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Validation of two LC—HRMS methods for large-scale untargeted metabolomics of serum samples: Strategy to establish method fitness-for-purpose

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ABSTRACT

Untargeted metabolomics by LC—HRMS is a powerful tool to enhance our knowledge of pathophysiological processes. Whereas validation of a bioanalytical method is customary in most analytical chemistry fields, it is rarely performed for untargeted metabolomics. This study aimed to establish and validate an analytical platform for a long-term, clinical metabolomics study. Sample preparation was performed with an automated liquid handler and four analytical methods were developed and evaluated. The validation study spanned three batches with twelve runs using individual serum samples and various quality control samples. Data was acquired with untargeted acquisition and only metabolites identified at level 1 were evaluated. Validation parameters were set to evaluate key performance metrics relevant for the intended application: reproducibility, repeatability, stability, and identification selectivity, emphasizing dataset intrinsic variance. Concordance of semi-quantitative results between methods was evaluated to identify potential bias. Spearman rank correlation coefficients (r_s) were calculated from individual serum samples. Of the four methods tested, two were selected for validation. A total of 47 and 55 metabolites (RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS, respectively) met specified validation criteria. Quality assurance involved system suitability testing, sample release, run release, and batch release. The median repeatability and within-run reproducibility as coefficient of variation% for metabolites that passed validation on RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS were 4.5 and 4.6, and 1.5 and 3.8, respectively. Metabolites that passed validation on RPLC-ESI$^+$-HRMS had a median *D*-ratio of 1.91, and 89 % showed good signal intensity after ten-fold dilution. The corresponding numbers for metabolites with the HILIC-ESI$^-$-HRMS method was 1.45 and 45 %, respectively. The r_s median ({range}) for metabolites that passed validation on RPLC-ESI$^+$- was 0.93 ($N = 9$ {0.69–0.98}) and on HILIC-ESI$^-$-HRMS was 0.93 ($N = 22$ {0.55–1.00}). The validated methods proved fit-for-purpose and the laboratory thus demonstrated its capability to produce reliable results for a large-scale, untargeted metabolomics study. This validation not only bolsters the reliability of the assays but also significantly enhances the impact and credibility of the hypotheses generated from the studies. Therefore, this validation study serves as a benchmark in the documentation of untargeted metabolomics, potentially guiding future endeavors in the field.

1. Introduction

The field of metabolomics investigates the complete range of primarily endogenous metabolites (small molecules ≤2 kDa), collectively known as the metabolome, within a biological system, such as an organism, cell, tissue, or bodily fluid [1,2]. The dynamic nature of the metabolome is influenced by both intrinsic factors like genetics, inflammation, and oxidative stress, and extrinsic factors including pathogens, environmental conditions, drugs, and nutritional status [2,3]. Understanding the interactions among these factors and their impact

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on the metabolic profile is crucial for advancing knowledge in pathophysiology, assessing treatment efficacy, and identifying biomarkers for various health conditions [2,4].

Metabolic profiling studies are conducted using either targeted or untargeted approaches, aiming to qualitatively and/or quantitatively assess changes in metabolites influenced by the aforementioned factors [1,5–7]. Common techniques for acquiring metabolomics data include nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). NMR-based metabolomics is known for its quantitative capabilities, cost-effectiveness, and rapid processing, yet it lacks sensitivity [8]. In contrast, MS-based metabolomics, particularly when combined with liquid chromatography (LC), offers enhanced sensitivity and a broader range of detectable metabolites [3,4,7–9]. High-resolution mass spectrometry (HRMS), has become the predominant platform in LC-MS untargeted metabolomics due to its sensitivity, selectivity, and broad-scope acquisition capabilities [4,7]. Both targeted and untargeted metabolomics have been used for large-scale clinical metabolomics studies [10–13].

Targeted metabolomics focuses on the identification and quantification of known metabolites related to specific pathways or biological activities, utilizing authentic reference standards [5,7]. Both untargeted NMR-based and targeted LC-MS/MS-based approaches are suitable for high-throughput applications in clinical laboratories, and these methodologies offer the benefits of comparability across studies and result traceability [11,12,14]. For the untargeted, semi-quantitative approaches, variable levels of information are acquired for all detectable molecules present in a sample, where known endogenous metabolites, exposome components, and even novel metabolites can be identified [13,15]. The data analysis is complex, the overall analysis time is longer, and result traceability is inferior compared with targeted approaches. Despite limitations, untargeted LC—HRMS metabolomics remains interesting for clinical studies as it covers more analytes than alternative strategies.

Quality management, encompassing quality assurance and quality control (QC), is critical for ensuring the reproducibility and reliability of bioanalytical results [16–19]. This includes system suitability testing and comprehensive documentation of method performance. While specific guidelines exist for the validation of quantitative assays [16,20], there is a lack of standardized validation protocols for semi-quantitative, untargeted metabolomics. Previous studies have tested parameters such as method robustness and repeatability across batches [21]. Method validation is a continuous and iterative process that serves to test method robustness, document performance, and allows analysts to gain experience with a given procedure [16]. It is an essential component of evaluating method fitness-for-purpose. Determining the fitness-for-purpose of bioanalytical assays typically also involves adherence to proficiency testing. Unfortunately, such schemes are also not commercially available for semi-quantitative, untargeted metabolomics. While attempts have been made to harmonize metabolomics research reporting criteria ranging from minimum to preferred requirements [17–19,22], method validation is uncommon in the field. There are however neighboring disciplines using similar methods, where we can look for inspiration for validation strategies.

The workflows applied in untargeted metabolomics data acquired by LC—HRMS closely resemble the workflows applied in environmental and forensic drug screening. All use targeted [23–28], semi-targeted/suspect [13,25,27–29], and non-targeted [13,15,23,26,30] data analysis workflows for the screening of thousands of analytes, each with variable levels of identification confidence. In each of these environmental and forensic drug screening workflows (targeted, semi-targeted/suspect, or non-targeted), it is common practice to employ a validation strategy involving validating the methods for a representative set of analytes with targeted data analysis, and then deducing fitness-for-purpose for a wider application of the method [23,25–27,29,31–34]. The availability of blank matrices in untargeted environmental and forensic drug screening studies allows for the investigation of reproducibility of identifications around reporting limits [Anon., 35] or decision points [36], and extensive matrix interference studies with multiple blank samples. Despite the absence of blank matrices for untargeted metabolomics, the validation strategies used in environmental and forensic drug screening remain pertinent and were therefore adopted in this study.

This study aimed to establish an analytical platform for a long-term clinical metabolomics study and assess the method's fitness-for-purpose. The objectives were to i) develop and test four analytical methods for untargeted metabolomics by LC—HRMS, ii) establish a quality assurance system including QC samples, iii) select the most suitable methods for comprehensive and robust analysis of serum, iv) evaluate the concordance between the methods, and finally v) produce validation reports.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile, water, methanol, isopropanol, ammonium formate, ammonium acetate, formic acid, and sodium hydroxide were of LC-MS grade and purchased from Fisher Scientific (Pittsburg, USA). The mass spectrometry metabolite library (MSMLS 230–01) panel was purchased from IROA technologies (Sea Girt, USA). SRM 1950 and Internal standards (ISTD): Acetyl-l-carnitine-D_3, Hexadecanoyl(palmitoyl)-l-carnitine-D_3, l-Leucine-5,5,5-D_3, l-Tryptophan-(indole-D_5), l-Methionine-(methyl-D_3), Stearic acid-18,18,18-D_3, Chenodeoxycholic-2,2,3,4,4,6,6,7,8-D_9 acid, 18:0-D_{35} Lyso PC, Dopamine-1,1,2,2-D_4 hydrochloride were purchased from Merck Life Science (Darmstadt, Germany).

For system suitability, quercitin, amitriptylin, histidine, arginine, labetalol, doxepin, proline, and tryptophan in at least reagent grade were purchased from Merck Life Science (Darmstadt, Germany) or VWR (Radnor, USA).

2.2. Samples

Mixed and anonymized human serum samples from a previous study were used for method development, and prepared samples from this material were further frozen and used for column conditioning throughout the study.

