

Development and application of an LC-MS/MS method for the combined quantification of oxysterols and bile acids

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Abstract Oxysterols and bile acids are interconnected bioactive lipids playing pivotal roles in diverse physiological and pathological processes. For this reason, they are increasingly studied together for their implications in various diseases. However, due to analytical challenges inherent to the nature of these analytes, very few methods have been developed for the simultaneous analysis of these lipids. We here report the development of a sensitive LC-MS/MS method for the combined quantification of 18 oxysterols, 11 unconjugated, 15 conjugated bile acids, and 1 bile acid precursor, using 8 isotope-labeled internal standards, addressing the need for a more comprehensive analysis of these interesting lipid families. During the method development, we investigated different extraction protocols, set up a purification step, and achieved chromatographic separation for these lipids, overcoming challenges such as the large number of analytes, isomers, and wide range of polarity across the analytes. Finally, the method was successfully applied to the analysis of preclinical and clinical samples, quantifying 12 oxysterols and 14 bile acids in human plasma, 10 oxysterols and 18 bile acids in mouse plasma from the vena cava, and 10 oxysterols and 20 bile acids in mouse plasma from the portal vein within a single chromatographic run.

Supplementary key words lipidomics • lipids • sterols • cholesterol metabolism • cytochrome P450 • liquid chromatography mass spectrometry • 25-hydroxycholesterol • 4 β -hydroxycholesterol • ursodeoxycholic acid • deoxycholic acid

Oxysterols and bile acids are derivatives of cholesterol. Cholesterol can be oxidized through enzymatic processes, mainly involving cytochromes P450, or through the action of reactive oxygen species leading to the formation of oxysterols (1). Oxysterols play a role in many physiological and pathological processes such as metabolism, immunity, or inflammation (2–6). Furthermore, they also serve as crucial intermediates in the biosynthesis of bile acids (7, 8). The dual role of oxysterols, both as metabolic intermediates and lipid

mediators renders them interesting subjects of investigation in both physiological and pathological contexts.

Albeit primarily known for their involvement in the digestion and absorption of dietary fats, facilitating the emulsification and solubilization of lipids in the intestine, bile acids are also full-fledged bioactive lipids, modulating various metabolic pathways, including cholesterol homeostasis and glucose metabolism, through interactions with specific receptors (9, 10). Structurally, bile acids exhibit remarkable diversity, characterized by variations in hydroxylation patterns and conjugation states. This structural heterogeneity leads to the formation of distinct bile acid species with a wide range of polarity.

Due to their properties, there is a growing interest in the study of oxysterols and bile acids. In this context, the quantification of these lipid mediators brings valuable information to biological studies. While several methods to quantify oxysterols or bile acids have been reported (11–15), the development of a method allowing for their simultaneous quantification has significant advantages. Indeed, the utilization of distinct analytical approaches for oxysterols and bile acids not only requires additional time but also demands larger amounts of tissue samples (16). Conversely, considering the intrinsic relationship between oxysterols and bile acids biosynthesis, a comprehensive combined quantification of both families holds the potential to provide invaluable insights into the study of pathophysiological processes.

However, the development of such a method comes with its share of challenges. The large number of analytes, the presence of isomers, as well as the broad spectrum of polarity ranging from hydrophobic oxysterols to hydrophilic conjugated bile acids is a challenge for every step of the analysis, from the extraction to the detection by mass spectrometry.

We report here the development of a sensitive LC-MS/MS method for the combined quantification of 18 oxysterols, 11 unconjugated, 15 conjugated bile acids,

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1 bile acid precursor, and its subsequent application to preclinical and clinical samples.

MATERIALS AND METHODS

Materials

7-keto-25-hydroxycholesterol (7-keto-25-OHC), 7-keto-27-hydroxycholesterol (7-keto-27-OHC), 7 α ,24(S)-dihydroxycholesterol (7 α ,24(S)-diOHC), 7 α ,25-dihydroxycholesterol (7 α ,25-diOHC), 7 α ,27-dihydroxycholesterol (7 α ,27-diOHC), 5 α ,6 β -dihydroxycholesterol (5 α ,6 β -diOHC), 7 α ,12 α -dihydroxycholestenone (7 α ,12 α -diOHCone), 7-ketocholesterol (7-ket ochol), 4 β -hydroxycholesterol (4 β -OHC), 7 α -hydroxycholesterol (7 α -OHC), 22(R)-hydroxycholesterol (22(R)-OHC), 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC), 7 α -hydroxycholestenone (7 α -OHCone), 5 α ,6 α -epoxycholesterol (5 α ,6 α -epoxychol), 5 β ,6 β -epoxycholesterol (5 β ,6 β -epoxychol), 24(S),25-epoxycholesterol (24(S),25-epoxychol), [25,26,26,27,27,27-²H₇]4 β -hydroxycholesterol (d₇-4 β -OHC) and [25,26,26,26,27,27,27-²H₇]24(R/S)-hydroxycholesterol (d₇-24-OHC) were purchased from Avanti Polar Lipids.

α -muricholic acid (α -MCA), β -muricholic acid (β -MCA), ω -muricholic acid (ω -MCA), hyocholic acid (HCA), cholic acid (CA), chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), lithocholic acid (LCA), tauro- α -muricholic acid (T α -MCA), tauro- β -muricholic acid (T β -MCA), tauro- ω -muricholic acid (T ω -MCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurohyodeoxycholic acid (THDCA), tauroursodeoxycholic acid (TUDCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycohyodeoxycholic acid (GHDCA), glyco-ur-seodeoxycholic acid (GUDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), [2,2,4,4-²H₄] cholic acid (d₄-CA), [2,2,4,4-²H₄]chenodeoxycholic acid (d₄-CDCA), [2,2,4,4-²H₄]lithocholic acid (d₄-LCA), [2,2,4,4-²H₄]taurocholic acid (d₄-TCA), [2,2,4,4-²H₄]glycocholic acid (d₄-GCA) were purchased from Cayman Chemical.

Trihydroxycholestanic acid (THCA) and deuterated trihydroxycholestanic acid (d₃-THCA) were obtained from Professor Herman J. ten Brink's lab.

LC-grade dichloromethane, methanol, ethanol, isopropanol, *n*-propanol, *n*-hexane, acetone, acetonitrile and methyl *tert*-butyl ether, purchased from VWR, were used for lipid extractions and purifications, whereas MS-grade methanol, acetonitrile, acetic acid and formic acid, also purchased from VWR, were used for LC-MS and LC-MS/MS analyses. EDTA and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich. Water was bidistilled in-house. Silica columns for solid-phase extractions (silica weight = 501 $\pm$ 5 mg per column) were made in-house from silica gel (particle size = 40–63 μ m, VWR) in glass Pasteur pipettes (VWR).

Methods

Tissue sampling.

Human Plasma. Blood from six donors was collected in EDTA-coated tubes (Sarstedt) by venous puncture and directly centrifuged (2,000 $\times$ g, 10 min, 4°C). Plasma was recovered, aliquoted, and stored at –80°C until the day of analysis. Approval from the ethics committee was obtained

(B4032023000052) and informed consent was given by the participants.

Mouse Liver and Plasma. C57BL/6J male mice (8–10 weeks old; Janvier Labs) were anesthetized and blood collected before euthanasia by cervical dislocation. Collected blood was centrifuged at 1,500 $\times$ g, plasma recovered, and aliquoted. Livers were excised before freezing in liquid nitrogen. Livers and plasma were stored at –80°C until the day of analysis. The protocol was approved by the UCLouvain's animal studies ethics committee (2021/UCL/MD/066).

LC-MS/MS method for the combined analysis of oxysterols and bile acids. Human plasma samples (200 μ l) or mouse plasma samples (50 μ l) were placed in ice-cold acetone (1:5, v/v) containing 50 μ g BHT to reduce artificial production of analytes due to cholesterol oxidation. For analysis of pure standards, PBS containing 40 ng EDTA was used in place of plasma. After the addition of internal standards (100 pmol of d₇-4 β -OHC, d₇-24-OHC, d₄-CA, d₄-CDCA, d₄-LCA, d₄-GCA, d₄-TCA, d₃-THCA), samples were vortexed and sonicated in ice-cold water for 10 min, then stored at –20°C to allow for protein precipitation overnight. Samples were then centrifuged (20,000 $\times$ g, 20 min, 4°C), the supernatant recovered and dried under nitrogen stream. The organic residues were then resuspended in 50 μ l isopropanol and transferred on an unmodified silica column, previously conditioned in *n*-hexane. Cholesterol and other hydrophobic compounds were then removed using 2 ml *n*-hexane followed by 4 ml of *n*-hexane - isopropanol (99:1, v/v). Oxysterols and bile acids were then eluted using 4 ml of dichloromethane-methanol (1:1, v/v). Finally, samples were dried under nitrogen stream and resuspended in 20 μ l methanol for the LC-MS/MS analysis.

Samples were analyzed using a Xevo TQ-S tandem quadrupole mass spectrometer coupled to an Acquity H-Class UPLC system from Waters. After injection (3 μ l), chromatographic separation was achieved using an Ascentis Express C18 column (150 $\times$ 2.1 mm; 2 μ m, Sigma-Aldrich) fitted with a Universal RP HPLC guard column (4 $\times$ 2 mm, Macherey-Nagel), kept at 30°C. Mobile phases were composed of water-acetonitrile (95:5, v/v, phase A), acetonitrile (phase B), and methanol (phase C). Each phase contained 0.1% formic acid. The gradient and flow rates (Table 1) were optimized to obtain an adequate chromatographic separation while ensuring short runs and constant column backpressure. The mass spectrometer was fitted with an electrospray ionization (ESI) source used in positive mode. Capillary voltage was at 3.5 kV, cone voltage at 20 V, and source offset at 20 V.

TABLE 1. Gradient and flow rates for chromatographic separation of the oxysterols and bile acids

Time (min)	Flow rate (ml/min)	%A	%B	%C
0.0	0.200	70.0	20.0	10.0
5.0	0.200	48.3	31.7	20.0
10.0	0.300	26.6	53.4	20.0
15.0	0.400	5.0	55.0	40.0
20.0	0.400	5.0	35.0	60.0
25.0	0.400	5.0	5.0	90.0
25.1	0.200	70.0	20.0	10.0
30.0	0.200	70.0	20.0	10.0

Phase A is composed of a mixture of water and acetonitrile (95:5, v/v), whereas phases B and C are composed of acetonitrile and methanol, respectively. Each phase contains 0.1% formic acid.

Desolvation temperature was 500°C, gas flow rates were 1100 L/hour for the desolvation gas, 150 L/hour for the cone gas, and a pressure of 6.0 bars was applied for the nebulizer gas. For MS1, LM resolution was 2.9, HM resolution was 15.1 with an ion energy of 0.2 V. For MS2, LM resolution was 2.8, HM resolution was 15.0 with an ion energy of 0.7 V. The collision gas flow rate was set at 0.15 ml/min. Quantifier and qualifier multiple-reaction monitoring transitions, as well as their respective collision energy, are described in Table 2. Data were acquired using MassLynx (Waters; www.waters.com) and processed using TargetLynx (Waters; www.waters.com).

