Application note | 001586

Pharma

Generation of a custom spectral library for the identification of plant oil-based additives in extractables and leachables analyses

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Keywords

Extractables, leachables, mzVault custom spectral library, unknown identification, Compound Discoverer software, Orbitrap Exploris 120 mass spectrometer, high-resolution accurate mass (HRAM), Vanquish Horizon UHPLC system, plant oil additives

Application benefits

- Demonstration of a straightforward workflow for the creation of custom Thermo Scientific™ mzVault™ spectral libraries from identified compounds and known unknowns in Thermo Scientific™ Compound Discoverer™ 3.3 software and their curation in mzVault 2.3 software
- Confident compound identification and structural characterization of plant oil-derived compounds from data acquired with the Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer and with annotation tools available in Compound Discoverer software
- Rapid and confident annotation of extractables from a medical device component based on matching to the custom mzVault library to facilitate E&L data analysis

Goal

Demonstrate the workflow for creation of an MS^2 spectral library for plant oil-based additives and their degradants, generated under different stress conditions, and its application to the annotation of unknown extractable compounds from a PVC tubing extract

Introduction

Extractables and leachables (E&L) analysis is crucial in the development of pharmaceuticals and medical devices to ensure product safety and quality. Due to the chemical complexity of the base materials, many compounds with known or unknown origins need to be identified. Liquid chromatography high-resolution mass spectrometry (LC/HRMS) combined with software containing customized databases and spectral libraries is a powerful tool for compound identification of these unknowns.

Plant oils and modified plant oils have been widely used as plasticizers and lubricants due to their sustainability. However, these additives can degrade into a variety of E&L compounds during their usage and processing. Standards of these degradants are typically unavailable commercially.

One such example is epoxidized soybean oil (ESBO), which is known as Plastic Additive 15 in the U.S. Pharmacopeia or Plastic Additive 04 in the European Pharmacopoeia. It is commonly used as a plasticizer in polyvinyl chloride (PVC). The European Chemicals agency describes ESBO as a UVCB substance, which stands for "unknown or variable composition, complex reaction products or biological materials".[1] It is a complex mixture of triglycerides and related compounds with or without the epoxide functionality, derived from soybean oil.[2] The majority of fatty acids making up the triglycerides are linoleic acid "FA(18:2)", oleic acid "FA(18:1)", and linolenic acid "FA(18:3)". The structure of TG(54:6-eO), a typical component in ESBO derived from the triglyceride containing three linoleic acid substituents through epoxidation of the double bonds, is shown in Figure 1.

In this study, using the case of epoxidized soybean oil, we describe the identification of components in modified plant oil using LC/HRMS data and present a workflow for construction of a library of identified plant oil-based compounds and their degradants. The subsequent application of the library to the annotation of extractables from a medical device component is detailed.

Experimental

A commercial standard of epoxidized soybean oil (containing a mixture of compounds, mainly epoxidized triglycerides) was analyzed by LC/HRMS as obtained and after treatment with different conditions to induce degradation/transformation of the components. The compounds in the samples were detected and annotated with Compound Discoverer 3.3 SP1 software.

An mzVault library was generated based on confidently annotated compounds, and a PVC tubing sample was analyzed with the same LC/HRMS method to illustrate the application of the custom spectral library to identify compounds that could not otherwise be annotated from public data sources.

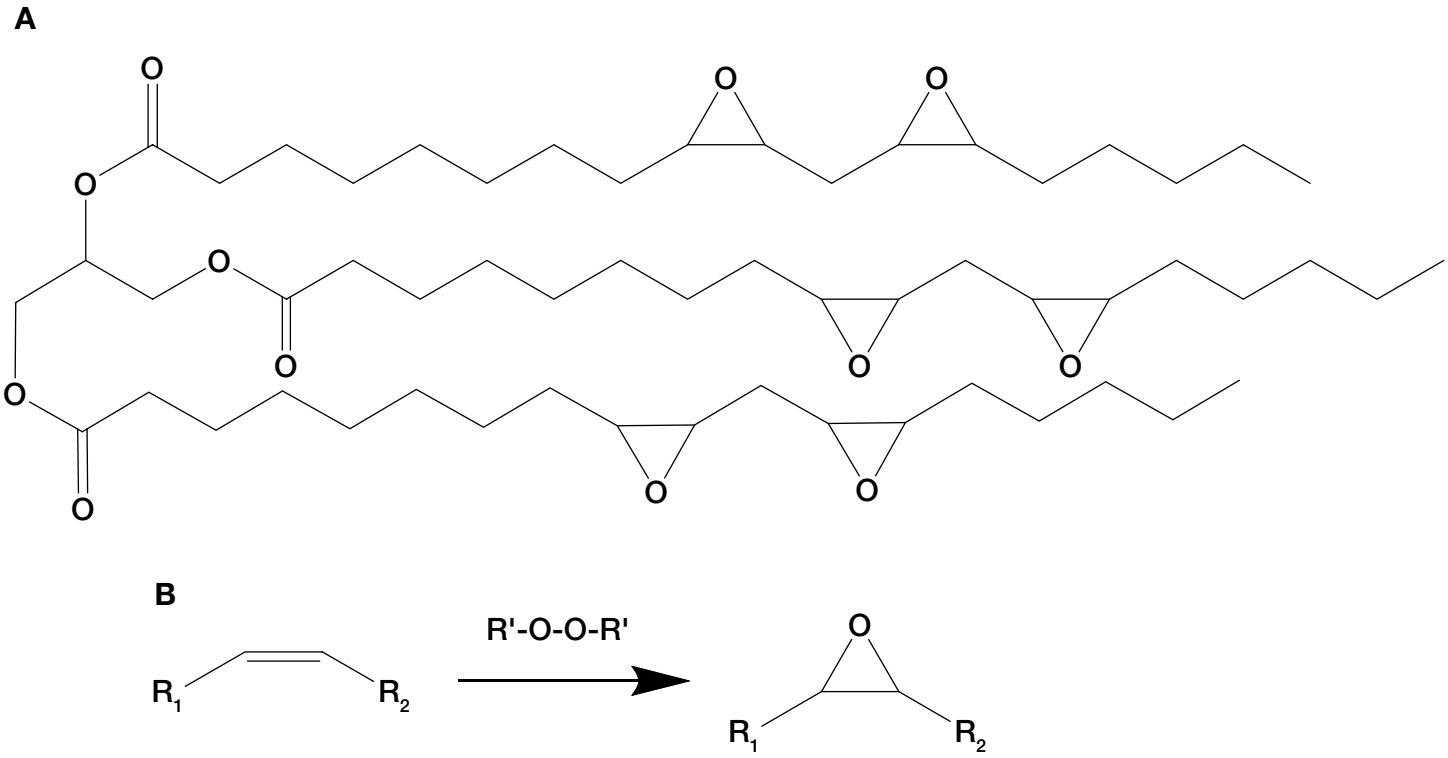

Figure 1. (A) Structure of ESBO component TG(54:6-eO), a triglyceride (TG) with 54 carbons in the side chains and 6 epoxide groups, and (B) epoxide formation using a peroxide reagent

