

Comprehensive characterization of cholesterol oxidation products formed during thermal processing through targeted quantification and untargeted annotation

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Abstract

Cholesterol oxidation products (COPs) are generated during the thermal processing of cholesterol-containing foods and have been implicated in a variety of biological processes. This study established a reproducible method for generating COPs from cholesterol and comprehensively characterized the resulting oxidation products. Pure cholesterol powder was converted to a thin film by rotary evaporation and heated at 145°C for 20 hours. Lipid peroxidation was measured by spectrophotometric assay, while six major COPs were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using authentic standards. Untargeted feature detection, annotation, and molecular networking were performed using full-scan MS1 and data-dependent MS/MS spectra processed through MZmine and GNPS feature-based molecular networking (FBMN). Thermal treatment increased lipid peroxide levels ~3.5-fold relative to unheated cholesterol and promoted the formation of all six targeted COPs. 7-ketocholesterol was the predominant oxidation product, followed by epoxycholesterols and hydroxycholesterols, whereas only trace levels were detected in unheated controls. Untargeted LC-MS/MS analysis identified 921 aligned features and revealed substantial chemical diversification following thermal oxidation. MS/MS library matching supported the annotation of additional sterol-derived compounds, including 7 α -hydroxy-4-cholesten-3-one and cholest-4,6-dien-3-one, while FBMN further revealed structurally related molecular families enriched in heated cholesterol and linked annotated COPs to numerous unannotated sterol-associated features. Together, these findings demonstrate that thermal oxidation of pure cholesterol generates a chemically complex mixture that extends beyond the limited set of COPs typically monitored in targeted analysis. This workflow provides a reproducible and analytically characterized approach for generating and profiling COP mixtures for future food chemistry and biological studies.

1. Introduction

Cholesterol is a naturally occurring lipid found in a wide array of animal-based foods, including meat, eggs, and dairy products. Fundamental for maintaining cell membrane integrity, cholesterol also serves as a precursor for steroid hormones, bile acids, and vitamin D3 (Craig et al. 2026). However, due to the presence of a reactive double bond at the C5-C6 position and an allylic hydrogen at C7, cholesterol in food is highly susceptible to oxidative degradation, particularly under conditions involving heat, oxygen, light exposure, or prolonged storage under suboptimal conditions (Schroepfer 2000). This oxidative transformation yields a diverse array of bioactive derivatives collectively referred to as cholesterol oxidation products (COPs) (Schroepfer 2000). These compounds differ structurally from native cholesterol and possess potent cytotoxic, pro-inflammatory, and pro-atherogenic properties (Vejux and Lizard 2009; Poli et al. 2013).

Modern food systems are increasingly dominated by ultra-processed foods (UPFs), which are often rich in cholesterol and frequently subjected to thermal processing that can accelerate the formation of COPs (Monteiro et al. 2013). Elevated dietary intake of UPFs has been associated with adverse health outcomes, including endothelial dysfunction and progression of metabolic diseases (Zarrouk et al. 2014; Grinshpan et al. 2024; Lane et al. 2024). Among the known COPs, 7-ketocholesterol (7-KC), hydroxycholesterols, and epoxycholesterols are the most frequently detected in both food and biological matrices (Vine et al. 1998; Derewiaka and Molińska (née Sosińska) 2015). Despite the growing recognition of the pathophysiological roles of COPs, systematic induction and quantification under controlled experimental conditions remain challenging. Thermal oxidation has emerged as a useful model for studying the formation kinetics and COP profiles; however, methodology remains inconsistent and poorly described. For instance, prior studies have varied widely in heating temperatures (ranging from 100°C to over 200°C), exposure times (from minutes to several days), oxygen availability, and sample preparation techniques, such as whether cholesterol was oxidized in bulk powder, in solution, or as a thin film (Vine et al. 1998; Derewiaka and Molińska (née Sosińska) 2015; Min et al. 2015). Aside from this, most studies used Gas Chromatography-Mass Spectrometry for COP analysis, which requires derivatization and may cause thermal degradation of labile COPs, leading to reduced accuracy and potential formation of artifacts. These discrepancies have resulted in significant variability in reported COP yields and profiles, making cross-study comparisons difficult, hindering reproducibility (McDonald et al. 2012).

High-resolution LC-MS/MS enables the integration of targeted quantification of known COPs with untargeted detection and annotation of additional oxidation products. While authentic standards allow high-confidence identification and quantification of selected COPs, thermal oxidation generates a chemically complex mixture of sterol-derived products that extends beyond those routinely included in targeted analytical panels. This limitation reflects both the limited availability of authentic standards and the incomplete structural characterization of many oxidation products. Furthermore, many oxidation products share identical elemental compositions and precursor masses, making structural annotation challenging when relying solely on targeted approaches. In this context, untargeted workflows incorporating feature detection, MS/MS spectral-library matching, and feature-based molecular networking (FBMN) provide complementary tools for identifying structurally related oxidation products and exploring their broader chemical landscape.

Therefore, the objectives of this study were to establish a reproducible thin-film thermal oxidation protocol for purified cholesterol and to comprehensively characterize the resulting oxidation profile using targeted and untargeted analyses. Specifically, we quantified six major COPs using authentic standards and evaluated lipid hydroperoxide formation. We also applied feature detection, spectral-library matching, and molecular networking to identify additional thermally generated sterol-derived products. We demonstrate that thin-film thermal oxidation of purified cholesterol produces a reproducible and chemically complex mixture of COPs, encompassing both major oxidation products and a broader spectrum of additional sterol-derived compounds revealed through untargeted LC-MS/MS and molecular networking. Together, these findings provide a reproducible and analytically characterized COP preparation while highlighting the substantial chemical diversity generated during cholesterol oxidation, supporting future food chemistry, food safety, and mechanistic biological studies of COPs.

2. Materials and methods

2.1. Preparation of COPs from cholesterol

Purified cholesterol (500 mg; Millipore Sigma, Catalog #C3045) was dissolved in 50 mL chloroform (Millipore Sigma, ACS grade, Catalog #319988) in a 500-mL round-bottom flask. The solvent was removed under vacuum using a rotary evaporator (35–40 rpm, 40°C water bath) to generate a uniform thin film of cholesterol on the inner surface of the flask. The dried cholesterol film was subsequently heated at 145°C for 20 h, resulting in the formation of a semi-solid amber-colored material enriched in COPs. This temperature was selected because it consistently generated abundant COPs while preserving sufficient residual cholesterol for quantitative analysis. For LC-MS/MS analysis, a portion of the heated material was dissolved in isopropanol at 2 mg/mL. For lipid hydroperoxide analysis, a separate aliquot was dissolved in chloroform (7:3, v/v) at 10 mg/mL. Unheated cholesterol was processed in parallel and served as a control for all analyses.

