APPLICATION NOTE 000419

Extractable analysis of rubber stoppers for pharmaceutical applications

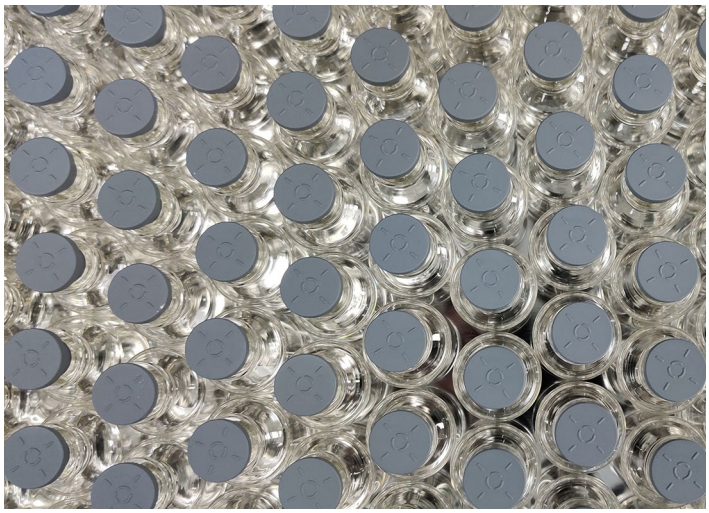
Using UHPLC coupled with an Orbitrap Exploris 120 mass spectrometer and Compound Discoverer software

Authors: Dujuan Lu[1], Danny Hower[1], Chongming Liu[1], Kate Comstock[2]

[1]SGS Health Science, Fairfield, NJ, USA

[2]Thermo Fisher Scientific, San Jose, CA

Keywords: Extractables and leachables analysis, high-resolution accurate mass (HRAM), Orbitrap Exploris 120 mass spectrometer, Vanquish Horizon UHPLC system, data processing software, databases, small molecule structure identification



Application benefits

- Perform high-throughput routine E&L analysis using UHPLC coupled with a Thermo Scientific™ Orbitrap™ mass spectrometer
- Achieve high-quality HRAM MS and MS^2 data acquired with high scan speed and fast polarity switching
- Obtain confident results using intelligent data processing software with parallel searching of multiple databases
- Increase overall E&L analysis quality and efficiency

Introduction

Extractables and leachables (E&L) analysis is essential to identify and quantitate harmful leachable impurities for a wide range of products including pharmaceutical, medical devices, and wearable electronics products. To ensure product and consumer safety, E&L analysis must be carried out at an early stage for toxicological risk assessment following regulatory requirements. The current ISO standards, USP chapters, European agencies, and FDA guidelines all have increased requirements and expectations for chemical characterization and toxicological risk assessments.[1]

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High-resolution accurate-mass (HRAM) mass spectrometry coupled with ultra high performance liquid chromatography (UHPLC) is routinely used for nonvolatile extractable and leachable analysis. HRAM MS delivers accurate mass and isotope fine structure for unambiguous determination of a compound's elemental composition, while the MS^2 and MS^n fragments provide crucial information on the molecular fingerprint for structure identification. In addition, data processing software and a high-quality database and spectral library are essential for obtaining confident E&L analysis results in a timely manner.

This application note presents a study for nonvolatile extractable analysis of rubber stoppers for pharmaceutical applications using a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system. The data processing was carried out using Thermo Scientific™ Compound Discoverer™ small molecule identification software.

Experimental

Sample preparation

Commercially available rubber stoppers for pharmaceutical applications were extracted using isopropanol (IPA) and dichloromethane (DCM) at 50 °C for 72 hours following the ISO 10993-12 guideline[2]. The extract solutions were analyzed directly by LC/UV/MSMS for nonvolatile compounds.

Reagents and consumables

- Thermo Scientific™ Water, UHPLC-MS Grade (P/N W8-1)
- Thermo Scientific™ Methanol, UHPLC-MS Grade (P/N A4581)
- Fisher Chemical™ Formic Acid, Optima™ LC/MS (P/N A117-10X1AMP)
- Sigma-Aldrich, Ammonium Acetate (P/N 73594-25G-F)

Liquid chromatography

Chromatographic separations were carried out on the Vanquish Horizon UHPLC system consisting of the following modules:

- Vanquish Binary Pump H (P/N VH-P10-A)
- Vanquish Split Sampler HT (P/N VH-A10-A)
- Vanquish Column Compartment (P/N VH-C10-A)
- Vanquish Diode Array Detector FG (VF-D11-A)

A Thermo Scientific™ Hypersil GOLD™ VANQUISH™ C18 UHPLC column (2.1 × 100 mm, 1.9 µm, P/N 25002-102130-V) was used with the gradients specified below at a flow rate of 0.4 mL/min and a column temperature of 50 °C.

