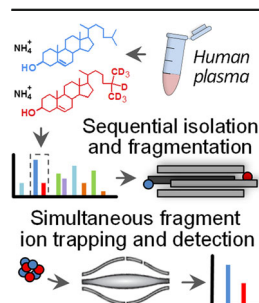CrossMark

RESEARCH ARTICLE

Easy, Fast, and Reproducible Quantification of Cholesterol and Other Lipids in Human Plasma by Combined High Resolution MSX and FTMS Analysis

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Abstract. Reliable, cost-effective, and gold-standard absolute quantification of non-esterified cholesterol in human plasma is of paramount importance in clinical lipidomics and for the monitoring of metabolic health. Here, we compared the performance of three mass spectrometric approaches available for direct detection and quantification of cholesterol in extracts of human plasma. These approaches are high resolution full scan Fourier transform mass spectrometry (FTMS) analysis, parallel reaction monitoring (PRM), and novel multiplexed MS/MS (MSX) technology, where fragments from selected precursor ions are detected simultaneously. Evaluating the performance of these approaches in terms of dynamic quantification range, linearity, and analytical precision showed that the MSX-based approach is superior to that of the FTMS and PRM-based approaches. To further show the efficacy of this approach, we devised a simple routine for extensive plasma lipidome characterization using only 8 μL of plasma, using a new commercially available ready-to-spike-in mixture with 14 synthetic lipid standards, and executing a single 6 min sample injection with combined MSX analysis for cholesterol quantification and FTMS analysis for quantification of sterol esters, glycerolipids, glycerophospholipids, and sphingolipids. Using this simple routine afforded reproducible and absolute quantification of 200 lipid species encompassing 13 lipid classes in human plasma samples. Notably, the analysis time of this procedure can be shortened for high throughput-oriented clinical lipidomics studies or extended with more advanced MS^{ALL} technology (Almeida R. et al., J. Am. Soc. Mass Spectrom. **26**, 133–148 [1]) to support in-depth structural elucidation of lipid molecules.
Keywords: Cholesterol, Multiplexing, Shotgun lipidomics, High resolution mass spectrometry, Orbitrap

Introduction

Shotgun lipidomics platforms using nanoelectrospray ionization and high resolution mass spectrometry (MS) technology afford comprehensive lipidome analysis with confident identification and accurate quantification of several hundred lipid molecules in a single sample [1–6]. Applications of this technology have prompted mechanistic insights into the regulation of lipid metabolism [2, 4, 7, 8], membrane-related

processes [9–12], and lipid–protein interactions [13, 14]. In addition, the technology has also pinpointed reliable lipid biomarkers of cardiovascular disease [15, 16]. Notably, a prerequisite for global lipidome analysis is that each sample should be analyzed by a series of mass spectrometric routines executed in both positive and negative ion mode (e.g., MS^{ALL} [1]). However, in this analytical setting it is challenging to quantify non-esterified cholesterol (cholestenol) since it ionizes poorly by (nano)electrospray ionization and undergoes in-source fragmentation to produce a positively charged cholestadiene fragment ion.

Several solutions are available for circumventing this issue and enabling gold-standard absolute quantification of cholesterol (e.g., pmol/μL plasma) by shotgun lipidomics. One option is to improve the ionization efficiency of cholesterol, and an

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appropriate internal standard such as $^2H_7$-cholesterol, by using chemical derivatization with either sulfur trioxide or acetyl chloride to produce sulfate or acetate derivatives, respectively [17, 18]. Notably, these approaches entail extra sample handling steps, and an additional sample injection and mass spectrometric analysis. An alternative and simpler approach is to use high resolution Fourier transform (FT) MS analysis on Orbitrap-based machines and monitor the cholesterol-derived in-source cholestadiene fragment ions with m/z 369.3516 and m/z 376.3955 released from endogenous cholesterol and the internal standard $^2H_7$-cholesterol, respectively [19]. The latter approach, however, is not specific for non-esterified cholesterol and can prompt inaccurate quantification since cholesteryl esters (CEs) also undergo in-source fragmentation to produce the cholestadiene fragment ion with m/z 369.3516 (Supplementary Figure S1).

