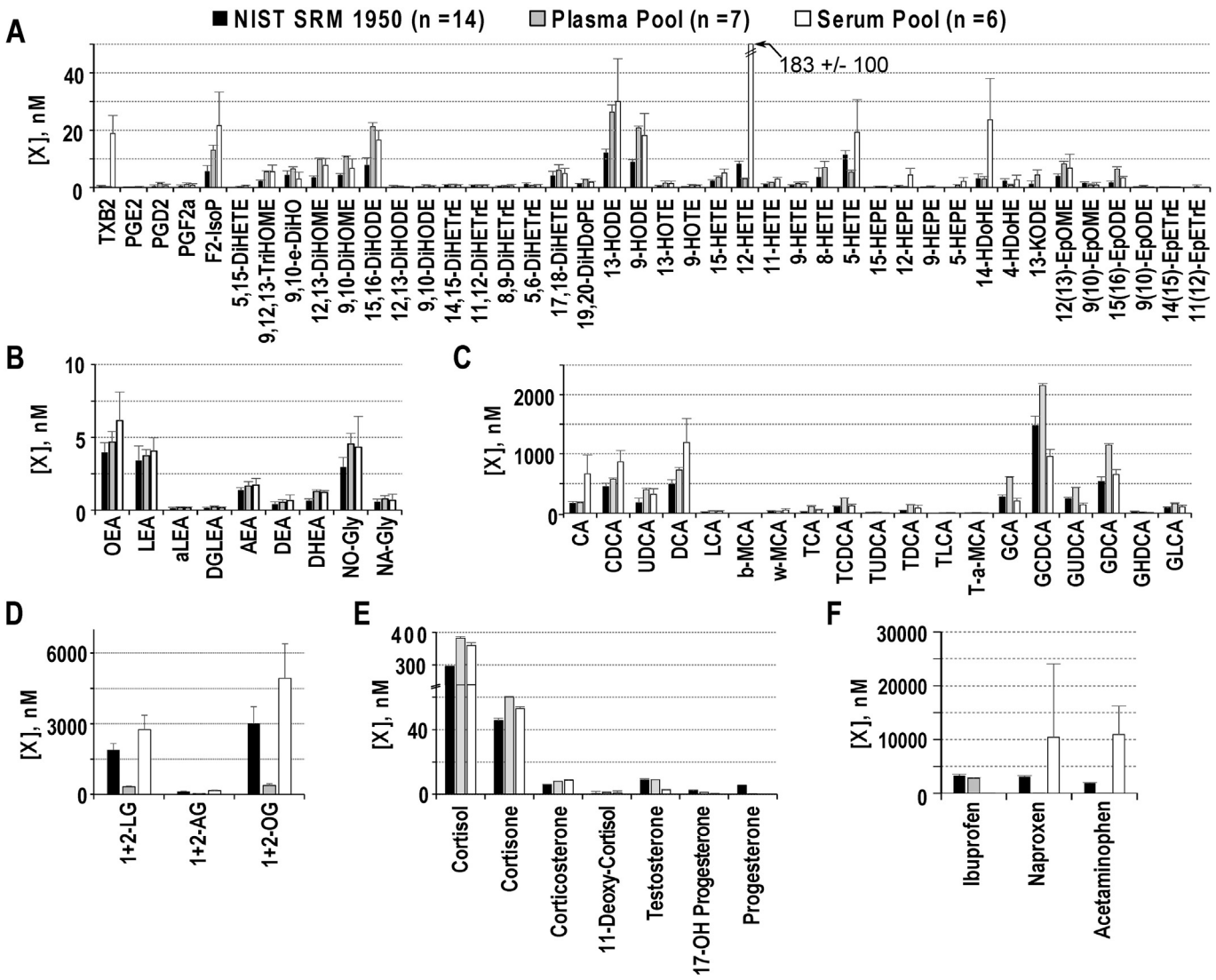


Fig. 5. Comparison of average metabolite concentrations in NIST SRM 1950, with independent pools of plasma and serum: A) Oxylipins; B) N-acylethanolamides; C) Bile acids; D) Monoacylglycerols; E) Steroids; F) Nonsteroidal anti-inflammatory drugs. NIST SRM 1950 is representative of the constructed study specific plasma pool from 10 individuals. Serum pools from 10 separate individuals show significantly larger concentrations of oxylipins associated with clotting. Matrix-dependent differences in bile acids, monoacylglycerols, steroids, and NSAIDs likely reflect differences in the underlying pooled subject.