Mobile phase A: H_2O/10 mM ammonium acetate

Mobile phase B: Methanol

Gradient	Time (min)	%B
	0	5
	1.0	5
	20.0	99
	30.0	99
	30.1	5
	35.0	5

Mass spectrometry

The mass spectrometry analysis was carried out on an Orbitrap Exploris 120 mass spectrometer (P/N BRE725531) equipped with a Thermo Scientific™ OptaMax™ NG ion source.

Source parameters

Parameter	Value
Ion source	OptaMax NG electrospray ion source
Ionization mode	ESI positive/negative
Scan range (Full MS) (*m/z*)	125–1250
Spray voltage (kV)	+3.5 (positive)/-2.5 (negative)
Ion transfer tube temp. (°C)	300
S-lens RF level	70.0
Vaporizer temp. (°C)	400
Sheath gas (Arb)	40
Aux gas (Arb)	10
Sweep gas (Arb)	1

MS method parameters

Parameter	Value
Resolution (Full Scan/MS^2)	60,000/15,000
AGC target value (Full MS/MS^2)	70/Standard
Max injection time (Full MS/MS^2)	100/Auto
Data-dependent MS^2 ($ddMS^2$) acquisition	Top 4 for data acquisition using positive/negative polarity switching
Isolation window (*m/z*)	1.4
Normalized HCD (%)	15, 30, 50

An EASY-IC internal calibration was employed for all data acquisitions to ensure high mass accuracy throughout.

The MS data acquisition methods were set up using the method template in method editor. Figure 1 shows the method of Full Scan MS followed by top 4 data-dependent MS^2 with polarity switching and EASY-IC internal calibration.

Results and discussion

High-resolution Full Scan/ddMS² data with polarity switching acquisition for untargeted screening

For routine untargeted E&L analysis, high-resolution accurate-mass (HRAM) Full Scan and MS^2 data acquisition with polarity switching is necessary for elemental composition determination and structure characterization, which also ensures the detection of structurally diverse compounds and increases the analysis throughput.

In this study, the data acquisitions were carried out using Full Scan MS followed by top 4 data dependent MS^2 with polarity switching at resolution 60,000 (Full MS) and 15,000 (MS^2), respectively. The high scan speed of the Orbitrap Exploris 120 mass spectrometer enabled a duty cycle of ~1 second for 10 scan events data acquisition (Figure 2).

The obtained high quality HRAM Full Scan and higher-energy collisional dissociation (HCD) MS^2 data with sub-ppm mass accuracy and fine isotope pattern enabled confident elemental composition assignments, which warrant the accuracy of database search result.

Unknown compound identification is part of untargeted screening of E&L analysis. The accurate mass with fine isotope pattern, and information-rich HCD MS^2 spectra greatly assisted the structure elucidation of unknown compounds (Figures 3 and 4).

Figure 1. Instrument method – High-resolution Full Scan ddMS² with polarity switching and EASY-IC