With the recent development of more sensitive hybrid quadrupole-Orbitrap-based instruments (i.e., Orbitrap Fusion and Q-Exactive) it is now possible to use high resolution full scan FTMS analysis to monitor cholesterol and $^2H_7$-cholesterol as intact ammonium adducts (e.g., [cholesterol+NH$_4$]$^+$, m/z 404.3887), detected as low intensity ions but with high signal-to-noise ratios (Supplementary Figure S1) [6]. Moreover, it is also possible to quantify cholesterol levels using parallel reaction monitoring (PRM) of ammoniated cholesterol and $^2H_7$-cholesterol, and thereby consecutively monitor the intensity of cholestadiene and $^2H_7$-cholestadiene fragment ions, respectively, in two separate FTMS^2 (Fourier transform tandem mass spectrometry) scans [5]. Furthermore, a third approach is to monitor cholesterol and $^2H_7$-cholesterol using novel multiplexed MS/MS (MSX) technology [20–24]. In this approach, selected precursor ions are sequentially (1) isolated by a quadrupole mass analyzer, (2) fragmented in a collision cell, and (3) fragment ions from each of the selected precursor ions are trapped inside the collision cell prior to being routed together to an Orbitrap mass analyzer for simultaneous detection. Notably, at the present time there has been no effort to compare the performance of the three approaches available for direct cholesterol quantification on hybrid quadrupole-Orbitrap-based instruments.

Here, we report on the analytical merits of absolute quantification of cholesterol in human plasma using full scan FTMS, PRM, and MSX analysis. To this end, we optimized the performance of the three approaches and assessed their individual performances in terms of dynamic quantification range and analytical precision. This assessment demonstrated that both MSX and PRM analysis outperforms full scan FTMS in terms of dynamic range and linearity. Moreover, we also found that MSX-based cholesterol quantification is more precise compared with PRM and FTMS-based analysis. To demonstrate the efficacy of the MSX method, we devised a simple shotgun lipidomics routine for simultaneous and absolute quantification of cholesterol and other lipid molecules in human plasma. This routine uses an easy-to-use, commercially available internal standard mixture (SPLASH Lipidomix) that is spiked into human plasma, a single lipid extraction step and 6 min of combined high resolution FTMS and MSX analysis. Using this routine we were able to reproducibly quantify the absolute levels of 200 lipid species from 13 lipid classes in a single injection of a human plasma extract.

Materials and Methods

Chemicals and Lipid Standards

Chloroform, methanol, and 2-propanol were purchased from Rathburn Chemicals (Walkerburn, Scotland). Ammonium acetate and ammonium formate were from Sigma-Aldrich (Buchs, Switzerland). All solvents and chemicals were HPLC grade. SPLASH Lipidomix (containing 213 μM PC 15:0-18:1-$^2H_7$, 8.0 μM PE 15:0-18:1-$^2H_7$, 5.4 μM PS 15:0-18:1-$^2H_7$, 38.1 μM PG 15:0-18:1-$^2H_7$, 10.7 μM PI 15:0-18:1-$^2H_7$, 10.7 μM PA 15:0-18:1-$^2H_7$, 48.2 μM LPC 18:1-$^2H_7$, 10.9 μM LPE 18:1-$^2H_7$, 541 μM CE 18:1-$^2H_7$, 5.5 μM MAG 18:1-$^2H_7$, 16.0 μM DAG 15:0-18:1-$^2H_7$, 70.5 μM TAG 15:0-18:1-$^2H_7$-15:0, 41.9 μM SM 18:1-$^2H_9$, and 254 μM (25, 26, 26, 26, 27, 27, 27-$^2H_7$)-cholesterol) and (25, 26, 26, 26, 27, 27, 27-$^2H_7$)-cholesterol were from Avanti Polar Lipids (Alabaster, AL, USA). β-Sitosterol was from Sigma-Aldrich (Brøndby, Denmark).

Sample Collection

Human blood samples were collected from five healthy volunteers (three males; two females). Informed consent was obtained from all individuals before participation. The study was approved by The Regional Scientific Ethical Committees for Southern Denmark and performed in accordance with the Helsinki Declaration. The volunteers (body mass index 23.3–30.5 kg/m^2; 25–40 years of age) fasted overnight. Venous blood samples were collected into 4 mL K2 EDTA vacutainer tubes. EDTA plasma was separated by centrifugation (2500 g, 5 min, 4 °C), immediately snap-frozen in liquid nitrogen, and stored at –80 °C until further analyses.

Animal experiments were conducted in accordance with the Danish law on Animal Experiments (LBK no. 1306 - 23/11/2007, amendments § 1 nr. 612 - 14/06/2011) and approved by the Danish Animal Experiment Inspectorate. C57BL/6J mice of 12 wk of age were fasted for 2 h and subsequently anesthetized. Blood sampling was performed by cardiac puncture into K3 EDTA micro tubes (Sarstedt, Nümbrecht, Germany). EDTA plasma was separated by centrifugation (3000 g, 15 min, 4 °C), immediately snap-frozen in liquid nitrogen, and stored at –80 °C until further processing.

Lipid Extraction

Human and mouse plasma samples (8 μL) were subjected to lipid extraction at 4 °C as previously described [3, 25]. Briefly, plasma aliquots were diluted with 155 mM ammonium formate to a final volume of 200 μL and spiked with 12 μL of SPLASH Lipidomix. Subsequently samples were extracted with 990 μL of chloroform/methanol (10:1, v/v) and mixed for 120 min at

1400 rpm. After 3 min centrifugation at 1500 *g*, the lower organic phase was collected and vacuum evaporated.