2.2. Lipid hydroperoxide determination

Lipid hydroperoxide (LOOH) concentrations were determined according to the iron-based spectrophotometric method of Shantha and Decker with minor modifications (Shantha and Decker 1994). Briefly, lipid samples were diluted to a final concentration of 1 mg/mL in chloroform (7:3, v/v), and 25 μ L of each aqueous reagent was added according to the original protocol. Absorbance was measured at 500 nm using a UV-1280 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Lipid hydroperoxide concentrations were quantified using a standard curve prepared from cumene hydroperoxide (0–40 μ mol/L) and expressed as mmol hydroperoxide per kg cholesterol.

2.3. LC-MS/MS data acquisition

2.3.1. LC-MS/MS analysis

Cholesterol and COPs were analyzed using an Agilent 1290 Infinity II liquid chromatography system coupled to an Agilent 6546 quadrupole time-of-flight (Q-TOF) mass spectrometer. Chromatographic separation was achieved on a Waters BEH C18 column (2.1 $\times$ 100 mm, 1.7 μ m particle size). Mobile phase A consisted of 60% acetonitrile and 40% water containing 0.1% formic acid and 10 mM ammonium formate, while mobile phase B consisted of 90% isopropanol, 9% acetonitrile, and 1% water containing 0.1% formic acid and 10 mM ammonium formate. The injection volume was 2 μ L. Quantification was performed using high-resolution full-scan acquisition (m/z 50–1500) together with data-dependent MS/MS fragmentation of the most abundant precursor ions exceeding an intensity threshold of 1×10^4 counts to support both targeted quantification and untargeted molecular networking analyses. Quantification of cholesterol and COPs was performed using extracted ion chromatograms generated from diagnostic precursor ions identified using authentic standards. Compound identities were confirmed through agreement in accurate mass (<5 ppm mass error), retention time, and MS/MS fragmentation patterns.

2.3.2. Targeted quantification of COPs

Six-point calibration curves were generated for cholesterol and each of the six COP standards, including 7-ketocholesterol (7-KC) (Sigma-Aldrich), 5 α ,6 α -epoxycholesterol (Cayman Chemicals), 5 β ,6 β -epoxycholesterol (Cayman Chemicals), 7 α -hydroxycholesterol (Cayman Chemicals), 7 β -hydroxycholesterol (Cayman Chemicals), and cholest-4-en-3-one (Cayman Chemicals), using 0, 1.56, 3.125, 6.25, 12.5, and 25 ng injected on-column. All standards were analyzed in triplicate. Cholesterol-d7 (Avanti Polar Lipid) was used as an internal standard and added at the same concentration to all standards and samples.

For targeted COP quantification, 200 ng of sample was injected for both heated and unheated cholesterol preparations. For cholesterol quantification, 10 ng and 20 ng were injected for unheated and heated cholesterol samples, respectively, to ensure detector responses remained within the linear range of the calibration curves. Data processing, peak integration, and quantification were performed using Skyline software (<https://skyline.ms/project/home/software/Skyline/begin.view>). A custom analyte library containing cholesterol and all target COPs was used for automated peak identification and integration. Quantification was performed using linear regression with the intercept constrained to zero. Compound identities were assigned based on concordance among accurate mass, retention time, and MS/MS fragmentation spectra. All raw and processed LC-MS/MS data have been deposited in the MassIVE repository under accession number MSV000099229.

2.3.3. Untargeted profiling and annotation of COPs via MZmine and GNPS feature-based molecular networking

Untargeted profiling of COPs was performed in positive-ion mode using an open-source data-processing pipeline applied identically to all injections (2 μ L injection volume; two technical replicates each of heated 1000 μ g/mL, heated 100 μ g/mL, and unheated 10 μ g/mL cholesterol). Raw data files were converted to the open mzML format with msconvert (ProteoWizard), applying vendor centroiding to the MS1 and MS/MS scans. Data were processed in MZmine 4.9.14 using a workflow consisting of mass detection, chromatogram construction, feature deconvolution, alignment, gap filling, and blank subtraction, followed by spectral-library matching. Following filtering and alignment, an aligned feature table containing approximately 921 features across all samples was generated.

Within MZmine, putative compound annotations were assigned by matching experimental MS/MS spectra against the public MSMS-Public spectral library (version VS19), requiring a minimum cosine similarity of 0.70 and at least six matched fragment ions. All feature-level annotations reported in this study (Table 2) derive from this MZmine MSMS-Public VS19 search; annotation confidence levels were assigned according to the framework proposed by Schymanski et al. (Schymanski et al. 2014).

To further evaluate structural relationships among detected features, the aligned MZmine feature table and associated MS/MS spectra were exported to GNPS (<https://gnps.ucsd.edu>) for Feature-Based Molecular Networking (FBMN) (Nothias et al. 2020). FBMN was used here solely as a complementary tool for visualizing structural relatedness among features and did not contribute to the annotations reported in this study. Prior to networking, MS/MS spectra were filtered by removing fragment ions within ± 17 Da of the precursor ion and retaining the six most intense fragment ions within each ± 50 Da window. Precursor and fragment ion mass tolerances were both set to 0.02 Da. Network edges (Feature-to-feature matching) required a cosine similarity score above

0.70 and at least six matched fragment ions, and were retained only when each node appeared within the other's ten most similar neighbors. Molecular families were limited to a maximum of 100 nodes by iterative removal of the lowest-scoring edges. As part of the standard FBMN workflow, network spectra were searched against the built-in GNPS spectral libraries (Wang et al. 2016) (Horai et al. 2010) and annotated using DEREPLICATOR (Mohimani et al. 2018), using the same cosine and match-ion thresholds; these network-level matches (Feature-to-library matching) were used only to validate our prior annotations thus were not reported. Molecular networks were visualized in Cytoscape (version 3.10.4) (Shannon et al. 2003).