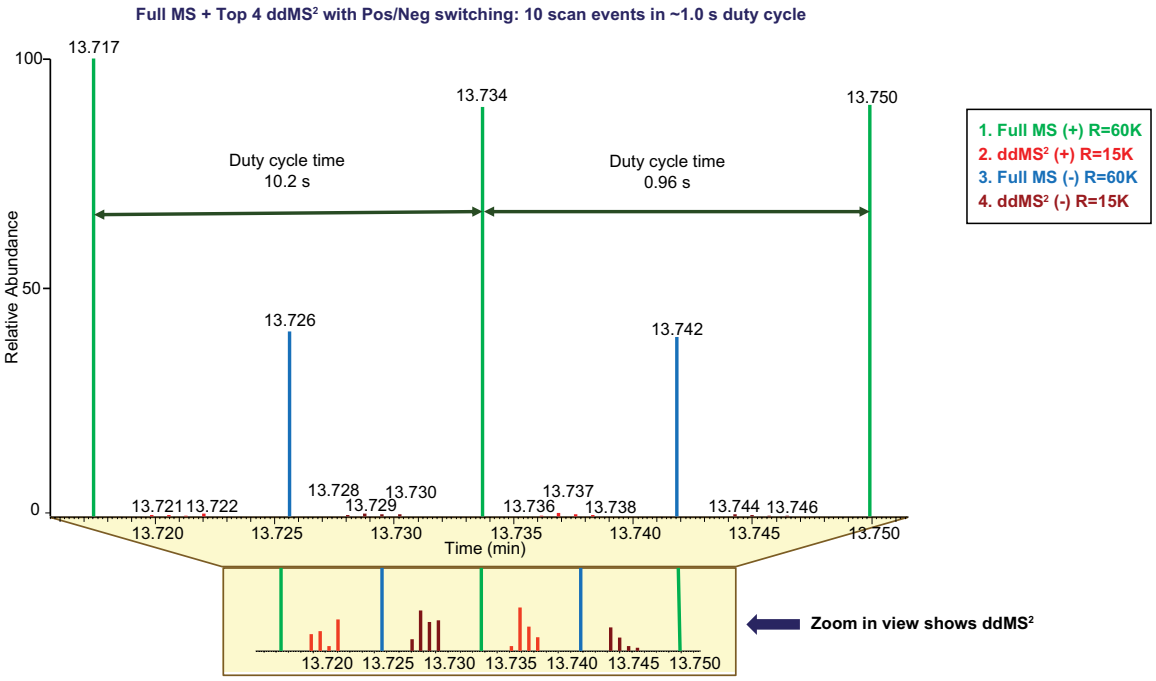

Figure 2. Short duty cycle time (~ 1 s) demonstrates fast scan speed for high-resolution Full MS and ddMS² with fast polarity switching acquisition.