estimated levels in NIST 1950, measured concentrations were again highly correlated (Fig. 4C), with some notable exceptions. In particular, the concentrations of the muricholic acids in our assay were considerably lower than those reported by other laboratories. Close inspection of chromatograms revealed subtle retention time shifts, as well as multiple substantial peaks in the scheduled MRM window for these compounds which rely on a parent to parent transition, due to the resistance of these compounds to collision induced fragmentation. Due to variable LC parameters, the low abundance of these metabolites in human samples, and the lack of specificity in the mass spectral detection for the muricholic acids, great care should be taken in the quantification and reporting of these metabolites. In addition, our measurements of the docosahexaenoic acid-derived alcohol, 14-HDoHE was significantly higher than levels reported by other laboratories, and more variable than oxylipins detected at similar concentrations (Fig. 4C and D). While peak relative retention times were stable, it is possible that measurements of this metabolite are either inaccurate or influenced by unknown interferences in the sample. Importantly, reagent blanks showed negligible background levels of this or any analytical target suggesting that the experimental materials were not the source of interferences. Therefore, if 14-HODHE levels are artificially elevated due to an interference, the increases are likely dependent on an endogenous compound. It is also possible that the concentration of the source material used to prepare the analytical calibration solutions themselves were lower than indicated by the commercial source, resulting in inflated concentration estimates. Regardless of the source, these results suggest that particular care should be taken in the evaluation and reporting 14-HDoHE results. In addition, the LA-derived epoxides (i.e. EpOMEs) and their corresponding 1,2 or vicinal diols (i.e. DiHOMEs) were also routinely found to be lower in our laboratory versus other reporting labs. Since variance estimates have yet to be provided for these compounds, it is unclear as to whether these values are substantially different than others, but it raises important issues regarding the need for high quality authentic standards and harmonization efforts to enhance the consistency of measures between laboratories [30].

3.4. Comparison NIST SRM 1950 to pooled plasma and serum

A comparison of concentrations measured in the NIST SRM 1950 and pooled plasma and serum are shown in Fig. 5. With the exception of the 1+2-MAGs and NSAIDs, the concentrations of measured targets in the NIST reference plasma and the measured pooled plasma were quite consistent, with NIST showing lower concentrations. As expected and discussed in Section 3.2, serum had significantly higher concentrations of metabolites known to be involved in coagulation. As also might be expected, the levels and distributions of the exogenous NSAIDs were quite different between these sample pools derived from independent cohorts. Together these findings support NIST 1950 as a representative reference material for these metabolites.

3.5. Analysis of plasma and serum in elderly cohorts

Applying the described method to clinical cohorts highlighted the point that oxylipin profiles can identify plasma samples which may have had platelet activation during collection, an important data quality check prior to bio statistical investigations of plasma oxylipins. Of the 281 plasma samples measured, 2 had levels of TXB2 >4 nM, 12-HETE >40 nM and 5-HETE >30 nM, reflecting serum-like oxylipin profiles. It would appear that platelet activation occurred during sample collection or preparation of these plasma samples. Besides the described impact of coagulation on the oxylipin profiles, the 1+2-MAGs were the only other suite of metabolites clearly impacted by the apparent platelet activation.