2.4. Statistical analysis

LOOH concentrations and targeted LC-MS/MS quantification data are presented as means ± standard deviation (SD). LOOH measurements were performed in duplicate for each sample. Heated cholesterol values represent two independently prepared thermally oxidized cholesterol preparations (biological replicates), while unheated cholesterol values represent technical replicates of the procured purified cholesterol. For targeted LC-MS/MS analysis, each independently prepared heated cholesterol sample was analyzed separately (n = 2 biological replicates), and values are reported as means ± SD. Untargeted LC-MS/MS analyses were performed using two technical replicate injections for each sample group (heated cholesterol at 1000 µg/mL, heated cholesterol at 100 µg/mL, and unheated cholesterol at 10 µg/mL). Feature abundance summaries, overlap analyses, and principal component analysis (PCA) were performed in Python (version 3.11.15) using the aligned feature table generated by MZmine (version 4.9.14).

3. Results

3.1 Lipid hydroperoxide analysis

Cholesterol is a sterol lipid that can undergo oxidation to form LOOHs, the primary products of lipid peroxidation. LOOH was measured in duplicate in each of two independent heated cholesterol preparations and in an unheated control sample, with values presented as means ± SD. As shown in Table 1, LOOH concentrations were ~ 3.5-fold higher in heated cholesterol (4.21 ± 0.04 mmol/kg) than in unheated cholesterol (1.19 ± 0.05 mmol/kg), confirming that thermal treatment drives primary lipid oxidation.

Table 1
Lipid peroxide concentrations and percentage in unheated and heated cholesterol.

Description	Lipid peroxide (mmol/kg cholesterol)	Lipid peroxide (%)
Unheated cholesterol	1.19 ± 0.05	0.04 ± 0.001
Heated cholesterol	4.21 ± 0.04	0.13 ± 0.01
<i>Data represent means ± SD. Unheated variation = Two technical replicates of the procured chemical; heated variation = two separate preparations (145°C, 20 h).</i>		

3.2 Targeted quantification of cholesterol and COPs

Cholesterol and six targeted COPs were quantified by LC-MS/MS using authentic standards. All targeted analytes met Schymanski Level 1 identification criteria based on agreement in accurate mass, retention time, and MS/MS fragmentation spectra (Schymanski et al. 2014) (13). Compound-specific precursor ions, adducts, retention times, confidence levels, and identification approaches are summarized in Table 2. Cholesterol, hydroxycholesterols, and epoxycholesterols were quantified using [M + H-H₂O]⁺ ions, whereas 7-KC and cholest-4-en-3-one were quantified using [M + H]⁺ ions. The isomeric compounds 7α- and 7β-hydroxycholesterol co-eluted under the chromatographic conditions employed (RT = 4.5 min) and were therefore reported as a combined value. Representative extracted-ion chromatograms for authentic standards and experimental samples are provided in **Figure S1**.

Table 2. Cholesterol and cholesterol oxidation products: identification and quantification summary across confidence levels.

Compound	Formula	Theor. m/z	Detected m/z	$\Delta m/z$ (ppm)	RT (min)	Adduct	^a Conf. level	Identification method	^b Std. quant % heated	Std. quant % unheated	^c Peak area heated 1000
Authentic standards — quantified against calibration curves (Schymanski Level 1)											
Cholesterol	C ₂₇ H ₄₆ O	369.3516	369.35	<5	6.9	[M+H-H ₂ O] ⁺	1	Authentic standard + RT + MS ²	33.18 ± 4.82	102.5 ± 13.0	—
7-Ketocholesterol (7-KC)	C ₂₇ H ₄₄ O ₂	401.3414	401.342	<5	4.6	[M+H] ⁺	1	Authentic standard + RT + MS ²	8.51 ± 1.04	0.008 ± 0.008	—
5 α ,6 α -Epoxycholesterol	C ₂₇ H ₄₆ O ₂	385.3465	385.347	<5	5.4	[M+H-H ₂ O] ⁺	1	Authentic standard + RT + MS ²	2.18 ± 1.31	0.004 ± 0.001	—
5 β ,6 β -Epoxycholesterol	C ₂₇ H ₄₆ O ₂	385.3465	385.347	<5	5.3	[M+H-H ₂ O] ⁺	1	Authentic standard + RT + MS ²	3.65 ± 2.02	0.004 ± 0.003	—
^d 7 α /7 β -Hydroxycholesterol	C ₂₇ H ₄₆ O ₂	385.3465	385.347	<5	4.5	[M+H-H ₂ O] ⁺	1	Authentic standard + RT + MS ²	1.90 ± 0.12	0.015 ± 0.002	—
Cholest-4-en-3-one	C ₂₇ H ₄₄ O	385.3465	385.347	1.4	6.51	[M+H] ⁺	1	Authentic standard + RT + MS ²	0.53 ± 0.28	0.004 ± 0.004	5.03e+05
Untargeted spectral library matches (Level 2)											
7 α -Hydroxy-4-cholesten-3-one	C ₂₇ H ₄₄ O ₂	401.3414	401.343	3.6	4.68	[M+H] ⁺	2a	MS-DIAL spectral library MS ² match (MSMS-Public VS19, cos 0.78)	—	—	9.74e+05
Cholest-4,6-dien-3-one	C ₂₇ H ₄₂ O	383.3308	383.333	4.6	6.22	[M+H] ⁺	2a	MS-DIAL spectral library MS ² match (MSMS-Public VS19, cos 0.85)	—	—	2.01e+06
Putative class-level isomers (Level 3)											
Putative C ₂₇ H ₄₄ O ₂ isomers (n=6)	C ₂₇ H ₄₄ O ₂	401.3414	401.342	<5	5.13, 6.53, 12.79, 14.33, 14.50, 14.64	[M+H] ⁺	3	Accurate mass + MS ² in COP network (oxidized sterol class)	—	—	^e see footnote
Putative C ₂₇ H ₄₆ O isomers (n=8)	C ₂₇ H ₄₆ O	385.3465	385.347	<5	4.62, 4.95, 5.40, 5.48, 13.04, 14.22, 14.53, 14.67	[M+H] ⁺	3	Accurate mass + MS ² in cholesterol network (sterol ketone/diene)	—	—	^f see footnote

^a Confidence (Conf.) levels following Schymanski et al. (13), where Level 1 = confirmed structure (authentic standard + RT + MS²), Level 2a = probable structure by MS² library match, and Level 3 = tentative candidate(s) narrowed to a chemical class/substructure.