Mass Spectrometric Analysis

Lipid extracts and synthetic lipid standards were dissolved in chloroform/methanol/2-propanol (1:2:4 v/v/v) containing 7.5 mM ammonium acetate and loaded in 96-well plates (Eppendorf, Hamburg, Germany). Samples (10 µL) were infused with the robotic nanoflow ion source TriVersa NanoMate (Advion Biosciences, Ithaca, NY, USA) using nanoelectrospray chips (flow rate of 200 nL/min) and analyzed in positive ion mode using an Orbitrap Fusion Tribrid (Thermo Fisher Scientific, San Jose, CA, USA). The instrument was tuned and calibrated approximately every 2 wk following manufacturer's recommended procedures. Ionization voltage was +0.96 kV and back pressure was 1.25 psi. The temperature of the ion transfer tube was 275 °C. S-lens radio frequency (rf) level was set to 60%. Positive ion mode MS analysis of the lipid extracts was performed by (1) high resolution FTMS analysis of the *m/z* range 345–605, (2) high resolution FTMS of the *m/z* range 470–1200, (3) MSX analysis of both *m/z* 404.4 ([cholesterol +NH$_4$]$^+$) and *m/z* 411.4 ([^{2}H$_7$-cholesterol +NH$_4$]$^+$) (Supplementary Figure S3), (4) PRM analysis of *m/z* 404.4 ([cholesterol +NH$_4$]$^+$), and (5) PRM analysis of *m/z* 411.4 ([^{2}H$_7$-cholesterol +NH$_4$]$^+$). Each sample was analyzed for 6 min. All full scan FTMS data were acquired in profile mode, using a max injection time of 100 ms, automated gain control for an ion target of 10^5, three microscans, and a target resolution setting of 500,000. FTMS^2 data were collected in profile mode within the range *m/z* 340–440 with the following settings: higher-energy collisional dissociation (HCD) fragmentation using normalized collision energy to 8%, maximum injection time of 600 ms, automated gain control for an ion target of 5 × 10^4, five microscans, a target resolution of 30,000, and a quadrupole ion isolation window of 1.5 u.

Lipid Identification and Quantification

Lipid species detected by high resolution FTMS, PRM, and MSX analysis with a mass accuracy less than 5 ppm were identified and quantified using ALEX software and SAS 9.3 [1, 26]. Lipid species were quantified by normalizing their intensity to the intensity of an internal lipid standard of identical lipid class and multiplying by the spike amount of the internal lipid standard [27]. Only lipid species detected in all technical replicates and present in all subjects (n = 5) are reported.

Results and Discussion

Monitoring of Cholesterol by MSX Analysis

Quantitative monitoring of cholesterol on hybrid quadrupole-Orbitrap-based mass spectrometers (i.e., Orbitrap Fusion and Q-Exactive) can in principle be achieved using full scan FTMS, PRM, and MSX analysis. To compare the performances of these strategies we injected a lipid extract of human plasma spiked with the internal

standard ^{2}H$_7$-cholesterol and simultaneously acquired FTMS, PRM, and MSX spectra (bundled into a single acquisition method) (Figure 1).

Full scan FTMS analysis (Figure 1a) demonstrated that (1) cholesterol and ^{2}H$_7$-cholesterol can be detected as ammonium adducts, but with a very low intensity compared with other ions (Figure 1a and Supplementary Figure S1C), (2) the intensity ratio between ammoniated cholesterol and ^{2}H$_7$-cholesterol was 2.8:1, and that (3) in-source fragmentation produces a highly abundant cholestadiene fragment ion (*m/z* 369.3516) and a low abundant ^{2}H$_7$-cholestadiene fragment ion (*m/z* 376.3955). The higher intensity of *m/z* 369.3516 corroborates the notion that CEs undergo in-source fragmentation (Supplementary Figure S1). The PRM analysis showed that (1) fragmentation of ammoniated cholesterol yields the cholestadiene fragment ion with *m/z* 369.3516 (Figure 1b), (2) fragmentation of ammoniated ^{2}H$_7$-cholesterol yields the ^{2}H$_7$-cholestadiene fragment ion with *m/z* 376.3955 (Figure 1c), and that (3) the intensity ratio between these fragment ions was 2.6:1. Furthermore, the MSX analysis showed that the cholesterol- and ^{2}H$_7$-cholesterol-derived fragment ions with *m/z* 369.3516 and *m/z* 376.3955 could be detected simultaneously in a single detection event (Figure 1d), having an intensity ratio of 2.6:1. These results highlight that it is possible, on a hybrid quadrupole-Orbitrap mass spectrometer, to directly detect cholesterol and ^{2}H$_7$-cholesterol using three different approaches and that these produce similar results. However, this simple comparison does not by itself demonstrate which approach is the most precise, accurate, and sensitive for routine applications.

