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Article

Rapid Assessment of Lipidomics Sample Purity and Quantity Using Fourier-Transform Infrared Spectroscopy

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Abstract: Despite the increasing popularity of liquid chromatography-mass spectrometry (LC-MS)-based lipidomics, there is a lack of accepted and validated method for lipid extract quality and quantity assessment prior to LC-MS. Fourier-Transform Infrared Spectroscopy (FTIR) has been reported for quantification of pure lipids, however, the sample complexity and purity complex lipid extract quantification in lipidomics experiments could be impact quantification accuracy. Here, we report comprehensive assessment of the sample matrix on the accuracy of lipid quantification using Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR-FTIR). Pure lipids are characterized by CH-and C=O-stretching vibrations on FTIR, with quantitative range of 40–3000 ng and a limit of detection of 12 ng. Sample extraction method and local baseline subtraction during FTIR spectral processing significantly impact lipid quantification by CH-stretching. To facilitate sample quality screening, we developed the Lipid Quality (LiQ) score from a spectral library of common contaminants, using a ratio of peak heights between CH-stretching vibrations maxima and the collective vibrations from amide/amine, CH-stretching minima and sugar moieties. We evaluated LiQ score as a rapid sample quality control method by comparing to total LC-MS intensity of targeted lipidomics of 107 human plasma lipid extracts. Exclusion of poor-quality samples based on LiQ score improved the correlation between FTIR and LC-MS quantification. Finally, the uncertainty of absolute quantification by FTIR was estimated using a 795 ng SPLASH LipidoMix standard to be <10%. In summary, this study identified key parameters for accurate FTIR-based quantification of complex lipid mixture, and developed a rapid workflow requiring only 1 μL of MS-ready sample and < 5 minutes for routine lipidomics sample quality and quantity assessment.

Keywords: lipids; phospholipids; sphingolipids; triglycerides; FTIR; mass spectrometry; chemical contaminants

1. Introduction

With advances in technology and development of analytical and lipid-specific informatics methods, mass spectrometry-based lipidomics has become the method of choice for lipid species identification and quantification. Lipids play diverse roles in biological systems [1]. While genomic, transcriptomic and proteomic information on lipid metabolism enzymes and transporters can infer the potential lipid metabolism capacity of a cell/tissue, direct measurement of lipids by lipidomics is essential to obtain high resolution data on the lipidome which is heavily influenced by diet and environmental factors [2]. Furthermore, a linear relationship does not exist between genotype and functional components of the lipidome, such as fatty acid chain length or level of unsaturation. Therefore, it is essential to conduct lipidomics as part of a multi-omics investigation in

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pathologies with suspected lipid dysregulation such as cancer, neurodegenerative and metabolic diseases [3-5].

In principle, the steps in the workflow for lipidomics should parallel the other more developed omics, and comprise sample extraction, quality control, and quantification steps. However, sample quality control is currently absent from the recommendations for good practice in lipidomics [6], possibly due to the lack of validated, fit-for-purpose, sample-preserving methods for lipid quality control prior to mass spectrometry analysis. The impact of lack of quality control and lipid quantity normalization steps on lipidomics experimental data quality is unknown. Without total lipid quantification, sample volume (e.g. serum/plasma) or weight (e.g. tissue) have been used as the basis of normalization. While this approach is appropriate for studies using well-characterized sample types in cross-sectional or well-controlled studies, a sample quality control and quantification step is essential for novel, less-controlled sample types, for example, materials derived from foodstuffs, plant samples and wildlife.

The current commercial product marketed for lipid quantification, the DirectDetect™ [7] uses Fourier-transform infrared (FTIR) spectroscopy with an external standard to quantify lipids based on CH symmetric stretching vibration between 2870 and 2840 cm^{-1} [8]. While this simple formula is accurate for pure lipids, the quantification accuracy of complex lipid samples/mixtures, and potential impact of different lipid extraction methods and biochemical contamination on total lipid quantification by FTIR have not yet been fully assessed. Since presence of other (bio)chemicals in the sample can influence IR absorbance for CH stretching due to masking effect [9], and lipidomics experiments almost always analyze complex (rather than pure) lipids, a technical assessment and validation of complex lipid quantification by FTIR is essential prior to adoption into the workflow.

Biochemical contamination of the extracted lipid sample may occur during sample preparation (e.g. incomplete metabolite removal, trace sucrose, detergents), or due to contaminants in the solvent or tubes (e.g. PEG). While bi-phasic preparation of lipidomics samples offer a theoretically pure lipid sample [10], technical errors and inaccurate pipetting can lead to contamination of lipid samples with no obvious indication of this incident until MS acquisition has taken place. Depending on the extent and type of contamination, the impact of biochemical contamination may cause inaccurate MS data and quantification due to matrix effect [11], and potentially, catastrophic contamination of the LC column and/or MS source requiring expensive repair/replacement of parts. These potentially adverse events could be prevented by incorporating a simple validated lipid sample quality control assay prior to LC-MS.

In this work, we evaluated Attenuate Total Reflectance (ATR)-FTIR for complex lipid sample quantification and contaminant detection. We chose ATR sampling method for FTIR because it requires just 1 microliter of MS-ready sample without additional sample preparation. The organic solvent from the lipid extracts readily evaporates on the ATR sensor, allowing rapid FTIR data acquisition. To establish the impact of sample extraction method and contaminants on FTIR spectra and the ensuing lipid quantification, we compared a cell extract using commonly used mono-phasic and bi-phasic extraction methods. In addition, we titrated a range of common biochemical contaminants to a pure lipid extract to assess the sensitivity ATR-FTIR for contaminant detection. Finally, we developed a lipid quality (LiQ) score and evaluated external lipid calibration standards for complex lipid quantification. The proposed lipidomics sample quality control workflow was then applied to a set of plasma samples with comparison to LC-MS-based lipidomics.