LC-HRMS method for the quantification of oxysterols. For set up purposes, the combined method was compared to a method for oxysterol analysis previously validated in our lab (11). In this method, samples were placed in glass vials containing deuterated internal standards (133.3 pmol d₇-4 β -OHC, 200 pmol d₇-24-OHC), dichloromethane, methanol (containing 10 μ g BHT) and water (containing 20 ng EDTA) (4:2:1, v/v/v). Samples were then sonicated in ice-cold water for 10 min prior to centrifugation (600 $\times$ g, 10 min, 4°C). Organic phases were recovered and dried under a stream of nitrogen. The lipid extracts were resuspended in dichloromethane and transferred on unmodified silica column for purification by solid-phase extraction. Cholesterol was eliminated with *n*-hexane - isopropanol (99:1, v/v) and oxysterols recovered with *n*-hexane - isopropanol (7:3, v/v). Eluates were recovered and dried under a stream of nitrogen. The resulting residues were resuspended in 30 μ l methanol and prepared for injection. The LC-high resolution mass spectrometry (HRMS) system consisted of an Accela LC system coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was achieved using an Ascentis Express C18 column (150 $\times$ 4.6 mm; 2.7 μ m, Sigma-Aldrich), kept at 15°C. Mobile phase was a gradient of methanol and water containing acetic acid. Mass spectrometry analysis was performed using an atmospheric pressure chemical ionization (APCI) source in the positive mode. Mass calibration was performed before each experiment. Data acquisition and processing were performed using Xcalibur (Thermo Fisher Scientific; www.thermofisher.com).

LC-HRMS method for the quantification of bile acids. The combined method was also compared to an already validated method for bile acid analysis for set up purposes. In this method, samples were homogenized in ice-cold distilled water and proteins were precipitated using acetone in presence of deuterated internal standards (200 pmol d₄-CA, 200 pmol d₃-DHCA, 200 pmol d₃-THCA). Samples were next centrifuged, supernatant recovered and evaporated to dryness. The resulting residue was resuspended in methanol and analyzed by LC-HRMS using an LTQ-Orbitrap XL mass spectrometer coupled to an Accela HPLC system (Thermo Fisher Scientific). Analyte separation was performed on an Ascentis Express C18 column (100 $\times$ 4.6 mm; 2.7 μ m, Sigma-Aldrich), kept at 40°C. Chromatographic separation was achieved using a gradient of water and acetonitrile in the presence of formic acid. Mass spectrometry analysis was performed using an ESI source used in negative mode. Mass calibration was performed before each experiment. Data acquisition and processing were performed using Xcalibur (Thermo Fisher Scientific).

Method validation. The method validation experiments of the present method were performed based on the International Council for Harmonisation (ICH) guideline M10 on bioanalytical method validation (<https://www.ema.europa.eu/en/ich-m10-bioanalytical-method-validation-scientific-guideline>, accessed April 1, 2024). Stock solutions for all oxysterols and bile acids, including deuterated standards, were prepared at concentrations between 2.10⁻² M and 10⁻³ M in methanol and stored at -80°C. Working solutions were prepared by different dilutions and were aliquoted in methanol. Calibration standard levels were chosen based on previous experiments and reported levels in human and mouse plasma (15, 17–21). Calibration curves were performed three times, on three different days, in triplicate. All calibration curves were subjected to the entire analytical process. Final calibration curves were found acceptable if the accuracy of the back-calculated concentrations of calibration standard was within $\pm$ 15% of the nominal value for at least 75% of the different levels (except $\pm$ 20% at lower limit of quantification (LLOQ) level), with a minimum of 6 calibration standard levels for each calibration curve. LLOQ and upper limit of quantification (ULOQ) were determined as the lowest and highest calibration standard meeting the above criteria, respectively. Experiments for the determination of between-run accuracies and precisions in a surrogate matrix (PBS) were performed three times, on three different days, in triplicate, while they were performed once, in quintuplicate for the determination of within-run accuracies and precisions. These parameters were assessed at 4 different levels: LLOQ, low (twice the LLOQ), mid (half of the ULOQ), and ULOQ. Moreover, experiments for the determination of between-run accuracies and precisions in diluted human plasma (20 μ l plasma in 180 μ l PBS) were performed three times, on three different days, in triplicate, while they were performed once, in quintuplicate for the determination of within-run accuracies and precisions. These parameters were assessed at 2 different levels (Q1 and Q2) (supplemental Table S1). Accuracy was considered acceptable by the ICH guideline M10 if it fell within $\pm$ 15% of the nominal value (except $\pm$ 20% at LLOQ level). Similarly, precision (% coefficient of variation) was found acceptable if it did not exceed 15% (except 20% at LLOQ level). Carry-over effects were determined by analyzing blank samples after calibration standards at ULOQ and were considered acceptable if the remaining signal in the blank was inferior to 20% of the LLOQ, or inferior to 5% of the initial signal for internal standards.

Recovery and matrix effect. Analytes recovery was calculated for the whole analytical process by extracting internal standards structurally representative of all the analytes. The samples were extracted, purified, and injected in the LC-MS/MS system. The obtained signal was compared to the signal obtained after direct injection of equal amounts of internal standards in the LC-MS/MS system. The matrix factor was assessed by comparing the signal of internal standards in the presence and absence of matrix. These experiments were performed three times, on three different days, in triplicate.

Statistical analysis. All data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using GraphPad Prism (version 10.0; www.graphpad.com). One-way ANOVA, or Kruskal-Wallis' test when relevant, coupled to Dunnett's or Dunn's test were used to compare more than two groups of

TABLE 2. Oxysterols and bile acids quantified with this method

Abbreviation	Common name	LM ID	RRT	Exact mass	Quantifier (CE)	Qualifier (CE)	IS
7-keto-25-OHC	7-keto-25-hydroxycholesterol	LMST01010449	1.263	416.32905	417.4 > 399.3 (10)	417.3 > 381.4 (14)	d ₇ -24-OHC
7-keto-27-OHC	7-keto-27-hydroxycholesterol	LMST04030180	1.317	416.32905	417.4 > 94.9 (24)	417.4 > 80.8 (30)	d ₇ -4β-OHC
7α,24 (S)-diOHC	7α,24 (S)-dihydroxycholesterol	LMST04030168	1.331	418.34470	401.3 > 383.3 (10)	383.4 > 104.9 (36)	d ₃ -THCA
7α,25-diOHC	7α,25-dihydroxycholesterol	LMST04030166	1.277	418.34470	383.4 > 104.9 (36)	383.4 > 365.2 (6)	d ₄ -GCA
7α,27-diOHC	7α,27-dihydroxycholesterol	LMST01010462	1.320	418.34470	383.4 > 104.9 (36)	401.3 > 383.3 (10)	d ₄ -GCA
5α,6β-diOHC	5α,6β-dihydroxycholesterol	LMST01010052	1.895	420.36035	385.4 > 94.9 (24)	385.4 > 367.2 (8)	d ₇ -24-OHC
7α,12α-diOHCone	7α,12α-dihydroxycholestenone	LMST04030114	1.685	416.32905	417.3 > 381.4 (14)	417.3 > 97.1 (30)	d ₇ -24-OHC
7-ketochol	7-ketcholesterol	LMST01010049	2.052	400.33413	401.3 > 80.8 (26)	401.3 > 96.9 (20)	d ₇ -24-OHC
4β-OHC	4β-hydroxycholesterol	LMST01010014	2.480	402.34978	385.4 > 367.2 (8)	385.4 > 94.9 (24)	d ₇ -4β-OHC
7α-OHC	7α-hydroxycholesterol	LMST01010013	2.007	402.34978	367.4 > 94.9 (24)	367.4 > 104.9 (36)	d ₇ -24-OHC
22 (R)-OHC	22 (R)-hydroxycholesterol	LMST01010086	1.521	402.34978	367.4 > 94.9 (24)	367.4 > 104.9 (36)	d ₇ -24-OHC
24 (S)-OHC	24 (S)-hydroxycholesterol	LMST01010019	1.688	402.34978	385.4 > 367.2 (8)	367.4 > 104.9 (36)	d ₇ -24-OHC
25-OHC	25-hydroxycholesterol	LMST01010018	1.610	402.34978	367.4 > 94.9 (24)	385.4 > 367.2 (8)	d ₇ -24-OHC
27-OHC	27-hydroxycholesterol	LMST01010057	1.736	402.34978	367.4 > 94.9 (24)	385.4 > 94.9 (24)	d ₇ -24-OHC
7α-OHCone	7α-hydroxycholestenone	LMST04030123	1.979	400.33413	401.3 > 96.9 (20)	401.3 > 383.3 (10)	d ₇ -24-OHC
5α,6α-epoxychol	5α,6α-epoxycholesterol	LMST01010011	2.334	402.34978	385.4 > 367.2 (8)	385.4 > 94.9 (24)	d ₇ -4β-OHC
5β,6β-epoxychol	5β,6β-epoxycholesterol	LMST01010010	2.259	402.34978	385.4 > 367.2 (8)	385.4 > 94.9 (24)	d ₇ -4β-OHC
24 (S),25-epoxychol	24 (S),25-epoxycholesterol	LMST01010012	1.800	400.33413	383.4 > 104.9 (36)	383.4 > 365.2 (6)	d ₇ -24-OHC
α-MCA	α-muricholic acid	LMST04010066	0.809	408.28757	373.3 > 355.3 (12)	373.3 > 159.1 (22)	d ₄ -CA
β-MCA	β-muricholic acid	LMST04010067	0.779	408.28757	373.3 > 355.3 (12)	373.3 > 159.1 (22)	d ₄ -CA
ω-MCA	ω-muricholic acid	LMST04010065	0.762	408.28757	373.3 > 355.3 (12)	373.3 > 159.1 (22)	d ₄ -CA
HCA	Hyochoolic acid	LMST04010064	0.905	408.28757	373.3 > 355.3 (12)	373.3 > 159.1 (22)	d ₄ -CA
CA	Cholic acid	LMST04010001	1.000	408.28757	373.3 > 355.3 (12)	373.3 > 159.1 (22)	d ₄ -CA
CDCA	Chenodeoxycholic acid	LMST04010032	1.195	392.29266	357.3 > 135.2 (24)	357.3 > 161.2 (18)	d ₄ -CDCA
HDCA	Hyodeoxycholic acid	LMST04010024	0.934	392.29266	357.3 > 161.2 (18)	357.3 > 135.2 (24)	d ₄ -CDCA
MDCA	Murideoxycholic acid	LMST04010025	0.857	392.29266	357.3 > 161.2 (18)	357.3 > 104.9 (40)	d ₄ -CDCA
UDCA	Ursodeoxycholic acid	LMST04010033	0.973	392.29266	357.3 > 161.2 (18)	357.3 > 135.2 (24)	d ₄ -CDCA
DCA	Deoxycholic acid	LMST04010040	1.228	392.29266	357.3 > 135.2 (24)	357.3 > 104.9 (40)	d ₄ -CDCA
LCA	Lithocholic acid	LMST04010003	1.447	376.29775	359.3 > 149.0 (20)	359.3 > 341.3 (5)	d ₄ -LCA
Tα-MCA	Tauro-α-muricholic acid	168408*	0.455	515.29167	480.3 > 126.0 (20)	480.3 > 462.3 (10)	d ₄ -TCA
Tβ-MCA	Tauro-β-muricholic acid	LMST05040012	0.473	515.29167	480.3 > 126.0 (20)	480.3 > 462.3 (10)	d ₄ -TCA
Tω-MCA	Tauro-ω-muricholic acid	118703092*	0.429	515.29167	480.3 > 126.0 (20)	480.3 > 462.3 (10)	d ₄ -TCA
TCA	Taurocholic acid	LMST05040001	0.753	515.29167	480.3 > 462.3 (10)	462.3 > 126.0 (20)	d ₄ -TCA
TCDCa	Taurochenodeoxycholic acid	LMST05040005	0.955	499.29676	464.3 > 126.0 (32)	464.3 > 105.1 (54)	d ₄ -TCA
THDCA	Taurohyodeoxycholic acid	119046*	0.643	499.29676	464.3 > 126.0 (32)	464.3 > 105.1 (54)	d ₄ -TCA
TUDCA	Tauroursodeoxycholic acid	LMST05040015	0.662	499.29676	464.3 > 126.0 (32)	464.3 > 105.1 (54)	d ₄ -TCA
TDCA	Taurodeoxycholic acid	LMST05040013	1.019	499.29676	464.3 > 126.0 (32)	464.3 > 105.1 (54)	d ₄ -TCA
TLCA	Taurolithocholic acid	LMST05040003	1.260	483.30184	466.3 > 126.0 (20)	466.3 > 149.0 (20)	d ₄ -TCA
GCA	Glycocholic acid	LMST05030001	0.823	465.30904	488.3 > 470.2 (24)	488.3 > 413.2 (26)	d ₄ -GCA
GCDCA	Glycochenodeoxycholic acid	LMST05030008	0.999	449.31412	472.3 > 98.1 (26)	472.4 > 454.2 (20)	d ₄ -GCA
GHCA	Glycohyodeoxycholic acid	114611*	0.731	449.31412	472.3 > 98.1 (26)	472.4 > 454.2 (20)	d ₄ -GCA
GUDCA	Glycoursodeoxycholic acid	LMST05030016	0.758	449.31412	472.3 > 98.1 (26)	472.4 > 454.2 (20)	d ₄ -GCA
GDCA	Glycodeoxycholic acid	LMST05030006	1.042	449.31412	472.4 > 454.2 (20)	472.4 > 397.2 (26)	d ₄ -GCA
GLCA	Glycolithocholic acid	LMST05030009	1.215	433.31921	456.4 > 97.8 (26)	472.4 > 454.2 (10)	d ₄ -GCA
THCA	Trihydroxycholestanic acid	122312*	1.290	450.33452	415.3 > 397.3 (10)	473.3 > 455.3 (20)	d ₃ -THCA
d ₇ -4β-OHC	d ₇ -4β-hydroxycholesterol		2.378	409.39372	374.2 > 104.9 (36)	392.4 > 374.2 (8)	
d ₇ -24-OHC	d ₇ -24 (R/S)-hydroxycholesterol		1.682	409.39372	392.4 > 374.2 (8)	374.2 > 104.9 (36)	
d ₄ -CA	d ₄ -cholic acid		0.998	412.31268	377.3 > 359.3 (13)	377.3 > 163.1 (22)	
d ₄ -CDCA	d ₄ -chenodeoxycholic acid		1.192	396.31777	361.3 > 165.0 (16)	361.3 > 361.3 (0) ^b	