Reagents and consumables

- Water, UHPLC grade, Thermo Scientific™ (P/N W8-1)
- Methanol, UHPLC grade, Thermo Scientific™ (P/N A458-1)
- Ammonium acetate, Optima™ LC/MS grade, Fisher Chemical™ (P/N A11450)
- Thermo Scientific™ SureSTART™ Screw Glass Vial, 2 mL, Level 3 (P/N 6PSV9-1PSS)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps, Level 3 (P/N 6PSC9TST)
- Epoxidized soya bean oil, Millipore Sigma™ Supelco™ (P/N 43956)
- Isopropanol, Optima™ LC/MS grade, Fisher Chemical™ (P/N A461-1)
- Hydrochloric acid, Optima™ grade, Fisher Chemical™ (P/N A466-250)
- Sodium hydroxide (5N solution), Fisher Chemical™ (P/N SS256-500)
- Hydrogen peroxide (30% solution), Thermo Scientific™ (P/N H325-100)

Instrumentation

The chromatographic separation was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system, consisting of:

- Thermo Scientific™ Vanquish™ System Base (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-03)
- Thermo Scientific™ Vanquish™ Diode Array Detector FG (P/N VF-D11-A-01) with Standard Flow Cell (10 mm, P/N 6083.0510)

This was connected to an Orbitrap Exploris 120 mass spectrometer (P/N BRE725531) equipped with a Thermo Scientific™ OptaMax™ NG HESI ion source.

Sample preparation

Sample treatment included gamma irradiation, autoclave, heat, acid, base, and oxidation. For the unstressed standard, epoxidized soybean oil was dissolved in isopropanol (IPA) at 1 mg/mL and then diluted to a working concentration of 10 µg/mL in methanol (MeOH).

For both gamma irradiation and autoclave treatment, neat ESBO was used. In the former case, a sample was irradiated with 27.6–35.1 kGy in an autosampler vial. In the latter, a sample of ESBO was subjected to 121 °C at 15 psig for 15 min. From these, aliquots were dissolved in IPA at 1 mg/mL and then diluted to 10 µg/mL in MeOH.

For heat treatment, a glass scintillation vial containing neat ESBO was incubated at 60 °C for 24 h, then dissolved in IPA at 1 mg/mL and diluted to 10 µg/mL in MeOH.

For acid, base, and oxidative stresses, samples of ESBO were first dissolved in IPA at 1 mg/mL before dilution to 100 µg/mL in MeOH. These were then diluted 1:10 in the respective stress solutions (0.001 M HCl in MeOH/0.001 M NaOH in MeOH/3% H_2O_2 in MeOH) and placed on a shaker plate at 400 RPM and 30 °C for 24 h.

All samples were transferred into SureSTART Level 3 autosampler vials for analysis.

The PVC tubing extract was obtained by incubating 1 g of previously sterilized tubing (using ethylene oxide treatment) in 10 mL of 50% IPA at 50 °C for 72 h in accordance with ISO recommendations.[3] The sample was diluted 1:10 in MeOH prior to analysis.

Liquid chromatography – mass spectrometry

The LC/MS analysis was carried out using the conditions listed in Tables 1 and 2.

Table 1. UHPLC experimental conditions

Parameter	Value
Column	Thermo Scientific™ Accucore C18™, 2.6 µm, 2.1 × 100 mm (P/N 17126-102130)
Mobile phase	A: 0.01% (w/V) ammonium acetate in water B: 0.01% (w/V) ammonium acetate in methanol
Flow rate	0.4 mL/min
Mixer volume	35 µL (10 µL static + 25 µL capillary mixer)
Autosampler temperature	10 °C
Injection volume	2 µL
Needle wash solvent	50% methanol
Column temperature	45 °C (still air mode)
Divert valve timing	Flow to waste from 0–0.5 min and 25–30 min
DAD settings	Wavelength 200–400 nm, 10 Hz acquisition speed

Table 2. UHPLC gradient conditions

Time (min)	Mobile phase B (%)	Time (min)	Mobile phase B (%)
0.0	5	18.0	100
0.5	5	25.0	100
4.0	25	25.1	5
10.0	90	30.0	5

Table 3. MS HESI source conditions

Parameter	Value
Spray voltage	+3,500 V / -2,800 V
Sprayer position	1.2, M/H, center
Vaporizer temperature	350 °C
Ion transfer tube temperature	300 °C
Sheath gas	40 a.u.
Aux gas	10 a.u.
Sweep gas	1 a.u.

Table 4. MS method parameters

Polarity Switching ddMS² method (Top 4)	
RF level, %	70
Easy-IC	Scan-to-Scan
MS resolution	30,000 @ *m/z* 200
MS mass range	*m/z* 100–1500
MS² resolution	15,000 @ *m/z* 200
HCD collision energies (normalized, %)	10, 30, 60
MS² isolation window (*m/z*)	1.8
Maximum injection time (ms)	50
Intensity threshold	1.0e5
Dynamic exclusion	5 s, exclude isotopes
Targeted mass exclusion	List of 50 most abundant background ions for each polarity, obtained from averaging MS¹ spectra of solvent blank injection between 0 and 25 min, respectively.

Untargeted LC/MS analyses of the ESBO samples and the PVC tubing extract were carried out using fast polarity-switching data-dependent MS² (ps-ddMS²) experiments on the Orbitrap Exploris 120 MS. The HESI source conditions and relevant MS method parameters are detailed in Tables 3 and 4.

Data processing

The acquired MS and MS² data from duplicate injections of each sample were processed using Compound Discoverer 3.3 SP1 software separately for each polarity, using a custom workflow based on the template "Degradants w Stats Related and Unknown ID w Database Searches" with modifications described below and depicted in Figure 2.

- Untargeted peak detection was set to detect all sample compounds at or above an intensity of 1e6, which were extracted from the select mass spectra, deisotoped and adducts grouped into compounds, with their elemental compositions predicted.
- Compounds were searched against the Thermo Scientific™ mzCloud™ spectral library, ChemSpider™ databases, and a custom mass list generated based on the known constituents of ESBO (such as TG(54:6-eO) in Figure 1) and potentially expected degradation products (diglycerides, free fatty acids, transesterification products of reaction with methanol solvent). ChemSpider and MassList searches were based on the molecular formula/accurate mass, while mzCloud and NIST™ searches (using the mzVault node) were based on MS² spectral matches with 5 ppm and 10 ppm mass tolerances, respectively.