2. Materials and Methods

2.1. Materials

Stearic acid (S4751), sucrose (84097), glucose (G8270), galactose (G0750), NP-40 (542334), Triton-X100 (T8787), sodium deoxycholate (D6750), 1-butanol (34867), 2,6-di-

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tert-butyl-4-methyl-phenol (BHT, B1378), tert-butyl-methyl-ether (TBME, 34875), 2-aminoanthracene (A38800) and Val-Tyr-Val (V8376) were purchased from Sigma Aldrich (Australia). RNA primer (Qiagen, MS00003556) was purchased from Qiagen (Australia). SPLASH LipidoMix (330707) was purchased from Avanti Polar Lipids, inc. (Alabaster, Alabama, USA). Agilent API-TOF Reference Mix (G1969-85001), 250 µl PP inserts with graduation (#5190-4073) and PTFE/silicone rubber septa (#5182-0731) were ordered from Agilent Technologies (Mulgrave, Vic, Australia). Methanol (A456-4), 2-propanol (A451-4) and acetonitrile (A955-4) were purchased from Thermo Fisher Scientific (Australia). The Synergy UV Water Purification System was used to filter MilliQ water (Merck Millipore).

2.2. Cell culture and lipid extract preparation

PC3 (RRID: CVCL_0035) cells were grown in 5% FBS (Bovogen, SFBS-FR) / RPMI-1460 (Gibco, 11875119) culture medium at 37°C, 5% CO_2. Cells were passaged into 10cm petri dishes, harvested by cell scraping, and counted using hemocytometer. To prepare three extracts with varying levels of contamination, aliquots of one million cells were prepared, pelleted and washed with PBS, and the processed with one of the following methods. For "Mixed sample" of all cellular components, the cell pellet was resuspended with 300 µL butanol:methanol followed by immediate analysis of the cell extract by FTIR spectroscopy. In case of the "BuMe" extract, the cell pellet was resuspended with 300 µL butanol:methanol, then incubated overnight at -20°C. Protein was then removed by centrifugation at 16,000 x g for 30 min at 4°C, and the supernatant was collected in a separate tube and analyzed by FTIR spectroscopy. For the "TBME" extract, the cell pellet was resuspended in 200 µL of chilled methanol, sonicated for 1 min and incubated overnight at -20°C. TBME (tributyl methylether, 700 µL) was added and the sample vortexed before adding 180 µL of milliQ water. After mixing, the sample was centrifuged at 16,000 x g for 30 min at 4°C and the upper (lipid extract) and middle (metabolite extract, for quality control analysis) phases were collected into individual tubes. The lipid extract was then dried, resuspended in 300 µL butanol:methanol and analyzed by FTIR spectroscopy. The remaining protein pellet was washed, resuspended (50 µL) in PBS, and the amount of protein quantified by BCA assay for use in quality control analyses.

2.3. Preparation of contaminant samples

Stock mixtures of each contaminant were generated by the following methods. Solid sucrose, glucose and galactose were resuspended in 10 µL of MilliQ water before dilution in 3:1 butanol:methanol to a final volume of 1mL and concentration of 1 µg/µL. Protein extracted from cell lysates was diluted in 3:1 butanol:methanol to provide a 1mL stock solution with a protein concentration of 1 µg/µL. Due to the precipitation of protein and sugars in organic solvents, these samples were thoroughly vortexed before each use measurement or dilution. Solid detergent (sodium deoxycholate) were resuspended in 3:1 butanol:methanol to a concentration of 1% (w/v), whereas liquid detergent (Triton X-100, NP-40) were diluted to 1% (v/v) in 3:1 butanol:methanol. Lyophilised RNA primer was resuspended in 10 µL MilliQ water and diluted to 1 µg/µL (200µL) in 3:1 butanol:methanol, and its concentration confirmed using Nanodrop (give details). Metabolites extracted from 10^7 cells (method described above) were collected from the aqueous phase of a TBME extraction (380 µL), dried and resuspended in 380 µL 3:1 butanol:methanol. Lipids purified by TBME extraction were then quantified by ATR-FTIR spectroscopy and diluted to 1µg/µL for use as pure lipid diluent for quality control analysis. Lipids extracted by the BuMe method were diluted using the same dilution factor as TBME lipids.

Dilutions for quality control analysis were prepared by spiking variable amounts of contaminant stock into 10 µL pure lipid extract, followed by drying and resuspension in 10 µL 3:1 butanol:methanol. This ensures a constant lipid concentration of 1 µg/µL without dilution from the contaminant that has been spiked-in.

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2.4. Human plasma sample collection

De-identified plasma samples from a cohort of patients with chronic liver disease were used for proof-of-concept application of lipid extract quality assessment. These samples were collected following informed consent from the participants and the work was approved by the research ethics committees of the Prince Charles Hospital (HREC/15/QPCH/202) and QIMR Berghofer Medical Research Institute (P2352), and abide by the Declaration of Helsinki principles. Blood was collected using EDTA as the anticoagulant. Following centrifugation of the samples, plasma was collected and stored at -80oC.

2.5. Human plasma sample preparation

Plasma samples were thawed on ice prior to metabolite and lipid extractions. All pipetting steps were performed in a cold-room or on ice. Plasma (30 µL) was mixed with 270 µL of butanol/methanol (1:1 v/v) containing 10mM ammonium formate, 50 µg/mL BHT and 1.5 µL SPLASH internal standard mixture. Samples were agitated using a thermomixer and sonicated (25°C, 850 rpm, 1 hour). Samples were centrifuged at 16,000g (20°C, 15 minutes) before aliquoting 100 µL of the supernatant for mass spectrometry analysis. For FTIR spectra acquisition, 100 µL of butanol:methanol lipid extracts were aliquoted into a Greiner V-bottom 96-well plate and dried using a evaporative sample concentrator (Genevac EZ-2, Marshall scientific). The samples were reconstituted in 12 µL of butanol:methanol (1:1).

2.6. ATR-FTIR spectroscopy

An Agilent Cary 630 fitted with ATR module was used for the acquisition of spectra. Mid-infrared spectra (4,000 – 650 cm^{-1}) were collected at a resolution of 8 cm^{-1} using 64 scans per acquisition. The detector stage was cleaned with 80% ethanol and the background spectra (ambient room air at 21°C) was collected. Lipid sample (1µL) or standard in butanol:methanol mixture was applied to the detector and allowed to air dry (~30 sec). Lipid standards were prepared by serial dilution of stearic acid and Avanti SPLASH LipidoMix in butanol:methanol. All spectra were baseline adjusted using the baseline algorithm built into the Agilent MicroLab Expert software with set regions 2031-1865 cm^{-1} and 3971-3799 cm^{-1}. The spectra were then exported from MicroLab Expert (Agilent) software as CSV files.