^b Std. quant % = analyte mass relative to total measured analyte (calibration curves of authentic standards, Skyline targeted analysis, n=2 biological replicates, mean ± SD).

Heated values are % of total analytes in heated cholesterol; unheated values are estimates as concentrations fell below the lowest calibration point.

^c Peak area heated 1000 = mean MS¹ feature area in heated 1000 µg/mL replicates from the MZmine-aligned untargeted run (n=2). This is a separate chromatography run from the targeted analysis; values are not interchangeable with Std. quant %.

^d 7 α - and 7 β -hydroxycholesterol co-eluted and are reported as a combined value.

^e Putative C₂₇H₄₄O₂ peak areas (heated 1000): id 426 (RT 5.13) 2.92e+05; id 514 (RT 6.53) 3.23e+04; id 748 (RT 12.79) 4.18e+04; id 873 (RT 14.33) 6.69e+04; id 893 (RT 14.50) 2.23e+04; id 912 (RT 14.64) 9.69e+04.

^f Putative $C_{27}H_{46}O$ peak areas (heated 1000): id 325 (RT 4.62) $4.02e+04$; id 402 (RT 4.95) $4.29e+04$; id 447 (RT 5.40) $1.70e+05$; id 455 (RT 5.48) $8.89e+04$; id 770 (RT 13.04) $6.15e+04$; id 867 (RT 14.22) $4.89e+04$; id 897 (RT 14.53) $1.02e+05$; id 918 (RT 14.67) $3.20e+04$

ND = not detected in unheated samples.

Unheated cholesterol contained only trace amounts of COPs. In contrast, thermal treatment generated measurable levels of all six targeted COPs and substantially reduced the proportion of native cholesterol. Cholesterol accounted for virtually all measured sterols in unheated samples ($102.5 \pm 13.0\%$ of total measured analytes, reflecting analytical variability) but decreased to $33.18 \pm 4.82\%$ following heating, indicating extensive oxidative conversion (Table 2). Among the quantified COPs, 7-KC was the predominant oxidation product, representing $8.51 \pm 1.04\%$ of total analytes in heated cholesterol. $5\beta,6\beta$ -epoxycholesterol and $5\alpha,6\alpha$ -epoxycholesterol accounted for $3.65 \pm 2.02\%$ and $2.18 \pm 1.31\%$ of total analytes, respectively, while combined $7\alpha/7\beta$ -hydroxycholesterol represented $1.90 \pm 0.12\%$. Cholest-4-en-3-one was detected at lower abundance ($0.53 \pm 0.28\%$). Collectively, the six quantified COPs accounted for approximately one-fourth of the quantified sterol pool in the heated samples, indicating that a large proportion of the oxidation-derived sterol species were not represented in the targeted analytical panel. These findings demonstrate that thermal oxidation substantially depletes native cholesterol while generating a chemically diverse mixture of oxidation-derived sterol products. Consequently, subsequent untargeted LC-MS/MS analyses were performed to further characterize the resulting sterol oxidation profile.

3.3 Untargeted feature profiling and group separation

To further characterize thermally generated features beyond those included in the targeted COP panel, all samples were subjected to untargeted LC-MS/MS feature extraction and alignment using MZmine. A total of 921 unique aligned features were detected across all six injections (two technical replicates each of heated cholesterol at $1000 \mu\text{g/mL}$, heated cholesterol at $100 \mu\text{g/mL}$, and unheated cholesterol at $10 \mu\text{g/mL}$). Thermal processing markedly increased chemical complexity, with 913 features detected in heated $1000 \mu\text{g/mL}$ samples and 615 features detected in the heated $100 \mu\text{g/mL}$ samples, compared with only 32 features in unheated cholesterol (Fig. 1A).

Feature-overlap analysis indicated that the majority of detected features were associated with thermal oxidation. Of the 921 aligned features, 305 features were unique to the heated $1000 \mu\text{g/mL}$ samples, while 584 features were shared between the two heated groups. In contrast, only a single feature was unique to unheated cholesterol, and just 24 features were common to all sample groups (Fig. 1B). PCA revealed extensive compositional differences between heated and unheated cholesterol samples. PC1 accounted for 94.2% of total variance and clearly separated thermally oxidized samples from unheated controls, while PC2 explained an additional 2.4% of the variance (Fig. 1C–D). Collectively, these findings indicate that thermal oxidation generates a substantially more diverse chemical landscape extending well beyond the six targeted COPs alone and highlight the presence of numerous thermally associated features requiring further structural characterization. This motivated subsequent annotation and molecular-networking analyses to further explore these structural relationships.

3.4 Spectral library annotation and MS/MS confirmation

Spectral-library matching was performed to further characterize the oxidation products detected in the untargeted LC-MS/MS dataset. Among the 921 aligned features, eight features received MZmine spectral-library matches corresponding to six unique compounds (Schymanski Level 2). Cholesterol itself did not return a spectral-library match; its identity in the untargeted dataset was assigned by manual interpretation of its MS/MS spectrum, supported by accurate mass and retention time agreement with the authentic cholesterol standard analyzed in the same batch (Level 1). Together, these analyses yielded nine annotated features (Table 2). The six Level 2 compounds included three sterol-related compounds – cholest-4-en-3-one, 7α -hydroxy-4-cholesten-3-one, and cholest-4,6-dien-3-one – as well as three non-sterol background compounds (a hindered-amine piperidine, a polyethylene glycol homolog, and erucamide). Among the six targeted COPs quantified using authentic standards, only cholest-4-en-3-one produced a high-confidence MS/MS spectral-library match (cosine similarity = 0.87; Fig. 2A), providing independent confirmation of its identity. The remaining five targeted COPs were not matched by the library spectra, likely reflecting the limited representation of COPs in current public spectral libraries and differences in instrumentation or acquisition parameters between the reference spectra and our experimental data.