(continued)

TABLE 2. Continued

Abbreviation	Common name	LM ID	RRT	Exact mass	Quantifier (CE)	Qualifier (CE)	IS
d ₄ -LCA	d ₄ -lithocholic acid		1.435	380.32285	363.3 > 153.0 (20)	363.3 > 363.3 (0) [§]	
d ₄ -TCA	d ₄ -taurocholic acid		0.746	519.31678	484.3 > 466.3 (10)	466.3 > 466.3 (0) [§]	
d ₄ -GCA	d ₄ -glycocholic acid		0.820	469.33415	492.3 > 474.2 (24)	492.3 > 417.2 (26)	
d ₅ -THCA	d ₅ -trihydroxycholestanoic acid		1.280	453.35335	418.3 > 400.3 (10)	476.3 > 476.3 (0) [§]	

The table shows the Lipid Maps ID (LM ID) for each analyte, as well as their relative retention time (RRT), quantifier (Q) and qualifier (q) MRM transitions and the internal standard used for their quantification.

*PubChem ID was entered when Lipid Maps ID was unavailable.

[§]For some internal standards, the qualifier transition is not the product of a fragmentation.

unpaired values. Spearman correlations were used for the analysis of oxysterol and bile acid levels found in human plasma. Statistical significance was set at $P < 0.05$.

RESULTS

Extraction

The physicochemical properties of the oxysterols and bile acids are quite different. Thus, during the development of this analytical method, we investigated different extraction protocols in order to maximize the coextraction of these analytes. These approaches included the following: (i) a classical liquid-liquid extraction protocol, (ii) a salting-out liquid-liquid extraction protocol using potassium chloride and calcium chloride, and (iii) an extraction protocol through protein precipitation using acetone.

Liquid-liquid extraction. In this section, we assessed the extraction efficiency of different solvent mixtures based on the work of Folch *et al.* (22, 23). In their work, Folch *et al.* used a mixture of chloroform, methanol, and water to extract lipids from matrices. In this setting, methanol will mainly go in the aqueous phase rather than the organic phase. In our experiments, we replaced chloroform by dichloromethane due to the presence of peroxides in chloroform, which induces artificial production of oxysterols during the extraction process (data not shown). Contrary to oxysterols, bile acids are poorly soluble in dichloromethane and highly soluble in methanol. In this setting, methanol is mainly extracted in the aqueous phase, giving a low extraction efficiency of these polar lipids using a mixture of dichloromethane, methanol, and water. For this reason, we opted to replace methanol for another solvent. This solvent would mainly be extracted in the organic phase and therefore facilitate the coextraction of oxysterols and bile acids.

As such, we extracted oxysterol and bile acid standards using dichloromethane (8 ml), water (2 ml), and another organic solvent (4 ml), namely ethanol, isopropanol, *n*-propanol, acetone, and acetonitrile. A condition using methanol was used as a reference. Moreover, in all the conditions we added HCl 2 M (300 μ l) to enhance the extraction of bile acids (Fig. 1). The amount of added acid was determined to obtain a low pH (1.0–1.2) in the aqueous phase, allowing for better extraction of bile acids with low pK_a values. For the sake of clarity, in this article, we chose to show the results for 4 oxysterols and 4 bile acids, which are structurally representative of all the analytes. However, the data for all the analytes are available in the supplemental materials.

The results show a decent extraction efficiency for most oxysterols compared to the direct injection of standards in the LC-MS system, except for the epoxycholesterols (supplemental Table S2). Our hypothesis is that epoxycholesterols are cleaved during the

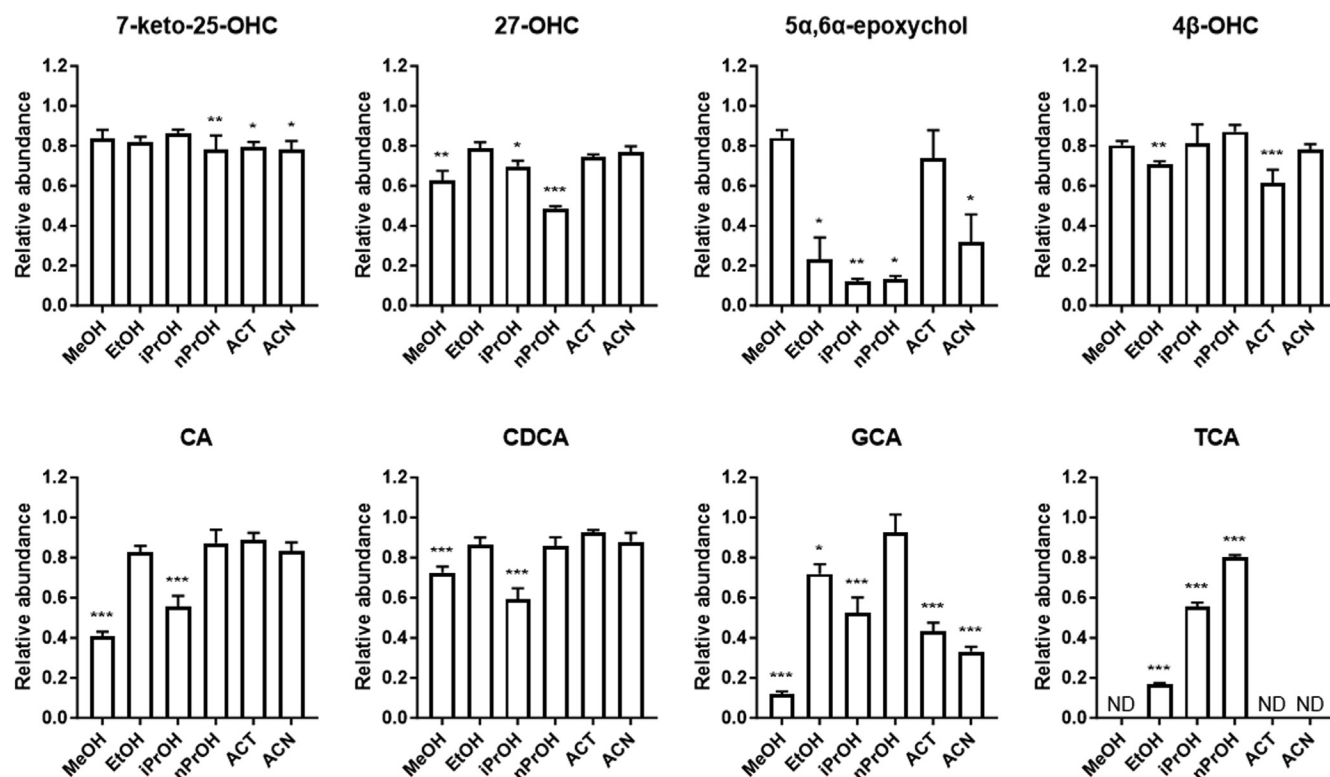


Fig. 1. Coextraction of oxysterols and bile acids by acid liquid-liquid extraction. Pure standards of oxysterols and bile acids were extracted by a mixture of dichloromethane (8 ml), water (2 ml), and a third solvent (4 ml) listed on the graphs, in the presence of hydrochloric acid (2 M, 300 μ l) before injection into the LC-HRMS system for oxysterol and the LC-HRMS system for bile acid analysis. Data are expressed as a relative abundance compared to the direct injection of equal amounts of pure standards, set at 1. Deuterated internal standards were spiked after the extraction step. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. N = 2; n = 3. ACN, acetonitrile; ACT, acetone; EtOH, ethanol; HRMS, high resolution mass spectrometry; iPrOH, isopropanol; MeOH, methanol; ND, not detected; nPrOH, *n*-propanol.

extraction process due to the presence of acid (24). This could lead to the artificial production of oxysterol during the process, although this has not been observed in this experiment. We also observed an acceptable extraction efficiency for most bile acids using ethanol, isopropanol, and *n*-propanol (supplemental Table S3). The use of methanol, acetone, or acetonitrile results in poor extraction yields for the most polar compounds, such as taurocholic acid.

Salting-out liquid-liquid extraction. Having demonstrated the incompatibility of acid use with the extraction of some oxysterols, we opted for a different approach with the aim of coextracting hydrophobic oxysterols and more hydrophilic bile acids. In this experiment, we extracted oxysterols and bile acids standards using a mixture of dichloromethane (8 ml), water (2 ml) containing (or not) potassium chloride or calcium chloride, and 4 ml of methanol, ethanol, or isopropanol, in the absence of acid. The results show a good extraction yield for oxysterols in all conditions (Fig. 2A and supplemental Table S4). On the other hand, conjugated bile acids show a poor extraction efficiency using methanol, which is increased by using ethanol or isopropanol (Fig. 2A and supplemental

Table S5). We also observe an extraction yield dependent on salt concentration, with potassium chloride showing better results compared to calcium chloride.

For the second experiment of salting-out liquid-liquid extraction, we focused on the extraction of bile acids using potassium chloride as a salt and increased its concentration until saturation. We also selected the solvents enabling the best extraction yields in the first experiment, namely ethanol, isopropanol, and *n*-propanol. The results show an increased extraction efficiency with potassium chloride concentration, especially for the more polar conjugated bile acids (Fig. 2B and supplemental Table S6). We also observe the limited extraction yield in the absence of salt, highlighting the importance of acidifying the aqueous phase when performing a liquid-liquid extraction (eg comparing GCA and TCA data in Figs. 1 and 2B).