2.7. LC-MS lipidomics

Targeted lipidomics experiments were performed following previously published methodologies [12]. Briefly, an Agilent Technologies 1290 Infinity II UHPLC system with an Agilent ZORBAX eclipse plus C18 column (2.1x100mm 1.8µm) (#959758-902) and guard column (#821725-901), coupled online to an Agilent 6470 triple quadrupole system was used for the targeted lipidomics experiments. The instrument was tuned in positive ionization mode and unit resolution. Buffer A contained 10mM ammonium formate in water/acetonitrile/isopropanol (50:30:20% v/v/v), whereas buffer B contained contains 10mM ammonium formate in water/acetonitrile/isopropanol (1:9:90% v/v/v). A multi-wash procedure was performed prior to each sample injection. In this procedure, the needle was washed and needle seat back flushed with isopropanol, MilliQ water and acetonitrile to reduce sample carryover.

The source nitrogen gas temperature was set to 175°C at a flow of 11 L/min and a sheath gas temperature of 250°C at a flow of 10 L/min. The capillary voltage was set to 3500 V and nozzle voltage to 0 V for positive mode and the nebulizer operated at 20 psi. Check tunes were performed in wide, unit and enhanced modes prior to each experiment

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to confirm the performance of the mass spectrometer. The quadrupole was tuned to reference masses 118.09, 322.05, 622.03, 922.01 and 1221.99 in positive ionization mode; 112.99, 302.00, 601.98, 1033.99 and 1333.97 in negative ionization mode.

The instrument was operated in dynamic MRM mode using the transitions published by Huynh et al. [12], including LipidoMix internal standards. Six microliters of sample were injected per acquisition. Acquired data were imported into Skyline (MacCoss Lab, Department of Genome Sciences, University of Washington) [13], peak integration was automated but manually confirmed and corrected if required. Internal standard retention time was used to confirm correct peak integration of lipids belonging to the same class. Peak areas were exported from Skyline for further analysis in R (R Foundation for Statistical Computing, Vienna, Austria) [14]. Total intensity (TI) values were generated by summing all lipid species intensities per acquisition and adjusted to reflect differences in concentration between MS measurements (6 µL of 100µL plasma lipid sample, 6% total sample measured per acquisition) and FTIR spectroscopy (1 µL of concentrated plasma lipid sample, 8.33% total sample measured per acquisition).

2.8. Data analysis and statistics

Method development and optimization analysis were completed using R (version 4.0.2) and Graphpad Prism software, where final application utilized MicroLab Quant and Expert software. Baselined spectra were analyzed using R studio and the DescTools package (0.99.44). The area under the curve (AUC) was calculated for each desired region using the trapezoid method via the AUC function. A local baseline was calculated using the minimum and maximum range values in the trapezoid area formula [area = ($Height_{Min}$ - $Height_{Max}$)/2 * ($Wavenumber_{Max}$ - $Wavenumber_{Min}$)] and subtracted from each AUC to result in a baselined AUC value. Mean baselined AUC and standard deviation were calculated across the technical replicates (n = 3 - 6) for each sample. Simple linear regression analysis and graphs were generated by Graphpad Prism (v8) for each standard lipid calibration curve. Signal to noise ratios (S/N), limits of detection (LOD) and quantitation (LOQ) were calculated using the International Conference on Harmonization method [15]. FTIR and mass spectrometry measures were compared by Pearson correlation (R value) and simple linear regression analysis (R^2 and p values). Graph collation and diagrams were generated by Inkscape.

3. Results

3.1. Impact of lipid sample purity on ATR-FTIR spectra – comparison of lipid extraction methods

First, we assessed the impact of lipid extraction methods. ATR-FTIR spectra were collected from different aliquots of the same cell pellet sample prepared using different extraction methods, and compared to spectra of a synthesized pure lipid mixture imitating human plasma composition (SPLASH LipidoMix, 794µg/µL). We chose two commonly used lipid extraction methods: mono-phasic butanol:methanol (BuMe) extraction, and bi-phasic tert-butyl-methyl-ether (TBME) extraction (Figure 1A). In addition, the full cell extract (BuMe suspension) comprising of all biomolecules was also analyzed (termed Mixed sample in Figure 1). Notably, the TBME extract and SPLASH LipidoMix had very similar spectra, indicating that TBME generates a highly pure lipid extract. The hydrocarbon (CH-stretching region in the 3,000 – 2,800 cm^{-1}) and ester (C=O, 1760 – 1730 cm^{-1}) peaks were detected with no observable neighboring peaks that could confound area under the curve (AUC)-based quantification (Figure 1B). A titration of the SPLASH LipidoMix mixture showed linearity for both the CH and C=O regions, comparable to a simple saturated lipid stearic acid (18 chain hydrocarbon and a terminal carboxyl group), indicating a linear relationship between these regions and lipid abundance (Figure 1C). We further characterized the detection limits and signal-to noise ratios (SNR) for absolute quantification using the CH and C=O peaks for each lipid calibration curve (Table 1). Good signal-to-noise ratios were observed, ranging from 2.525 (CH, steric acid) to 4.432 (C=O, steric acid),

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with SPLASH LipidoMix having consistent SNR of 3.071-3.333 for C=O and CH, respectively (Table 1). With the exception of C=O for stearic acid, excellent linearity was obtained in the measured range, with R^2 >0.96 (Figure 2C). The CH band has the better sensitivity, with very similar LOD (~12 ng) and LOQ with stearic acid and SPLASH LipidoMix calibration curves (~40 ng, Table 1).