Optimizing MSX-Based Cholesterol Quantification

To optimize the monitoring of cholesterol and ^{2}H$_7$-cholesterol by MSX and PRM analysis, we first evaluated how collision energy impacts the detection of the cholestadiene-based fragment ions with *m/z* 369.3516 and *m/z* 376.3955, respectively. To this end, each sterol was subjected to FTMS^2 analysis at different collision energies. This analysis demonstrated that optimal collision energy for detection of the cholestadiene-based fragment ions is 8% for both cholesterol and ^{2}H$_7$-cholesterol (Figure 2). Moreover, this result also shows that the deuterium atoms in the internal standard, ^{2}H$_7$-cholesterol, do not alter the fragmentation properties relative to that of endogenous cholesterol. Hence, ^{2}H$_7$-cholesterol is an appropriate standard for quantification of cholesterol. Furthermore, the result also highlights that users of Orbitrap-based instrumentations should carefully optimize collision energy for detection of lipid fragment ions.

To try to minimize the in-source fragmentation of cholesterol and ^{2}H$_7$-cholesterol, and thereby potentially improve the detection of intact ammoniated sterol molecules by FTMS, PRM, and MSX analysis, we also tested the influence of the instrument's ion entrance setting. This analysis showed that using an ion entrance S-lens radio frequency value of 60% was optimal for detection of intact ammoniated sterol molecules by full scan FTMS analysis (Supplementary Figure S2A) and also
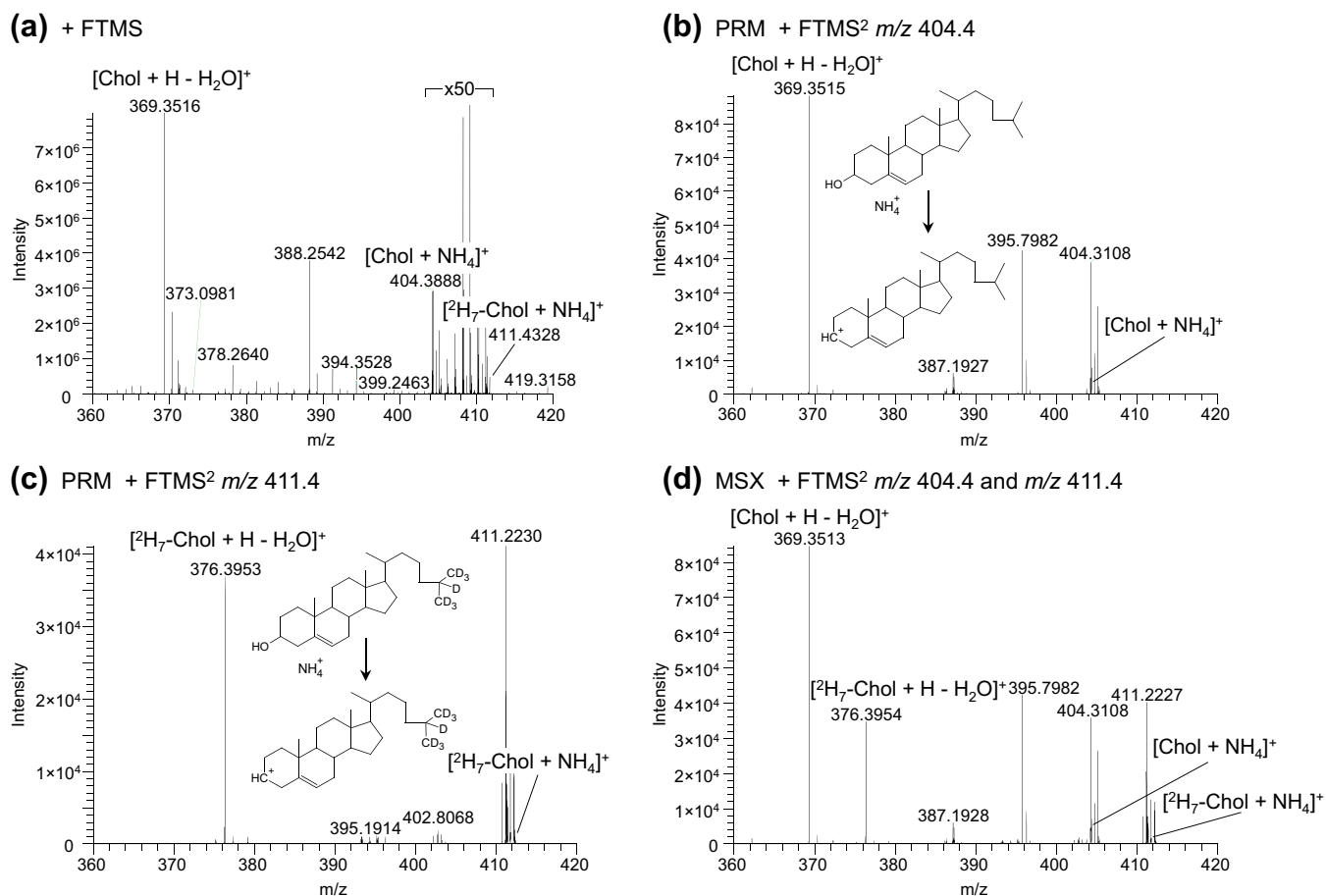
(a) + FTMS