For the validation, serum samples were obtained from 54 blood donors (26 female and 27 male). Serum was prepared by centrifuging the individual blood samples at 1500 *g* for 10 min at 4 °C. A subset of the collected serum samples was aliquoted for individual analysis ($N = 38$) as part of the validation. A QC_{pool} was prepared by mixing all serum samples in a large batch. QC_{noIS} was prepared as a QC_{pool} without the addition of ISTD. Method blank samples consisted of a water:MeOH (50:50, v/v%).

Levelled QC samples, a QC high (QC_H) and a QC low (QC_L), were prepared to evaluate between-run reproducibility. QC_H was prepared by freeze-drying 6 mL QC_{pool} for 48 h at −50 °C and 0.05 mBar and reconstitute in 4 mL LC-MS grade water. QC_L was prepared by diluting 2 mL QC_{pool} to 4 mL with LC-MS grade water. All QC samples were aliquoted into plastic tubes and stored at −80 °C

2.3. Sample preparation

Extraction was performed on a Tecan Fluent 780 liquid handler (Tecan, Männedorf, Switzerland), with the workflow presented in Fig. 1. Fifty μL sample was transferred to a 96-well plate and 450 μL cold ACN:MeOH (75:25, v/v%) with ISTDs was added while shaking (BioShake, QInstruments; 1300 RPM). After 3 additional minutes of shaking the plate was covered by a lid and centrifuged (Hettich universal 320 R) for 12 min at 4 °C and 887 *g* for pelleting the proteins. In the liquid handler, 50 μL of supernatant was transferred to the hydrophilic interaction

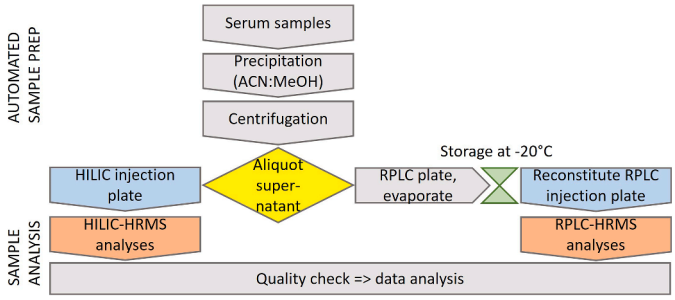

Fig. 1. Flowchart for sample preparation and analysis of each analytical batch. ACN: acetonitrile, HILIC: Hydrophilic interaction liquid chromatography, HRMS: High resolution mass spectrometry, MeOH: methanol, RPLC: Reversed-phase liquid chromatography.

liquid chromatography (HILIC) injection plate that was directly sealed and transferred to the LC-MS instrument for HILIC analyses. For the reversed phase LC (RPLC) analyses, 300 µL of the supernatant was transferred to another injection plate, evaporated to dryness at 35 °C under a gentle stream of nitrogen (Techne sample concentrator, Antylia Scientific, Vernon Hills, USA) and frozen at −20 °C until analysis. Before RPLC injections, evaporated samples were resuspended by adding first 10 µL of water:MeOH (50:50, v/v%), shaking the plate; adding 40 µL of LC-MS grade water, finished by another round of shaking. Final solution of the ISTDs in the quenching medium was in the range 9–222 µg/L, optimized for this application. The selection of ISTD was inspired by Zheng et al. [37]. All QC samples, with the exception of system suitability and instrument blanks, were prepared as samples. In the last batch, five additionally prepared QC_{pool}s were mixed in a vial to have sufficient volume for iterative MS/MS experiments.

2.4. Quality control

For *system suitability*, a system control (QC_{SS}) containing 0.1 mg/L quercetin, amitriptyline, histidine, arginine, labetalol, doxepin, proline, and tryptophan was analysed on each analytical method and evaluated based on retention time, signal intensity, and mass accuracy in full MS before and after sample analyses together with a system blank to identify system contamination by inspecting the total ion chromatogram. For *sample release*, the ISTD signals were monitored in each injected sample, QC, and blank to verify the correct injection and identify possible errors over the run. *Run release* was performed by monitoring selected endogenous metabolites in the QC_{pool}, QC_H, QC_L, and blank injections. *Batch release:* successful execution of AcquireX injections was verified by checking inclusion list reduction in the XML files that were generated by the vendor software to select features for iterative scans.

2.5. Instrumentation

LC-MS was performed with a Thermo Scientific Vanquish Horizon UHPLC system interfaced with a Thermo Scientific Orbitrap ID-X Tribrid Mass Spectrometer (Waltham, MA). Four analytical methods were employed based on HILIC or RPLC with negative or positive electrospray ionization (ESI^-or ESI^+). LC separation parameters are described in Table 1. The injection volume was 3 µL for all methods. Analytical columns were purchased from Waters Corp (Milford, USA) comprising an ACQUITY BEH amide (100 × 2.1 mm, 1.7 µm) for HILIC analyses, and an ACQUITY HSS T3 (150 × 2.1 mm, 1.8 µm) for RPLC analyses. The flow rate was 0.45 ml/min for HILIC-ESI^-, 0.40 ml/min for HILIC-ESI^+-HRMS, and 0.35 ml/min for both RPLC methods.

MS settings: Heated Electrospray Ionization source and ion transfer parameters applied were as follows: sheath flow rate (arbitrary units) = 50, auxiliary gas flow rate (arbitrary units) = 10, sweep gas flow rate (arbitrary units) = 1, spray voltage = 3.5 kV (positive) or −2.5 kV (negative), ion transfer tube temperature = 325 °C, vaporizer temperature = 350 °C. For relative quantification, full-scan MS data was acquired in the Orbitrap mass analyser with the following settings: Resolution = 60,000, scan range *m/z* 70–800, normalized AGC target = 25 %.

For compound identification, iterative scans (AcquireX) MS/MS was performed on QC_{pool} at the end of the last analyzed batch with automated inclusion and exclusion lists with the following settings: Resolution = 30,000, isolation window = 1.2, Normalized AGC target = 100 %, Collision energy mode = stepped (20, 35, 50 eV), with mass tolerances ± 3 ppm from inclusion list.

Table 1
LC method settings for the four analytical methods tested.

Mobile phases	Analysis (run)	Gradient (%B)
HILIC-ESI^-		
A: 10 mM AmAc pH=9, B: 90 % ACN with 10 mM AmAc pH=9	9.2 (14.7) min	5.5 min (100), inject, hold 2.5 min, gradient (60) 6.5 min, hold 0.2 min
HILIC-ESI^+		
A: 5 mM AmF pH=3.1*, B: 95 ACN + 5 mM AmF + 0.1 % FA	11 (17) min	6 min (100), inject, hold 2.5 min, gradient (70) 8.5 min
RPLC-ESI^-		
A: 5 mM AmF pH=3.1*, B: ACN + 0.1 % FA	15.5 (16.6) min	0.6 min (10), inject, hold 0.5 min, gradient (98) 13 min, hold 2 min, gradient (10) 0.5 min
RPLC-ESI^+		
A: 5 mM AmF pH=3.1*, B: ACN + 0.1 % FA	18.5 (19.6) min	0.6 min (10), inject, hold 0.5 min, gradient (98) 16 min, hold 2 min, gradient (10) 0.5 min

ACN: Acetonitrile, AmAc: ammonium acetate, AmF: ammonium formate, ESI+/-: Electrospray ionization, positive/negative, FA: formic acid, HILIC: Hydrophilic interaction liquid chromatography, HRMS: High resolution mass spectrometry, RPLC: Reversed-phase liquid chromatography.
* : measured prior to analysis.

2.6. Analytical runs and validation parameters

Validation experiments were conducted over three batches and twelve analytical runs. A batch refers to a collection of samples that after sample preparation is transferred to two injection plates each (HILIC or RPLC), each of which is analyzed with two different methods (Fig. 1). The injection order was reversed in batch #2 to evaluate stability in the autosampler. Two (RPLC) and five (HILIC) prepared serum samples were used for column conditioning after successful system suitability testing and before initiating the analytical runs. Analytical runs with the same method from different batches were never run consecutively, but always with at least one other analytical run between. Each analytical run had between 54 and 63 injections excluding system suitability testing and conditioning, with the sample lists presented in Table SIA.5 and SIB.5. The number of samples per batch is identical to what the platform is intended for. A total of six QC_{pool} samples were analyzed in each batch with a maximum of ten injections in between. Each batch had between 3 and 6 QC_H and QC_L.