The analysis of over 500 samples from two independent cohorts also shows the general utility of the method, and its ability to produce biologically meaningful results. A complete report of the detected geometric mean and range of concentrations in healthy elderly subjects can be found in Supplemental Tables S12-Table S18. Considering the analysis of sex steroids in both matrices, testosterone and 17-hydroxypregesterone were routinely observed in both plasma and serum, progesterone was not observed in serum from the ROSMAP cohort, which was a substantially older population (78 ± 7yr vs 69 ± 8 yr). Testosterone levels were generally found to be within the reported reference ranges for this steroid. Across the entire serum set and the 131 healthy plasma controls, 7 of 88 men (8%) had measured levels below the ~0.3 nM estimated age x sex specific reference ranges, while 2 had levels above the high range of ~30 nM [43,44]. Similarly, within this combined set of samples, 15 of 275 women (5%) showed evidence of low testosterone, and 4 (1%) in their 70s had substantially elevated levels for women [45]. Finally, a subset of metabolites was found to be clearly influenced by subject fasting state. As highlighted in Fig. 6, the NAEs were reduced in the non-fasted state, while a suite of conjugated bile acids and oxylipins derived from eighteen carbon fatty acids including oleic acid, LA, and ALA were increased. Many NAEs are known to influence appetite. In fact, in a small cohort of elderly women linoleoyelethanolamide (i.e. LEA) and the LEA:docosahexaenoylethanolamide ratio (LEA:DHEA) were found to negatively correlate with satiety, consistent with our findings [46]. Similarly, postprandial impacts on conjugated bile acids are well described in the literature [47], and postprandial increases in LA-derived oxylipins including the hydroxyoctadecadienoioc acids (i.e. HODEs) have also been reported after the consumption of a high fat meal [48]. How these systems interact to influence physiological responses is to the best of our knowledge unexplored. Together, these findings support the utility of the current methods to provide broad insight into various interacting aspects of metabolism.

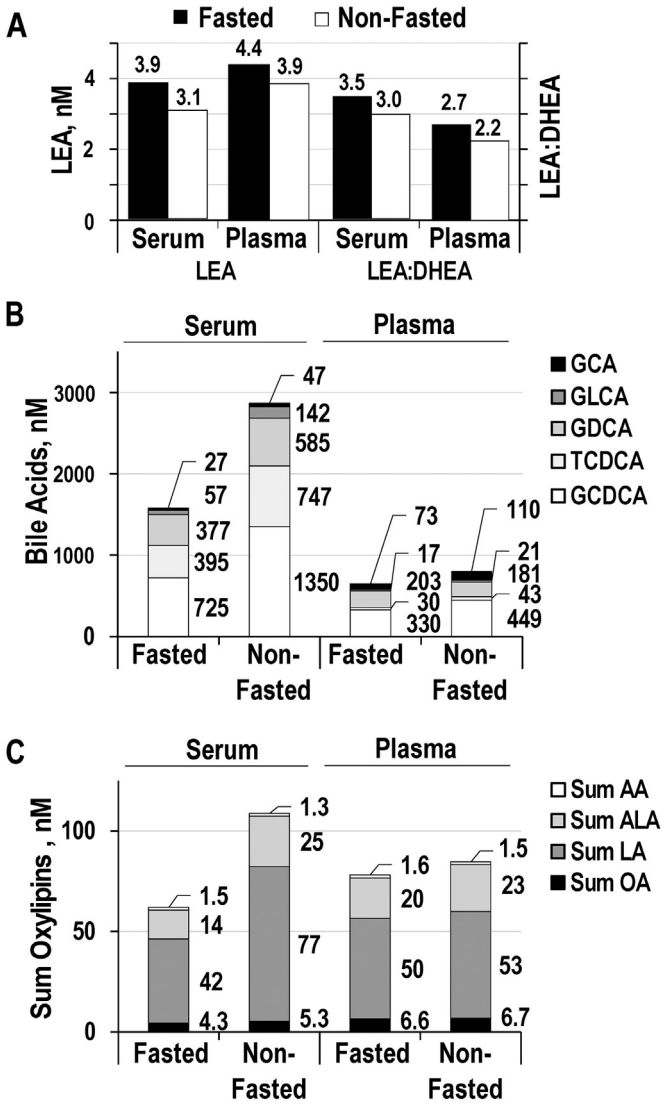


Fig. 6. Evidence of metabolites influenced by the fasted state of individuals in both serum and plasma. A) Linoleoylethanolamide (LEA) and the linoleoylethanolamide:docosahexaenoic acid ethanolamide (LEA:DHEA) ratio both declined in the non-fasted state. B) Conjugated bile acids in serum and plasma increased in the non-fasted state. C) Oleaic acids (OA), linoleic acid (LA), alpha-linolenic acid (ALA) and arachidonic acid (AA) derived metabolites seen to change are summed by parent fatty acids for simplification. All results are the geometric means of the described populations. Fasted serum – n = 84; non-fasted serum n = 146; fasted plasma – n = 81; non-fasted plasma – n = 50.