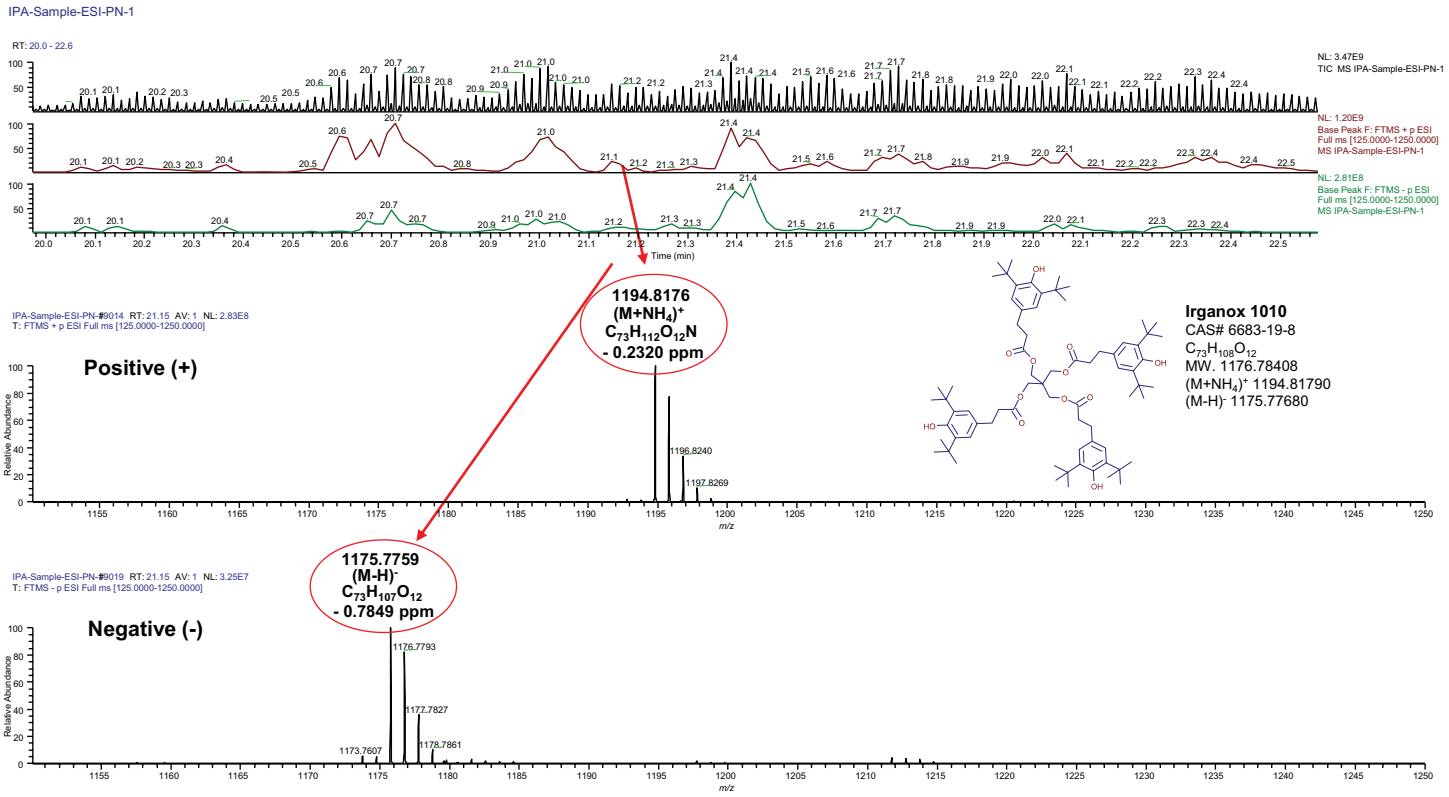

Figure 3. HRAM MS polarity switching with sub-ppm mass accuracy

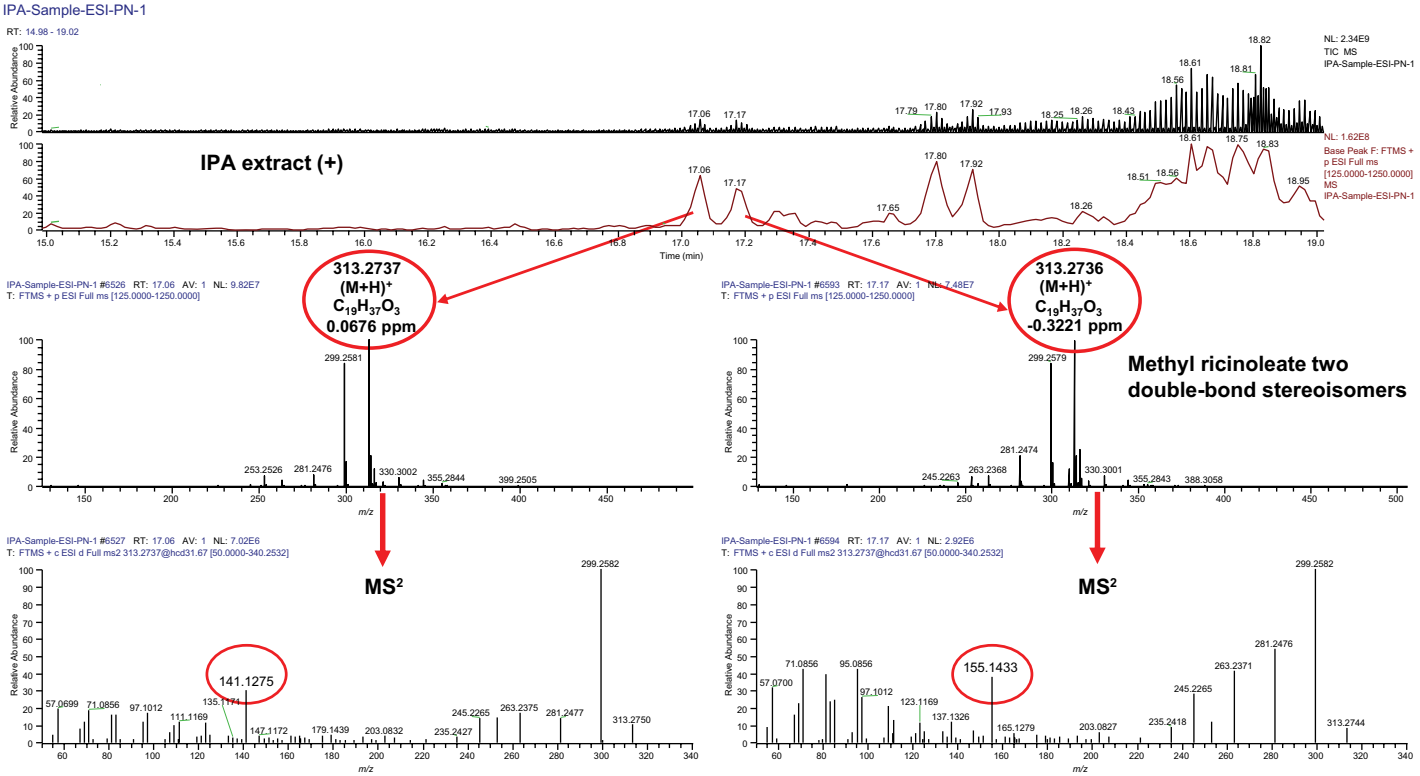

Figure 4. HRMS Full Scan and HCD ddMS² data for compound identification

Isopropanol (IPA) and dichloromethane (DCM) extraction results

The LC/MS results show that under the same extraction conditions, IPA and DCM rubber stopper extractions generated similar profiles but with different peak intensities (Figure 5); the DCM extract showed a higher intensity MS chromatogram and higher peak intensity for extracted compounds. It is worth mentioning that the compounds *m/z* 341.3049, $C_{21}H_{40}O_3$ at RT 19.95 and *m/z* 323.2580, $C_{20}H_{34}O_3$ at RT 19.92 were only observed from IPA extract. The variations were mostly caused by the polarity difference of IPA and DCM. The UV and MS total ion chromatograms of rubber stopper IPA and DCM are shown in Figures 5A and 5B.