Protein precipitation. The salting-out liquid-liquid extraction protocol showing relatively low extraction yield for the more polar bile acids, we decided to test another protocol based on protein precipitation using acetone. This protocol has already been tested and validated for bile acids analysis (data not published). Therefore, we focused on the extraction of oxysterols.

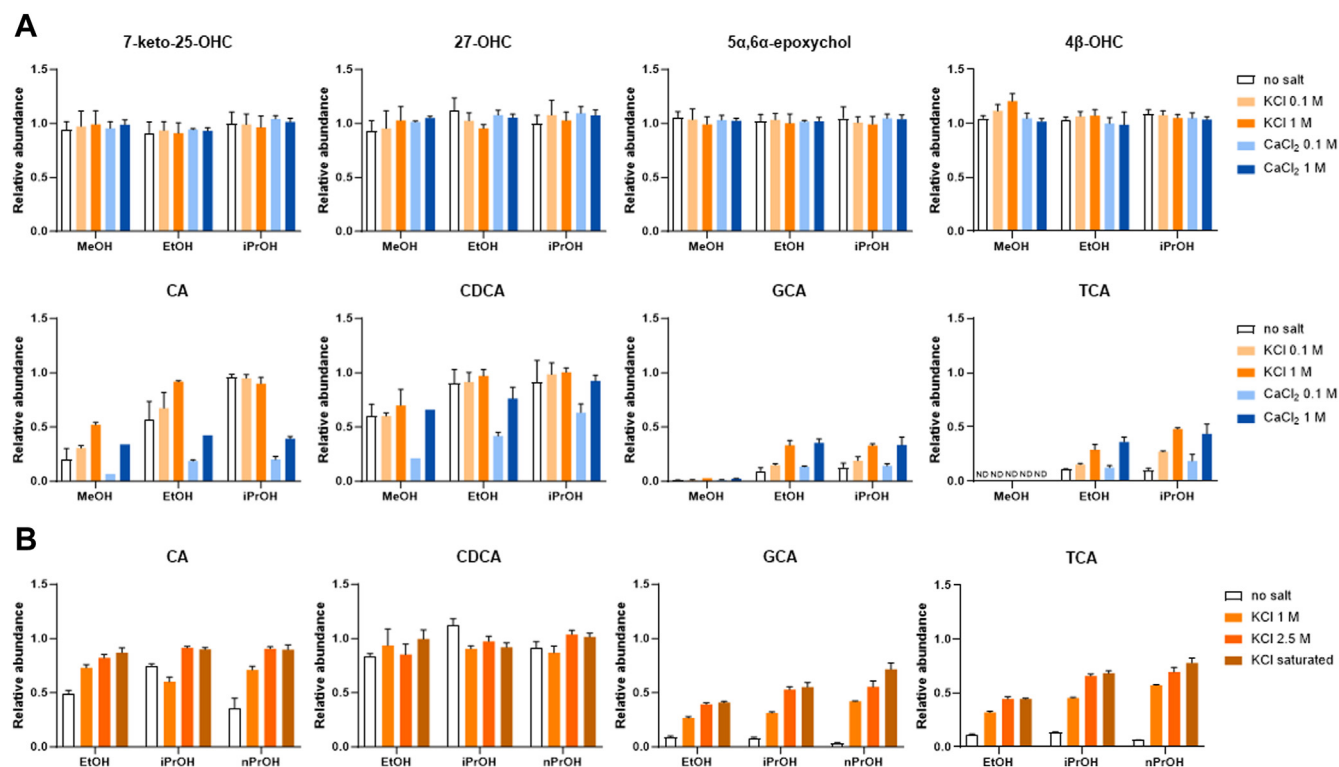


Fig. 2. Coextraction of oxysterols and bile acids by salting-out liquid-liquid extraction. A: Pure standards of oxysterols and bile acids were extracted by a mixture of dichloromethane (8 ml), water (2 ml, containing no salt, potassium chloride (KCl), or calcium chloride (CaCl₂) at 0.1 M or 1 M) and a third solvent (4 ml). B: Pure standards of bile acids were extracted by a mixture of dichloromethane (8 ml), water (2 ml, containing no salt or KCl at 1 M, 2.5 M or saturated) and a third solvent (4 ml). Deuterated internal standards were spiked after the extraction step. Analytes were then injected into the LC-HRMS systems for oxysterol and bile acid analysis. Data are expressed as a relative abundance compared to the direct injection of equal amounts of pure standards, set at 1. N = 3; n = 1. EtOH, ethanol; HRMS, high resolution mass spectrometry; iPrOH, isopropanol; MeOH, methanol; ND, not detected; nPrOH, *n*-propanol.

In this experiment, we compared the extraction efficiency of our previously validated method for oxysterol analysis using liquid-liquid extraction (11) to different protocols based on protein precipitation using acetone. Therefore, oxysterols were extracted from mouse liver homogenates using increasing volumes of acetone (Fig. 3). We also investigated the interest of successive extractions with acetone.

The results show similar oxysterol extraction yields when using 1000 μl acetone and our previously validated method for oxysterol analysis (supplemental Table S7). Therefore, we opted for this extraction protocol at this stage.

Purification

After setting up the extraction process, we moved on to the purification protocol. Indeed, while such a procedure is not a common practice for bile acid analysis, it is widespread in the case of oxysterols. The main goal of this step is to eliminate cholesterol from the matrix. Indeed, cholesterol is present in far greater amounts compared to oxysterols, and its presence may induce artificial oxysterol formation due to autooxidation during the analytical process. A common purification protocol for oxysterols relies on a solid-phase

extraction using normal silica as a stationary phase and mixtures of *n*-hexane and isopropanol for elimination of cholesterol and elution of oxysterols (25).

Therefore, we set up a purification protocol based on a solid-phase extraction using normal silica. After loading the samples on the silica using isopropanol, we eluted cholesterol (and other hydrophobic compounds) using *n*-hexane followed by *n*-hexane - isopropanol (99:1, v/v). Next, as oxysterols are eluted using *n*-hexane-isopropanol 7:3 (v/v) in these conditions, we increased the proportion of isopropanol to elute the analytes of interest from the column (11). However, as bile acids were not properly eluted by increasing the proportion of isopropanol up to a mixture of *n*-hexane-isopropanol 1:1 (v/v) (data not shown), we investigated the potential of using a mixture of dichloromethane and methanol for their elution.

In this experiment, we spiked PBS and human plasma with structurally diverse deuterated internal standards. After extraction using the protein precipitation protocol mentioned above, we loaded the samples on the silica column using isopropanol. We then recovered the *n*-hexane-isopropanol (99:1, v/v) and dichloromethane-methanol (1:1, v/v) fractions. After elution of the remaining lipids using methanol, we

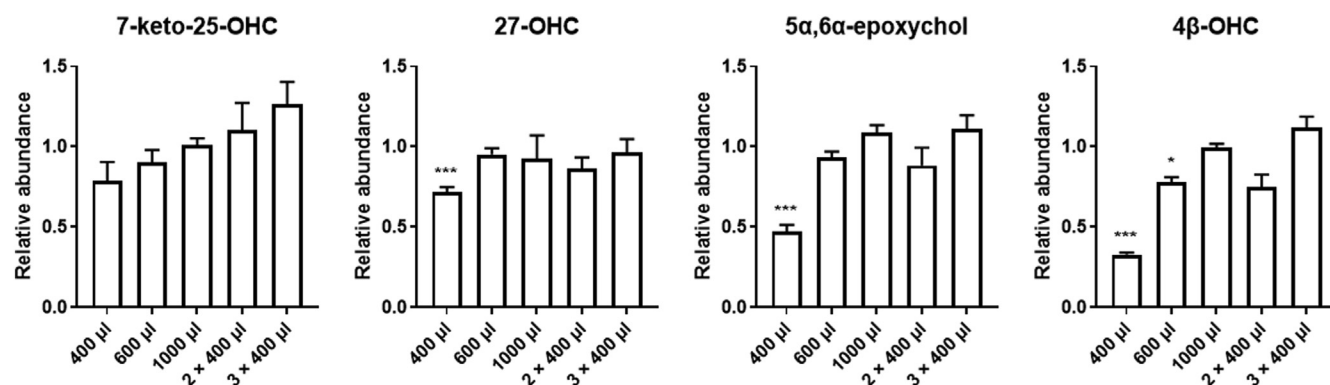


Fig. 3. Extraction of oxysterols following protein precipitation using acetone. Endogenous oxysterols were extracted from 30 mg mouse liver tissue following protein precipitation using increasing volumes of acetone. Data are expressed relatively to the signal obtained using the validated LC-HRMS method for oxysterol analysis (see method section), set at 1. Deuterated internal standards were spiked after the extraction step. * $P < 0.05$; *** $P < 0.001$. $N = 3$; $n = 3-6$. HRMS, high resolution mass spectrometry.

analyzed each fraction and compared the signal intensities to direct injections of equal amounts of internal standards in the LC-MS/MS system.

The results indicate minimal, if any, signal for the deuterated oxysterols and bile acids in the *n*-hexane-isopropanol fraction, supporting that this step can be used to decrease the amount of cholesterol in the samples (Fig. 4). Although some deuterated standards are also detected in the methanol fraction, a majority of the internal standards' signal is detected within the fraction of interest (namely the dichloromethane-methanol fraction). Furthermore, the signal

distribution among the various fractions appears unaffected by the presence of the matrix.

This experiment allows us to determine the recovery rates for each internal standard. This is achieved by comparing the signal intensities obtained through spiked PBS with those obtained via direct injection into the LC-MS/MS system (Table 3). Additionally, this experiment enables us to evaluate the influence of the matrix on our measurements by comparing signal intensities in the presence and absence of matrix (Table 3). The recovery rates for each internal standard exhibited a range from 64.2% to 103.9%, while the

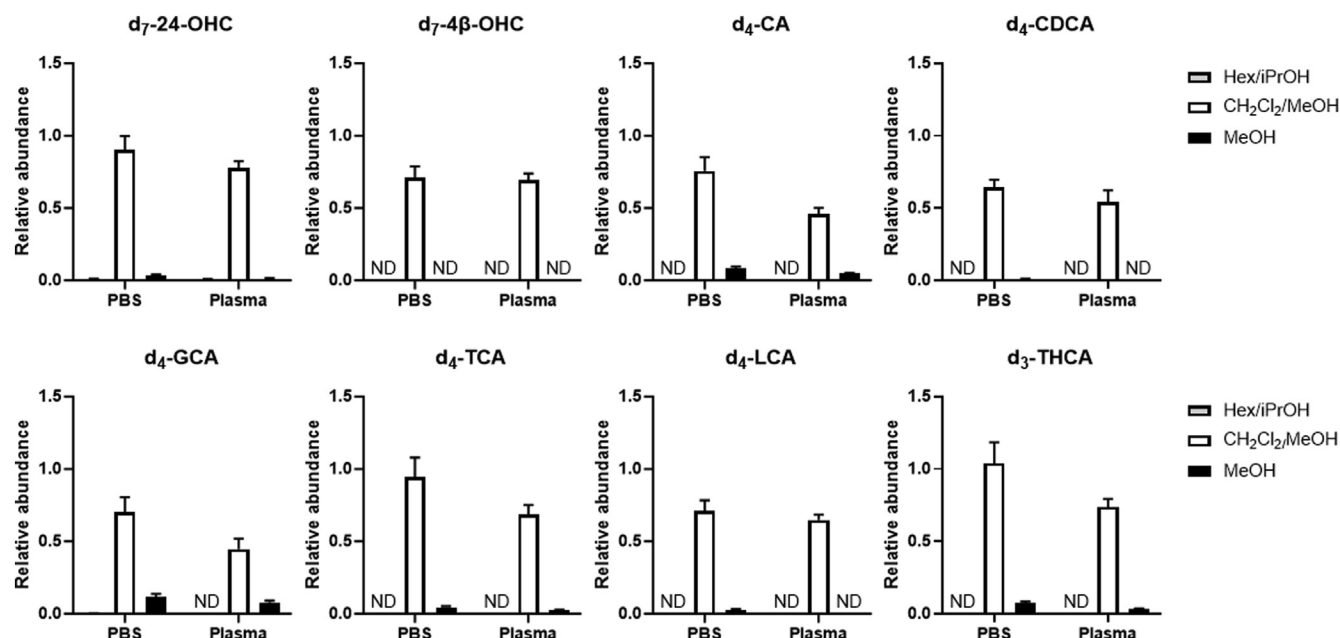


Fig. 4. Copurification of deuterated oxysterols and bile acids by solid-phase extraction. Deuterated oxysterols and bile acids were extracted following protein precipitation using water and acetone (1:5, v/v), and loaded onto a silica column for purification by solid-phase extraction (SPE). Three SPE fractions were eluted: (i) *n*-hexane-isopropanol (99:1, v/v, Hex/iPrOH), (ii) dichloromethane-methanol (1:1, v/v, CH₂Cl₂/MeOH), (iii) methanol (MeOH). Data are expressed as a relative abundance compared to the direct injection of equal amounts of pure deuterated standards, set at 1. Murideoxycholeic acid standard (200 pmol) was spiked in each SPE fraction after the elution and was used as an internal standard. Samples were then injected into the LC-MS/MS system for the combined detection of oxysterols and bile acids. $N = 3$, $n = 3$.