Validation criteria are presented in Table 2. Tested validation parameters were repeatability, reproducibility (within- and between-run), compound identification selectivity (to the library and to the SRM 1950), carry-over and stability in both the autosampler and after repeated freeze-and-thaw cycles. Freeze-and-thaw stability was tested with 6 QC_{pool} samples, that were frozen and thawed for ten cycles with individual aliquots after each cycle. Linearity and *D*-ratio [38] was also evaluated. Full specifications of calculations are available in the validation reports (Supporting Information A and B). Samples used for validation included individual serum samples (N = 38), QC_{pool}, QC_H, QC_L, blank samples, and SRM 1950. To pass validation, metabolites had to meet set criteria for repeatability, reproducibility (within- and

Table 2
Validation parameters for each metabolite measured in each analytical method, with how it is measured and set criteria.

Variable	Test	Sample	Set criteria
Repeatability	6 consecutive injections	QC_{pool}	< 15 % CV
Reproducibility, within-run	Sample prepared 6 times	QC_{pool}	< 20 % CV
Reproducibility, between-run	Prepared 3–6 times, across 3 runs	QC_L and QC_H	< 25 % CV
Carry-over	Solvent after sample	SRM 1950	< 10 % ratio on absolute areas
Stability	Freeze-and-thaw stability (x10)	QC_{pool}	Flagged if degraded >30 %
Selectivity, to library	Measurement uncertainty and match to mass spectral library	QC_{pool}	Δmass <3 mDa, ΔRT <0.7/0.3 min. MS/MS evaluated
Selectivity, to SRM 1950	Measurement uncertainty	SRM 1950 and QC_{pool}	Δ RT: < 0.1 min
Linear range	Response linearity and detection for validated analytes.	Pooled from previous study	Evaluated
D-ratio [38]	$\frac{\sigma_{i,\ QCpool}}{\sqrt{\sigma^2_{i,\ QCpool} + \sigma^2_{i,\ samples}}} * 100\%$	Individual serum samples (N = 38)	Evaluated

CV: coefficient of variation, HILIC: Hydrophilic interaction liquid chromatography, MS/MS: tandem mass spectrometry, RPLC: Reversed-phase liquid chromatography, RT: retention time, SRM: standard reference material, QC: Quality control.

between-run), and compound identification selectivity to library. The parameters freeze-and-thaw stability, *D*-ratio, linearity, and selectivity to the SRM 1950 were just evaluated. Finally, the concordance of semi-quantitative results for validated metabolites detected on more than one method was evaluated.

2.7. Data analysis

System suitability, sample and run release were evaluated with Skyline [39]. Compound identification was made with local mass spectral libraries curated in mzVault 2.3 (Thermo Fisher Scientific, Waltham, USA). The spectral libraries had MS/MS spectra and retention time for each of the four tested analytical methods and were based on injection of metabolites from the MSMLS in neat solution. Only metabolites with level 1 identification were considered in this study [17, 22]. Compound Discoverer 3.3 (Thermo Scientific, Waltham (MA), USA) was used for peak picking, feature alignment, and metabolite identification with details available in SIA (page 10) and SIB (page 10). Data from all three batches were analyzed together, for each method, as ISTD-corrected metabolite areas. A maximum of one outlier per metabolite per QC type was removed from the data sets, resulting in a minimum metabolite retention of 92 % for QC_H and QC_L and 94 % for QC_{pool}. All subsequent data analysis was performed with Microsoft excel (Microsoft Corporation, Redmon (WA), USA) separately for each method. Variance is given as coefficient of variance (CV), i.e. relative standard deviation in percent. Formulas for variables tested are available in the validation reports in supporting information A and B.

Spearman rank correlation coefficients (r_s) were calculated from ISTD-corrected areas. QC_{pool} normalization was used for visual presentation of concordance results.

2.8. Validation design

The validation design was inspired by the validation approach commonly applied for LC—HRMS-based untargeted environmental and forensic drug screening methods. This involved paying extra attention to a representative set of metabolites (identification level 1), including careful, manual evaluation of every library match. The targeted data analysis in this validation study is based on untargeted data acquisition and untargeted peak-picking; but in contrast to semi-targeted/suspect and non-targeted data analysis only the features identified at level 1 are evaluated.

Semi-quantitation and metabolite identification are the two central data variables from untargeted metabolomics data sets, both of which ideally should be evaluated from spiking representative blank samples, or parallel and/or proficiency testing. While no complementary detection techniques were evaluated in the present study, it was possible to evaluate concordance between methods. The four different methods tested in this study relied on two different types of column material, different mobile phase buffer systems, two different ionization modes, and four different mass spectral libraries.

Semi-quantitative results from different methods are not expected to show linear correlation, but discrepancy in rank order could indicate systematic bias from an interference. The non-parametric Spearman rank correlation coefficient was thus selected to evaluate concordance. Concordance in semi-quantitative results obtained with complementary separation and detection systems improves confidence in both metabolite identification and semi-quantitative results: The risk of co-eluting matrix interference and/or misidentifications from different mass spectral libraries is smaller with two methods than one.

3. Results

3.1. Method development

With the goal of developing four analytical methods (HILIC-ESI^--, HILIC-ESI^+-, RPLC-ESI^+-, and RPLC-ESI^--HRMS) that could span a broad range of metabolites, different methods parameters were evaluated. The HILIC methods were guided by vendor application notes [40]. Sample preparation processes, including the concentration of internal standards (ISTDs), choice of quenching agent, and the ratio of quenching agent to serum, were optimized for metabolite peak shape and method sensitivity. A detailed discussion of method development and optimization is beyond the scope of this study. A quenching ratio of ACN:MeOH (75:25, v/v%) at 1:10 serum to quenching agent proved effective for pelletization and chromatographic performance in HILIC. Automated sample preparation involved precipitation for HILIC and subsequent evaporation and reconstitution for RPLC analysis. Liquid class settings for all solvents were optimized on the automated liquid handler.

3.2. Validation analytical runs

The validation experiments spanned three batches, each analyzed with four analytical methods, resulting in a total of twelve analytical runs, in addition to MS/MS acquisition. The sample lists and thus samples and injection order, for analytical runs across methods were identical. Batch overviews are presented in Tables SIA.2–4 and SIB.2–4. Only samples and runs that successfully passed quality control were used for validation. This included evaluating the ISTD signals in all injections and assessing the signals of endogenous metabolites in QC samples, as graphically represented in Fig. 2. Representative plots for sample release (Tables SIC.1 and SIC.2) and run release (Table SIC.3) are included in the supporting information.

A notable variance in ISTDs was observed in the second run from each injection plate, notably in HILIC runs with higher organic content in the sample aliquot. From the sample release a retention time drift of up to 0.1 min for HILIC and up to 0.1 min for RPLC were observed across all analytical runs. The retention time range at peak max of the latest eluting metabolite in HILIC-ESI^--HRMS was up to 0.5 min across the three runs. To verify successful MS/MS acquisition, the reduction of the

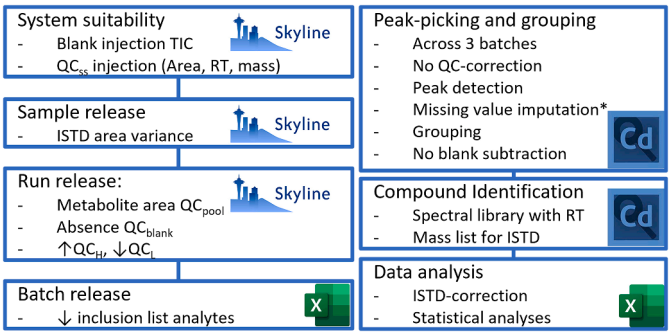

Fig. 2. Quality assurance and data analysis workflow with logo for corresponding software used. ISTD: internal standard, MS/MS: tandem mass spectrometry, RT: retention time, TIC: total-ion chromatogram, QC: quality control.

inclusion list that is defined by the instrument software to tag potential metabolite features, was used as an indicator. Generally, most of the chromatographically retained inclusion list features were acquired already in the first iterative MS/MS scan.