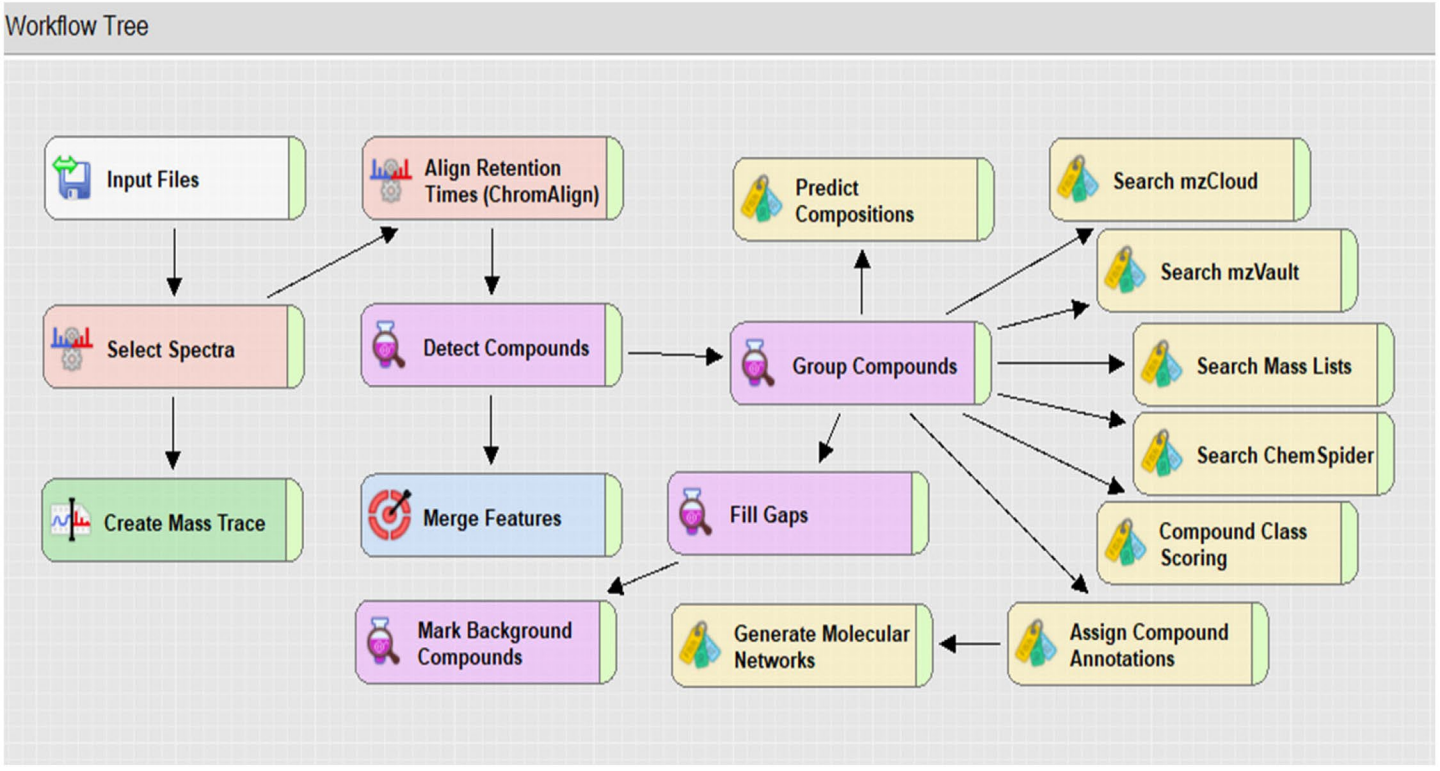

Figure 2. Node-based data processing workflow used in Compound Discoverer 3.3 SP1 software for the detection and identification of compounds in the ESBO samples for library generation

The exported mzVault spectral libraries were curated in mzVault 2.3 SP1 software to merge the data from both polarities into a single library, and spectral average as well as noise threshold the MS^2 data, before reimporting into Compound Discoverer software.

The tubing sample data was searched using the default workflow template 'E and L Unknown ID with Online and local Database Searches', with the addition of the custom mzVault library for the identified known and unknown components of ESBO, with a retention time tolerance of 1 min.

Results and discussion

Analysis of untreated and stressed samples of ESBO

The total ion chromatograms (TICs) of the stressed standards in positive mode are shown in Figure 3. The TICs of the autoclaved and gamma irradiated ESBO standards are comparable to that of the untreated ESBO standard, suggesting negligible effects of both sterilization methods on the compound profile, while heat and oxidation treatment showed the increased abundance of more polar (earlier eluting) compounds, with acid and base treatment creating the largest difference in the TIC profiles.

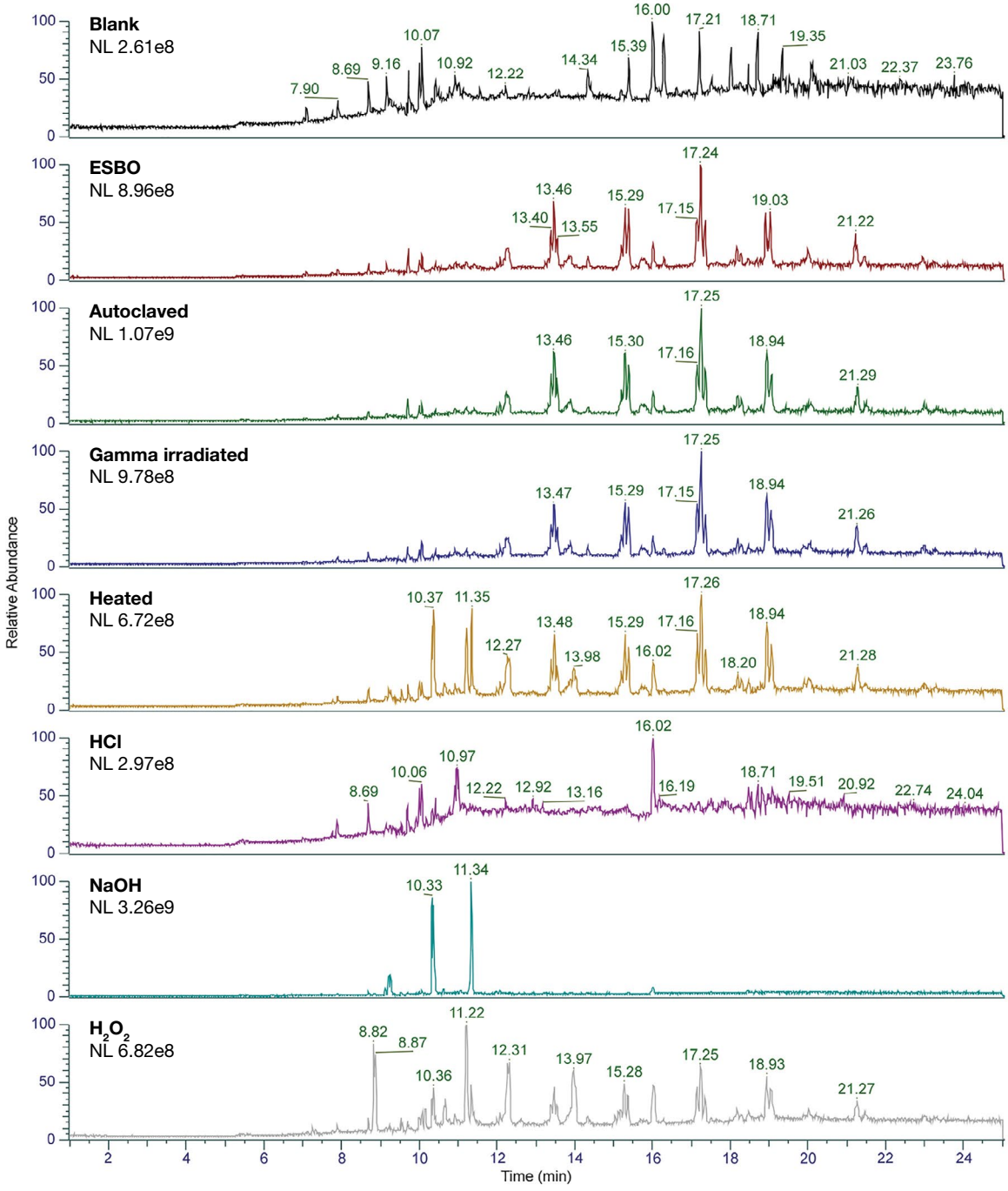

Figure 3. Overview of ESI (+) TICs of solvent blank, untreated, and stressed ESBO standards shown with local normalization