Importantly, the untargeted workflow identified two additional cholesterol-derived compounds, cholest-4,6-dien-3-one and 7α -hydroxy-4-cholesten-3-one, which were not included in the targeted quantification panel (Fig. 2B and C). These compounds received cosine similarity scores of 0.85 and 0.78, respectively (Table 2). Head-to-tail mirror plots comparing experimental and reference spectra against the reference library spectra demonstrated strong agreement in fragmentation patterns for these two sterols, supporting their annotation. In addition, two putative groups of sterol-related features were assigned to the molecular formula $C_{27}H_{44}O_2$ (six features) and $C_{27}H_{46}O$ (eight features) based on accurate mass and MS/MS fragmentation patterns, but could not be assigned specific structures (Schymanski Level 3; Table 2). The remaining library matches corresponded to non-sterol background features, including a hindered-amine piperidine, a polyethylene-glycol homolog, and erucamide. Although the hindered-amine piperidine (4-hydroxy-1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidine; m/z 202.180, $[M + H]^+$) produced a high-confidence spectral match (cosine similarity = 0.87; Fig. 2D), its molecular formula ($C_{11}H_{23}NO_2$) contains nitrogen and therefore cannot arise from cholesterol oxidation. The feature was detected only in heated samples and increased with sample concentration, suggesting introduction from the cholesterol stock or migration of polymer additives during heating rather than formation as a COP. Because the feature persisted after blank subtraction and was inconsistent with cholesterol-derived chemistry, it was classified as an exogenous contaminant and excluded from the downstream analysis.

Collectively, spectral-library matching identified additional sterol-derived compounds beyond those included in the targeted COP panel while distinguishing unrelated background contaminants. These findings indicate that thermally oxidized cholesterol contains a broader repertoire of sterol-derived species than captured by targeted analyses and provided the basis for further investigation of structural relationships using feature-based molecular networking (FBMN).

3.5 Feature-based molecular networking (FBMN)

FBMN was performed to evaluate structural relationships among compounds detected in the untargeted LC-MS/MS dataset and to determine whether unannotated features were associated clustered with annotated cholesterol-derived compounds. The GNPS workflow organized 49 clustered MS/MS features into seven network components connected by 107 spectral-similarity edges. Three of these components were consistent with non-biological background, and were excluded from the displayed network: a polyethylene glycol (PEG) series, a high-mass regularly spaced polymer series, and a polymer-additive series eluting near the void volume. The four remaining components (Clusters 1–4), comprised 26 features connected by 28 edges (Fig. 3).

The largest components were composed of cholesterol-related features and oxidized sterols. Cluster 2 was centered on cholesterol (feature 522; identified by manual MS/MS interpretation rather than by spectral-library matching) and contained structurally related features exhibiting mass differences consistent with dehydration, dehydrogenation, and other putative cholesterol-derived modifications. Cluster 1 contained the annotated oxidized sterols 7 α -hydroxy-4-cholesten-3-one (feature 336) and cholest-4,6-dien-3-one (feature 488), together with several structurally related but unannotated features, suggesting the presence of additional cholesterol-derived compounds related to thermal oxidation. Cluster 3 comprised a further family of sterol-associated features that was likewise strongly enriched in thermally oxidized samples, although these compounds could not be confidently annotated. The remaining displayed component cluster 4 consisted of two low-abundance, unannotated features (526, 527) that were not connected to the sterol families and could not be assigned. Consequently, no further structural or biological interpretation was assigned to this cluster. Finally, node pie charts scaled by relative MS¹ peak area showed that the sterol-associated families (clusters 1–3) were abundant in heated cholesterol and largely absent from unheated samples.

Overall, FBMN organized annotated and unannotated sterol-associated features into distinct molecular families and revealed extensive structural relationships among compounds enriched in thermally oxidized cholesterol. These findings provide additional evidence that thermal oxidation generates a substantially broader repertoire of cholesterol-derived compounds than is captured by targeted analysis alone.

4. Discussion

In this study, we established a standardized thermal oxidation protocol to generate and characterize COPs. By combining a thin-film oxidation approach with targeted LC-MS/MS quantification and untargeted MS/MS analysis, we demonstrated that heating cholesterol at 145°C for 20 h produces not only the commonly monitored COPs, but also a substantially broader spectrum of cholesterol-derived oxidation products. Consistent with previous studies, heating cholesterol at temperatures ranging from 100–200°C increases both LOOH levels and COP formation, confirming heat as a major driver of cholesterol degradation (Derewiaka and Molińska (née Sosińska) 2015). However, substantial variability in COP yields has been reported across studies due to differences in oxidation conditions and experimental methodology (Vine et al. 1998; Staprans et al. 2000; Yan et al. 2022). For instance, one study showed that film fragments of cholesterol oxidized at a faster rate in aqueous suspensions than when oxidized in the dry state (Kim and Nawar 1993). Accordingly, cholesterol was dissolved in chloroform, and thin films were produced using a rotary evaporator to maximize the exposed surface area and promote uniform thermal oxidation. Using this approach, heated samples exhibited a ~3.5-fold increase in LOOH levels relative to unheated controls. These hydroperoxides are key intermediates in cholesterol oxidation and serve as precursors for more stable COPs, including 7-ketocholesterol and epoxycholesterols (Derewiaka and Molińska (née Sosińska) 2015).

Targeted LC-MS/MS analysis enabled quantification of six major COPs and demonstrated substantial depletion of native cholesterol following thermal oxidation. Among the quantified products, 7-KC was the predominant COP, consistent with its formation via C7 oxidation and its greater stability to intermediate oxidation products (Lathe 2002; Rodriguez-Estrada et al. 2014; Anderson et al. 2020). Initially, cholesterol undergoes peroxide formation followed by dehydration to 7-KC (Ghosh 2025). Given its stability, 7-KC tends to accumulate in both *in vitro* and *in vivo* conditions; hence, it is recognized as the most prevalent nonenzymatically produced COP (Helmschrodt et al. 2013; Anderson et al. 2020). The substantial reduction in cholesterol content to approximately one-third of total quantified analytes in heated samples further supports extensive conversion of cholesterol into oxidation products. This conversion pattern mirrors findings from Derewiaka et al. (Derewiaka and Molińska (née Sosińska) 2015), who reported a rapid decline in cholesterol at temperatures exceeding 120°C, with complete degradation at 200°C. Epoxycholesterols (5 α ,6 α - and 5 β ,6 β -epoxycholesterol) and 7 α /7 β -hydroxycholesterols were also detected, consistent with previous reports describing these compounds as secondary products arising from cholesterol hydroperoxides under thermal and oxidative conditions (Zmysłowski and Szterk 2019). These findings confirm that the oxidation protocol generates major COPs commonly used in experimental studies.