Data processing using Compound Discoverer software with multiple databases and spectral library searching

E&L analysis data processing is generally carried out by searching internal and commercial databases followed by verification with in-house expertise. Good data processing software incorporating database and spectra library searches can significantly improve both efficiency and accuracy of extractable compounds identification. In this study, the data was processed using Compound Discoverer 3.2 software (OPTON-30925).

Compound Discoverer 3.2 software processes high-resolution accurate-mass (HRAM) data exclusively, and its advanced algorithm requires HRAM data and fine isotope pattern for accurate elemental formula and formula mass prediction. The software uses a flexible and customizable node-based processing workflow for component extraction followed by online and local database/spectral library search.

The data processing workflow was set up following the "New Study and Analysis Wizard" using workflow template *"E and L Unknown ID with Online and Local Database Searches".* The databases used in this workflow included Thermo Scientific™ mzVault™ E&L library, mzCloud™ spectral library, NIST HRMS ESI tandem mass spectral library, ChemSpider, and E&L compound database (Figure 6).

The processing results are shown in "Result View" (Figure 7). The result contains comprehensive information on extracted compounds and multiple database search results. The chromatographic and spectra properties of compounds can be inspected using the interactive "Compounds" table with predicted formula and accurate mass and the "Chromatograms" and "Mass Spectrum" views. The "Related Tables" contain the related information for each compound.

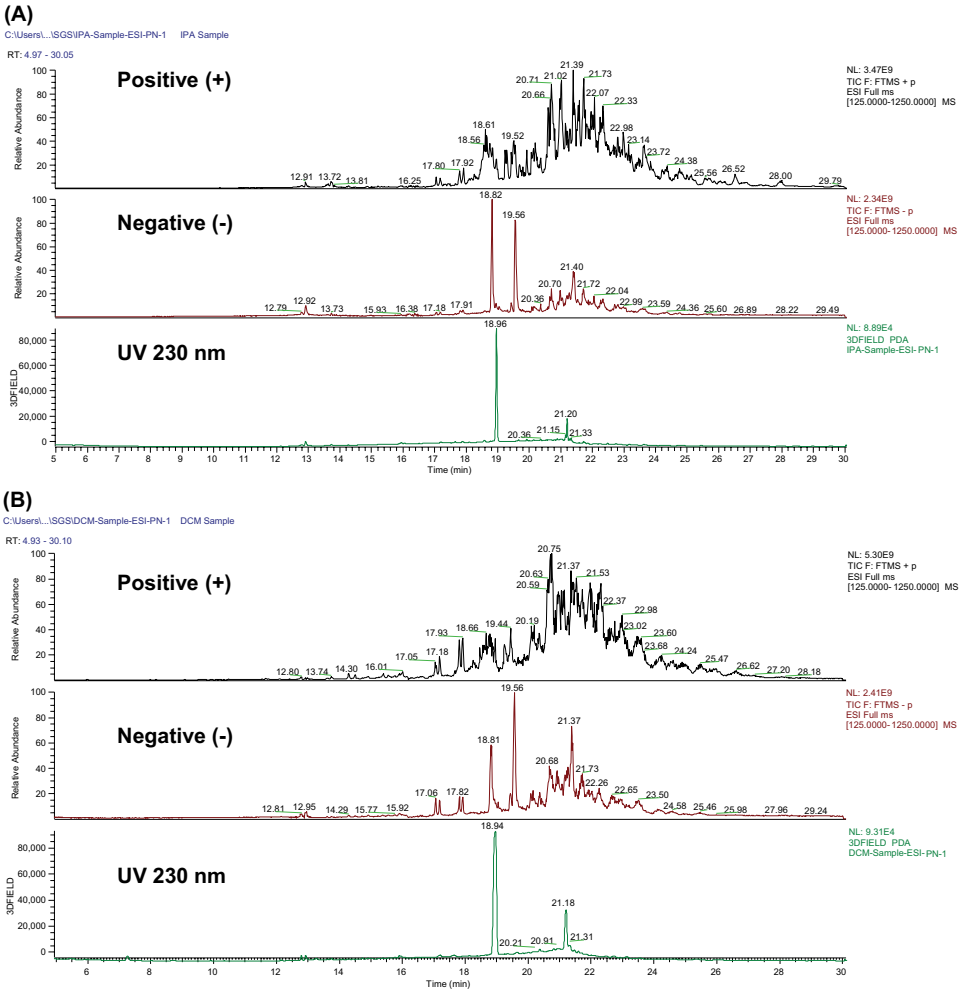

Figure 5. (A) UV and MS total ion chromatogram of rubber stopper IPA extract and (B) UV and MS total ion chromatogram of rubber stopper DCM extract