The similarity and/or difference between the treatment conditions could also be observed in the PCA plot (Figure 4), which shows that both acid and base treatment induced that largest change in the chemical composition of the ESBO sample.

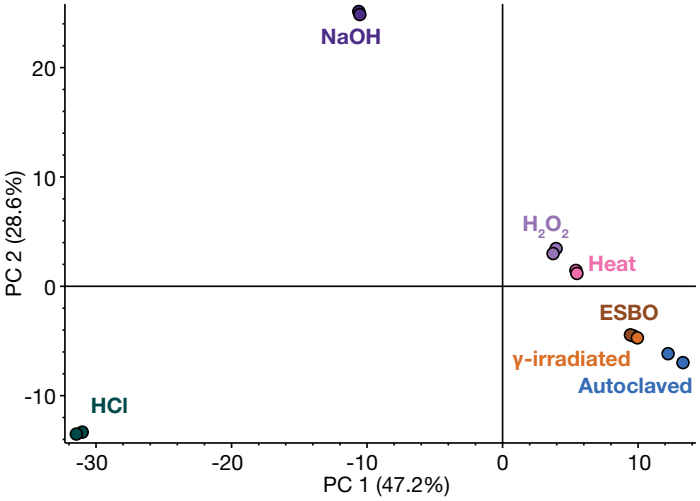

Figure 4. PCA plot generated in Compound Discoverer software based on the detected compounds in both positive and negative ion modes, showing clustering of the different samples based on similarity, and excellent technical reproducibility of the duplicate injections

In the untreated ESBO sample, the compound mixture was found to be made up predominantly of triglyceride-derived compounds with variation in chain lengths and degree of epoxidation, in agreement with previous reports (Compound I in Figure 5).[2]

In the autoclaved and gamma irradiated samples, these compounds were found to be mostly unchanged in terms of their relative abundance, except for an increase in free fatty acids (e.g., epoxidized linoleic acid "FA(18:2-eO)", Compound V in Figure 5, also detected in the negative mode data) in the gamma irradiated sample.

Multiple early-eluting peaks appeared in the TICs of the heat and oxidation stressed ESBO standards, indicating more hydrophilic degradants were generated. These could be identified as epoxidized diglycerides (Compound II, Figure 5) and further degradation products thereof, based on the elemental composition and inspection of their fragmentation spectra. Specifically, in the heat-treated sample, the presence of trans-esterified fatty acid methyl esters (Compound III, Figure 5), and increased abundance of diglyceride and monoglyceride (Compound IV, Figure 5) transformation products were observed, along with a slight decrease in the abundance of epoxidized triglycerides. This is likely the result of the heat-induced methanolysis of some ester bonds with the solvent employed in the stress condition leading to formation of the methyl esters.

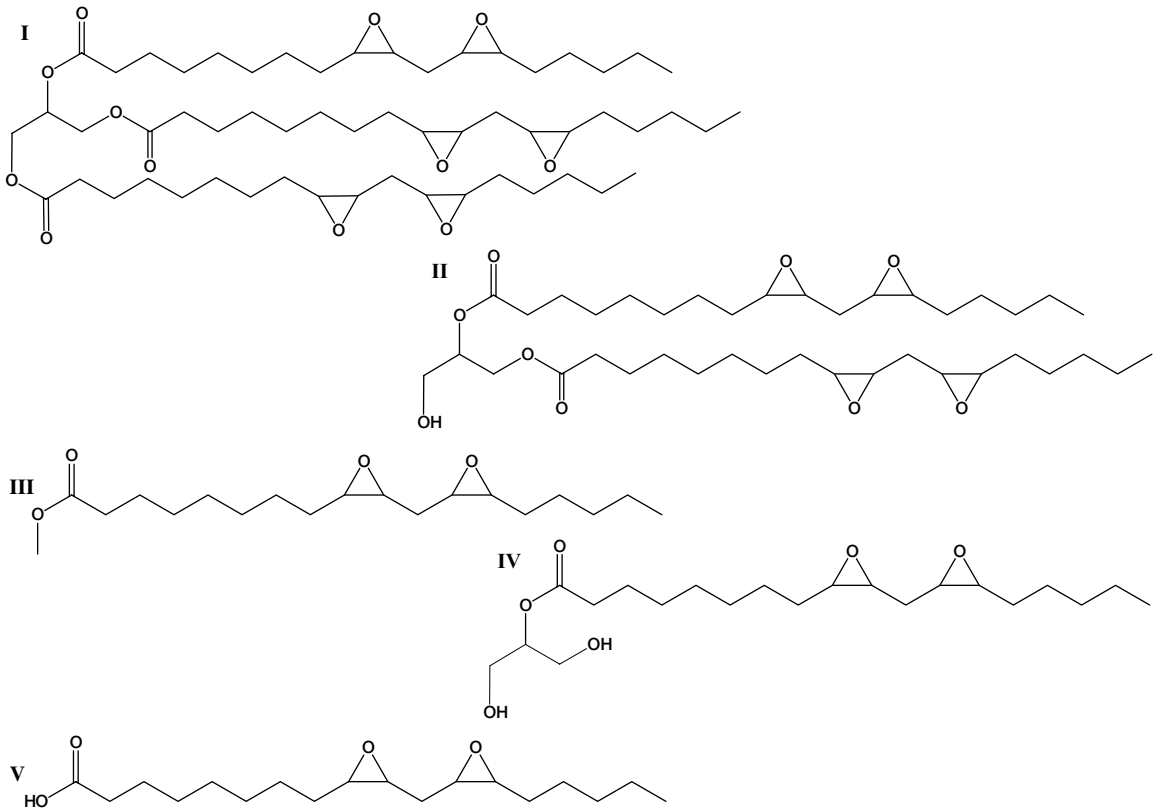

Figure 5. Overview of representative compounds from the different compound classes identified in the untreated and stressed ESBO samples: I – epoxidized triglyceride, II – epoxidized diglyceride, III – fatty acid methyl ester, IV – epoxidized monoglyceride, V – free fatty acid, using the example of epoxidized linoleic acid "FA(18:2-eO)" in all cases

Like the heat-treated sample, the oxidation treatment resulted in degradation of triglycerides through cleavage of ester bonds, but with higher amounts of free epoxidized fatty acids detected, likely as a result of suppressed methyl ester formation.