A key finding of this study is that targeted quantification substantially underestimated the chemical diversity generated during thermal oxidation of cholesterol. Although six major COPs were quantified, untargeted LC-MS/MS analysis detected 921 aligned features, revealing a far more complex oxidation landscape than captured by conventional targeted approaches. MS/MS spectral library matching identified additional high-confidence sterol-derived compounds, including 7 α -hydroxy-4-cholesten-3-one and cholest-4,6-dien-3-one, with spectral matches, while molecular formula prediction and network analysis revealed multiple Level 3 sterol-associated families that could not be assigned definitive structures. Interestingly, 7 α -hydroxy-4-cholesten-3-one is more commonly recognized as a bile acid biosynthetic intermediate (Eusufzai et al. 1993) than as a thermally generated COP, highlighting the need for further structural confirmation of this annotation. FBMN further organized these compounds into structurally related molecular families enriched in heated samples, supporting the presence of numerous chemically related sterol-derived products associated with thermal oxidation. Overall, these findings suggest that thermally oxidized cholesterol contains a substantially broader spectrum of sterol-derived products beyond the limited set of COPs typically monitored in targeted analyses. Although only a small proportion of the sterol-associated features could be assigned high-confidence identities using currently available spectral libraries, the large number of remaining unannotated sterol features highlights the limited coverage of COPs and related sterol oxidation products in existing public databases, and emphasizes the need for expanded reference libraries.

This broader chemical landscape has important implications for both food and biomedical research. Most studies investigating the biological effects of oxidized cholesterol focus on a limited number of COPs, for instance, 7-KC, hydroxy or epoxycholesterols. Our findings suggest that exposure to thermally oxidized cholesterol involves a substantially more complex mixture of sterol-derived compounds, many of which remain chemically uncharacterized and

whose biological activities are largely unknown. Consequently, integrating untargeted LC-MS/MS with FBMN provides a valuable complement to target analyses by expanding the detectable repertoire of COPs and their biological implications.

Several limitations should be acknowledged. First, 7 α - and 7 β -hydroxycholesterol could not be chromatographically resolved and were therefore quantified as a combined peak, although this approach has been used previously in LC-MS/MS workflows (McDonald et al. 2012). Second, although targeted standards provided Schymanski Level 1 confidence for the quantified COPs, most newly observed untargeted features remain Level 2 or Level 3 annotations and require authentic standards or orthogonal structural confirmation for definitive identification. Future studies should isolate these individual compounds and confirm their structures using orthogonal analytical approaches, such as NMR-based structural elucidation. Finally, while the present study utilized purified cholesterol rather than a complex food matrix, this reductionist approach enabled reproducible COP generation and comprehensive analytical characterization without confounding effects arising from matrix composition. However, we were unable to evaluate the influence of water activity, antioxidants, transition metals, or co-oxidizing lipids on COP formation, which should be considered in future studies.

Overall, this study provides a reproducible platform for generating, characterizing, and quantifying thermally derived COPs from purified cholesterol. By integrating lipid hydroperoxide analysis, targeted quantification, untargeted feature discovery, and feature-based molecular networking, this workflow captures both established and previously uncharacterized products of cholesterol oxidation. These findings provide a foundation for future studies aimed at defining the occurrence, biological activity, and health consequences of COPs, particularly in the context of diet-induced metabolic disorders (Leonarduzzi et al. 2002).

Declarations

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Authors contributions

Md Amran Gazi: Writing – Original draft, Methodology, Formal analysis, Data curation. **Xin Qing**: Methodology, Formal analysis, Data curation, Writing – review & editing. **Greg Barrett-Wilt and Sean Baker**: Methodology, Validation, Data Curation, Writing – review & editing. **Mark P. Richards and Vanessa A. Leone**: Funding acquisition, Conceptualization, Supervision, Writing – review & editing. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing financial or personal interests that influenced the reported work.

Declaration of generative AI and AI-assisted technologies

During the preparation of this manuscript, ChatGPT, Claude Code, and Claude.ai were used to aid in formatting and organizing the manuscript outline, and Grammarly was applied to improve the readability and clarify the language. The authors wrote, reviewed, and edited the manuscript, and take full responsibility for the content of the published article.

Data availability

All raw LC-MS/MS data generated in this study have been deposited in the MassIVE repository (Mass Spectrometry Interactive Virtual Environment) under accession number MSV000099229 (<ftp://massive-ftp.ucsd.edu/v10/MSV000099229/>). Feature-Based Molecular Networking results, including the MS/MS spectral summary, feature quantification table, and molecular network, are publicly available through the associated GNPS job at <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=b2f1ab175a524a2c811f45fb713f9365>. Additional data supporting the findings of this study are provided within the manuscript.

Code availability

All analysis code, the MZmine 4.9.14 processing batch, the FBMN visualization style and network (GraphML/Cytoscape) files, the figure-generation scripts, and the supporting feature/annotation tables, metadata, and reference-standard MS² spectra are available at <https://github.com/xqing3/cop-lcms-analysis>.