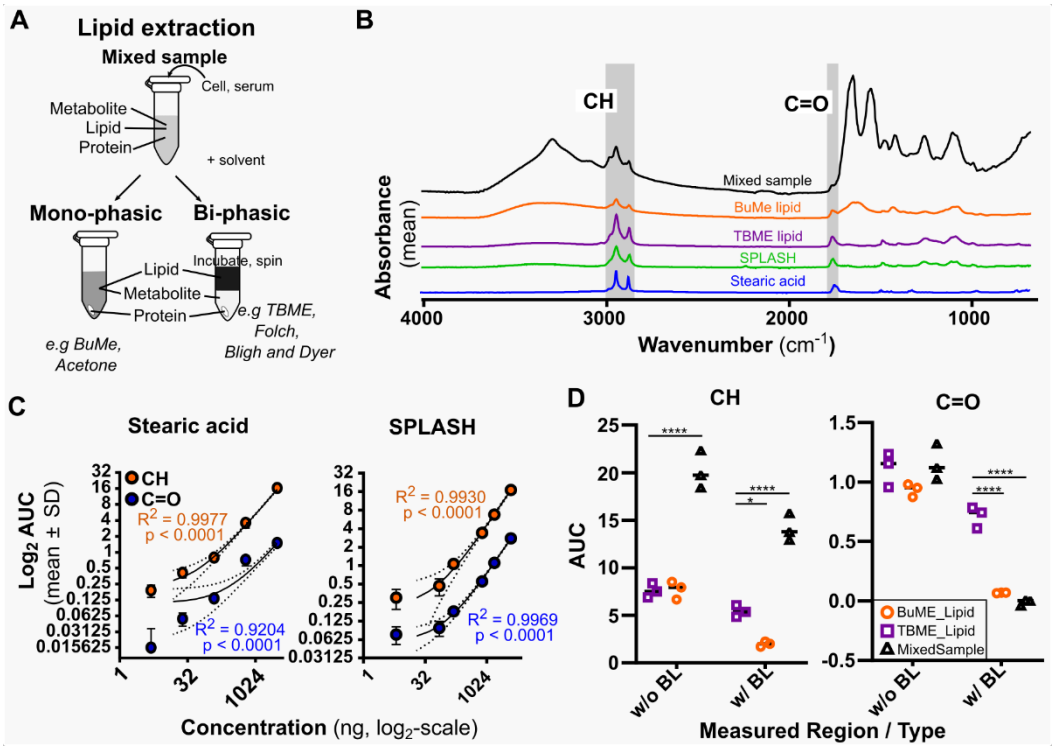

Figure 1. Assessment of lipid quantification by ATR-FTIR spectroscopy – impact of different extraction methods. **A**) Depiction of macromolecular separation by mono- and bi-phasic lipid extraction methods. Cell or biofluid samples were homogenized in solvent/s and macromolecules separated into multiple phases by centrifugation. **B**) Analysis of spectral features in extracted (BuMe, TBME) or commercial lipid (SPLASH, stearic acid) samples. Hydrocarbon (3,000 – 2,800 cm-1) and ester (1,760 – 1,710 cm-1) regions are highlighted in grey. **C**) Titration of lipid standard by simple fatty acid (stearic acid) or complex lipid mixtures (SPLASH), expressed as a log-log graph. Linear regression analysis was performed between the area under the curve (AUC) of the CH and C=O regions and known lipid quantities. N = 3. Linear regression was fitted, displaying 95% confidence interval. **D**) Area under the curve (AUC) measurements of the CH and C=O regions either with (w/ BL) or without (w/o BL) baseline subtraction. Two-sided one-way ANOVA compared between measurements, where * $p < 0.05$, *** <0.001, **** <0.0001.

Table 1: Characteristics of two different ATR-FTIR lipid calibration curves.

	Stearic acid		SPLASH LipidoMix	
	CH	C=O	CH	C=O
SNR	2.5	4.4	3.3	3.1
LOD	12.7 ng	18.8 ng	11.6 ng	29.5 ng
LOQ	42.5 ng	62.8 ng	38.7 ng	98.6 ng
Linear equation	0.005162x + 0.2286	0.000464x + 0.1159	0.004206x + 0.215	0.000681x + 0.05761

CH (3000-2800cm-1), C=O (1760-1710cm-1).
SNR Signal-to-noise ratio. LOD Limit of detection, calculated by 3*Sdblank /linear regression gradient. LOQ limit of quantitation, calculated by 10*Sdblank /gradient.

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While these calibration curves demonstrate the quantification of pure lipid samples by FTIR, two of the complex lipid samples (Mixed sample and BuMe extract) present confounding neighboring peaks in both the CH and the C=O regions. Although TBME provided the most pure lipid extract, the biphasic extraction method is not easily adapted to high throughput procedures, and is prone to accidental contamination during the procedure. In contrast, BuMe extraction is liquid handler-friendly and has been widely used in lipidomics. Therefore, we next compared the relative quantification accuracy for either CH or C=O regions of the different extraction methods, using TBME extraction as benchmark. BuMe, TBME and Mixed samples are expected to have the same total lipid quantity, and same CH/C=O AUC, as lipids were extracted from the aliquots of the same batch of cells. As shown in Figure 1D (left), the unadjusted AUC of the CH region provided consistent relative quantification between TBME and BuMe extraction, but gives an overestimation in the Mixed sample due to the large contribution of neighboring broad peak (3,500 – 2,500 cm^{-1}). Surprisingly, the unadjusted C=O peak yields similar AUCs across all three samples despite the contribution of neighboring peaks in the BuMe and Mixed samples (Figure 1D, right). However, the AUC for the C=O peak is drastically lower than the CH region.

Finally, we evaluated local baseline subtraction as it is often used as a standard data processing step in FTIR-based quantification [16, 17]. Surprisingly, quantification in both the CH and C=O regions was less effective when baseline subtraction was employed, resulting in significantly different AUC values between TBME and other extractions (Figure 1D). Therefore, our results caution against using local baseline subtraction, and suggest the potential use of the C=O peak for quantification of non-pure lipid extracts.

3.2. ATR-FTIR spectroscopy differentiates lipid from other biological materials for quality control assessment

Next, to delineate spectral features that can be used as a measure of contamination and lipid quality, we catalogued FTIR spectra of common contaminants that may be present in lipid extracts, namely the common lysis detergents Triton X-100, NP-40 and sodium deoxycholate (SDC)(all 1% v/v), the sugars sucrose, glucose and galactose (all 1μg/μL), and possible co-extracted biological compounds including protein (1μg/μL) from cultured cells, RNA (1μg/μL) from a synthesized primer and a metabolite extract from extracted from cultured cells. The lipid signature CH and C=O regions are shaded in grey in Figure 2A for comparison. While, like lipids, some detergents display large CH peaks, in lipids these peaks are much more sharply defined. This is most consistently characterized by a larger hydrocarbon minimum ("CH_{min}", 2,888 cm^{-1}, Figure 2A). Both protein and nucleic acid samples showed distinctive peaks in the 1,700 – 1,550 cm^{-1} region (labelled "Amide I", Figure 2A), typically considered to be a characteristic of amide and amine functional groups [18]. The most distinctive feature in sugar-containing samples is the large CO-stretching contribution (a mix of COH and single bonded CO, labelled "sugar" region, 1,200 – 1,000 cm^{-1}). Metabolite samples presented with features of pure sugar and RNA spectra.