4. Conclusions

The described high-throughput 96-well plate sample preparation and fused UPLC-MS/MS-based analysis for oxylipins, acylethanolamides, bile acids, glucocorticoids, testosterone, 17-hydroxy progesterone, progesterone and NSAIDs was found to have good precision for most residues, performing better for plasma than for serum. Labeled surrogate recovery accuracy and precision estimates were improved by correction with the use of instrument internal standards and found to be stable across 7 analytical batches of both plasma and serum. For the majority of metabolites, intra- and inter-batch variability was <25% for metabolites above the LOQ, with precision decreasing as measured values approached the estimated LOD. Analytical results generally compared well with

NIST 1950 certified values. While bile acids were precise and highly correlated with the certified values, our measures tended to exceed the certified concentrations by 20–50%, and a source of this difference has not been identified. Results from pooled serum were more variable than plasma for oxylipins associated with coagulation, and some not classically associated with these processes. However, many oxylipins, and most other metabolites were either not, or marginally, effected by this process, and the stable surrogate performance suggests that the high variance in the measured serum pool may be related to ongoing biological activity in these samples, and not inherent analytical variability. Application of the method to two independent cohorts of plasma and serum produced results consistent with literature reports, and highlighted the utility of such broad-based metabolic profiling in biomedical research.

CRediT authorship contribution statement

Theresa L. Pedersen: Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing, Writing. **Ira Gray:** Investigation, Methodology. **John W. Newman:** Conceptualization, Supervision, Resources, Formal analysis, Writing - review & editing, Writing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Dr. Kamil Borkowski at UC Davis for technical support and review of the final manuscript. The authors would also like to specifically thank Drs. Rima Kadoura-Daouk at Duke University and David Bennett at Rush University and the Alzheimer's Disease Metabolomics Consortium (ADMC) for providing samples in support of this validation effort. The primary method development was funded by the USDA [Intramural Projects2032-51530-022-00D, 2032-51530-025-00D] and the NIH [U24 DK0971540]. The validation data presented here are in whole or in part based on data obtained from the AMP-AD Knowledge Portal (https://adknowledgeportal.synapse.org/#/Explore/Data). Metabolomics data is provided by the ADMC and funded wholly or in part by the following grants and supplements thereto: NIA R01AG046171, RF1AG051550, RF1AG057452, R01AG059093, RF1AG058942, U01AG061359, U19AG063744 and FNIH: #DAOU16AMPA awarded to Dr. Kaddurah-Daouk at Duke University in partnership with a large number of academic institutions. As such, the investigators within the ADMC, not listed specifically in this publication's author's list, provided data but did not participate in analysis or writing of this manuscript. A complete listing of ADMC investigators can be found at: https://sites.duke.edu/adnimetab/team/. Serum samples were provided by the Religious Orders and the Rush Memory and Aging studies (ROS/MAP) funded by NIA [P30AG10161, R01AG16819, R01AG17917, U01AG61356]. ROSMAP data can be requested at https://www.radc.rush.edu The content is solely the responsibility of the authors and does not represent the official views of the USDA or NIH. The USDA is an equal opportunity employer and provider.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.11.019.