TABLE 3. Calculated recovery and matrix factors

Name	% Recovery	% Matrix factor
d ₇ -4 β -OHC	71.1	98.2
d ₇ -24-OHC	90.4	88.1
d ₄ -CA	75.7	63.4
d ₄ -CDCA	64.2	88.6
d ₄ -LCA	71.1	98.0
d ₄ -TCA	94.3	76.7
d ₄ -GCA	70.1	62.2
d ₃ -THCA	103.9	75.1

These were calculated for each internal standard used in this method.

matrix factors varied between 60.7% and 97.7%. Although we observe a significant loss of signal for some internal standard during the analysis, it should be noted that the wide range of polarity across oxysterols and bile acids complicates the complete recovery of the analytes. Moreover, it should be noted that this phenomenon is taken into account in the overall method as the calibration curves are submitted to the entire analytical process.

LC-MS/MS analysis

Oxysterols and bile acids are lipid families comprising numerous isomers (Table 2). Therefore, the chromatographic separation is essential when using a mass spectrometer as a detector. In this method, we set up a gradient of water, acetonitrile, and methanol in the presence of formic acid to chromatographically separate our compounds of interest using a C18 column as a stationary phase (Table 1). Methanol, a protic solvent, was found to be a strong eluent for bile acids, impairing the chromatographic separation of these analytes. However, its presence was essential for effectively separating oxysterols (data not shown). These observations lead us to setup a “double gradient” for oxysterols and bile acids chromatographic separation. The first phase is the increase of acetonitrile (an aprotic solvent) in the mixture to allow for bile acids separation, which is followed by a second phase, increasing the proportion of methanol, replacing acetonitrile for a better separation of oxysterols (Fig. 5 and supplemental Fig. S1). The flow rate has also been optimized to reduce the time of analysis while conserving a constant backpressure on the system.

We investigated the use of both electrospray (ESI) and APCI probes and found that bile acids were not detected in our hands using an APCI probe. Subsequently, we explored the possibility of using negative or positive ionization modes and found that oxysterols were poorly detected in the negative mode (data not shown). Consequently, we used an ESI probe used in positive ionization mode and optimized the multiple-reaction monitoring transitions for all the lipids of interest. These transitions were chosen based on their signal intensity after infusing each compound individually in the mass spectrometer along with a mobile

phase mixture based on the gradient and the retention time of each compound. The transition that yielded the highest signal intensity was employed as the quantifier (Q) transition, while the second highest signal intensity transition was utilized as the qualifier (q) transition (Table 2). For oxysterols and bile acids, the precursor ion of the quantifier transition is often $[M + H - H_2O]^+$ or $[M + H - 2H_2O]^+$, which are both signatures of in-source fragmentation resulting in a loss of one or two water molecules. Contrastingly, glycoconjugated bile acids produce $[M + Na]^+$ as a precursor ion. The loss of an additional molecule of water is often detected after the precursor ion's fragmentation into the product ion. For tauroconjugated bile acids, the ion 126.0 is often detected as the product ion, which is the signature of the loss of a taurine moiety, whereas 98.1 represents the detection of a glycine moiety with a sodium adduct for glycoconjugated bile acids.

Method validation

We built calibration curves for each oxysterol and bile acid with at least six calibration levels per analyte, based on reported levels in human and mouse plasma. We spiked PBS with constant amounts of deuterated standards and different amounts of standards. After extraction, purification and injection of the samples into the LC-MS/MS system, we generated plots for each analyte's area under the curve relative to its corresponding internal standard against the nominal amount of standard per sample. The corresponding internal standards, as well as the quadratic regression model and the 1/x weighting were selected based on their ability to provide the most accurate back-calculated results for each compound (Table 4). The R^2 value was above 0.94 for each compound. The LLOQ was determined as described in the method section and ranged from 0.39 to 3.91 pmol per sample for all our lipids of interest. These LLOQ support the sensitivity of the developed method, especially considering the low injection volume (3 μ l from a 20- μ l sample). Within-run and between-run accuracies and precisions were determined as described in the method section and are reported in Tables 4 and 5. Carry-over effects were considered negligible as the analysis of blank samples after injection of calibration standards at the ULOQ did not exceed 20% of the LLOQ (or 5% of the initial signal for internal standards) (data not shown).

Oxysterol and bile acid quantification in human and mouse plasma

Next, we applied the developed method to the analysis of the plasma from six human donors (4 females and 2 males), in which 12 oxysterols and 14 bile acids were successfully detected and quantified in a single chromatographic run (Fig. 6A). We also applied the method to the analysis of mouse plasma from the vena cava (Fig. 6B) and the portal vein (Fig. 6C),

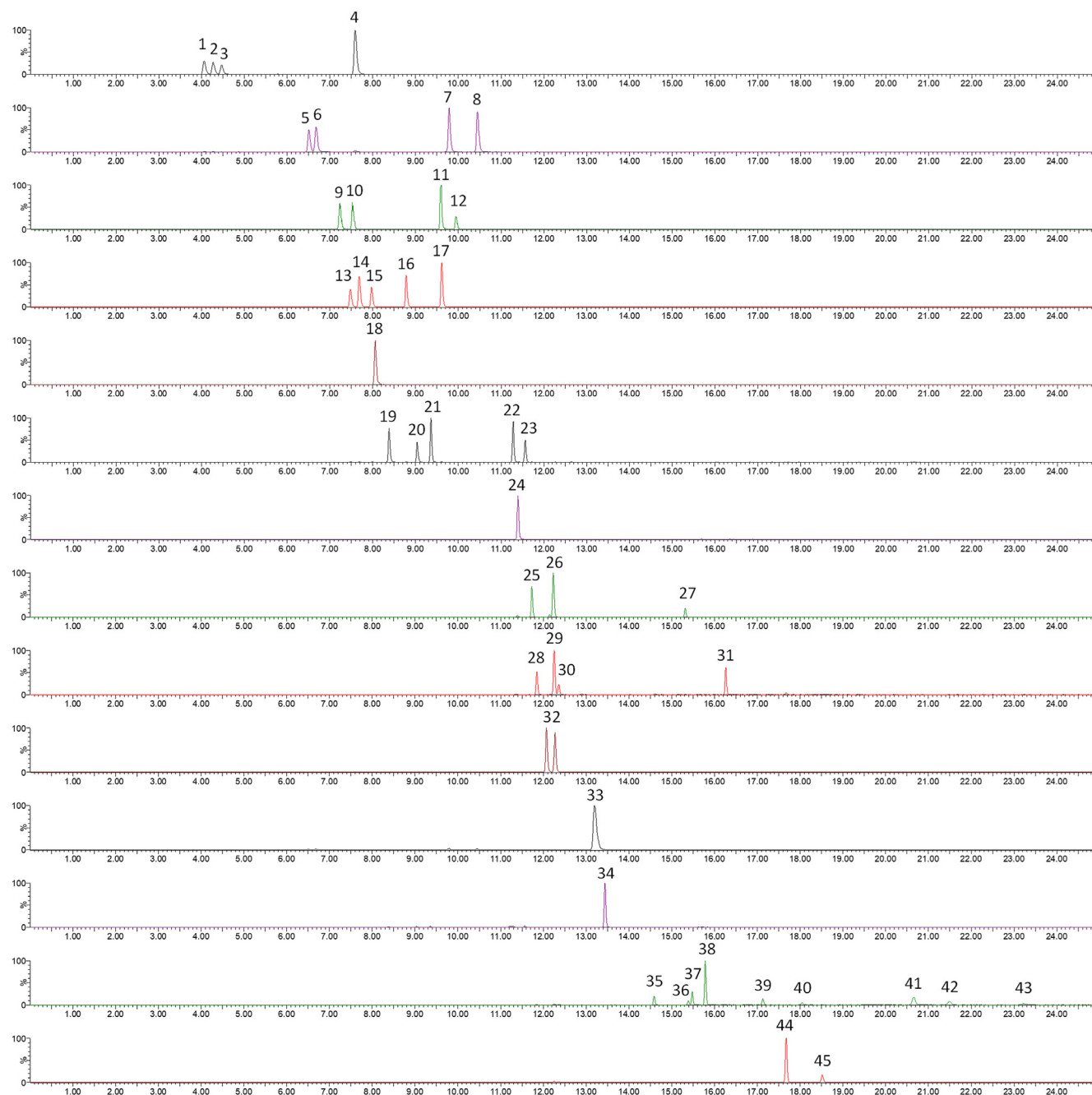


Fig. 5. Representative chromatogram of oxysterols and bile acids analyzed using the described method. Pure standards (1 pmol) were injected in the LC-MS/MS system described in the method section. 1: tauro- ω -muricholic acid, 2: tauro- α -muricholic acid, 3: tauro- β -muricholic acid, 4: taurocholic acid, 5: taurohyodeoxycholic acid, 6: tauroursodeoxycholic acid, 7: taurochenodeoxycholic acid, 8: taurodeoxycholic acid, 9: glycohyodeoxycholic acid, 10: glycooursodeoxycholic acid, 11: glycochenodeoxycholic acid, 12: glycodeoxycholic acid, 13: ω -muricholic acid, 14: α -muricholic acid, 15: β -muricholic acid, 16: hyocholic acid, 17: cholic acid, 18: glycocholic acid, 19: murideoxycholic acid, 20: hyodeoxycholic acid, 21: ursodeoxycholic acid, 22: chenodeoxycholic acid, 23: deoxycholic acid, 24: glycolithocholic acid, 25: 7-keto-25-hydroxycholesterol, 26: 7-keto-27-hydroxycholesterol, 27: 7 α ,12 α -dihydroxycholestenone, 28: 7 α ,25-dihydroxycholesterol, 29: 7 α ,24(*S*)-dihydroxycholesterol, 30: 7 α ,27-dihydroxycholesterol, 31: 24(*S*),25-epoxycholesterol, 32: trihydroxycholestanoic acid, 33: tauroolithocholic acid, 34: lithocholic acid, 35: 22(*R*)-hydroxycholesterol, 36: 25-hydroxycholesterol, 37: 24(*S*)-hydroxycholesterol, 38: 27-hydroxycholesterol, 39: 7 α -hydroxycholesterol, 40: 5 α ,6 β -dihydroxycholesterol, 41: 5 β ,6 β -epoxycholesterol, 42: 5 α ,6 α -epoxycholesterol, 43: 4 β -hydroxycholesterol, 44: 7 α -hydroxycholestenone, 45: 7-ketocholesterol.

demonstrating its versatility for the analysis of clinical and preclinical samples ([supplemental Table S8](#)).