3.3. Data analysis

With the goal of correcting for injection volume and evaporation, nine ISTDs were evaluated for each chromatographic method. From these, one to three were selected for data analysis based on their chromatographic retention, signal intensity, peak shape, and detection reproducibility in Compound Discoverer. Each metabolite was corrected for ISTD in each sample. When multiple ISTDs were used, an averaged factor was applied. The final ISTD used for data analysis were: HILIC-ESI$^-$-HRMS: l-Leucine-5,5,5-D_3 and l-Methionine-(methyl-D_3), HILIC-ESI$^+$-HRMS: l-Methionine-(methyl-D_3), Hexadecanoyl(palmitoyl)-l-carnitine-D_3, and l-Tryptophan-(indole-D_5), RPLC-ESI$^+$-HRMS: l-Leucine-5,5,5-D_3, Hexadecanoyl(palmitoyl)-l-carnitine-D_3, and l-Tryptophan-(indole-D_5), RPLC-ESI$^-$-HRMS: Chenodeoxycholic-2,2,3,4,4,6,6,7,8-D_9 acid.

Compound Discoverer was used for peak-picking, alignment, and library matching. The ISTDs were identified using a mass list approach (i.e. targeted detection), extracting analytes based on mass and retention time in full MS, in contrast to the non-targeted feature detection of metabolites. Only metabolites detected with peak rating higher than 4.5 in at least half of the samples and MS/MS spectrum matching a metabolite in the mass spectral library were evaluated. Each identification was visually confirmed in Compound Discoverer. The data analysis workflow is summarized in Fig. 2.

3.4. Selecting methods for validation

Initial inspection of the validation data set revealed an overlap in metabolites detected across the four analytical methods, but most analytes were identified on HILIC-ESI$^-$- and RPLC-ESI$^+$-HRMS. Due to overlaps, as well as time and robustness considerations, only metabolites from these methods underwent full validation. RPLC-ESI$^+$-HRMS covered metabolites in the medium-range polarity containing nitrogen including carnitines, bile acids, amino acids, and analytes from tryptophan metabolism. Conversely, HILIC-ESI$^-$-HRMS covered hydrophilic metabolites including sugars, organic acids, and hydrophilic amino acids. 5816 features were detected in RPLC-ESI$^+$-HRMS, of which 4733 had an MS/MS spectrum, and 64 matched with a library entry. The corresponding numbers for HILIC-ESI$^-$-HRMS were 4482 features, of which 3566 had an MS/MS spectrum, and 101 matched with a library entry. Fig. 3 presents stripplots illustrating each metabolite that passed validation, where each QC and serum sample is normalized to the batch-averaged QC_{pool}. Stripplots from the initial evaluation of analytical methods not considered for full validation (HILIC-ESI$^+$-HRMS and RPLC-ESI$^-$-HRMS) are further available in Figure SIC.1 and Figure SIC.2.

3.5. Method validation

The full validation reports for RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS are available in supporting information A and B, respectively. Only metabolites that were detected in serum were evaluated. Mass spectral libraries for RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS encompassed 251 and 384 metabolites, from which 47 and 55 metabolites met specified validation criteria, of which 33 and 40 could be unambiguously identified, respectively. Seventeen (RPLC-ESI$^+$-HRMS) and 63 (HILIC-ESI$^-$-HRMS) features matched a library item, but failed validation, as presented in Tables SIA.6 and SIB.6. Metabolites that passed validation together with key validation results are presented in Tables 3 and 4. Validated metabolites are presented with one annotation in Tables 3 and 4 and Figs. 3 and 4, the other metabolites that could fit ambiguously identified metabolites are listed in Tables SIA.6 and SIB.6.

3.5.1. Repeatability and reproducibility

The repeatability (set criteria: < 15 % CV) and within-run reproducibility (set criteria: < 20 % CV) of validated metabolites with the RPLC-ESI$^+$-HRMS method were below 10 %, except for one and two metabolites, respectively, that were in the range 10–20 % (Table 3). The number of metabolites measured by HILIC-ESI$^-$-HRMS with a repeatability and reproducibility in the range 10–20 % were two and five, respectively (Table 4). The ratio between within-run QC_{pool} and QC_L and QC_H, respectively, was evaluated for between-run reproducibility (set criteria: < 25 % CV). In RPLC-ESI$^+$-HRMS, the CV% between batches of QC_L/QC_{pool} and QC_H/QC_{pool} were below 15 % except for two metabolites in each that were between 15 and 25 % (Table 3). The corresponding number of exceptions in HILIC-ESI$^-$-HRMS was five in each falling between 15 and 25 % (Table 4).

3.5.2. Selectivity

Compound identification selectivity (Tables 3 and 4) was tested against the local mass spectral library and retention time deviation was further compared with an injection of SRM1950. The quality of metabolite identifications was visually checked in Compound Discoverer. If a feature matched with multiple isomers in a retention time window, each possible identification was evaluated, and the metabolite identification was labelled "ambiguous" if more than a single library entry matched the measured feature. In Fig. 3, ambiguous identifications are labelled with an asterisk.

3.5.3. Stability

Freeze-and-thaw stability was evaluated, but not used to filter metabolites. Metabolites were not much affected by repeated freeze-and-thaw cycles with few exceptions, such as inosine (Tables SIA.8 and SIB.8). Unstable metabolites (flag criteria: <30 % reduction after ten freeze-and-thaw cycles) are labelled with a hashtag in Fig. 3.

3.5.4. Linearity and D-ratio

Seventy% of metabolites validated in the RPLC-ESI$^+$-HRMS method had a good signal and chromatographic peak shape after diluting the serum two orders of magnitude (Table 3) while only 45 % of metabolites measured by HILIC-ESI$^-$-HRMS showed good chromatographic peak shape after diluting one order of magnitude (Table 4). Detectability over extended dilution ranges indicates sensitive and robust detection of metabolites. On the other hand, 93 % and 44 % of the metabolites validated in HILIC-ESI$^-$-HRMS have *D*-ratio below 10 and 1 %, respectively (Table 4). The corresponding numbers for RPLC-ESI$^+$-HRMS are 87 % and 28 %, respectively (Table 3). Metabolites measured with low *D*-ratio will be more sensitive for smaller, but significant, biological perturbations. The dynamic range appeared smaller around the front elution in the RPLC-ESI$^+$-HRMS chromatography, around 1 min, as