Significant changes to the peak profile could be observed in the base and acid stressed ESBO samples. Notably, the main components in the NaOH stress sample were found to be methyl esters of the epoxidized free fatty acids linoleic acid, oleic acid, and linolenic acid, presumably due to transesterification (i.e., cleavage of the triglycerides and subsequent esterification with the solvent methanol).

In the acid stress sample, the triglyceride compounds were also found to be nearly completely degraded, resulting in the presence of several compounds for which a structure could not be readily elucidated. These were included in the subsequent spectral library generation as known unknowns, as only their elemental composition could be determined with confidence.

Overall, in the positive mode, Compound Discoverer software detected 484 compounds in the ESBO standards after background filtering, with a peak rating threshold of 4.0 in at least two samples. The polarity-switching FullMS-ddMS^2 method provided excellent fragmentation coverage, with 459 compounds (95%) having associated fragmentation spectra.

In the analysis of the negative mode data, a smaller fraction of compounds could be detected from the untreated and stressed ESBO standards. In part, this was expected due to the lower ionization efficiency of the triglycerides and related compounds without a readily abstractable proton. Still, a total of 40 compounds could be detected as acetate adducted ions, with the majority being epoxidized free fatty acids, triglycerides, and diglycerides that were also observed in the positive mode. Some additional degradation products were detected in the H_2O_2 and NaOH samples, however their structures could not be readily identified. All 40 compounds detected above the peak detection threshold of 1e6 in the ESI(–) data were triggered for MS^2 acquisition in the raw data, providing complete fragmentation coverage.

As detailed above, the major components of ESBO were identified as epoxidized triglycerides with various degrees of epoxidation and chain lengths, detected as their ammonium and sodium adducts in the positive mode and acetate adducts in the negative mode. An overview of some of the identified compounds from the different compound classes is given in Figure 5. These were generally present as multiple peaks eluding at different retention times, due to the multiple structural isomers, as well as the diastereomeric epoxidation. This is illustrated in Figure 6a, which shows the six most abundant isomer clusters.

While the diastereomers resulting from the epoxidation of the unsaturated fatty acyl chains in the ESBO components cannot be distinguished from MS^2 fragments, Figures 6b and 6c show the differentiation of structural isomers due to the different lengths of fatty acyl chains. Here, two isomers eluting at 13.46 min and 13.83 min, denoted as isomers A and B, respectively, could be distinguished based on the minor differences in the fragments as annotated in the figure. The observed fragments indicated that the TG(54:6-eO) isomers A and B contained TG(18:2_18:2_18:2-eO) and TG(18:3_18:2_18:1-eO), respectively.

Spectral library generation

After the compound annotations were reviewed, spectral libraries representing both annotated components, as well as highly abundant but not readily identifiable compounds (“known unknowns”) from the untreated and stressed ESBO samples, were generated using the Export function in Compound Discoverer software, for positive mode and negative mode data, respectively. The two libraries containing data for 27 compounds in the negative mode and 91 compounds in the positive mode could be merged in the mzVault 2.3 SP1 software based on compound name and retention time matches. For compound entries with duplicate spectra, spectral averaging was performed before thresholding of the spectra (using 3% relative intensity cutoff) for noise removal. The resulting curated spectral library contained 96 compounds with 118 MS^2 spectra, with 27 compounds including negative mode data.

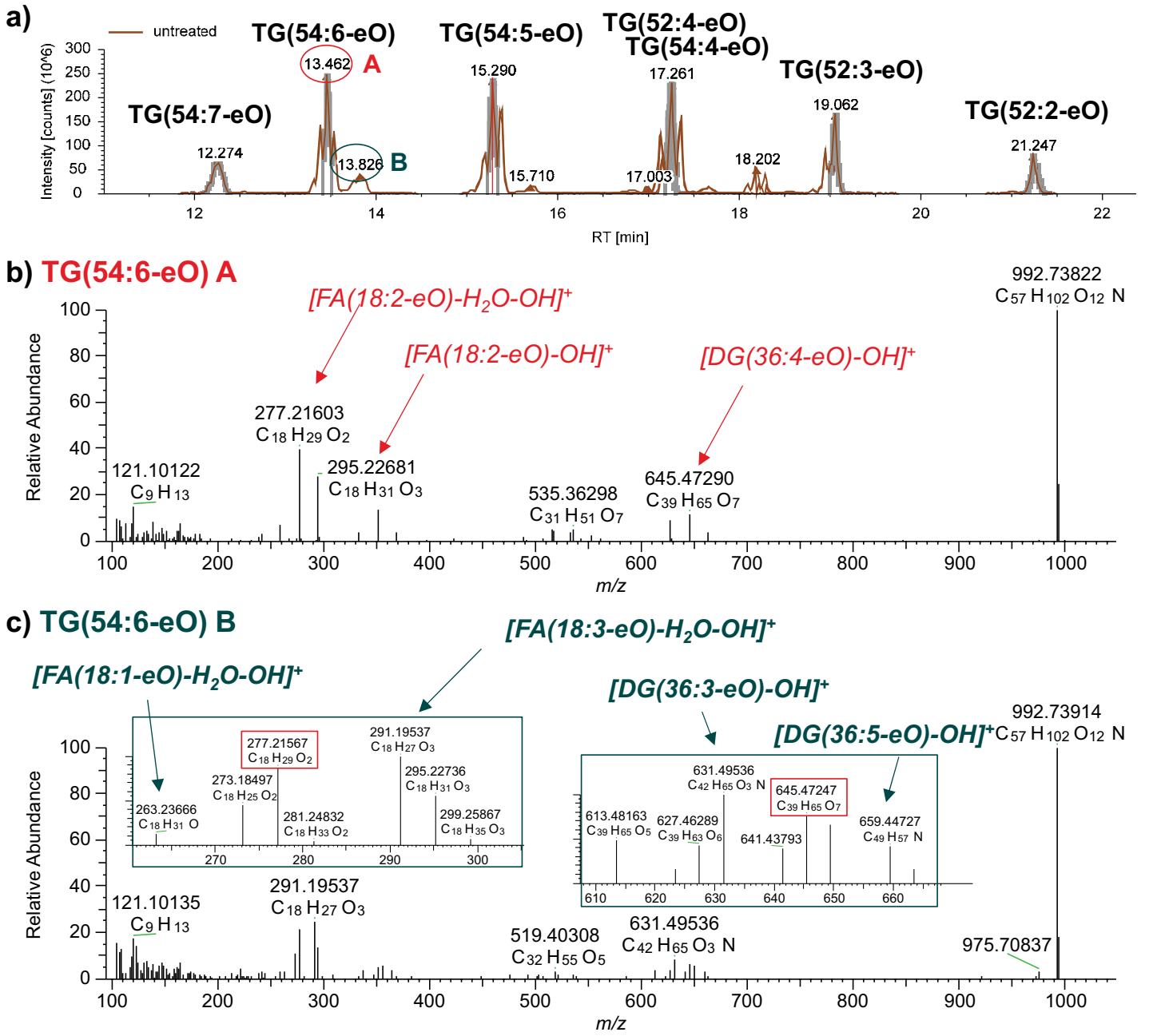

Figure 6. a) ESI (+) XICs of several abundant epoxidized triglyceride isomers found in ESBO; b) and c) fragmentation spectra for isomers A and B of TG(54:6-eO) eluting at 13.46 min and 13.83 min, respectively, with annotated fragment ions indicating the different fatty acyl compositions

MzVault software also allows the inclusion of additional meta information such as CAS identifiers and mol structures or other relevant information. For example, compound origin or sample treatment condition could be added at this point. A screenshot of the ESBO library in the mzVault software is shown in Figure 7.