This method enables the production of extensive data in a single chromatographic run, allowing for

subsequent collective analysis, such as through Spearman correlations ([Fig. 7](#)), thereby providing further insights into the results. For instance, using results from human plasma, some correlations are

TABLE 4. Calibration curve parameters, accuracies, and precisions of the described method

Name	Range (pmol)		Calibration curve equation ($y = ax^2 + bx + c$)				Within-run accuracy (% bias)				Within-run precision (% CV)				Between-run accuracy (% bias)				Between-run precision (% CV)			
	LLOQ	ULOQ	a	b	c	R^2	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
7-keto-25-OHC	0.39	50	1.92E-04	0.0680	0.0092	0.975	-6.6	8.6	-12.2	-9.5	5.5	14.8	3.7	2.5	7.7	6.1	5.1	3.5	17.8	18.0	10.9	10.1
7-keto-27-OHC	0.39	50	3.09E-04	0.1509	-0.0052	0.990	17.4	12.7	1.8	11.9	8.1	4.5	8.4	4.5	14.9	8.7	1.3	-8.6	12.8	15.7	8.2	9.2
7 α ,24 (S)-diOHC	1.95	250	-3.51E-06	0.0038	0.0122	0.971	6.7	13.4	13.2	13.8	9.2	3.3	6.3	12.0	5.7	11.3	-1.5	-10.4	17.3	13.2	11.8	16.2
7 α ,25-diOHC	1.95	250	2.83E-05	0.0110	-0.0042	0.973	7.4	3.8	-2.8	-7.0	10.3	5.7	9.4	7.8	13.3	1.1	7.1	7.8	18.9	11.7	12.5	4.8
7 α ,27-diOHC	1.95	250	1.69E-05	0.0050	0.0011	0.986	2.3	0.6	-3.0	1.7	13.5	2.9	8.6	6.6	16.3	11.6	7.4	12.5	13.6	12.5	10.7	5.7
5 α ,6 β -diOHC	1.95	250	-2.82E-06	0.0021	0.0035	0.980	5.4	-1.3	-28.2	-14.0	17.1	16.5	10.1	12.6	24.6	12.0	-7.9	-17.6	21.9	13.8	11.9	5.8
7 α ,12 α -diOHCone	0.39	50	-1.33E-05	0.0613	0.0101	0.969	-12.8	-12.6	-11.7	-6.7	24.2	4.5	15.3	15.2	-15.8	18.1	-5.3	-14.6	13.7	15.7	24.9	17.5
7-ketochol	1.95	250	-5.97E-07	0.0032	0.0271	0.947	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 β -OHC	3.91	500	-1.82E-06	0.0066	0.0241	0.978	12.8	21.2	1.3	13.9	4.1	7.7	7.2	1.1	-18.4	10.3	0.8	8.7	16.3	17.3	15.4	11.8
7 α -OHC	1.95	250	1.56E-06	0.0042	0.0081	0.944	-13.7	-14.0	7.2	19.0	17.4	6.3	2.1	2.1	-13.0	-10.2	2.7	11.5	19.6	19.5	15.7	10.5
22 (R)-OHC	1.95	250	1.05E-06	0.0028	0.0016	0.991	2.9	3.0	-4.0	-8.9	12.3	10.3	2.3	4.5	20.2	9.7	3.4	1.5	12.0	18.1	8.1	4.7
24 (S)-OHC	1.95	250	-5.63E-06	0.0066	0.0033	0.992	-9.8	10.0	2.5	2.7	5.0	5.5	5.0	2.4	-13.8	-1.3	5.4	6.4	10.1	9.6	5.1	6.9
25-OHC	1.95	250	2.31E-06	0.0035	-0.0002	0.997	12.1	3.7	4.3	18.2	13.0	5.5	7.5	5.0	10.4	-0.8	2.4	0.1	7.9	7.3	3.6	2.5
27-OHC	1.95	250	-5.84E-07	0.0078	-0.0036	0.997	18.8	15.3	2.4	10.5	10.8	7.2	7.7	6.6	17.6	2.2	2.6	-1.5	3.2	4.1	4.9	4.0
7 α -OHCone	1.95	250	1.23E-05	0.0062	-0.0039	0.953	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 α ,6 α -epoxychol	3.91	500	-8.45E-06	0.0227	0.0332	0.990	-4.1	14.4	-8.2	12.8	17.5	10.2	5.4	4.1	-2.0	2.9	-7.9	0.1	7.8	9.3	8.4	9.5
5 β ,6 β -epoxychol	3.91	500	-2.13E-05	0.0451	0.1436	0.982	8.1	30.1	10.6	9.5	9.3	7.2	7.2	1.2	-17.9	2.0	-0.2	-0.8	27.2	16.9	13.7	13.5
24 (S),25-epoxychol	3.91	500	4.57E-07	0.0007	0.0003	0.984	14.2	-1.8	13.8	-4.6	17.0	22.7	8.0	12.8	10.3	16.1	6.2	-4.8	18.3	11.2	10.8	15.2
α -MCA	1.95	250	1.85E-06	0.0048	-0.0039	0.994	28.4	10.2	4.9	-2.1	2.1	2.0	3.2	3.0	19.8	6.5	8.3	1.4	6.2	7.7	5.5	4.2
β -MCA	1.95	250	1.41E-05	0.0023	0.0027	0.967	32.5	26.5	7.1	8.0	2.6	4.5	6.7	2.1	15.6	16.5	4.9	12.4	19.8	7.4	5.6	2.1
ω -MCA	1.95	250	1.33E-05	0.0030	0.0029	0.985	15.4	19.5	8.9	3.9	6.9	4.0	4.9	1.9	3.0	12.1	4.1	8.2	19.9	11.5	5.1	3.6
HCA	1.95	250	-1.29E-06	0.0073	-0.0058	0.997	29.4	12.8	6.8	-7.5	5.9	3.0	3.7	4.2	22.3	5.3	9.9	1.4	7.6	4.6	4.7	4.5
CA	1.95	250	1.49E-05	0.0078	0.0083	0.993	-17.2	2.2	6.7	2.8	2.7	1.5	3.4	1.9	-17.0	-3.6	4.5	2.9	7.2	10.9	5.1	4.1
CDCA	1.95	250	3.09E-05	0.0125	-0.0002	0.997	17.6	14.3	6.3	17.9	4.9	6.0	3.7	2.6	12.4	5.2	7.2	2.1	12.1	5.4	2.7	1.2
HDCA	1.95	250	2.77E-05	0.0035	0.0051	0.989	25.9	17.4	3.7	0.5	4.2	4.2	7.5	1.7	18.2	12.0	4.2	4.6	10.9	8.1	7.7	3.0
MDCA	1.95	250	2.85E-05	0.0079	-0.0011	0.994	-1.6	8.5	8.0	19.9	17.5	4.7	5.0	3.1	8.8	2.2	8.1	2.6	22.8	6.4	4.8	3.3
UDCA	1.95	250	6.53E-05	0.0091	0.0096	0.989	2.4	6.2	1.4	-1.6	4.0	6.2	5.3	2.7	18.6	10.0	3.2	3.7	21.8	10.9	4.7	3.2
DCA	1.95	250	3.56E-05	0.0046	0.0052	0.986	19.4	16.3	4.9	2.1	8.6	4.3	4.7	1.9	22.6	12.8	3.2	5.3	9.9	15.7	3.3	4.5
LCA	1.95	250	2.37E-05	0.0151	0.0010	0.996	-3.3	5.1	5.6	18.6	8.2	5.9	6.2	1.8	15.0	5.7	8.0	3.6	9.0	7.8	4.4	2.9
T α -MCA	3.91	500	7.72E-06	0.0080	-0.0103	0.983	9.9	8.1	1.1	4.3	3.3	4.3	4.1	1.3	-9.0	-9.6	-0.1	-1.5	12.5	13.1	8.9	6.0
T β -MCA	3.91	500	4.53E-06	0.0039	0.0017	0.990	2.6	10.9	8.5	1.9	5.4	2.7	1.3	8.4	-0.3	-1.5	3.9	4.0	16.7	18.5	6.6	5.5
T ω -MCA	3.91	500	2.19E-06	0.0017	0.0018	0.989	12.8	17.0	4.9	1.9	12.7	3.0	6.7	2.8	7.2	13.8	5.3	5.9	14.7	12.8	7.3	4.1
TCA	3.91	500	2.67E-06	0.0063	-0.0028	0.998	6.2	2.0	3.8	18.1	6.6	4.8	3.8	1.6	9.9	3.3	4.8	3.2	12.8	3.5	3.5	1.3
TCDCa	3.91	500	5.32E-06	0.0053	0.0038	0.987	-7.4	2.9	2.5	-2.3	15.2	2.7	2.9	9.8	-6.8	-2.6	2.5	0.4	8.5	13.1	6.6	7.1
THDCA	3.91	500	6.14E-06	0.0035	-0.0002	0.989	-2.6	5.7	4.2	-1.4	15.7	4.7	1.8	9.5	-5.3	-2.4	3.3	0.3	16.5	19.0	3.9	5.9
TUDCA	3.91	500	5.89E-06	0.0029	-0.0036	0.971	-17.2	18.6	6.4	11.0	8.3	3.7	5.0	1.9	-4.0	-10.8	-0.1	0.2	11.7	17.4	11.8	6.5
TDCA	3.91	500	5.56E-06	0.0045	-0.0054	0.979	-7.8	23.5	6.5	10.1	17.9	5.3	6.5	1.5	-8.0	-11.2	-0.9	0.0	12.0	13.9	7.3	5.2
TLCA	3.91	500	7.02E-06	0.0117	0.0071	0.982	-1.8	2.3	4.2	-3.7	18.1	4.9	4.2	11.3	1.6	9.1	0.5	-0.2	9.9	16.2	12.3	10.8
GCA	3.91	500	1.90E-06	0.0061	0.0012	0.997	16.5	3.4	1.1	15.0	8.5	4.0	3.3	1.9	18.5	7.4	4.3	2.2	6.8	5.0	3.5	4.6
GCDCA	3.91	500	-1.08E-06	0.0023	0.0021	0.986	19.9	18.2	8.8	1.3	15.5	6.2	7.3	12.2	8.0	12.4	3.9	6.1	16.8	6.9	17.7	15.9
GHDCA	3.91	500	-1.72E-08	0.0013	0.0002	0.987	9.1	14.1	15.3	1.2	4.5	8.2	4.8	7.9	4.3	2.6	12.6	5.3	20.5	9.6	11.1	8.0
GUDCA	3.91	500	9.41E-07	0.0011	-0.0011	0.989	-11.8	5.7	13.1	15.1	16.0	13.2	4.6	1.8	-7.8	-3.5	6.6	2.0	16.9	11.9	6.4	5.4
GDCA	3.91	500	2.99E-06	0.0068	-0.0045	0.983	-6.3	2.0	2.3	6.8	9.4	4.0	5.9	2.4	4.8	-2.9	6.9	6.8	12.3	11.4	8.5	9.3
GLCA	3.91	500	2.90E-06	0.0013	0.0003	0.975	-16.5	13.7	0.8	14.6	20.2	3.4	10.6	1.8	3.4	9.9	7.3	8.1	17.3	7.0	6.5	6.0
THCA	3.91	500	-1.91E-06	0.0099	-0.0041	0.997	15.7	-2.5	-6.6	-5.4	3.5	4.0	2.8	1.8	17.6	3.9	2.6	0.1	3.6	2.6	4.0	5.8

This table shows the dynamic range (pmol per sample) and parameters of each calibration curve, as well as the within-run and between-run accuracies (expressed as % bias), and precisions (expressed as % CV).