(b) PRM + FTMS^2 m/z 404.4

(c) PRM + FTMS^2 m/z 411.4

(d) MSX + FTMS^2 m/z 404.4 and m/z 411.4

Figure 1. Detection of cholesterol by high resolution FTMS, PRM and MSX analysis. **(a)** Positive ion mode FTMS spectrum of human blood plasma spiked with ^{2}H$_7$-cholesterol. Cholesterol and ^{2}H$_7$-cholesterol are detected as ammonium adducts having m/z 404.3887 and m/z 411.4326, respectively. Cholesterol and CE undergo in-source fragmentation to produce the fragment ion of m/z 369.3516 (Supplementary Figure S1). **(b)** Positive ion mode FTMS^2 spectrum of m/z 404.4, corresponding to ammoniated cholesterol. **(c)** Positive ion mode FTMS^2 spectrum of m/z 411.4, corresponding to ammoniated ^{2}H$_7$-cholesterol. **(d)** Positive ion mode multiplexed FTMS^2 spectrum of m/z 404.4 and m/z 411.4, corresponding to ammoniated cholesterol and ^{2}H$_7$-cholesterol, respectively. All FTMS^2 spectra were acquired using a collision energy of 8%

for the detection of cholestadiene-based fragment ions by FTMS^2-based analysis (Supplementary Figure S2B).
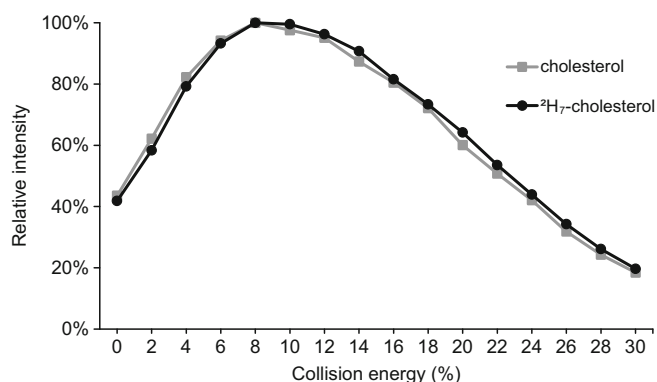

Figure 2. Optimization of collision energy for detection of the cholestadiene-based fragment ions. Relative intensity of fragment ions m/z 369.3516 ([cholesterol+H-H$_2$O]$^+$) and m/z 376.3955 ([^{2}H$_7$-cholesterol+H-H$_2$O]$^+$) as function of collision energy

Dynamic Quantification Range of MSX Analysis

Next, we evaluated the dynamic quantification range of the three different approaches. To this end, we prepared a dilution series where the synthetic standard ^{2}H$_7$-cholesterol was titrated relative to a constant amount of the synthetic standard β-sitosterol (which has the same sterol ring structure as cholesterol, but has an ethyl group in its aliphatic side chain). This dilution series was spiked into human plasma samples, which were subjected to lipid extraction and analyzed by MSX, and also by FTMS and PRM analysis (bundled into a single acquisition method). To evaluate the dynamic quantification range, we plotted the intensity ratio between the precursor or fragment ions of ^{2}H$_7$-cholesterol and β-sitosterol as a function of their molar ratio and for each of the three acquisition procedures (Figure 3). This assessment demonstrated that the responses of the MSX- and PRM-based analysis are linear with a slope value of approximately one across three orders of magnitude and having detection limit of approximately 150 nM (corresponding to the molar ratio value of 0.015 in Figure 3).
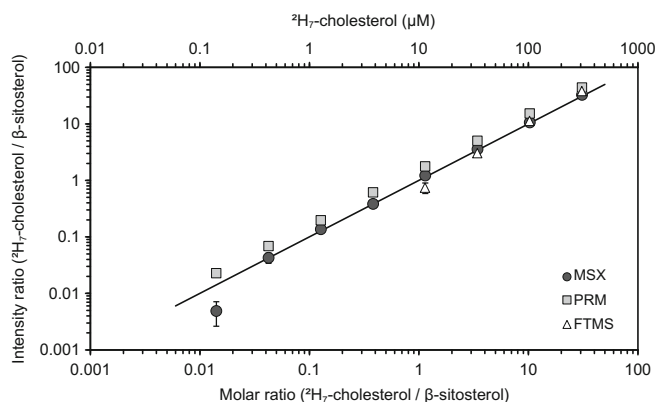
Figure 3. Dynamic quantification range of cholesterol analysis by FTMS, PRM and MSX-based analysis. ^{2}H$_7$-cholesterol was titrated (from 16 nM to 324 μM) relative to a constant amount of β-sitosterol (10 μM), spiked into human plasma (n = 3) and subjected to lipid extraction. The lipid extracts were analyzed by FTMS, PRM and MSX analysis. The upper x-axis shows the absolute concentration of ^{2}H$_7$-cholesterol. The lower x-axis shows the concentration of ^{2}H$_7$-cholesterol relative to the concentration of β-sitosterol (i.e., molar ratio). The y-axis shows the intensity of m/z 376.3955 ([^{2}H$_7$-cholesterol+H-H$_2$O]$^+$) relative to the intensity of m/z 397.3829 ([β-sitosterol+H-H$_2$O]$^+$). The line indicates the linear function with slope 1. Data points represent the average ± SD of three biological replicates