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Figures

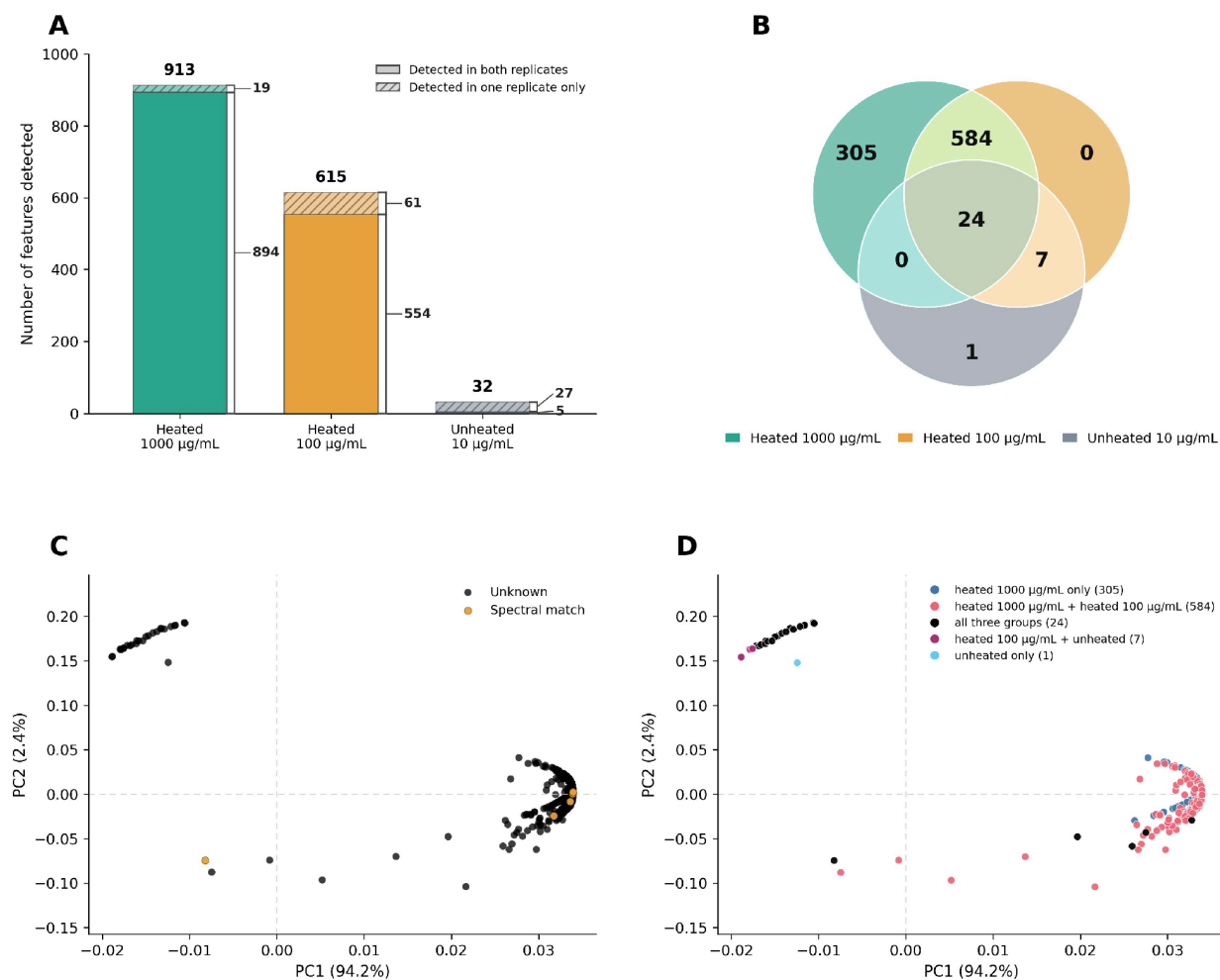


Figure 1

Untargeted feature profiling of heated vs. unheated cholesterol. **(A)** Aligned feature counts per sample group. The number above each bar is the total features detected in the group (≥ 1 replicate); right-side brackets split this into the both-replicate count (solid segment) and the one-replicate-only count (hatched segment). **(B)** Overlap of the 921 aligned features among heated cholesterol (1000 $\mu\text{g/mL}$), heated cholesterol (100 $\mu\text{g/mL}$), and unheated cholesterol (10 $\mu\text{g/mL}$), where a feature is counted in a group if detected in ≥ 1 replicate. **(C)** PCA of compounds by annotation status (yellow = spectral library match, black = unannotated). **(D)** The same PCA with each compound colored by the exact set of treatment group(s) in which it is detected (legend gives the membership and feature count). PC1 and PC2 explain the indicated percentage of variance.

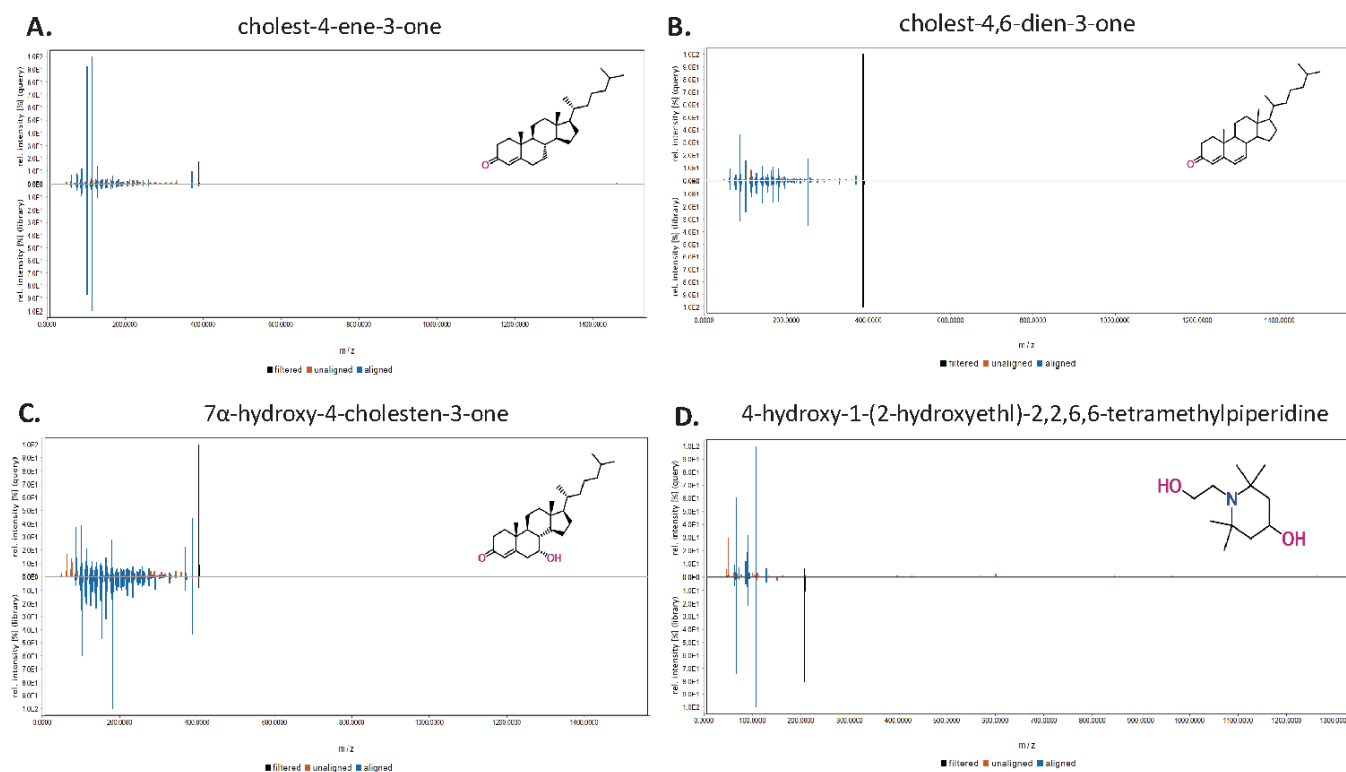


Figure 2

Representative MS² mirror plots of annotated compounds (query, top) against reference library spectra (bottom): (A) 4-cholesten-3-one (cosine 0.87), (B) 4-Hydroxy-1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidine (cosine 0.87), (C) cholest-4,6-dien-3-one (cosine 0.85), and (D) 7 α -hydroxy-4-cholesten-3-one (cosine 0.78). Blue = aligned fragments; Orange = unaligned fragments.