Notably, all tested compounds show hydrocarbon-specific peaks (3,000 – 2,800 cm^{-1}), a broad OH/NH peak (3,500 – 2,500 cm^{-1}), or a large amine/amide peak (1,645 cm^{-1} maxima, variable range), all of which could contribute background signal to the lipid-specific CH and C=O peaks. To estimate the effect that these contaminants have on the lipid quantitation, we conducted a titration assay by combining varying concentrations of these contaminants in a constant amount of lipid (1μg/μL). AUCs were calculated for both the CH and C=O regions without baseline subtraction (Figure 2B). Large amounts of contaminating material led to inaccurate AUCs in both regions, highlighting the need for sample contamination/purity assessment prior to quantification.

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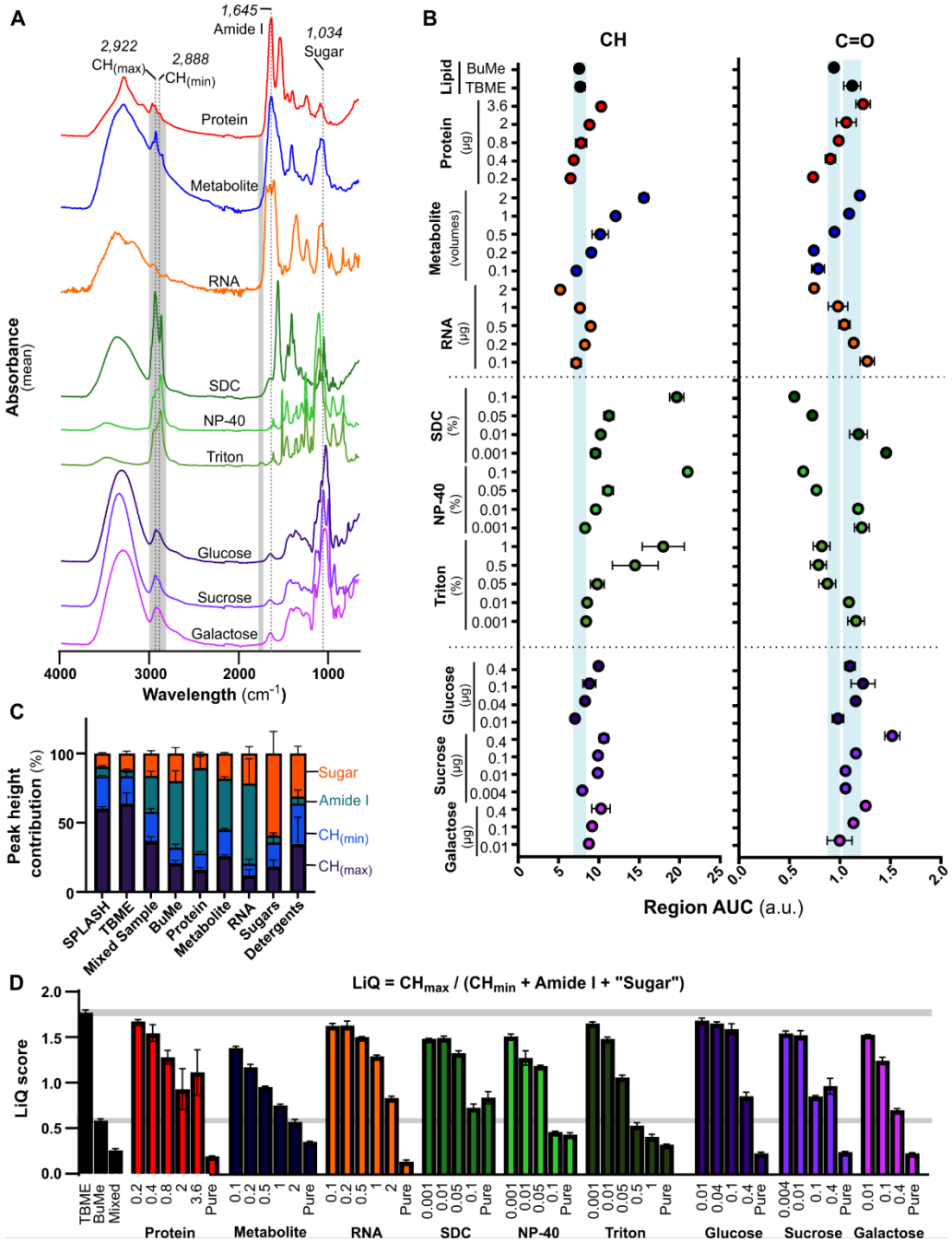

Figure 2: Lipid Quality (LiQ) score development. **A**) FTIR spectra of common lipid contaminants: protein (1µg/µL) extracted from cultured cells, RNA (1µg/µL) from a synthesized primer, metabolites extracted from cultured cells, the detergents Triton X-100, NP-40 and sodium deoxycholate (SDC, all 1% v/v), and the sugars sucrose, glucose and galactose (all 1µg/µL). Lipid characteristic regions are highlighted in grey, and characteristic contaminant bands are labelled. Three technical measurements were acquired and averaged. **B**) Quantification of lipid peaks (CH and C=O) by AUC measurements revealed the effects of contaminants on accurate quantitation. Varying concentrations of contaminant were spiked-into pure lipid samples (1µg per measurement). Three technical replicates were acquired for each sample, and error bars represent standard deviation. Light blue regions highlight the expected AUC from TBME- and BuMe-extracted lipids. **C**) Identification and contribution of prominent peaks in FTIR spectra of biological materials. Prominent spectral regions identified in panel A were evaluated by peak heights and averaged across all replicates. All contaminants considered, including glucose, sucrose and galactose sugars, and the detergents SDC, Triton-X100 and NP-40, were averaged across the group. The contribution of each peak height is summarized as an averaged percentage and standard deviation. Each contaminant without lipid was also

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measured ("neat"). **D**) Contaminated lipid samples evaluated using the LiQ score. Peak heights at CH_{max} were compared to the sum of the CH_{min}, Amide I and sugar peaks. Grey highlighted regions represent the expected LiQ scores for pure lipid or BuMe extracted lipids, within one standard deviation.