Analysis of PVC tubing extract

The TIC of the tubing extract acquired in positive and negative ionization mode, respectively, are shown in Figure 8, overlaid with the solvent blank control. The data were analyzed in Compound Discoverer software using the standard E&L workflow template, with the addition of the mzVault library described in the data processing section. The mzVault search was based on MS^2 spectral matches with retention time tolerance of 1 minute.

Table 5 shows a summary of the annotations for the 17 most intense compounds detected in the tubing extract. While several compounds could be annotated based on the mzCloud library match, ChemSpider, or MassList search, the majority of highlighted abundant compounds in the extract could only be confidently annotated based on fragmentation spectral matches to the custom library built from the stressed standards of ESBO.

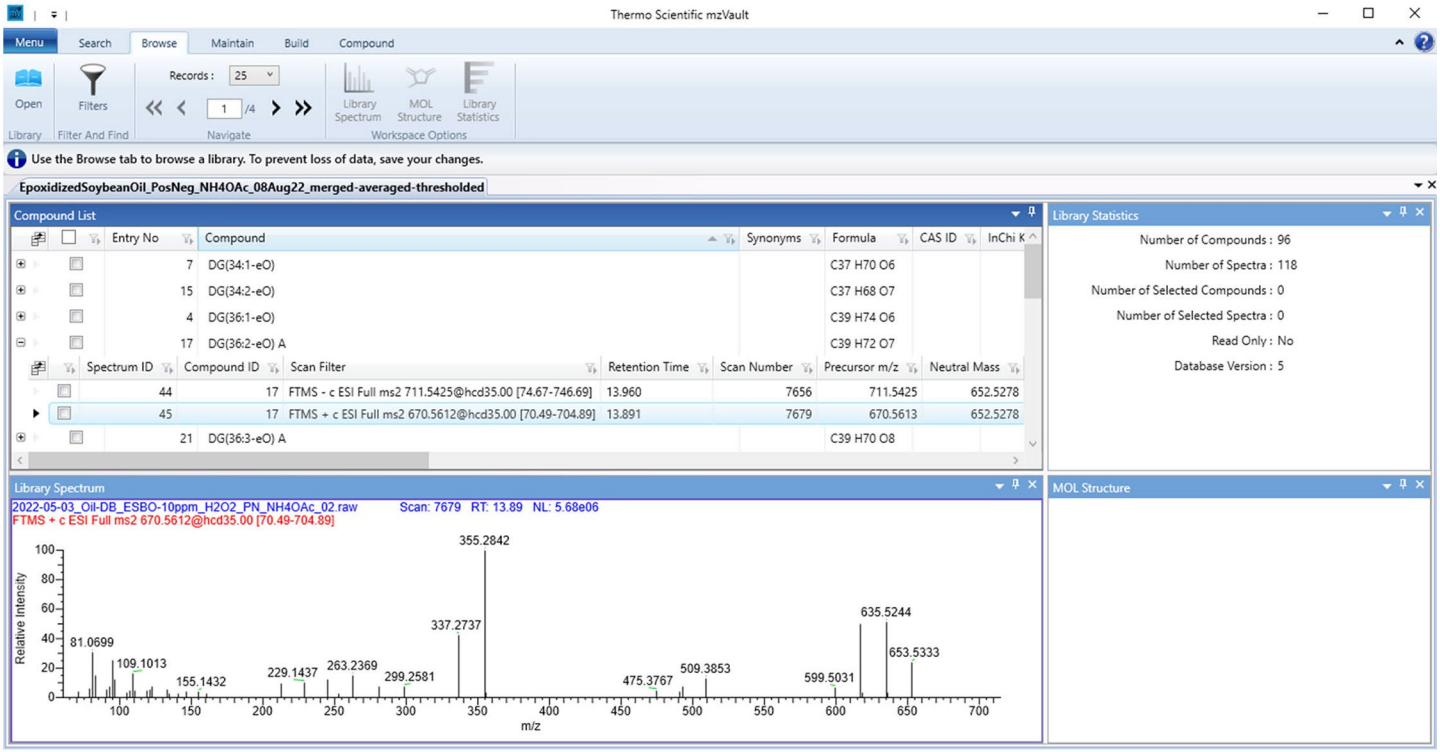

Figure 7. mzVault library view of the generated ESBO spectral library, showing the list of 96 included compounds and the MS/MS spectrum of the selected entry 'DG(36:2-eO) A'