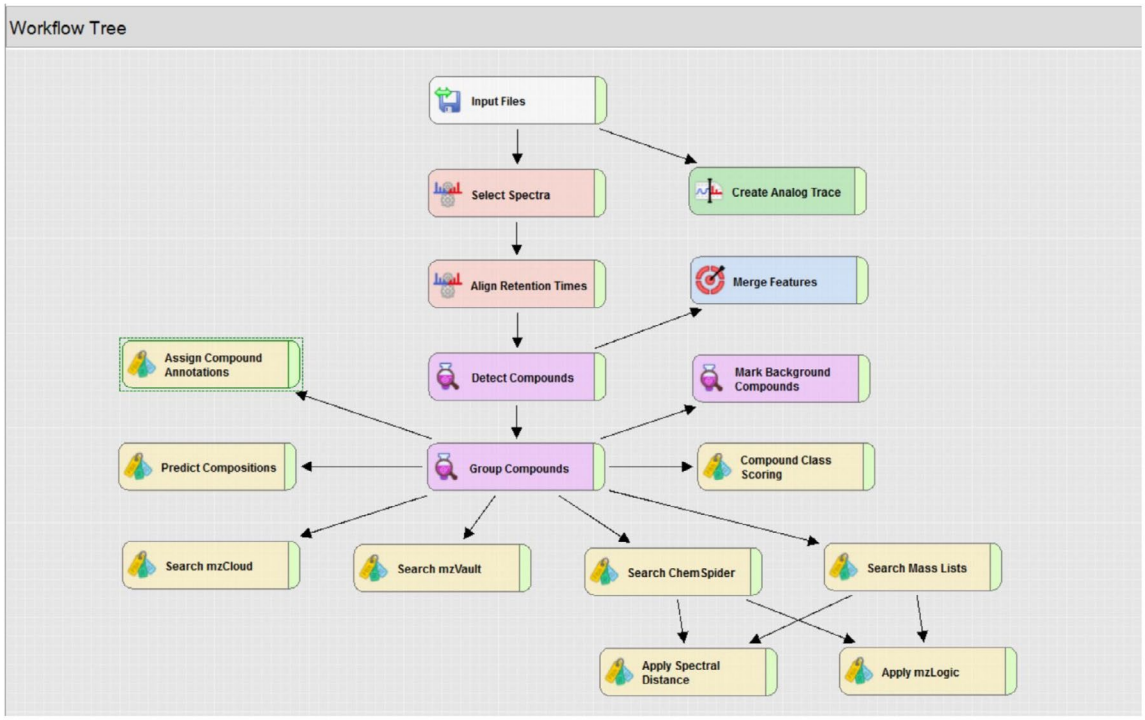

Figure 6. Compound Discoverer 3.2 software node-based processing workflow for E&L analysis