References

[1] J.W. Newman, T.L. Pedersen, V.R. Brandenburg, W.S. Harris, G.C. Shearer, Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects, PloS One 9 (2014), e111471.
[2] G.C. Shearer, W.S. Harris, T.L. Pedersen, J.W. Newman, Detection of omega-3 oxylipins in human plasma and response to treatment with omega-3 acid ethyl esters, J. Lipid Res. 51 (2010) 2074–2081.
[3] R. Fan, J. Kim, M. You, D. Giraud, A.M. Toney, S.H. Shin, S.Y. Kim, K. Borkowski, J.W. Newman, S. Chung, alpha-Linolenic acid-enriched butter attenuated high fat diet-induced insulin resistance and inflammation by promoting bioconversion of n-3 PUFA and subsequent oxylipin formation, J. Nutr. Biochem. 76 (2020) 108285.
[4] S.C. Dyall, Interplay between n-3 and n-6 long-chain polyunsaturated fatty acids and the endocannabinoid system in brain protection and repair, Lipids 52 (2017) 885–900.
[5] G.C. Shearer, K. Borkowski, S.L. Puumala, W.S. Harris, T.L. Pedersen, J.W. Newman, Abnormal lipoprotein oxylipins in metabolic syndrome and partial correction by omega-3 fatty acids, Prostaglandins Leukot. Essent. Fatty Acids 128 (2018) 1–10.
[6] S.C. Mills, A.C. Windsor, S.C. Knight, The potential interactions between polyunsaturated fatty acids and colonic inflammatory processes, Clin. Exp. Immunol. 142 (2005) 216–228.
[7] C.A. Rouzer, L.J. Marnett, Endocannabinoid oxygenation by cyclooxygenases, lipoxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways, Chem. Rev. 111 (2011) 5899–5921.
[8] A.D. McNeilly, D.P. Macfarlane, E. O'Flaherty, D.E. Livingstone, T. Mitic, K.M. McConnell, S.M. McKenzie, E. Davies, R.M. Reynolds, H.C. Thiesson, O. Skott, B.R. Walker, R. Andrew, Bile acids modulate glucocorticoid metabolism and the hypothalamic-pituitary-adrenal axis in obstructive jaundice, J. Hepatol. 52 (2010) 705–711.
[9] V. Theiler-Schwetz, A. Zaufel, H. Schlager, B. Obermayer-Pietsch, P. Fickert, G. Zollner, Bile acids and glucocorticoid metabolism in health and disease, Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1865 (2019) 243–251.
[10] M.N. Hill, J.G. Tasker, Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis, Neuroscience 204 (2012) 5–16.
[11] R.J. Sebaldt, J.R. Sheller, J.A. Oates, L.J. Roberts 2nd, G.A. FitzGerald, Inhibition of eicosanoid biosynthesis by glucocorticoids in humans, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 6974–6978.
[12] I. Vachier, C. Chavis, M. Majori, M. Farce, J. Bousquet, P. Godard, P. Chanez, Effects of glucocorticoids on endogenous and transcellular metabolism of eicosanoids in asthma, J. Allergy Clin. Immunol. 107 (2001) 824–831.
[13] R. Comitato, A. Saba, A. Turrini, C. Arganini, F. Virgili, Sex hormones and macronutrient metabolism, Crit. Rev. Food Sci. Nutr. 55 (2015) 227–241.
[14] D. Ruiz, V. Padmanabhan, R.M. Sargis, Stress, sex, and sugar: glucocorticoids and sex-steroid crosstalk in the sex-specific misprogramming of metabolism, J Endocr Soc 4 (2020) bvaa087.
[15] T.L. Pedersen, J.W. Newman, Establishing and performing targeted multiresidue analysis for lipid mediators and fatty acids in small clinical plasma samples, Methods Mol. Biol. 1730 (2018) 175–212.
[16] K. Strassburg, A.M. Huijbrechts, K.A. Kortekaas, J.H. Lindeman, T.L. Pedersen, A. Dane, R. Berger, A. Brenkman, T. Hankemeier, J. van Duynhoven, E. Kalkhoven, J.W. Newman, R.J. Vreeken, Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery, Anal. Bioanal. Chem. 404 (2012) 1413–1426.
[17] C. Gladine, A.I. Ostermann, J.W. Newman, N.H. Schebb, MS-based targeted metabolomics of eicosanoids and other oxylipins: analytical and inter-individual variabilities, Free Radic. Biol. Med. 144 (2019) 72–89.
[18] S. Gouveia-Figueira, M. Karimpour, J.A. Bosson, A. Blomberg, J. Unosson, J. Pourazar, T. Sandstrom, A.F. Behndig, M.L. Nording, Mass spectrometry profiling of oxylipins, endocannabinoids, and N-acylethanolamines in human lung lavage fluids reveals responsiveness of prostaglandin E2 and associated lipid metabolites to biodiesel exhaust exposure, Anal. Bioanal. Chem. 409 (2017) 2967–2980.
[19] S. Gouveia-Figueira, J. Spath, A.M. Zivkovic, M.L. Nording, Profiling the oxylipin and endocannabinoid metabolome by UPLC-ESI-MS/MS in human plasma to monitor postprandial inflammation, PloS One 10 (2015), e0132042.
[20] Z.X. Yuan, S. Majchrzak-Hong, G.S. Keyes, M.J. Iadarola, A.J. Mannes, C.E. Ramsden, Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 410 (2018) 6009–6029.
[21] M. Scherer, C. Gnewuch, G. Schmitz, G. Liebisch, Rapid quantification of bile acids and their conjugates in serum by liquid chromatography-tandem mass spectrometry, J Chromatogr B Analyt Technol Biomed Life Sci 877 (2009) 3920–3925.
[22] M. Peitzsch, T. Dekkers, M. Haase, F.C.G.J. Sweep, I. Quack, G. Antoch, G. Siegert, J.W.M. Lenders, J. Deinum, H.S. Willenberg, G. Eisenhofer, An LC–MS/MS method for steroid profiling during adrenal venous sampling for investigation of primary aldosteronism, J. Steroid Biochem. Mol. Biol. 145 (2015) 75–84.
[23] J.W. Honour, Mass spectrometry for steroids, Ann. Clin. Biochem. 51 (2014) 309–311.
[24] C.N. Serhan, S. Yacoubian, R. Yang, Anti-inflammatory and proresolving lipid mediators, Annu. Rev. Pathol. 3 (2008) 279–312.