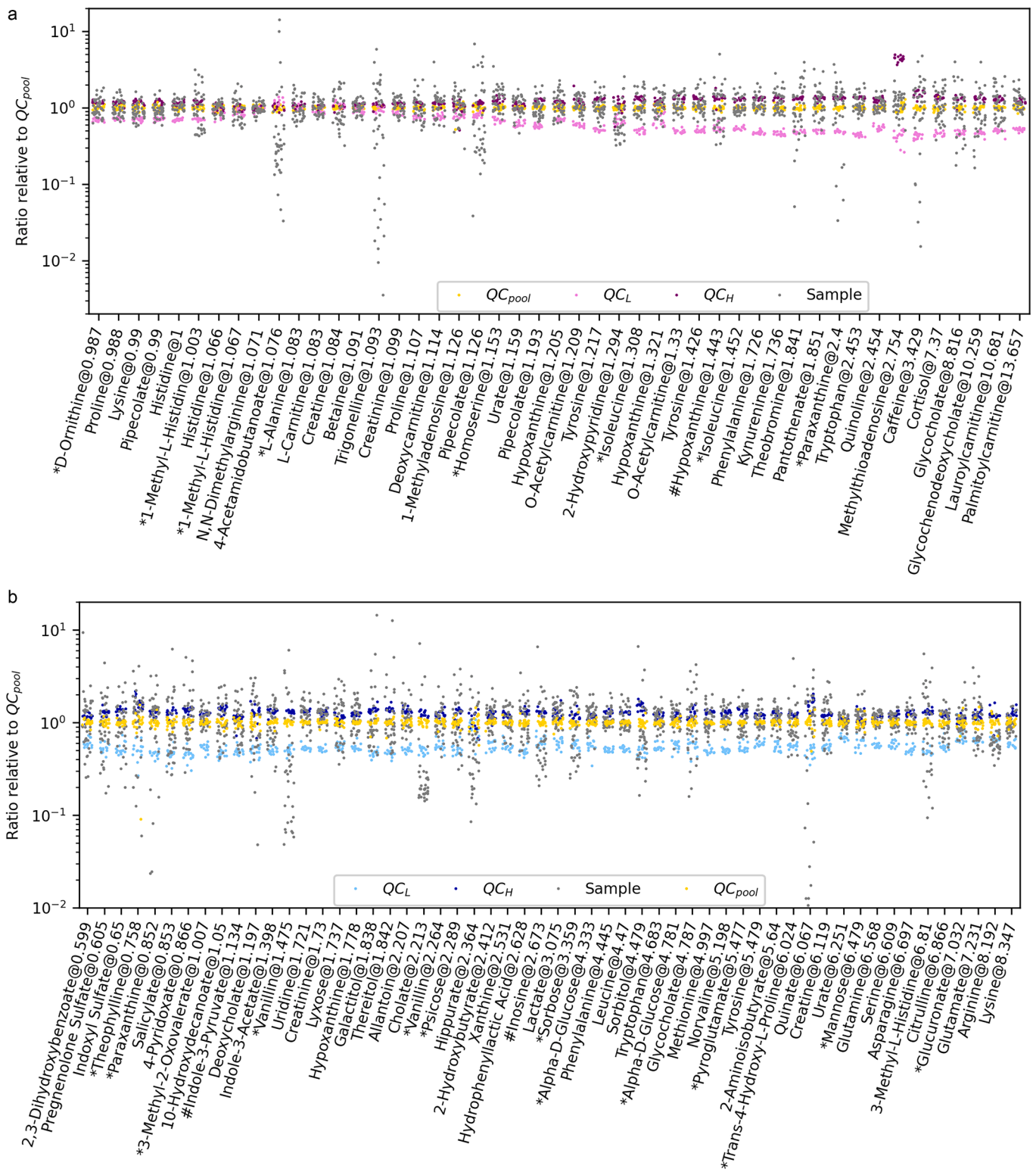

Fig. 3. Stripplots for validated metabolites in serum by RPLC-ESI$^+$-HRMS (top) and HILIC-ESI$^-$-HRMS (bottom). Each dot represents a single detection from a single sample, as ratio relative to within-run QC_{pool}. Each metabolite is given as library name at a given retention time (min). QC measurements are collected across three analytical runs, and individual samples in grey ($N = 38$) were analyzed in a single analytical run. QC_{pool}: yellow dots, QC_H: dark purple (top) or dark blue (bottom), QC_L: light purple (top) or light blue (bottom). *: ambiguous identification, #: unstable after repeated freeze-and-thaw cycles.

observed in Fig. 3 with a smaller relative distance between the QC_L and QC_H to QC_{pool}.

3.5.5. *Concordance of semi-quantitative results between analytical methods*

Rank correlations between analytical methods for validated metabolites in individually measured serum samples are presented in Fig. 4. Metabolites that presented with more than one peak were not tested for concordance between methods. When validated on both methods, metabolites from RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS were compared, otherwise results from the HILIC-ESI$^+$- and RPLC-ESI$^-$-HRMS

Table 3
Key validation results for metabolites measured in human serum by reversed-phase liquid chromatography - positive electrospray ionization - high-resolution mass spectrometry (RPLC-ESI$^+$-HRMS). Linearity is given as% dilution of a pooled serum sample. Asterisk: ambiguous identification, hashtag: unstable after repeated freeze-and-thaw cycles.

	Repeatability	Reproducibility			Selectivity			Linearity	*D*-ratio
Metabolite		Within-run	Between-run, QC_L	Between-run, QC_H	Library		SRM 1950		
Name@Retention time	CV%	CV%	CV%	CV%	ΔMass, ppm	ΔRT, min	ΔRT, min	Max-Min, %	%
*D-Ornithine@0.987	4.1	0.7	4.1	5.2	−1.95	0.07	0.01	111–22.2	1.81
Proline@0.988	4.9	5.4	3.4	5.2	−1.87	0.00	0.01	111–22.2	7.11
Lysine@0.99	5.5	6.4	4.5	5.0	−1.52	0.09	0.01	111–1.1	28.94
Pipecolate@0.99	4.8	6.7	4.2	5.0	−1.56	−0.07	0.01	111–1.1	29.28
Histidine@1	5.1	7.1	3.3	4.8	−1.32	0.08	0.01	111–1.1	7.52
*1-Methyl-l-Histidin@1.003	4.5	6.0	2.6	4.6	−1.33	0.09	0.01	111–1.1	0.48
Histidine@1.066	6.2	10.1	12.0	9.6	−1.03	0.15	0.01	111–1.1	15.98
*1-Methyl-l-Histidine@1.067	3.5	5.9	7.7	7.0	−1.11	0.18	0.01	111–1.1	2.29
N,N-Dimethylarginine@1.071	3.7	5.4	6.4	5.5	−1.08	0.13	0.01	111–1.1	16.83
4-Acetamidobutanoate@1.076	5.2	9.9	9.2	7.3	−1.83	0.06	0.01	111–1.1	0.01
*L-Alanine@1.083	4.1	4.9	6.2	5.6	−1.85	0.14	0.01	111–0.1	0.94
L-Carnitine@1.083	4.5	3.2	5.2	4.1	−1.64	0.12	0.01	111–0.1	2.97
Creatine@1.084	4.4	2.6	7.3	5.0	−1.66	0.10	0.01	111–0.1	0.72
Betaine@1.091	4.5	3.7	6.1	4.7	−2.00	0.09	0.01	111–1.1	4.38
Trigonelline@1.093	4.7	4.2	7.6	5.6	−1.58	0.10	0.01	111–0.1	0.10
Creatinine@1.099	4.4	2.8	5.5	4.3	−1.53	0.13	0.01	111–0.1	3.97
Proline@1.107	4.9	2.8	4.0	4.9	−1.73	0.12	0.01	111–0.1	2.92
Deoxycarnitine@1.114	4.4	4.1	6.5	4.6	−1.89	0.13	0.01	111–0.1	0.44
1-Methyladenosine@1.126	4.7	3.6	8.4	17.8	−0.83	0.11	0.01	111–5.6	31.05
Pipecolate@1.126	5.9	6.0	6.0	6.4	−1.40	0.06	0.01	111–5.6	0.29
*Homoserine@1.153	5.0	4.3	5.9	8.8	−2.09	0.20	0.01	111–1.1	1.68
Urate@1.159	4.7	3.0	7.7	6.3	−1.42	−0.19	0.01	111–1.1	1.37
Pipecolate@1.193	4.4	2.9	5.0	9.6	−1.73	0.13	0.01	111–1.1	1.22
Hypoxanthine@1.205	4.7	5.8	3.6	5.6	−1.78	0.08	0.01	111–1.1	2.19
O-Acetylcarnitine@1.209	7.0	4.2	5.1	14.2	−1.60	0.11	0.01	111–0.1	1.33
Tyrosine@1.217	4.3	5.9	5.2	4.2	−1.42	−0.12	0.01	111–5.6	1.26
2-Hydroxypyridine@1.294	7.2	4.4	10.2	20.2	−2.03	−0.19	0.01	111–66.6	1.92
*Isoleucine@1.308	4.7	5.1	6.4	4.7	−2.05	0.10	0.01	111–11.1	1.51
Hypoxanthine@1.321	4.1	7.3	15.3	7.5	−1.57	−0.04	0.01	111–0.1	7.60
O-Acetylcarnitine@1.33	4.4	5.8	5.2	4.9	−1.65	0.23	0.01	111–0.1	3.36
Tyrosine@1.426	4.7	4.6	5.8	6.3	−1.49	0.09	0.01	111–1.1	3.35
#Hypoxanthine@1.443	3.7	3.0	11.8	12.7	−2.01	0.20	*	111–1.1	1.91
*Isoleucine@1.452	5.0	3.6	4.5	3.3	−1.91	−0.04	0.01	111–1.1	3.08
Phenylalanine@1.726	3.8	3.8	5.1	3.2	−1.77	0.07	0.01	111–1.1	5.85
Kynurenine@1.736	4.0	3.3	6.4	5.6	−1.44	0.13	0.01	111–5.6	4.23
Theobromine@1.841	3.0	5.0	3.5	3.0	−1.67	−0.03	0.01	111–1.1	0.59
Pantothenate@1.851	4.4	2.4	4.3	4.7	−1.45	0.16	0.01	111–1.1	1.27
*Paraxanthine@2.4	11.0	2.7	3.1	3.3	−1.65	0.16	0.01	111–5.6	0.68
Tryptophan@2.453	4.1	5.1	4.5	2.9	−1.62	0.16	0.01	111–1.1	1.49
Quinoline@2.454	5.5	7.2	6.7	5.5	−1.90	−0.30	0.01	111–5.6	1.05
Methylthioadenosine@2.754	7.1	10.1	17.6	7.6	−1.46	0.19	0.01	111–22.2	24.59
Caffeine@3.429	3.8	2.3	7.4	9.5	−1.86	0.14	0.01	111–1.1	0.26
Cortisol@7.37	5.5	17.0	6.1	5.8	−1.26	0.07	0.01	111–5.6	2.81
Glycocholate@8.816	4.3	6.6	5.3	9.0	−1.58	0.07	0.01	111–1.1	0.35
Glycochenodeoxycholate@10.259	2.1	7.1	5.3	5.2	−1.69	0.06	0.01	111–1.1	0.28
Lauroylcarnitine@10.681	2.5	4.3	6.7	5.2	−1.35	0.16	0.01	111–1.1	0.48
Palmitoylcarnitine@13.657	9.8	4.1	4.2	5.6	−1.60	0.12	0.01	111–5.6	6.97