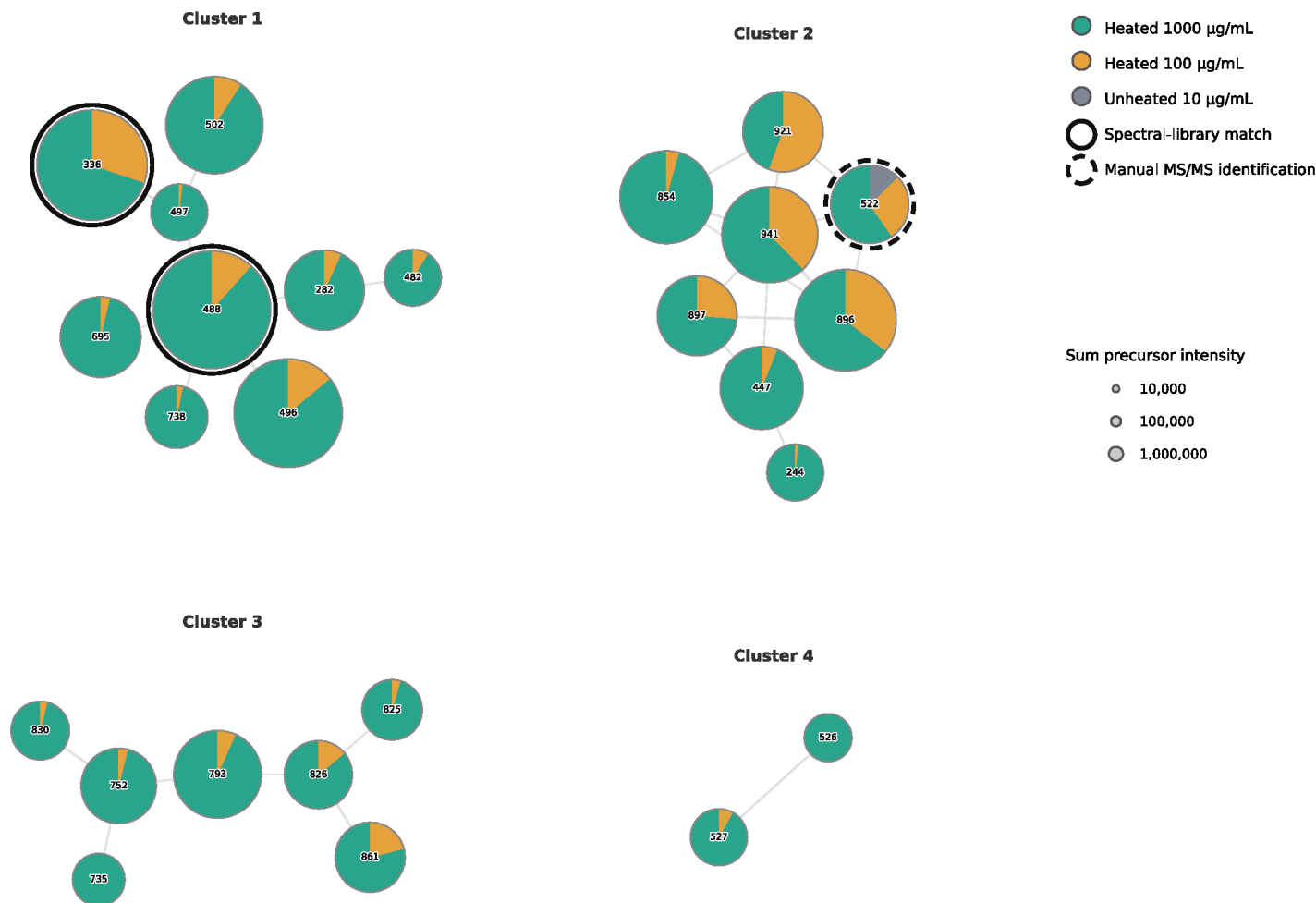


Figure 3

Feature-based molecular network (FBMN) of heated versus unheated cholesterol.

MS/MS spectra from all injections were assembled into a molecular network on GNPS from the MZmine ion-identity (IIMN) export. Each node represents an ion-identity-collapsed feature labeled by its MZmine feature ID. Node size is proportional to the summed MS¹ precursor intensity, and pie charts indicate the relative contribution of the heated 1000 µg/mL (green), heated 100 µg/mL (yellow), and unheated 10 µg/mL (grey) sample groups. Edges connect features with a modified cosine similarity ≥ 0.70 , with line width proportional to spectral similarity. Only connected molecular families are displayed; isolated (singleton) features are omitted, including the library-matched cholest-4-en-3-one (feature 507) and erucamide (feature 425). Clusters are numbered (Clusters 1–4) for reference in the text. Identified features carry a bold outline drawn just outside the node: a solid outline denotes an MZmine spectral-library match, and a dashed outline denotes manual MS/MS identification.

Identified features. Solid outline (MZmine spectral-library match): 336 = 7 α -hydroxy-4-cholesten-3-one and 488 = cholest-4,6-dien-3-one (cholesterol oxidation products, Cluster 1). Dashed outline (manual MS/MS identification): 522 = cholesterol (Cluster 2).

Removed components. Three connected components were identified as non-biological background and excluded from the displayed network: (i) a polyethylene glycol (PEG) series (features 69, 70, 73, 75, 76, 78, 80, 193; feature 75 = library-matched 3,6,9,12-tetraoxatetracosan-1-ol), (ii) a high-mass, regularly spaced polymer series (features 587, 623, 651, 652, 691, 724, 725, 767, 769, 784, 786, 810), and (iii) a polymer-additive series eluting near the void volume (features 1, 2, 4; feature 1 = library-matched 4-hydroxy-1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidine).

Supplementary Files

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- [FigureS1.pdf](#)