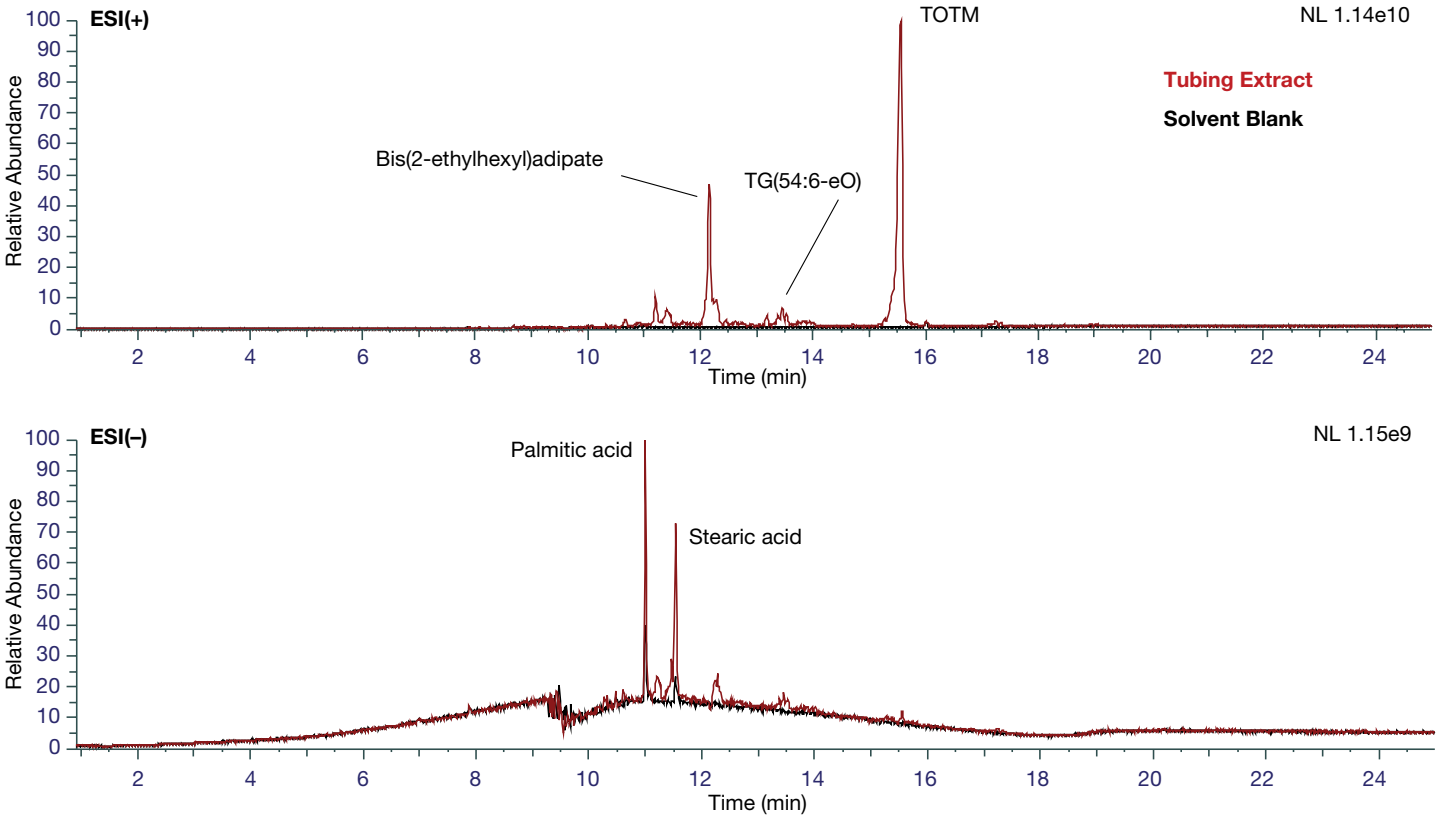

Figure 8. Overlays of the ESI(+) and ESI(–) TICs for the PVC tubing extract and solvent blank samples, with some identifications for high intensity compounds denoted

Table 5. Major compounds detected in the PVC tubing extract sample, sorted by peak areas and denoting the respective source of annotation

Entry	RT (min)	Calc. MW (Da)	Reference ion	Formula	Compound annotation	mzCloud match	ChemSpider hit	E&L MassList hit	mzVault match
1	15.5	546.3911	$[M+H]^+$	$C_{33}H_{54}O_6$	Trioctyl trimelitate (TOTM)		X	X	
2	12.2	370.3078	$[M+H]^+$	$C_{22}H_{42}O_4$	Bis(2-ethylhexyl)adipate	X	X	X	
3	12.3	988.6842	$[M+NH_4]^+$	$C_{57}H_{96}O_{13}$	TG(54:7-eO) A				X
4	11.2	680.4851	$[M+NH_4]^+$	$C_{39}H_{68}O_9$	DG(36:4-eO)				X
5	11.5	284.2715	$[M–H]^–$	$C_{18}H_{36}O_2$	Stearic acid	X	X	X	
6	13.5	974.7049	$[M+NH_4]^+$	$C_{57}H_{98}O_{12}$	TG(54:6-eO) A				X
7	11.4	1002.663	$[M+NH_4]^+$	$C_{57}H_{94}O_{14}$	TG(54:8-eO)				X
8	12.1	390.2765	$[M+H]^+$	$C_{24}H_{38}O_4$	Isooctyl phthalate or isomer		X	X	
9	13.8	974.705	$[M+NH_4]^+$	$C_{57}H_{98}O_{12}$	TG(54:6-eO) B				X
10	13.4	974.7045	$[M+NH_4]^+$	$C_{57}H_{98}O_{12}$	TG(54:6-eO) A				X
11	12.3	666.5062	$[M+NH_4]^+$	$C_{39}H_{70}O_8$	DG(36:3-eO) A				X
12	13.2	390.2765	$[M+NH_4]^+$	$C_{24}H_{38}O_4$	Dioctyl phthalate or isomer		X	X	
13	15.3	960.7252	$[M+NH_4]^+$	$C_{57}H_{100}O_{11}$	TG(54:5-eO) A				X
14	13.5	974.7046	$[M+NH_4]^+$	$C_{57}H_{98}O_{12}$	TG(54:6-eO) A				X
15	14	624.4955	$[M+NH_4]^+$	$C_{37}H_{68}O_7$	DG(34:2-eO)				X
16	12.2	992.714	$[M+NH_4]^+$	$C_{57}H_{100}O_{13}$	TG(54:6-eO) "+H_2O" Unknown				X
17	10.7	694.4646	$[M+NH_4]^+$	$C_{39}H_{66}O_{10}$	DG(36:5-eO)				X

In total, 70 of the 439 compounds detected with a peak rating of 5.0 or higher in the tubing extract (16% of compounds) could be annotated with the mzVault library at a match score threshold of 40. Of these, the majority were epoxidized tri- and diglycerides, along with monoglyceride and fatty acids. Additionally, several known unknown compounds initially detected in the oxidation and heat stress samples, could be detected in the tubing extract as well, which is consistent with the presence of diglyceride degradation products. These findings illustrate the need for the analysis of potential degradation products under stressed conditions, to build a comprehensive spectral library of potential extractables, which were absent from the untreated ESBO sample.

The PVC tubing in question was sterilized by ethylene oxide treatment prior to extraction, and based on the results above, this treatment produced compounds consistent with heat and oxidation stress.

Figure 9 shows an example of one of the spectral matches to the custom spectral library, namely between the MS^2 spectrum of Compound 3 from Table 5 (MW 988.6842 Da) in the tubing extract sample and the spectral library entry for 'TG(54:7-eO) A'. In addition to the fragmentation match of 95.5, the retention time of 12.26 min in the tubing extract sample was a close match to that of the library entry at 12.30 min, further increasing the confidence in the identification.