TABLE 5. Accuracies and precisions of the developed method in diluted human plasma

Name	Within-run accuracy (% bias)		Within-run precision (% CV)		Between-run accuracy (% bias)		Between-run precision (% CV)	
	Q1	Q2	Q1	Q2	Q1	Q2	Q1	Q2
7-keto-25-OHC	-10.5	6.1	3.5	2.9	17.0	15.1	2.7	6.1
7-keto-27-OHC	-26.9	0.0	5.0	4.7	17.2	28.6	3.6	11.1
7 α ,24 (S)-diOHC	-2.3	-0.6	3.9	6.5	-9.0	-12.2	14.6	17.2
7 α ,25-diOHC	-9.4	-6.8	2.2	6.0	12.1	10.4	10.0	9.6
7 α ,27-diOHC	-12.3	-7.4	10.8	6.0	5.5	11.4	10.2	10.7
5 α ,6 β -diOHC	-31.9	-21.2	1.9	3.5	4.5	10.6	5.8	11.0
7 α ,12 α -diOHCone	-26.9	0.0	5.0	4.7	17.2	28.6	3.6	11.1
7-ketochol	-5.7	-16.2	10.1	3.9	-10.9	9.2	22.6	13.6
4 β -OHC	13.4	2.3	4.9	9.2	6.6	5.6	11.6	13.0
7 α -OHC	-	-	-	-	-	-	-	-
22 (R)-OHC	-1.4	-0.6	3.2	3.8	14.3	12.7	6.0	8.0
24 (S)-OHC	-14.1	-18.4	1.3	4.5	7.4	9.7	7.3	8.6
25-OHC	-12.8	-11.4	1.3	3.5	6.8	9.5	8.7	12.5
27-OHC	-8.4	-10.7	1.8	2.7	3.7	8.7	7.5	10.5
7 α -OHCone	-12.1	-3.1	1.9	2.1	4.4	15.5	10.3	11.0
5 α ,6 α -epoxychol	6.6	-9.5	8.9	10.6	15.4	-6.9	23.1	24.7
5 β ,6 β -epoxychol	-5.7	-17.3	8.9	9.5	16.4	10.0	30.1	28.1
24 (S),25-epoxychol	-4.3	9.1	9.3	7.7	6.6	18.0	7.5	10.1
α -MCA	-0.1	-7.1	3.6	2.9	-6.4	-1.7	10.0	10.5
β -MCA	3.7	-4.5	4.1	3.1	-4.4	-0.5	8.7	7.8
ω -MCA	-2.2	-7.5	3.4	2.7	-7.4	-2.9	6.6	7.0
HCA	-9.4	-11.1	3.6	2.9	-9.9	-0.8	11.7	13.0
CA	-4.9	-1.2	0.9	2.1	-2.4	-0.3	9.2	7.9
CDCA	-13.3	-11.8	2.6	0.9	-5.3	2.1	10.1	9.4
HDCA	-17.9	-19.4	3.2	2.2	-8.3	0.8	11.5	14.0
MDCA	-17.0	-18.8	3.3	4.0	-13.4	-5.0	8.6	11.6
UDCA	-22.3	-17.7	3.3	3.1	-13.2	-2.0	10.5	14.8
DCA	-38.0	-23.0	1.8	15.1	-8.8	-4.4	11.9	10.5
LCA	-6.4	-8.4	3.0	4.2	-1.5	2.7	1.7	3.6
T α -MCA	-12.5	-7.8	2.7	1.2	-9.6	-6.1	2.7	5.1
T β -MCA	-13.4	-13.0	3.0	2.5	-10.4	-2.9	3.3	9.6
T ω -MCA	-14.2	-10.8	4.7	1.8	-7.7	-5.3	4.9	5.8
TCA	-7.0	-6.0	1.0	1.8	-2.3	-0.5	2.1	4.0
TCDCa	-23.6	-18.6	0.5	0.9	-5.6	-3.6	1.9	8.3
THDCA	-25.7	-4.9	2.9	2.2	-10.6	-4.9	3.4	13.0
TUDCA	-18.4	-14.3	2.7	1.2	-6.5	-3.1	2.3	10.1
TDCA	-25.2	-21.4	1.9	1.6	-6.8	-2.9	1.9	9.3
TLCA	-14.6	-13.1	0.8	1.1	5.1	-0.2	2.0	3.7
GCA	-10.6	-8.1	0.5	3.0	-7.3	-5.2	11.7	10.1
GCDCA	-13.3	-2.9	6.9	4.8	-0.7	1.3	15.7	15.8
GHDCA	-12.8	-14.1	4.1	2.8	4.2	0.0	13.3	11.5
GUDCA	-16.9	-15.1	3.3	1.8	-4.1	-8.1	10.6	9.4
GDCA	-17.4	-17.5	1.2	5.9	-0.6	-2.2	9.0	8.7
GLCA	2.5	-4.8	7.1	2.2	8.2	0.5	10.0	7.8
THCA	4.3	4.9	1.5	2.9	-6.3	-6.0	11.2	8.7

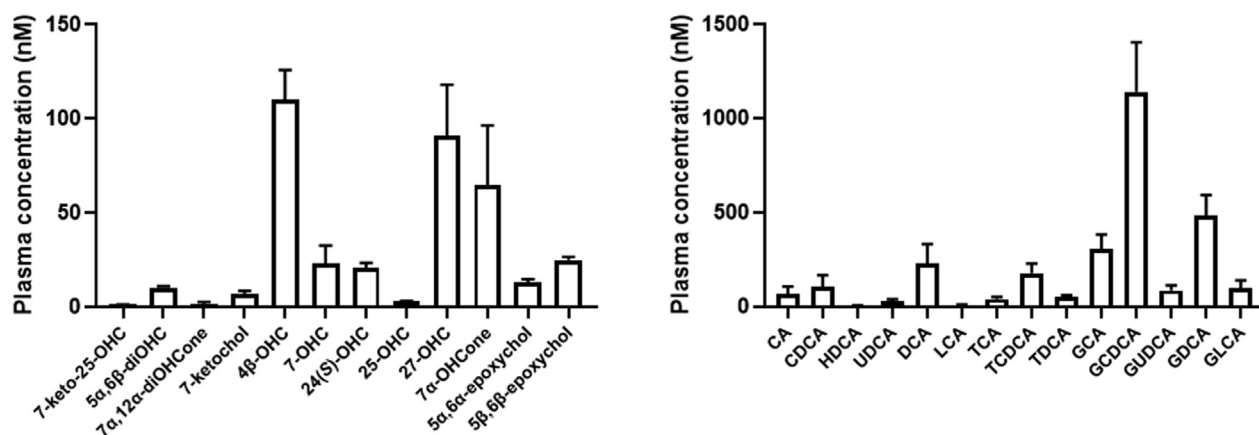
This table shows the within-run and between-run accuracies (expressed as % bias) and precisions (expressed as % CV) of the described method in diluted human plasma (20 μ l in 180 μ l PBS). Spiked amounts (Q1 and Q2) of pure standards are available in [supplemental Table S1](#).

expected, such as between 7-hydroxycholesterol and its direct metabolite 7 α -hydroxycholestenone ($r = 0.89$) or between primary bile acids CA and CDCA ($r = 0.94$), but others are more surprising, such as between 24(S)-hydroxycholesterol and tauroconjugated bile acids ($r = 0.83$ – 0.89), or the negative correlation between 4 β -hydroxycholesterol and glycoconjugated bile acids ($r = 0.43$ – 0.83). Using results from mouse plasma from the vena cava, we globally observe positive correlations between bile acids, and negative correlations between oxysterols and bile acids ([supplemental Fig. S2](#)). While the method we developed allows to establish potentially interesting correlations, broader studies should be conducted to elucidate the nature of these correlations and explore the underlying mechanisms.

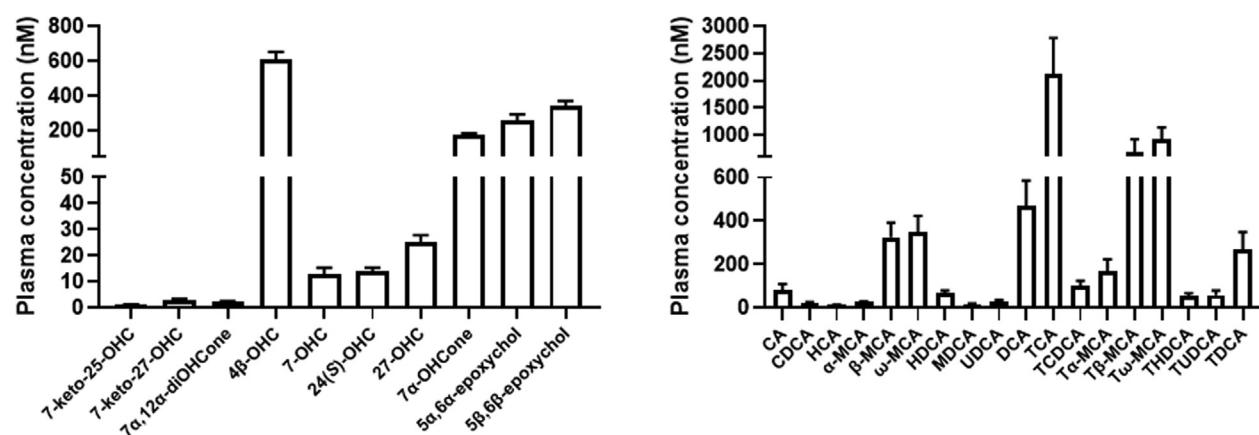
DISCUSSION

In this article, we report the development of an analytical method for the simultaneous quantification of oxysterols and bile acids. After investigating three coextraction protocols for oxysterols and bile acids, namely the acid liquid-liquid extraction, the salting-out liquid-liquid extraction and the protein precipitation, we then focused on the purification of our samples to eliminate hydrophobic compounds including cholesterol. This step is crucial as cholesterol may artificially produce oxysterols by autooxidation during the analytical process. We also worked on the chromatographic separation using liquid chromatography. Despite the high number of isomers and the wide range of polarity

A



B



C

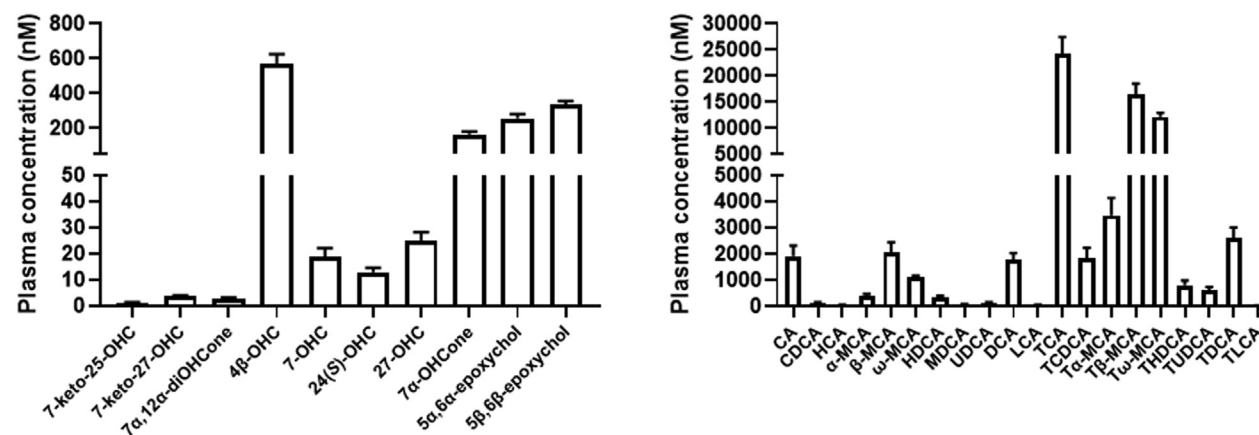


Fig. 6. Oxysterol and bile acid levels in human and mouse plasma. The described LC-MS/MS method for the combined quantification of oxysterols and bile acids was applied to the analysis of (A) human plasma (200 μ l) from six donors (4 females and 2 males), as well as (B) plasma from the vena cava and (C) plasma from the portal vein (50 μ l) from six C57BL/6J male mice. Figures from each matrix were split in two graphs for clarity purposes and illustrate oxysterol and bile acid quantified from a single chromatographic run. Common names of analytes are available in [Table 2](#). Calculated concentration of 7-OHC is the addition of 7 α -hydroxycholesterol and 7 β -hydroxycholesterol, as these analytes could not be separately analyzed in this method, in contrary to 4 β -hydroxycholesterol, which was chromatographically separated from 4 α -hydroxycholesterol (RRT = 2.48 and RRT = 2.37, respectively). Analytes absent from this figure were not detected using the described method. All numeric values are available in [supplemental Table S7](#). N = 6, n = 1–3. RRT, relative retention time.