To facilitate high throughput lipid quality assessment, we developed a sample Lipid Quality (LiQ) score algorithm based on peak heights which can be applied automatically to exported spectra in Excel. This score evaluates the ratio between lipid peaks to non-lipid spectral features present in common contaminants. Several features were selected based on common peak changes identified in the contaminant dataset, namely, CH-stretching peak maxima (CH_{max}, 2,922 cm^{-1}), CH minima (CH_{min}, 2,888 cm^{-1}), amide I maxima (1,645 cm^{-1}) and "sugar" maxima (1,034 cm^{-1}, COH and CO vibration). Descriptions of common spectral features are listed in Table S1. As shown in Figure 2C, the pure lipid samples SPLASH and TBME are more enriched in CH regions than other sample types, except for detergents (Figure 2C). Compared to lipids, detergents have a higher proportion of CH_{min} than CH_{max}. Therefore, we devised the LiQ Score as the peak height of the CH_{max} divided by (CH_{min} + amide I maxima + "sugar" maxima). While species of biological lipids will also contain amines, sugars and phosphodiester functional groups, the relative abundance of these groups does not outweigh or exceed the contribution of hydrocarbon or lipid ester bond due to the chemical stoichiometry of these molecules. Thereby, an accepted range that evaluates the tipping point between lipid sample and non-lipid content was determined. The LiQ Score was calculated for the range of samples evaluated so far (Figure 2D). TBME extracted lipids generated a LiQ Score of 1.7 ± 0.03, which does not overlap with the contaminated samples even at the lowest contaminant concentrations. BuMe extraction produced a lower LiQ Score of 0.5 ± 0.01, reflecting metabolite co-extraction. For analysis of suspect pure lipids, a LiQ score over 1.7 is recommended as an index for high quality lipid preparation. Due to varying metabolite quantities in different samples types [19], monophasic extractions are likely to have differing LiQ scores, where a LiQ of 0.35 corresponds to metabolite only samples.

High concentration galactose (0.4 μg galactose/μg lipid) and high concentration Triton-X100 (0.5% Triton with 1μg lipid) produced similar LiQ scores to the BuMe lipids, however, more detailed inspection of the spectra is needed to delineate the specific nature of the contamination. We provide some methods for doing this in Figure S1.

3.3. Evaluation of lipid quality and quantity in human plasma samples

The methods we developed to assess lipid quality and quantity were evaluated on a batch of 107 human plasma lipid samples. They were analysed by a high throughput lipidomics method that measures all major plasma lipid classes by multiple reaction monitoring-MS after BuMe extraction [12], with equal plasma volume loading. The total intensity (TI) from MRM-MS data shows a spread of values, indicative of differing plasma lipid concentrations and the lack of equal lipid loading (Figure 3A). After MS data acquisition, ATR-FTIR spectra were acquired for each sample, averaged and shown in Figure 3B. For each sample, technical replicates were averaged and then LiQ score and lipid quantity were calculated using CH, C=O and both stearic acid and SPLASH LipidoMix calibration curves, with and without exclusion of low scoring samples. The mean LiQ score for all samples was 0.4353 (range 0.2631 – 0.6804 (Figure 3C). As a conservative approach for excluding low purity samples, we used the pure metabolite LiQ score of 0.3514 as a cut-off (thick line in Figure 3C), which flagged 25 samples as low quality. Comparing quantification by CH or C=O region with MS total intensities identified linear relationships for each method, with a slightly stronger trend in CH regions ($R = 0.7808$, $R^2 = 0.6095$, $p < 0.0001$) than C=O ($R = 0.705$, $R^2 = 0.4970$, $p < 0.0001$), (Figure 3D, 3E). Excluding low quality samples led to improvements in the linear relationship seen for both the CH ($R = 0.8211$, $R^2 = 0.6743$, $p < 0.0001$) and C=O ($R = 0.7303$, $R^2 = 0.5333$, $p < 0.0001$) regions (Figure 3F, 3G).

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The total lipid quantity for each sample was calculated using both stearic acid and SPLASH LipidoMix calibration curves, using either the CH or C=O regions (Figure 3H). The two calibration curves generated similar results, but the calculated lipid quantity is highly dependent on the spectral region used, with the C=O region consistently predicting lower quantity than the CH region. To determine whether CH or C=O is more accurate for absolute quantification, we ran 794 ng of SPLASH LipidoMix on MS, then used either the SPLASH LipidoMix or stearic calibration curve to calculate lipid amount. As shown in Figure 3I, the CH AUC provides a closer estimate (921.5 ng and 748.3 ng) compared to the C=O region (398.9ng and 489.9 ng). While SPLASH LipidoMix calibration over-estimated the quantity by 16%, stearic acid underestimated the quantity by 9%. Taken together, the CH region without baseline correction provides the most accurate lipid quantification in high quality samples without interfering contaminants.