This limit of detection is similar to that of approaches based on chemical derivatization [17, 18] and adequate for clinical analysis of non-esterified cholesterol since it is highly abundant (micromolar range) in plasma samples. In comparison, the dynamic quantification range of the FTMS-based analysis was lower and only linear with a slope value of one across two orders of magnitude. Taken together, this assessment shows that MSX analysis, and also PRM analysis, afford sensitive and accurate quantification of non-esterified cholesterol in human plasma. Moreover, it also shows that quantification of cholesterol by MSX- and PRM-based analysis is preferred over FTMS-based analysis.

MSX Analysis Improves Analytical Precision

To further assess the performance of the three approaches, we determined their analytical precision for quantification of cholesterol in human and mouse plasma, two sample

matrices having different concentrations of non-esterified cholesterol. To this end, we spiked a human plasma sample with ^{2}H$_7$-cholesterol and subjected this sample to lipid extraction followed by six repeated mass spectrometric analyses with recording of full scan FTMS, PRM, and MSX data. This experiment was repeated on three different days to assess interday precision.

The precision of MSX-based cholesterol quantification on individual days (intraday precision) featured a coefficient of variation (CV) ranging from 0.8% to 1.1% (Table 1). In comparison, the intraday precision of full scan FTMS and PRM analysis featured CV values in the order of 11.6% and 2.7%, respectively. This demonstrates that precision of the MSX-based approach for cholesterol quantification in human plasma is 12- and 3-fold better compared with using FTMS and PRM analysis, respectively. Furthermore, evaluation of the day-to-day (interday) precision showed that the MSX-based analysis yielded a CV of 7.3%, whereas that of the FTMS- and PRM-based analysis featured CV values of 12.4% and 6.7%, respectively. These results show that the interday precision of the MSX-based routine is similar to that of FTMS- and PRM-based analysis. Furthermore, these results also show that the precision of the MSX-based analysis, and that of FTMS and PRM analysis, are similar to that of the chemical acetylation-based approach developed by Liebisch et al., which features intraday and interday CV values of 2.3% and 8.3–9.6%, respectively [18]. We note that similar performance characteristics were obtained when using mouse plasma (Table S1).

Quantification of Cholesterol in Human Plasma by MSX Analysis

Next, we evaluated the efficacy of the MSX-based routine for absolute quantification of non-esterified cholesterol in plasma samples from several human subjects. To this end, we spiked plasma from five healthy subjects with ^{2}H$_7$-cholesterol, performed lipid extraction, and analyzed the sample extracts using the MSX approach. This analysis showed an average concentration of 993 ± 169 μM cholesterol in the plasma from the five human subjects (Figure 4). This concentration is in excellent agreement with values previously reported, with estimates of ~1000 μM of non-esterified

Table 1. Intraday and Interday Precision of Cholesterol Quantification in Human Plasma Samples

		Intraday						Interday	
		Day 1 (n=6)		Day 2 (n=6)		Day 3 (n=6)		(n=3×6)	
		μM ± SD	CV (%)	μM ± SD	CV (%)	μM ± SD	CV (%)	μM ± SD	CV (%)
Human	MSX	999 ± 10	1.0	874 ± 7	0.8	1032 ± 12	1.1	968 ± 71	7.3
	PRM	988 ± 17	1.8	884 ± 25	2.8	1022 ± 28	2.7	965 ± 64	6.7
	FTMS	1078 ± 78	7.2	946 ± 59	6.2	1174 ± 136	11.6	1066 ± 132	12.4