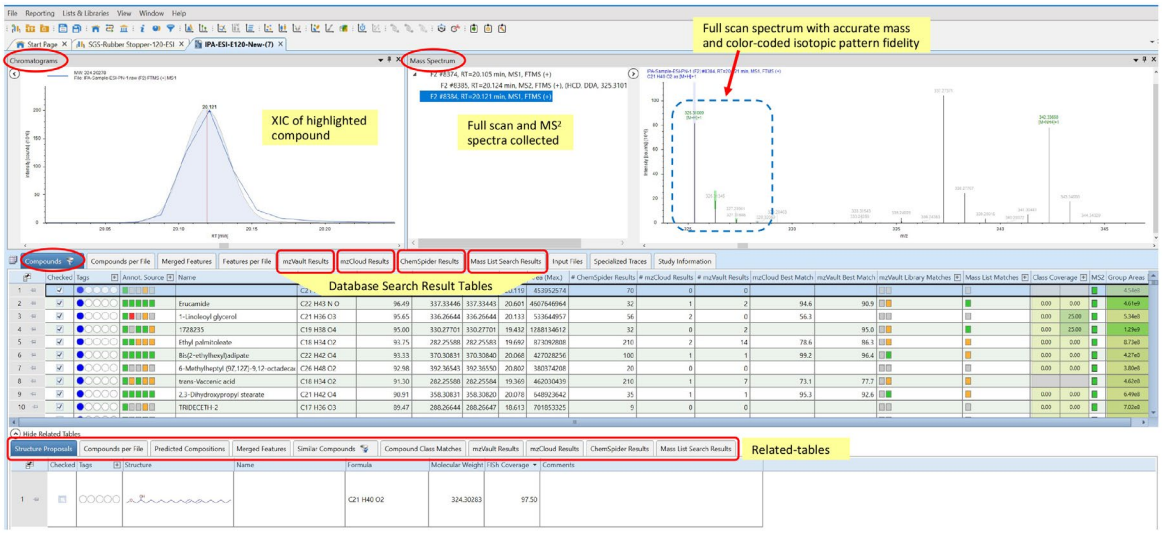

Figure 7. Compound Discoverer 3.2 software Processing Result view

The data processing was started by filtering the result tables through the flexible "Result Filter" to extract the most relevant data, then reviewing and confirming multiple database search results to identify any known compounds. Unknown compound identification was accomplished by proposing structures and checking the plausibility of the proposed structures through "FISh Scoring" (FISh = Fragment Ion Search). In addition, the "Class Coverage", "mzLogic Analysis", and "Partially Reprocessing" features greatly assisted obtaining optimum results in a timely manner.

The two approaches used were known compounds identification by databases search results and unknown compounds structure elucidation by utilizing Structure Proposal and FISh Scoring features.

Known compound identification by spectral library searches with fragment matching

By directly comparing with spectral library search results, the known extractables were readily identified through accurate mass and MS2 fragment ion matching. The annotated mirror plot of MS^2 spectra of identified compound and library standard shows the matching result for each hit. The mirror plot of the mzCloud search result of bis(2-ethylhexyl)adipate indicates confident identification (Figure 8). The higher Best Match score indicates a high degree of matching.

Unknown compound identification using Structure Proposals and FISh Scoring features

Unknown structure identification was carried out using Compound Discoverer 3.2 software's "Structure Proposals" and "FISh Scoring" features. Based on the predicted formula, molecular weight, and MS^2 fragmentation, putative structures were proposed in the Structure Proposals "Compound Annotation Editor" dialog box, followed by "FISh Scoring", an algorithm for in silico fragment prediction using both fragmentation rules and "Fragmentations and Mechanisms Library" search. The fragmentations and mechanisms library contains comprehensive known reactions and library mechanisms to support the predictions. Any matching fragments were automatically annotated with fragment structures, formula, accurate mass, and charge state. The FISh Coverage score indicated the percentage of fragment ion matching between experimental data and fragmentation libraries (Figure 9).

mzLogic Analysis assists structure determination from ChemSpider candidates

A ChemSpider search can generate many potential structure candidates, but without any fragment information. The "mzLogic Analysis" feature was used to rank the structures from ChemSpider candidates by comparing the experimental fragments to the extensive, fully curated MS^n fragment ions in the mzCloud mass spectral library and searching compounds with similar fragments.