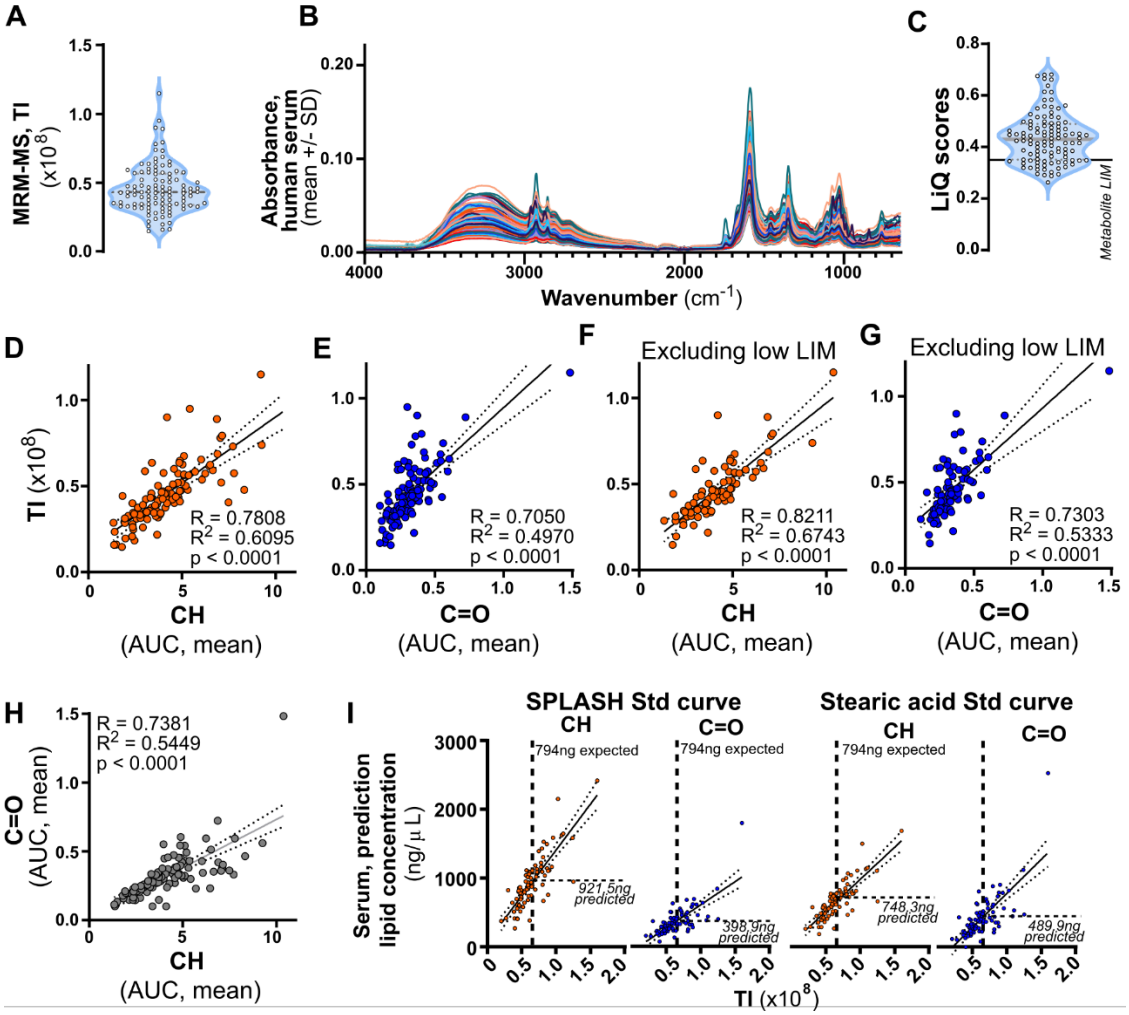

Figure 3: Comparison of ATR-FTIR quantification and mass spectrometry, and quality control analysis of human plasma lipidomics samples. Lipids were extracted by the BuMe method from 107 human plasma samples and analyzed by ATR-FTIR spectroscopy and multiple-reaction monitoring mass spectrometry (MRM-MS). **A**) Violin plot of all total intensities (TI) measured by mass spectrometry for each sample. **B**) Absorbance spectra acquired by ATR-FTIR spectroscopy for 107 human plasma lipidomics samples. Technical replicates were averaged. **C**) Lipid quality (LiQ) score for each plasma sample. The LiQ score for pure metabolite samples (LiQ = 0.351) is shown as a thick black line. **D-G**) The CH and C=O regions were measured by AUC and compared to MS by simple linear regression (R^2 and p values) and Pearson correlation (R values). **D, E**) Comparison for all 107 human plasma lipidomics samples. **F, G**) Comparison for 82 human plasma lipid quantification after removal of low LiQ scoring samples. **H**) Linear relationship between the AUC of the CH and

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C=O regions detected by FTIR spectrometry of human plasma lipid samples. **I)** Absolute quantification of human plasma lipid samples using SPLASH Lipidomics or stearic acid standard curves. The CH (orange) and C=O (blue) regions were measured for both standards and compared to TI by linear regression analysis. Quantification of a 794ng/µL lipid control was also carried outby MS and compared to the FTIR regression equation to calculate predicted absolute quantities by different standards and regions.

4. Discussion

Here we present a comprehensive evaluation of ATR-FTIR for complex lipid extract quantification and propose an integrated, simple, sample-conserving method to assist in lipidomics sample quality assessment prior to mass spectrometry analysis (**Figure 4**). The described ATR sampling method requires just one microliter of sample, conserving the majority of sample for downstream analysis. The method is simple and rapid. Drying of the sample and spectra acquisition requires < 2 minutes per sample. Sample purity can be assessed by visual inspection of the spectra using the instrument software (MicroLab or similar), or collecting peak heights from the software and calculating the LiQ score algorithm using Excel or manually. The same FTIR spectra can be used with an external calibration curve for quantification using AUC. As spectral absorbance properties of molecules are stable, a further advantage of the FTIR method is that the calibration curve can be saved on the instrument without re-acquisition every time.

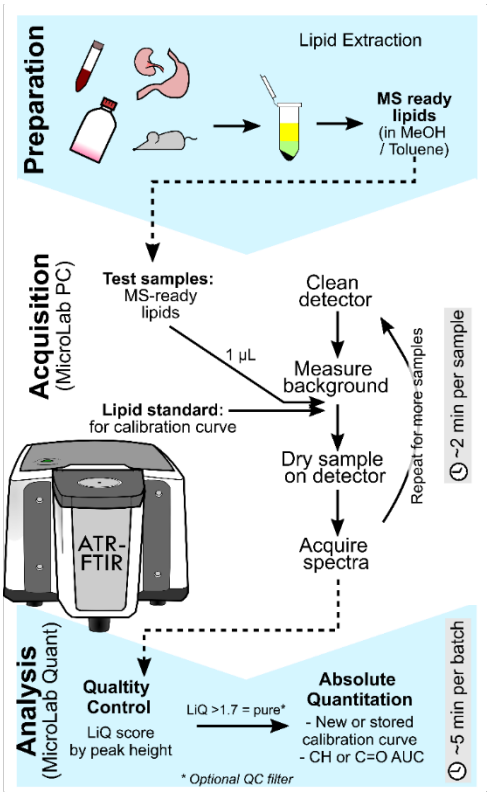

Figure 4. Proposed workflow for lipidomics sample quality and quantity assessment via ATR-FTIR spectroscopy. The extracted lipid sample (1 µL) is subjected to ATR-FTIR spectroscopy after drying on the detector. The Lipid Quality score is calculated in MicroLab Quant software for the Cary 630 spectrometer (Agilent), or equivalent software for other models, by obtaining peak heights and calculating the ratio of the wavenumbers 2,922, 2,888, 1,645 and 1,034 cm^{-1} manually. Accurate absolute quantification can be obtained for samples with Lipid Quality (LiQ) score >1.7 for pure extractions or >0.35 for BuMe extraction. The area under the curve for the CH band (3,000 – 2,800 cm^{-1}) can be applied for absolute quantification using a stored or new external calibration curve of stearic acid.