[25] M.E. Goetz, J.J. Hanfelt, S.E. John, S.H. Bergquist, D.W. Loring, A. Quyyumi, G.D. Clifford, V. Vaccarino, F. Goldstein, T.M. Johnson Nd, R. Kuerston, M. Marcus, A.I. Levey, J.J. Lah, Rationale and design of the emory healthy aging and emory healthy brain studies, Neuroepidemiology 53 (2019) 187–200.
[26] D.A. Bennett, A.S. Buchman, P.A. Boyle, L.L. Barnes, R.S. Wilson, J.A. Schneider, Religious Orders study and Rush memory and aging project, J Alzheimers Dis 64 (2018) S161–S189.
[27] U.S. EPA, EPA 821-R-16-006: Definition and Procedure for the Determination of the Method Detection Limit, Revision 2, Washington, DC, 2016, pp. 1–6.
[28] A. Pastor, M. Farre, M. Fito, F. Fernandez-Aranda, R. de la Torre, Analysis of ECs and related compounds in plasma: artifactual isomerization and ex vivo enzymatic generation of 2-MGs, J. Lipid Res. 55 (2014) 966–977.
[29] C. Garst, M. Fulmer, D. Thewke, S. Brown, Optimized extraction of 2-arachidonyl glycerol and anandamide from aortic tissue and plasma for quantification by LC-MS/MS, Eur. J. Lipid Sci. Technol. 118 (2016) 814–820.
[30] T.N. Dunn, A.H. Keenan, A.P. Thomas, J.W. Newman, S.H. Adams, A diet containing a nonfat dry milk matrix significantly alters systemic oxylipins and the endocannabinoid 2-arachidonoylglycerol (2-AG) in diet-induced obese mice, Nutr. Metab. 11 (2014) 24.
[31] K. Strassburg, A.M.L. Huijbrechts, K.A. Kortekaas, J.H. Lindeman, T.L. Pedersen, A. Dane, R. Berger, A. Brenkman, T. Hankemeier, J. van Duynhoven, E. Kalkhoven, J.W. Newman, R.J. Vreeken, Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery, Anal. Bioanal. Chem. 404 (2012) 1413–1426.
[32] K.N. Ikei, J. Yeung, P.L. Apopa, J. Ceja, J. Vesci, T.R. Holman, M. Holinstat, Investigations of human platelet-type 12-lipoxygenase: role of lipoxygenase products in platelet activation, J. Lipid Res. 53 (2012) 2546–2559.
[33] K.M. Rund, F. Nolte, J. Doricic, R. Greite, S. Schott, R. Lichtinghagen, F. Gueler, N.H. Schebb, Clinical blood sampling for oxylipin analysis – effect of storage and pneumatic tube transport of blood on free and total oxylipin profile in human plasma and serum, The Analyst 145 (2020) 2378–2388.
[34] P.C. Norris, S. Libreros, N. Chiang, C.N. Serhan, A cluster of immunoresolvents links coagulation to innate host defense in human blood, Sci. Signal. 10 (2017).
[35] D. Helsel, Much ado about next to nothing: incorporating nondetects in science, Ann. Occup. Hyg. 54 (2010) 257–262.
[36] B.A. Watkins, J. Kim, A. Kenny, T.L. Pedersen, K.L. Pappan, J.W. Newman, Circulating levels of endocannabinoids and oxylipins altered by dietary lipids in older women are likely associated with previously identified gene targets, Biochim. Biophys. Acta 1861 (2016) 1693–1704.