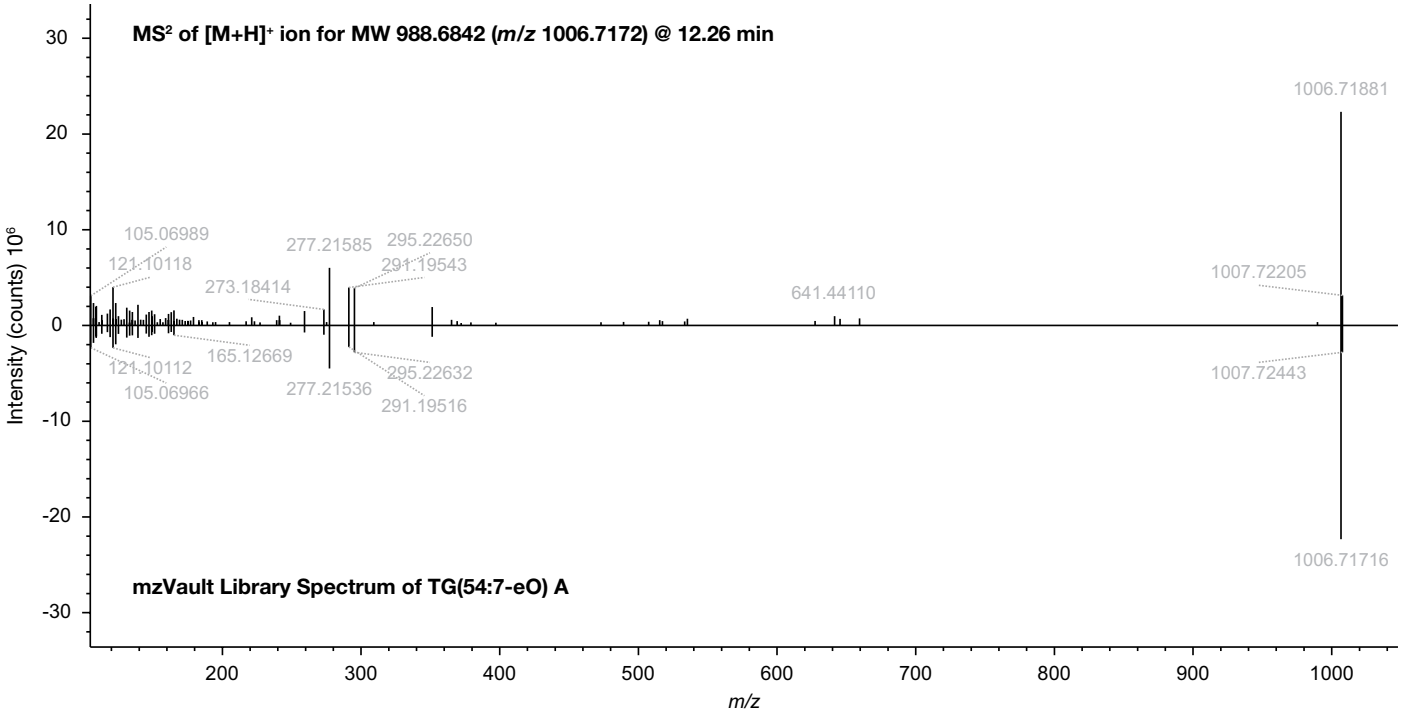

Figure 9. Mirror plot of the fragmentation spectrum for MW 988.6842 @ 12.26 min (*m/z* 1006.7172) and its mzVault library match 'TG(54:7-eO) A' with a match score of 95.5

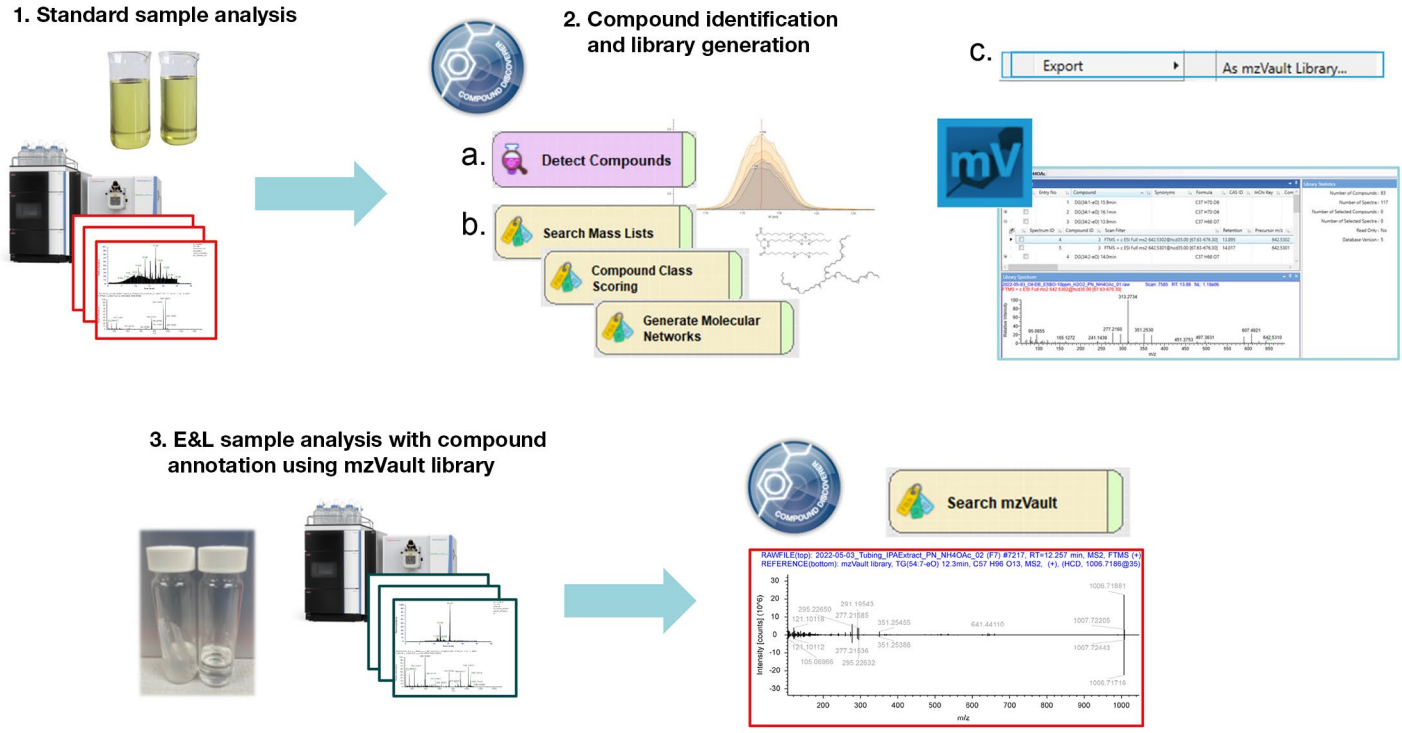

Figure 10. Summary of the workflow for the generation and application of a custom MS² spectral library from a complex standard mixture for the compound annotation of extractable compounds

Conclusion

A commercial standard of ESBO was subjected to autoclave, gamma irradiation, heat, acid, base, and oxidative stress conditions, followed by LC-HRMS analysis (Figure 10). Compounds detected and identified were compiled into a high-resolution mass spectral library. This library was subsequently used to streamline the compound identification in the solvent extract of a PVC tubing used in an on-body delivery system.

- The Orbitrap Exploris 120 MS coupled with the Vanquish Horizon UHPLC system, in combination with Compound Discoverer 3.3 SP1 software and mzVault 2.3 SP1 software, provides a comprehensive workflow for E&L analysis and the generation of custom spectral libraries from high quality fragmentation spectra.
- The annotation of ESBO-derived extractables in a PVC tubing extract could be drastically accelerated with the use of the custom spectral library generated from untreated and stressed ESBO standards.

References

1. Guidance in a Nutshell for identification and naming of substances under REACH and CLP, European Chemicals Agency, 2017 (doi: 10.2823/46787).
2. Toxicity Review for Epoxidized Soybean Oil (ESBO), Consumer Product Safety Commission, 2019. https://www.cpsc.gov/s3fs-public/ToxicityReviewforEpoxidizedSoybeanOil062019.pdf
3. ISO 10993-12 Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials.

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