There are several important reasons to evaluate lipid sample quality early in the lipidomics workflow. Firstly, certain contaminants, such as detergents, can confound MS data and even impact LC-MS performance for later samples in the sequence. Early detection of contaminated samples will allow elimination of these sample from the analysis, or re-extraction of the sample. Secondly, contamination can also confound total lipid quantification by FTIR, as illustrated in Figure 2, and applied in Figure 3. While we have devised a simple LiQ Score to facilitate screening for exclusion of low quality samples, closer analysis and interpretation of spectral features can provide additional information on the

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potential contaminants in these samples. To this end, we have offered three follow-on ratiometric analyses in Figure S1. These additional peak height ratios compare the contribution of lipid peak versus detergent, protein or sugar peaks individually to clarify the composition of the sample and potentially inform the source of contaminants.

Our evaluation of lipid quantification by FTIR highlights a detrimental impact of baseline subtraction on the CH region AUC, which we determined to be more accurate than the alternative C=O peak. Baseline subtraction is also referred to as baseline correction, or anchoring, and can be a default parameter in FTIR spectral processing software [16, 17]. While useful in some applications, quantification of peaks in complex mixtures with overlapping spectra faces augmentation of the local baseline absorbance. This bisects the target peak and results in partial exclusion of peak areas that are masked by the non-target peaks, leading to underestimates of quantity. More sophisticated methods such as spectral deconvolution could be employed to accurately separate the target from non-target peaks, as demonstrated in inorganic chemistry applications [20], however, attempts for complex biological features have been met with difficulty [21]. We caution users about the use of baseline correction for FTIR AUC-based lipid quantification calculations especially in instances of non-pure lipid samples.

Our detailed assessment of the calibration curve of a simple lipid (stearic acid) and a complex (SPLASH LipidoMix) lipid mixture showed similar signal-to-noise ratio, linearity, limit of detection and quantification. SPLASH LipidoMix is produced to reflect the human plasma composition, comprising of physiologically accurate concentrations of phospholipids, sphingolipids, cholesterol, cholesterol esters and glycerides, whereas stearic acid merely reflects a simple fatty acid of 18 carbon length. The difference in lipid composition of the two standards is evident in the full spectra (Figure 1B), where amine, phosphodiester and sugar groups are present in the complex mixture reflecting an expected biological sample. Prediction of lipid quantities was not impacted by this added complexity, instead highly dependent on the hydrocarbon chain length and number of chains (bound to head group by lipid ester group) of the chosen lipid standards and sample. Use of a simple lipid standard should be chosen to reflect the average lipid chain and number of the sample for the closest accuracy due to this relationship. Other FTIR-based analyses have been proposed to delineate lipid class composition and shifts in lipid spectra in disease [22, 23], whereby use of different species of lipid as standard may also provide some frame of reference for specific lipid classes or disease information.

Compared to the commercial product DirectDetect, which uses transmission mode for spectra acquisition, there are several advantages of our proposed ATR-FTIR lipid quality and quantity assessment workflow. DirectDetect outputs the calculated lipid quantity using the AUC from the CH region 2,870 – 2,840 cm^{-1} with baseline anchoring, which is narrower than our CH band (3,000–2,800cm^{-1}) and the recommendation to not baseline subtract. As far as we are aware, DirectDetect does not allow the opportunity to modify the quantification method or ranges to customize use [24]. The stated limit of detection for DirectDetect is 250 ng/μL, which is ten-fold higher than the proposed ATR-FTIR workflow [7]. No additional consumables are required in our workflow, although analysis is done one sample at a time. The DirectDetect sample cards hold 4 samples at a time, but it takes several minutes to read each card [24]. Our workflow requires approximately 2 minutes for each sample. While 1μL of lipid sample was demonstrated as feasible in the ATR-FTIR workflow as an effort to minimize sample wastage, pipetting of small volumes of organic solvent can be difficult. However, analysis of the ATR-FTIR technical replicates from the 107 plasma samples showed coefficient of variance of <5% (Figure S2), indicative of high reproducibility. For the best accuracy, we recommend using air-displacement pipettes, keeping samples at low temperature during workflow, and conditioning the pipette tip by aspirating and dispensing the sample at least twice before transferring sample to the detector. Technical replicates may also be advised to gauge pipetting accuracy.

One of the benefits of the ATR-FTIR workflow is the customization of the parameters for other applications. Here, we assessed CH and C=O region as two possible ranges for

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lipid quantification based on their prevalence in complex biological samples for lipidomics experiments. However, some applications and sample sources may require adjustment of these parameters. While lipidomics samples for mass spectrometry are prepared in organic solvent, other applications may find lipid in aqueous suspensions and thus more suitable to use the C=O region for quantification due to the overlapping water signal hindering CH region detection [25]. Unsaturation of lipids results in an additional CH peak between 3,010–3,000cm^{-1} and a reduction of the CH-region (3,000–2,800cm^{-1}, Figure S3). While in human-derived samples the unsaturation signal is minor compared to saturated signals, samples rich in unsaturated lipid may require an extension of the CH region to encompass this 3,010 cm-1 peak. This analysis of CH peaks may be extended as a method for measuring lipid unsaturation in the future. Additionally, fatty acids contain a carboxyl peak (~1,710cm^{-1}) as opposed to a lipid ester peak (~1,740cm^{-1}) and therefore extension of the C=O region may be beneficial for highly fatty acid enriched samples.

5. Conclusions

This study establishes the key technical parameters for use of ATR-FTIR as a simple method for complex lipid sample quality control and quantification, highlighting contaminants and local baseline correction as sources of error in lipid quantification. Furthermore, we developed the simple LiQ score to facilitate sample quality screening, and also offer more complex ratiometric assessments to further characterize contaminants. As this validated method fills the lipid sample quality control gap, requires no additional consumables and uses minimal sample, we anticipate it to be useful in lipidomics workflows.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Distinguishing spectral features in biological molecules; Figure S1: Complementary measurements for LiQ score quality control analysis; Figure S2: Variation in technical and biological replicates. Figure S3: Variations in the CH region by unsaturated fatty acids.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Research Ethics Committees of the Prince Charles Hospital (HREC/15/QPCH/202) and QIMR Berghofer Medical Research Institute (P2352).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: FTIR spectra are available via Zenodo (DOI:10.5281/zenodo.6592243). Mass spectrometry data are available via Panorama and can be accessed at https://panoramaweb.org/LiQscoreEvaluation.url. Reviewer access currently enabled using the login panorama+reviewer124@proteinms.net with password: lLkOhbtV.

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