Values are average concentrations of non-esterified cholesterol (μM) in human plasma. Lipids were extracted from 8 μL of plasma and analyzed for assessment of intraday precision using six replicate measurements. Lipid extraction and mass spectrometric analysis was done on three consecutive days and six replicate measurements per day for assessment of interday precision
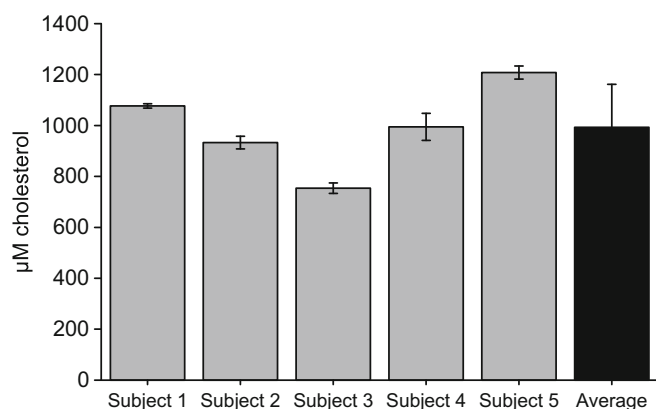
Figure 4. Concentration of non-esterified cholesterol in human blood plasma determined by MSX analysis. Grey bars represent mean ± SD of n = 3 replicate measurements per subject. The black bar is the average ± SD of n = 5 subjects

cholesterol in human plasma [5, 6, 28, 29]. Moreover, the analysis also showed pronounced inter-individual

differences in the absolute level of cholesterol, which highlights that the approach can be useful for applications in personalized medicine. Taken together with the above-described performance characteristics, we conclude that MSX-based analysis is a very simple, precise, and accurate approach for quantitative cholesterol analysis in human plasma.

Profiling the Plasma Lipidome by High Resolution FTMS and MSX Analysis

To demonstrate the usefulness and applicability of the MSX method, we next combined it together with high resolution full scan FTMS analysis, which is a fast and simple approach for quantifying sterol esters, glycerolipids, glycerophospholipids, and sphingolipids [1, 4, 6]. To support extensive lipidome quantification, we devised a simple routine based on using only 8 μL of plasma, which is spiked with a new commercially available ready-to-use standard mixture containing defined
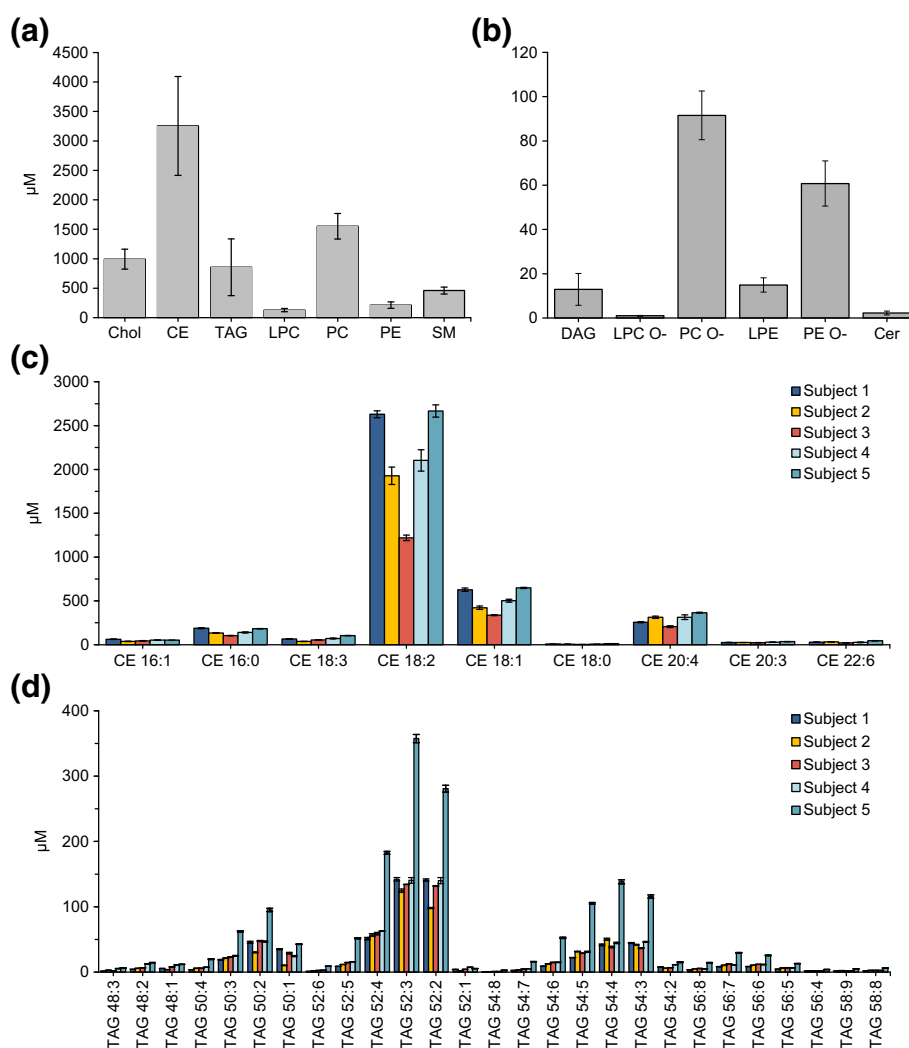