[37] B.G. Keevil, LC–MS/MS analysis of steroids in the clinical laboratory, Clin. Biochem. 49 (2016) 989–997.
[38] J.G. Bollinger, W. Thompson, Y. Lai, R.C. Oslund, T.S. Hallstrand, M. Sadilek, F. Turecek, M.H. Gelb, Improved sensitivity mass spectrometric detection of eicosanoids by charge reversal derivatization, Anal. Chem. 82 (2010) 6790–6796.
[39] S.W. Meckelmann, S. Hellhake, M. Steuck, M. Krohn, N.H. Schebb, Comparison of derivatization/ionization techniques for liquid chromatography tandem mass spectrometry analysis of oxylipins, Prostag. Other Lipid Mediat. 130 (2017) 8–15.
[40] Q. Liu, Q. Chi, R.T. Fan, H.D. Tian, X. Wang, Quantitative-profiling method of serum steroid hormones by hydroxylamine-derivatization HPLC-MS, Nat Prod Bioprospect 9 (2019) 201–208.
[41] T.-F. Yuan, J. Le, S.-T. Wang, Y. Li, An LC/MS/MS method for analyzing the steroid metabolome with high accuracy and from small serum samples, JLR (J. Lipid Res.) 61 (2020) 580–586.
[42] J.A. Bowden, A. Heckert, C.Z. Ulmer, C.M. Jones, in: U.D.o. Commerce (Ed.), NISTIR 8185 - Lipid Concentrations in Standard Reference Material (SRM) 1950: Results from an Interlaboratory Comparison Exercise for Lipidomics, 2017.
[43] T.G. Travison, H.W. Vesper, E. Orwoll, F. Wu, J.M. Kaufman, Y. Wang, B. Lapauw, T. Fiers, A.M. Matsumoto, S. Bhasin, Harmonized reference ranges for circulating testosterone levels in men of four cohort studies in the United States and europe, J. Clin. Endocrinol. Metab. 102 (2017) 1161–1173.
[44] X. Chen, K. Wang, Letter to the editor: "harmonized reference ranges for circulating testosterone levels in men of four cohort studies in the United States and europe, J. Clin. Endocrinol. Metab. 103 (2018) 698.
[45] R. Haring, A. Hannemann, U. John, D. Radke, M. Nauck, H. Wallaschofski, L. Owen, J. Adaway, B.G. Keevil, G. Brabant, Age-specific reference ranges for serum testosterone and androstenedione concentrations in women measured by liquid chromatography-tandem mass spectrometry, J. Clin. Endocrinol. Metab. 97 (2012) 408–415.
[46] A.N. Friedman, J. Kim, S. Kaiser, T.L. Pedersen, J.W. Newman, B.A. Watkins, Association between plasma endocannabinoids and appetite in hemodialysis patients: a pilot study, Nutr. Res. 36 (2016) 658–662.
[47] K. Linnet, Postprandial plasma concentrations of glycine and taurine conjugated bile acids in healthy subjects, Gut 24 (1983) 249–252.
[48] K. Strassburg, D. Esser, R.J. Vreeken, T. Hankemeier, M. Muller, J. van Duynhoven, J. van Golde, S.J. van Dijk, L.A. Afman, D.M. Jacobs, Postprandial fatty acid specific changes in circulating oxylipins in lean and obese men after high-fat challenge tests, Mol. Nutr. Food Res. 58 (2014) 591–600.