CV: coefficient of variation, QC: Quality control, RT: retention time, SRM: standard reference material.

methods were used. 24 metabolites that passed validation could be compared with concordance between methods, nine on RPLC-ESI$^+$- and 22 on HILIC-ESI$^-$-HRMS. The axis' color code in Fig. 4 shows which method the concordance evaluation is based on. The r_s-values ranged from 0.55 to 1, where the value 1 indicates a perfect rank correlation of the data points. Although the semi-quantitative results are not expected to show a linear correlation, the slope is presented to indicate comparative sensitivity of the methods. For metabolites with a high r_s-value, a slope coefficient above 1 could indicate that the methods plotted on the y-axis is more sensitive. RPLC results were used for eighteen metabolites in the concordance study. Median r_s was 0.95 (range{0.69–0.97}) for the seven metabolites with retention times earlier than 1.3 min in the RPLC methods, whereas the 11 metabolites with retention times after 1.3 had a median r_s of 0.96 (range{0.55–0.99}).

4. Discussion

The study aimed to establish a comprehensive and robust analytical platform for a long-term, untargeted metabolomics study. Two analytical methods were selected for the analytical platform. The reasoning behind the selection was the complementarity in chemical space covered, and most importantly, the sample release showed higher variance in the ISTD-signals from the second injection of each injection plate attributed to evaporation. The total time in the autosampler for the last injections was up to 40 h, which might not be sufficiently robust for a long-term study of precious sample material. Robustness was here prioritized over a broader analyte coverage. The validation passed with dual injection from the injection plates, and it was deduced that, in case of instrument malfunction, the samples can be stored for up to 24 h in the autosampler before initiating the analytical run.

Table 4
Key validation results for metabolites measured in human serum by Hydrophilic interaction liquid chromatography - negative electrospray ionization - high-resolution mass spectrometry (HILIC-ESI$^-$-HRMS). Linearity is given as% dilution of a pooled serum sample. Asterisk: ambiguous identification, hashtag: unstable after repeated freeze-and-thaw cycles.

	Repeatability	Reproducibility			Selectivity			Linearity	*D*-ratio
Metabolite		Within-run	Between-run, QC_L	Between-run, QC_H	Library		SRM 1950		
Name@Retention time	CV%	CV%	CV%	CV%	ΔMass, ppm	ΔRT, min	ΔRT, min	Max-Min, %	%
2,3-Dihydroxybenzoate@0.599	6.0	3.2	9.6	10.4	−2.0	−0.20	0.01	100–10	0.2
Pregnenolone Sulfate@0.605	5.7	6.2	9.8	8.9	−1.9	0.01	0.01	100–25	0.1
Indoxyl Sulfate@0.65	1.8	4.3	14.7	7.4	−2.2	−0.01	0.01	100–3	1.4
*Theophylline@0.758	1.9	6.5	20.2	15.1	−2.0	0.05	0.01	100–100	2.5
*Paraxanthine@0.852	0.9	2.6	6.9	5.6	−1.9	0.09	0.01	100–5	5.2
Salicylate@0.853	3.7	2.9	14.5	8.7	−2.0	0.05	0.01	100–5	0.7
4-Pyridoxate@0.866	2.6	3.8	14.2	5.7	−2.1	0.10	0.01	100–10	0.6
*3-Methyl-2-Oxovalerate@1.007	2.4	1.9	9.1	5.1	−2.4	0.04	0.01	100–3	1.6
10-Hydroxydecanoate@1.05	7.2	2.8	9.2	11.4	−1.5	0.26	0.01	100–25	1.9
#Indole-3-Pyruvate@1.134	5.3	7.2	8.1	10.2	0.0	0.00	0.01	100–25	4.3
Deoxycholate@1.197	10.6	4.7	13.7	17.8	−1.2	0.26	0.01	100–100	4.7
Indole-3-Acetate@1.398	3.3	3.0	10.4	5.7	−1.6	0.15	0.01	100–10	3.7
*Vanillin@1.475	1.6	2.2	8.5	2.7	−1.6	−0.42	0.01	100–75	0.1
Uridine@1.721	1.0	1.4	5.8	3.7	−2.1	0.15	0.01	100–3	0.7
Creatinine@1.73	1.2	1.6	4.8	5.1	−1.4	0.19	0.01	100–10	5.1
Lyxose@1.737	3.8	6.2	7.0	7.9	−1.6	−0.40	0.01	100–75	0.8
Hypoxanthine@1.778	1.1	2.6	8.1	3.2	−1.4	0.18	0.01	100–10	0.4
Galactitol@1.838	0.9	2.5	7.3	9.2	−1.7	0.07	0.01	100–50	0.4
Thereitol@1.842	1.4	2.4	7.1	8.8	−1.8	0.11	0.01	100–50	0.6
Allantoin@2.207	1.4	9.7	6.9	5.9	−1.4	0.14	0.03	100–10	1.3
Cholate@2.213	4.1	2.6	8.0	7.8	−0.7	0.17	0.02	100–100	0.4
*Vanillin@2.264	2.5	9.2	11.0	7.9	−1.2	0.29	0.03	100–50	3.3
*Psicose@2.289	5.6	6.9	12.5	11.4	−1.5	0.13	0.03	100–100	1.5
Hippurate@2.364	1.5	17.5	20.1	23.2	−1.6	0.34	0.01	100–5	15.0
2-Hydroxybutyrate@2.412	0.8	2.3	11.5	9.2	−1.5	0.35	0.02	100–10	1.2
Xanthine@2.531	0.6	3.4	10.1	4.3	−1.4	0.26	0.02	100–10	6.0
Hydrophenyllactic Acid@2.628	0.6	3.9	8.2	8.5	−1.7	0.37	0.03	100–25	3.9
#Inosine@2.673	0.6	2.5	12.6	5.1	−2.0	0.14	0.03	100–25	0.6
Lactate@3.075	1.8	3.7	10.7	13.1	−3.3	0.25	0.01	100–25	9.5
*Sorbose@3.359	1.1	2.0	14.2	9.5	−1.7	0.24	0.01	100–75	4.3
*Alpha-d-Glucose@4.333	3.6	7.1	13.7	5.8	−2.4	−0.28	0.01	100–25	0.6
Phenylalanine@4.445	0.6	4.1	4.7	3.9	−2.2	0.08	0.04	100–3	0.5
Leucine@4.47	1.0	3.8	5.3	2.6	−2.2	0.14	0.03	100–10	0.8
Sorbitol@4.479	10.2	13.5	10.2	11.0	−2.0	0.25	0.03	100–50	0.0
Tryptophan@4.683	0.9	3.9	8.0	5.5	−2.2	0.09	0.03	100–3	1.1
*Alpha-d-Glucose@4.781	0.4	10.5	10.1	6.0	−2.1	0.17	0.01	100–3	3.2
Glycocholate@4.787	4.6	9.4	17.7	13.2	−0.6	0.11	0.02	100–75	0.1
Methionine@4.997	0.8	2.1	5.8	2.7	−2.3	0.10	0.01	100–25	0.4
Norvaline@5.198	1.5	4.3	8.4	4.6	−2.2	0.29	0.01	100–10	1.5
*Pyroglutamate@5.477	0.4	2.0	9.6	6.4	−1.7	0.22	0.01	100–10	0.1
Tyrosine@5.479	0.8	3.0	6.0	3.5	−2.1	0.08	0.01	100–10	0.3
2-Aminoisobutyrate@5.64	5.7	4.4	10.2	5.4	−2.5	0.09	0.01	100–25	2.4
*Trans-4-Hydroxy-l-Proline@6.024	1.6	5.5	4.7	4.2	−2.2	0.10	0.01	100–50	0.5
Quinate@6.067	1.9	18.3	13.8	15.1	−2.1	0.34	0.02	100–75	0.8
Creatine@6.119	0.9	3.7	6.9	3.2	−2.4	0.08	0.01	100–10	0.3
Urate@6.251	1.4	4.6	10.7	4.1	−2.4	0.20	0.01	100–3	8.6
*Mannose@6.479	2.6	16.9	12.9	13.6	0.0	0.00	0.01	100–50	2.2
Glutamine@6.568	1.8	1.9	6.5	4.3	−2.3	0.12	0.01	100–3	5.6
Serine@6.609	1.5	2.6	7.4	5.0	−2.1	0.07	0.01	100–25	1.9
Asparagine@6.697	1.3	2.1	13.4	4.7	−2.3	0.07	0.01	100–25	8.8
3-Methyl-l-Histidine@6.81	0.8	3.0	8.9	4.5	−2.2	0.10	0.01	100–50	0.3
Citrulline@6.866	1.3	3.6	11.3	5.1	−2.3	0.08	0.01	100–25	10.2
*Glucuronate@7.032	2.9	9.8	21.4	11.3	−2.3	0.08	0.01	100–100	24.4
Glutamate@7.231	2.2	6.6	16.9	8.6	−2.0	0.23	0.03	100–10	8.3
Arginine@8.192	0.9	4.3	9.6	15.7	−2.3	−0.28	0.03	100–25	68.5
Lysine@8.347	0.6	3.0	6.5	8.4	−2.2	−0.26	0.04	100–50	10.1