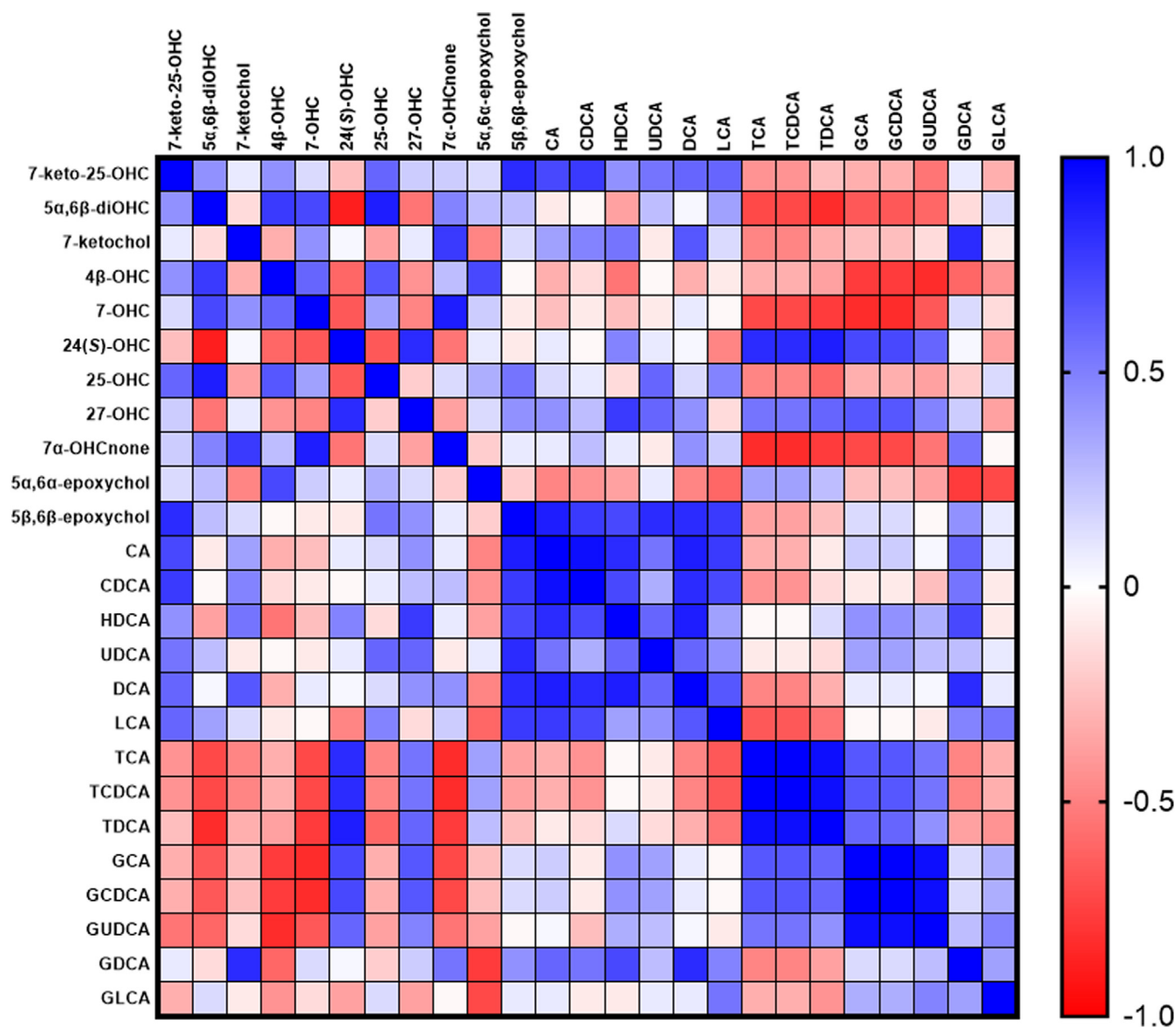


Fig. 7. Spearman correlations of oxysterol of bile acid levels quantified in human plasma. Positive correlations (r) are in *blue*, whereas negative correlations are in *red*. 7 α ,12 α -diOHCone was not detected in each donor's plasma and is therefore not included in the figure. $N = 6$, $n = 3$.

across our analytes complicating their coextraction, purification, and separate detection, we managed to develop an LC-MS/MS method for the sensitive detection of the 53 lipids of interest including eight deuterated internal standards using a tandem quadrupole mass spectrometer. Internal standards were selected to cover the wide range of polarity across the analytes, from hydrophilic conjugated bile acids to hydrophobic oxysterols, including a C_{27} bile acid precursor. However, only two of these deuterated standards are oxysterols (d_7 -4 β -hydroxycholesterol and d_7 -24(*R/S*)-hydroxycholesterol). Although these oxysterols were selected based on the position of their hydroxy moiety (ie, on the sterol backbone or its side chain), this could be regarded as a limitation of the method. Indeed, when commercially available, using isotope-labeled

internal standard with the same chemical structure as the analyte should be regarded as a best practice for LC-MS methods, as the coelution of the internal standards with the molecules of interest ensures a better performance of the method.

Nevertheless, we assessed within-run accuracies and precisions for most compounds of interest in a surrogate matrix (PBS) as well as in a diluted matrix (human plasma), based on the ICH guideline M10 on bioanalytical method validation, and investigated carry-over effects which were deemed negligible. We also assessed the recovery and matrix effect of the analytes using the surrogate analyte approach for eight deuterated standards. This approach provides highly reliable results but is limited by the commercial availability of stable isotope-labeled analogs. Indeed, although the observed

recoveries are fully taken into account as the calibration curves of this method are submitted to the entire analytical process, different matrix effects could be observed for individual analytes. Finally, we applied this quantification method to the analysis of clinical and preclinical samples, successfully quantifying 12 oxysterols and 14 bile acids in human plasma, 10 oxysterols and 18 bile acids in mouse plasma from the vena cava, and 10 oxysterols and 20 bile acids in mouse plasma from the portal vein within a single chromatographic run.

The measured levels of oxysterols and bile acids are consistent with the data from the literature, with the most abundant oxysterols found in human being 4 β -hydroxycholesterol, 27-hydroxycholesterol, and 7 α -hydroxycholestenone, the most abundant conjugated bile acids being glycochenodeoxycholic acid, glycodeoxycholic acid, and the most abundant unconjugated bile acids being deoxycholic acid and chenodeoxycholic acid. In mouse plasma, the most abundant oxysterol is 4 β -hydroxycholesterol, with little difference between plasma from the vena cava or the portal vein. On the other hand, we observe significant differences in bile acid levels in mouse plasma, with increased levels in the portal vein carrying bile acids reabsorbed from the gut, compared to plasma from the vena cava. While these results represent a major step forward in sterol analysis, not all the analytes described in the method were detected in our samples. This is expected, as the method was developed to analyze both human and murine samples from various matrices. Considering the wide range of concentrations between tissues, notably due to the enterohepatic circulation of bile acids, it is expected to find highly different analyte profiles depending on the matrix of interest (eg, plasma from vena cava or portal vein, liver, and feces).

Analytical methods for oxysterol or for bile acid analysis have already been reported. However, the sequential analysis of oxysterols and bile acids demands significant resources and time. Moreover, the implementation of two distinct analytical methods for these lipids often requires higher amounts of tissue, which is not always desirable, especially when analyzing precious samples. A few methods have already been reported for the concurrent analysis of oxysterols and bile acids (26–29). However, they are often limited to the most abundant molecules, provide poor chromatographic separation of isomers, or involve sample fractionation and subsequent derivatizations, hindering a direct and thorough analysis of these lipids.

Method validation, albeit a complex and imperfect process, especially for closely related endogenous compounds, is important as it enables the determination of key parameters such as accuracy, precision, and sensitivity. In this article, we chose to base our validation experiments on the recently harmonized ICH guideline M10 on bioanalytical method validation. However, it should be noted that validation guidelines are primarily established for the detection of a few

exogenous compounds, and not for dozens of closely related endogenous analytes. Therefore, in our field, it is not uncommon to see rigorous, yet unvalidated analytical methods, or method validations based on different guidelines but using their own acceptance criteria. While our results do not fully comply with the acceptance criteria of the ICH guideline M10 on bioanalytical method validation, we deem them satisfactory, considering the number, the endogenous nature, and physicochemical differences among our analytes. Nevertheless, our results demonstrate the high sensitivity of the method, with LLOQs ranging from 0.39 to 3.91 pmol per sample. Considering the injection volume, these LLOQs correspond to less than 60–600 fmol on column, allowing for the quantification of a new range of analytes in minute amounts.

The development of this innovative method for the analysis of neutral oxysterols, as well as unconjugated, taurine-conjugated and glycine-conjugated bile acids represents a powerful tool for the field of sterols. Indeed, it will support more frequent simultaneous analysis of these lipids, facilitating research on the interconnections between oxysterols and bile acids, and on their roles in physiological and pathological contexts. As a matter of fact, a more systematic and comprehensive analysis of oxysterols and bile acids could provide invaluable insights into the preclinical study of physiological and pathological processes, considering the close relationship between these lipid families. Moreover, the described method could serve as a compelling tool in a clinical setting, potentially enabling better diagnosis of diseases linked to deficiencies in bile acid synthesis.

Data availability

All data are contained within the manuscript and supporting information. 

Supplemental data

This article contains [supplemental data](#).

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Author contributions

M. R. and G. G. M. methodology; M. R. and G. G. M. validation; M. R. and G. G. M. formal analysis; M. R. and G. G. M. investigation; M. R. and G. G. M. data curation; M. R. and G. G. M. writing—original draft; M. R. and G. G. M. writing—review and editing; M. R. and G. G. M. visualization; G. G. M. conceptualization; G. G. M. resources; G. G. M. supervision; G. G. M. project administration; G. G. M. funding acquisition.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

APCI, atmospheric pressure chemical ionization; BHT, butylated hydroxytoluene; CA, cholic acid; CDCA, chenodeoxycholic acid; GCA, glycocholic acid; ESI, electrospray ionization; HRMS, high resolution mass spectrometry; ICH, International Council for Harmonisation; LCA, lithocholic acid; LLOQ, lower limit of quantification; MCA, muricholic acid; MRM, multiple-reaction monitoring; TCA, taurocholic acid; ULOQ, upper limit of quantification; UDCA, ursodeoxycholic acid.

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