Figure 5. Lipid composition of human blood plasma. (**a**), (**b**) Abundance of lipid classes in human plasma. Data represent mean ± SD (n = 5 subjects). (**c**) Concentration of major CE species in human blood plasma. (**d**) Concentration of major TAG species in human blood plasma. Individual histograms represent the average ± SD of three replicate measurements per subject

amounts of 14 different lipid standards labeled with deuterium atoms (SPLASH Lipidomix), and subjected it to lipid extraction and 5 min of simultaneous FTMS and MSX analysis (total run time 6 min). Using this routine to characterize the lipidome composition of plasma from five healthy subjects demonstrated the identification and absolute quantification of 200 lipid molecules from 13 lipid classes (Data file F1). Among the monitored lipids we observed that CE is the single most abundant lipid class in human plasma (Figure 5a), with CE 18:2 comprising over 50% of the molecules within this class (Figure 5c). Furthermore, we also found that glycerophospholipids represent over half of all the quantified lipid species (109 species). Of these, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipids were the most abundant in human plasma (Figure 5a). Triacylglycerols (TAGs) also encompass a high proportion of the total pool of lipid molecules in human plasma. Notably, we found that approximately 50% of all TAGs had a total of 52 carbon atoms in the acyl chains, where the TAG 52:2 and 52:3 species were observed to be the most abundant (Figure 5d). We note that our estimated concentrations of lipid species in human plasma are in good agreement with previous reports [5, 6, 28].

Conclusions

Here, we evaluated the use of novel MSX technology for gold-standard absolute quantification (e.g., µM or mg/dL) of non-esterified cholesterol in human plasma samples. We demonstrate that the performance of the MSX-based approach is comparable to PRM-based analysis in terms of accuracy, linearity, and dynamic quantification range, and better in terms of analytical precision. Moreover, we also show that cholesterol quantification by both MSX- and PRM-based analysis is superior to that of full scan FTMS analysis. Although these shotgun lipidomics-based approaches, as well as techniques based on chemical derivatization [17, 18], provide easy and fast analytical strategies for quantification of cholesterol, they all have the same limitation, which is the assumption that the intact precursor ion with m/z 404.3887 and the fragment ion with m/z 369.3516 are derived from only one cholestenol isomer, namely cholest-5-en-3-ol (i.e., cholesterol). As such, users of these approaches should be aware that the accuracy of the cholesterol quantification can potentially be hampered by the presence of other cholestenol isomers, such as cholest-7-en-3-ol (i.e., lathosterol). This, however, should only be of concern when studying cohorts of patients or cells having severe defects in de novo sterol biosynthesis. In such scenarios it could be worthwhile to explore whether the MS[3] fragmentation capabilities of the herein used instrumentation (Orbitrap Fusion) could enable specific detection of isomeric cholestenols. Alternatively, quantification of cholesterol (and other sterols) can also be done (with higher sensitivity) by combing chemical derivatization, reversed-phase chromatography, and selected ion monitoring on a triple quadrupole mass spectrometer [30]. However, compared with the 6 min MSX-based analysis, this approach

entails additional sample preparation (~1 h for derivatization and solvent evaporation) and a ~40 min chromatography run per sample (plus additional time for column reconditioning and blank runs to clean the column).

To demonstrate the efficacy of the MSX-based cholesterol quantification, we devised a simple routine that capitalizes on using only a few microliters of plasma that is spiked with a novel, commercially available ready-to-use internal standard mixture (SPLASH Lipidomix) and a single sample injection that affords cholesterol quantification by MSX analysis and quantification of sterol esters, glycerolipids, glycerophospholipids, and sphingolipids by full scan FTMS analysis. A key advantage of using the MSX-based method is that it does not require extra sample preparation steps with chemical derivatization to enhance ionization efficiency of non-esterified cholesterol and that it can be easily integrated with a palette of other acquisition procedures available on hybrid quadrupole-Orbitrap-based mass spectrometers (e.g., MS[ALL] [1]). As such, we note that the analysis time of this routine can be shortened to ~2 min for high throughput-oriented analysis or alternatively be combined with MS[ALL] technology with an analysis time of ~20 min to allow in-depth structural characterization of molecular lipid species (e.g., decipher the composition of fatty acyl moieties in detected lipid molecules). Finally, we note that the MSX-based approach is also applicable for quantifying cholesterol levels in tissue biopsies [4].

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