CV: coefficient of variation, QC: Quality control, RT: retention time, SRM: standard reference material.

The integration of an automated liquid handler in sample preparation offers great advantages in terms of less repetitive, manual tasks, error minimization, and scalability for larger studies. In the employed methodology, HILIC supernatants were directly injected, while RPLC samples underwent evaporation and reconstitution before initiating the RPLC analytical runs. This approach mitigated risks associated with the evaporation of volatile metabolites for the HILIC injections. The methods' complementarity and the flexibility they afford for laboratory scheduling are advantageous for large-scale studies.

The starting conditions of 10 % aqueous mobile phase for the RPLC methods is higher than for other untargeted methods [41–43]. This was decided in an attempt to avoid precipitation in the autosampler and improve method performance for medium-polarity metabolites. The RPLC gradients favor a chemical space where few endogenous

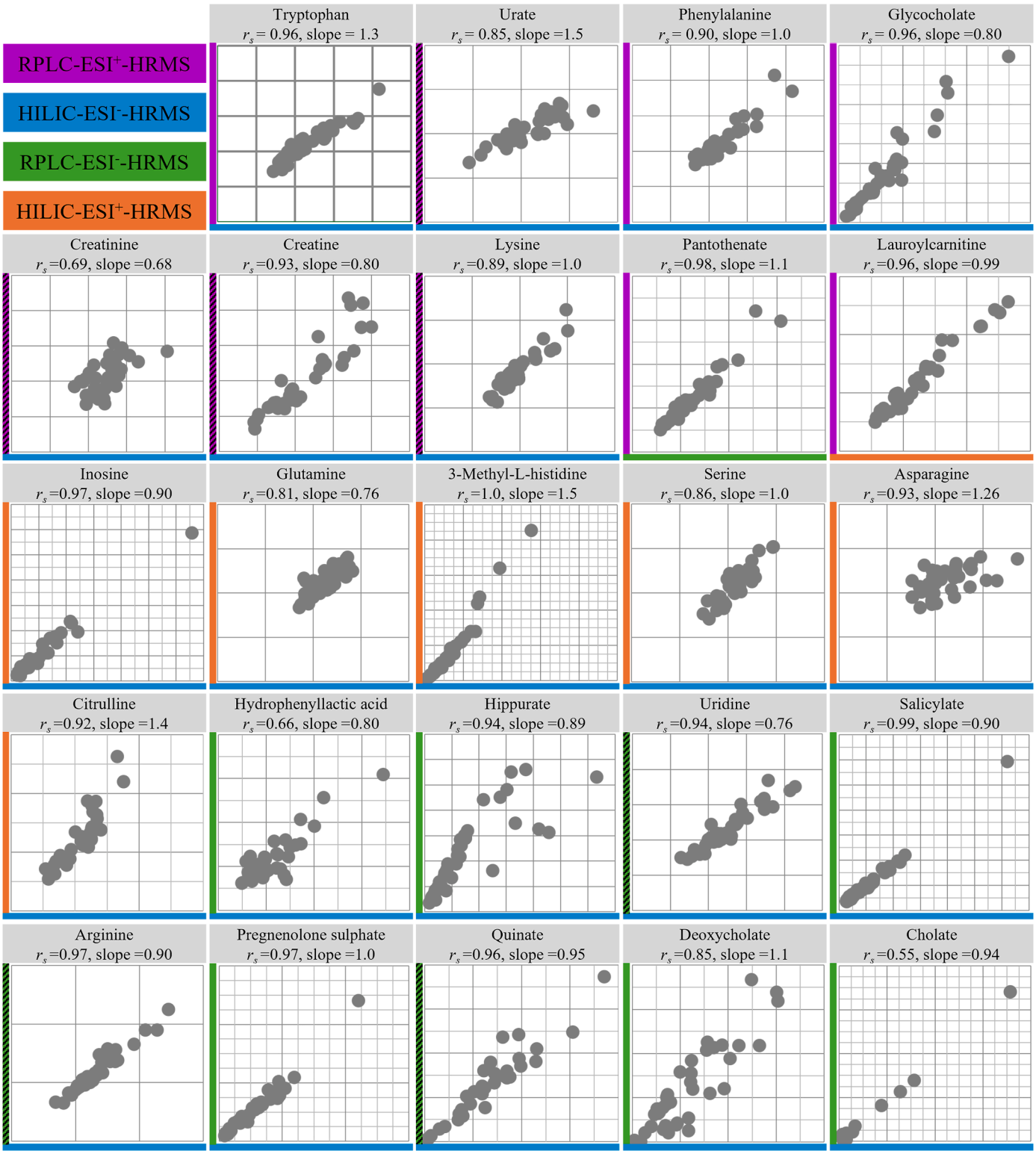

Fig. 4. Concordance of semi-quantatative results for validated metabolites measured on more than one methods. Results are from individually measured serum samples ($N = 38$), as internal standard-corrected areas. Results are further normalized to QC_{pool} for plotting, where each grid line is 0.5 for all methods, and each plot originates at (0,0). Striped bars presents a retention time below 1.3 min for the RPLC methods. r_s: Spearmans rank correlation coefficient.

metabolites elute, as observed by the retention time of validated metabolites, but it is hypothesized that exposome analytes will elute here. The two methods that were discarded, i.e. HILIC-ESI^+- and RPLC-ESI^--HRMS, may have performed better with different gradients and buffer systems; for instance with a steeper chromatography for HILIC-ESI^+-HRMS [41,43], and at neutral pH of the RPLC-ESI^--HRMS to improve detection and retention of fatty acids [43]. This validation study revealed some important issues with the methods tested. Most notably the drift of the HILIC retention time for the latest eluting metabolites, which is corrected for future applications by increasing the column equilibrium time by an additional 0.5 min. Furthermore, the ISTDs (Acetyl-l-carnitine-D_3, Stearic acid-18,18,18-D_3, 18:0-D_{35} Lyso

PC, and Dopamine-1,1,2,2-D_4) not used in sample release will not be used in future applications.

Analytical parameters were validated to *i*) guide the selection of the most suitable combination of methods for our application, to *ii*) evaluate and document the laboratory's competence in acquiring dependable untargeted metabolomics results, and finally to *iii*) get experience in running this type of study before commencing analysis of valuable samples. Chau et al. mentioned that parallelism, dilution linearity, and precision should be evaluated for semi-quantitative assays for biomarkers, and that accuracy cannot be evaluated [16]. The focus point of this study was to explore intrinsic variance in untargeted metabolomics data sets from selected methods, and concordance studies were used for parallelism. Analytical results from untargeted metabolomics are mostly used to identify metabolic perturbations, and the method is acceptable when the analytical variance is smaller than the biological variance for a given metabolite. Precision was thus defined as a key parameter to test in this validation. QC_{pool}-correction, commonly employed in untargeted metabolomics [19,38], was not applied in this study, because we sought to evaluate intrinsic dataset variance and analytical robustness. Metabolomics data analysis workflows include the removal of low-quality features [19], usually features with a variance lower than 20 to 30 % and detected in at least 70 % of the QC_{pool} [38]. In this study, reproducibility acceptance criteria in CV%, were set to 20 % within- and 25 % between-run, respectively. The QC_{pool} detection rate was at least 94 %. *D*-ratio is another commonly used parameter used to filter metabolites with a high analytical to biological variance [38]. In this validation study, the linear range of the RPLC method compared with HILIC was wider, but the lower *D*-ratio of validated metabolites in the HILIC method could indicate better performance for detection of biological variance. D-ratios were evaluated but not used as a validation criterium in the present work, since the biological variance in healthy, recently fed blood-bank donors, is not a good surrogate for biological variance associated with health and disease states. Metabolites with minor biological variance should be allowed to pass validation if the method performs well. Interesting examples are the metabolites arginine and cholate, both passed validation in HILIC-ESI$^-$-HRMS. The calculated *D*-ratios are 68.2 and 0.4 (Table 4), respectively, whereas the r_s values are 0.97 and 0.55 (Fig. 4). Although a small biological variance of arginine is detected, the rank of semi-quantitative results measured on two methods with complementary column material and buffer systems correlates. Arginine evidently performs well on the HILIC method, although a *D*-ratio higher than 50 % would have eliminated the metabolite from normal untargeted metabolomics data filtering [38]. The low r_s value of cholate could look problematic when comparing with the stronger correlation for the other metabolites. It is however evident from cholate in Fig. 4 that the high values correlate well, and no systematic bias is apparent.

Validation was only performed on level 1 identified metabolites, meaning metabolites identified with a local library from accurate mass, MS/MS, and retention time [17,22]. Multiple features could in some instances be identified as the same metabolite, some due to peak-splitting of early chromatographic elution as for histidine in RPLC-ESI$^+$-HRMS, and others possibly due to stereoisomers, misidentified isomers, or *in-source* fragment ions. Even with curated, local libraries, metabolite identification is not unambiguous and challenges related to compound identification in untargeted metabolomics are well-known [44]. The local mass spectral libraries used for this study were based on the commercially available set of metabolite standards (MSMLS), without filtering or grouping of the library entries. For instance, alanine and ornithine are validated as ambiguous identifications in RPLC-ESI$^+$-HRMS because both l- and d-enantiomers are in the MSMLS. On the contrary, the drug allopurinol that is not in MSMLS, would probably be identified as hypoxanthine as they are closely related isomers. An inherent problem with even the best curated, mass spectral libraries is that the operator must be aware of possible (de)limitations or caveats for the entries. We adopted the classification system commonly used in the field of metabolomics [17,22], but highlight that identification should be done with caution although identifications are level 1. Recovery studies, where analytical standards are spiked into real samples, as performed by Zelena et al. [21] would be an alternative approach to improve confidence in compound identification. Proficiency testing and/or ring trials are however the best way to address this problem.

The study also aimed to develop and test a quality assurance system, including quality control samples. QCs that are prepared as and run together with unknown samples are deemed more representative of the process. A QC_H and QC_L was thus developed in this study to evaluate between-run reproducibility, inspired by soaked QC samples used in hair testing [45]. The QC_L and QC_H were further used for run release, where it is tested for key metabolites that $QC_H > QC_{pool} > QC_L$. The performance of QC_H and QC_L across batches were satisfactory, even without QC_{pool} correction. Performance of levelled QCs should however be tested against current alternatives, i.e. variable injection volume [13] and QC_{pool} supernatant dilution rows [46]. Going forward, these levelled QCs will be monitored together with dilution series and variable injection volumes to compare performance and test if it can not only evaluate, but also guide correction for between-run variance in linearity.

The validated analytical methods will be applied on population study serum samples, that will have undergone a number of freeze-and-thaw cycles. This parameter was thus tested. Degradation after repeated freeze-and-thaw cycles was not a problem, but an increase in signal of the amino acids phenylalanine and glutamine was observed (Table SIA8 and SIB9, respectively), which is in line with previous findings for the long-term stability of metabolites in plasma at −80 °C [47].

While only a subset ($N = 24$) of validated metabolites could be evaluated for concordance in multiple methods, the evaluated metabolites did correlate well. It should be noted that although the RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS methods are only validated with a targeted data analysis workflow, the methods and acquired data will also be used in semi-targeted/suspect and non-targeted workflows. The validation data was based on untargeted acquisition and a non-targeted peak-picking was used to find metabolite features, which transverse the different workflows. In future studies, metabolite structures can be identified using tools like SIRIUS [48], if a non-targeted data analysis pipeline highlights unidentified features that clearly discriminate between cases and controls. The validation data can be revisited for *post-hoc* validation of features not currently matching metabolites in the applied mass spectral library, which is particularly valuable in studies with limited sample sizes. This *post-hoc* validation may allow for early filtering of lower-quality hits, in case there is a systematic difference in freeze-and-thaw cycles of serum samples evaluated with these methods or enhancing confidence in biomarker candidates for further investigation.

5. Conclusion

We here present validation reports for untargeted metabolomic profiling of clinical samples by LC—HRMS, for level 1 identified metabolites. Sample preparation was performed with an automated liquid handler, and the validation experiments were conducted for four analytical methods, across three batches. Out of four tested methods, full validation reports are presented for RPLC in positive and HILIC in negative electrospray ionization mode. The methods were amongst other evaluated for repeatability, reproducibility, stability, linearity, and identification selectivity with a particular emphasis on dataset intrinsic variance. 47 and 55 level 1 metabolites were validated in serum, for RPLC-ESI$^+$- and HILIC-ESI$^-$-HRMS, respectively. The semi-quantitative results for metabolites that passed validation and was detected on more than one method ($N = 24$) were tested with Spearman rank correlation and no apparent bias was observed. A quality assurance system was set up and tested encompassing system suitability testing and sample, run, and batch release. Levelled QC_{pool} samples were

introduced as a potential mean to evaluate variance in response linearity across batches.

Based on this validation, the presented analytical platform is concluded fit-for-purpose for untargeted metabolomics of metabolites measured in serum samples that passed validation across multiple batches.

Untargeted metabolomics, frequently cited as a hypothesis-generating field, benefits immensely from the pre-validation of method performance before commencing analysis of precious samples. Such validation not only bolsters the reliability of the assays but also significantly enhances the impact and credibility of the hypotheses generated from the studies. Therefore, this validation study serves as a benchmark in the documentation of LC—HRMS-based untargeted metabolomics method performance aimed for clinical application, potentially guiding future endeavors in the field.

Supporting Information

SIA: Full validation report for RPLC-ESI$^+$-HRMS, including tables with results and detailed information of validation parameters and design

SIB: Full validation report for HILIC-ESI$^-$-HRMS, including tables with results and detailed information of validation parameters and design

SIC: Quality assurance and results for RPLC-ESI$^-$-HRMS and HILIC-ESI$^+$-HRMS with representative plots for sample release and run release. Stripplots for the chromatographic runs not used for full validation

Declaration of generative AI and AI-assisted technologies in the writing process

Statement: During the preparation of this work the author(s) used openAI in order to improve language flow and reduce sentence length. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Sietske Grijseels: Conceptualization, Formal analysis, Software, Investigation, Methodology, Writing – review & editing. **Terje Vasskog:** Conceptualization, Project administration, Writing – review & editing. **Pia J. Heinsvig:** Writing – review & editing. **Torbjørn N. Myhre:** Data curation. **Terkel Hansen:** Conceptualization, Funding acquisition. **Marie Mardal:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Validation, Visualization, Writing – original draft.

Declaration of competing interest

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

Data availability

The authors do not have permission to share data.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465230.

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