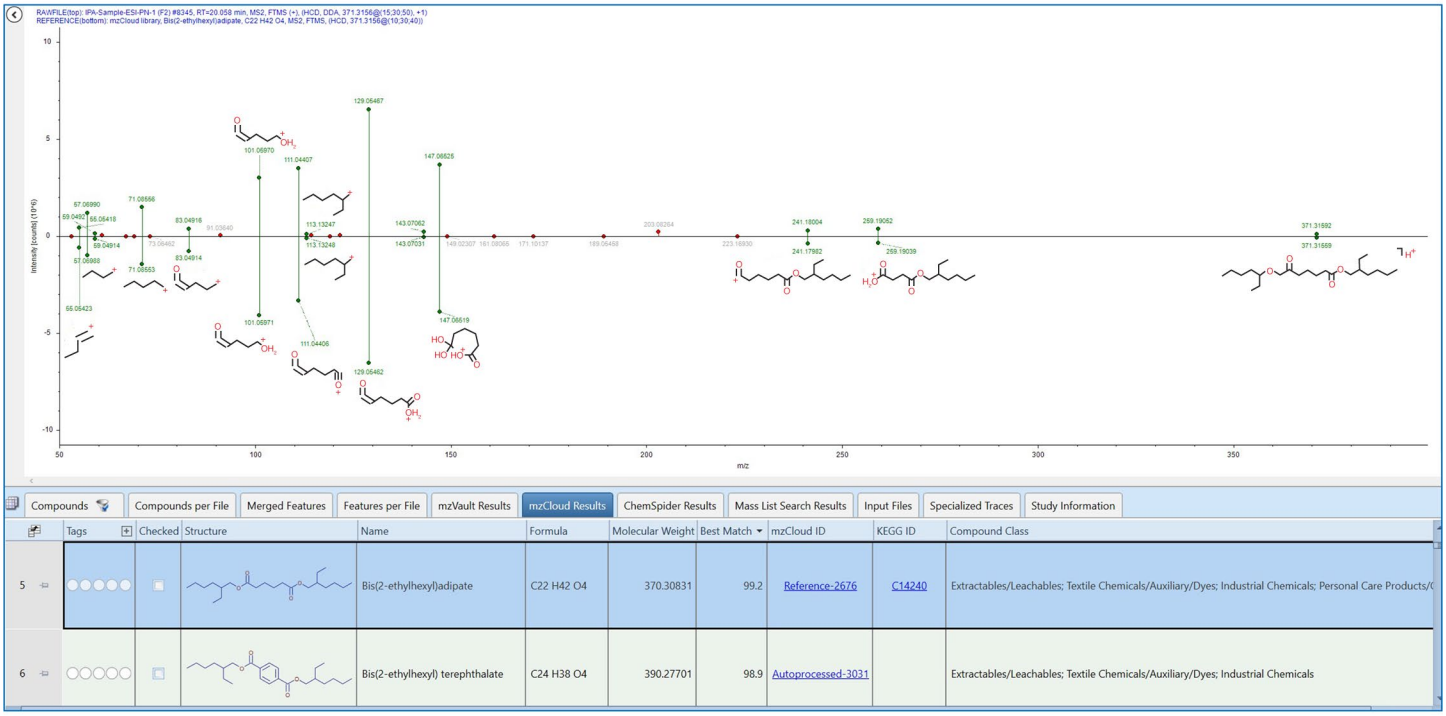

Figure 8. Mirror plot of MS² spectra of identified compound with reference standard

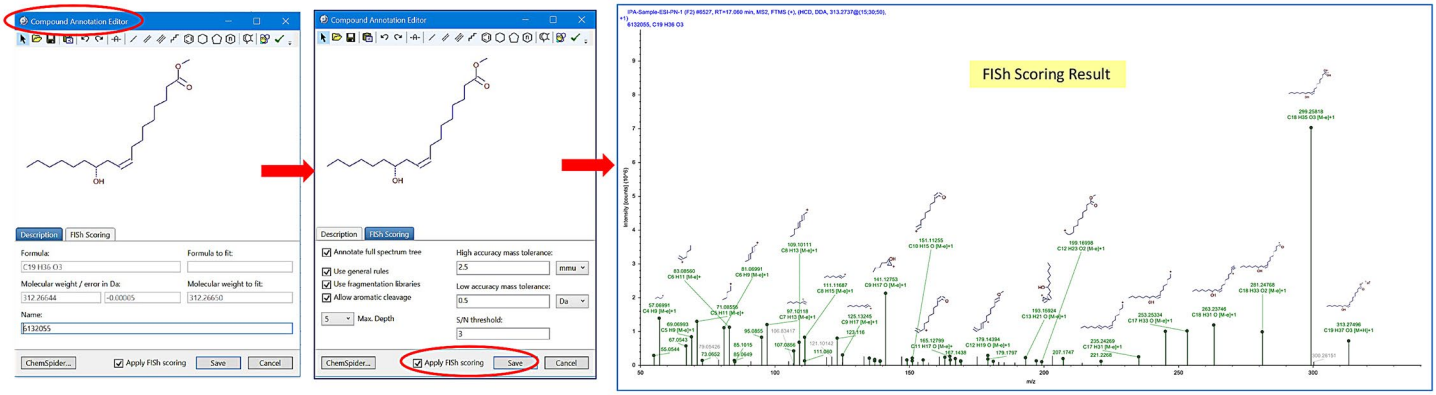

Figure 9. Structure proposal and FISh scoring for unknown structure elucidation

Figure 10 shows the mzLogic Analysis view. For ChemSpider candidates, mzCloud Similarity search results showed the similar structure in the mzCloud mass spectral library with 18.01057 Da mass difference; the common substructure was highlighted in blue. The candidate structure was added to the Structure Proposal table and modified based on the common substructure, followed by FISh Scoring. The FISh Coverage score was 95.65.

Fine tuning the analysis result using the flexible "Partially Reprocessing" feature

While reviewing the initial processing result, common MS^2 fragments (*m/z* 277.2165, 333.2429, 313.2737, and 355.2842) were observed in numerous peaks. To identify the compounds sharing the same fragments, two compound class lists were created using these fragment ions. The "Compound Class" node was added to the workflow tree. The modified workflow was submitted for partial reprocessing. The improved result was obtained instantly.

These fragments led to identifying compounds with structures that are related to epoxidized soybean oil (ESBO) and glyceryl ricinoleate, which are commonly used as a plasticizer and a stabilizer in rubber stopper production.

Data reporting

The result report was generated using the Compound Discoverer report template. For each identified compound, its structure, name (if available), formula, calculated MW, TIC chromatogram, Full Scan MS with isotope pattern, annotated MS^2 spectrum, and FISh Coverage were included in the report (Figure 11).

Compound Discoverer 3.2 software also provides a tool kit to create customized report formats.

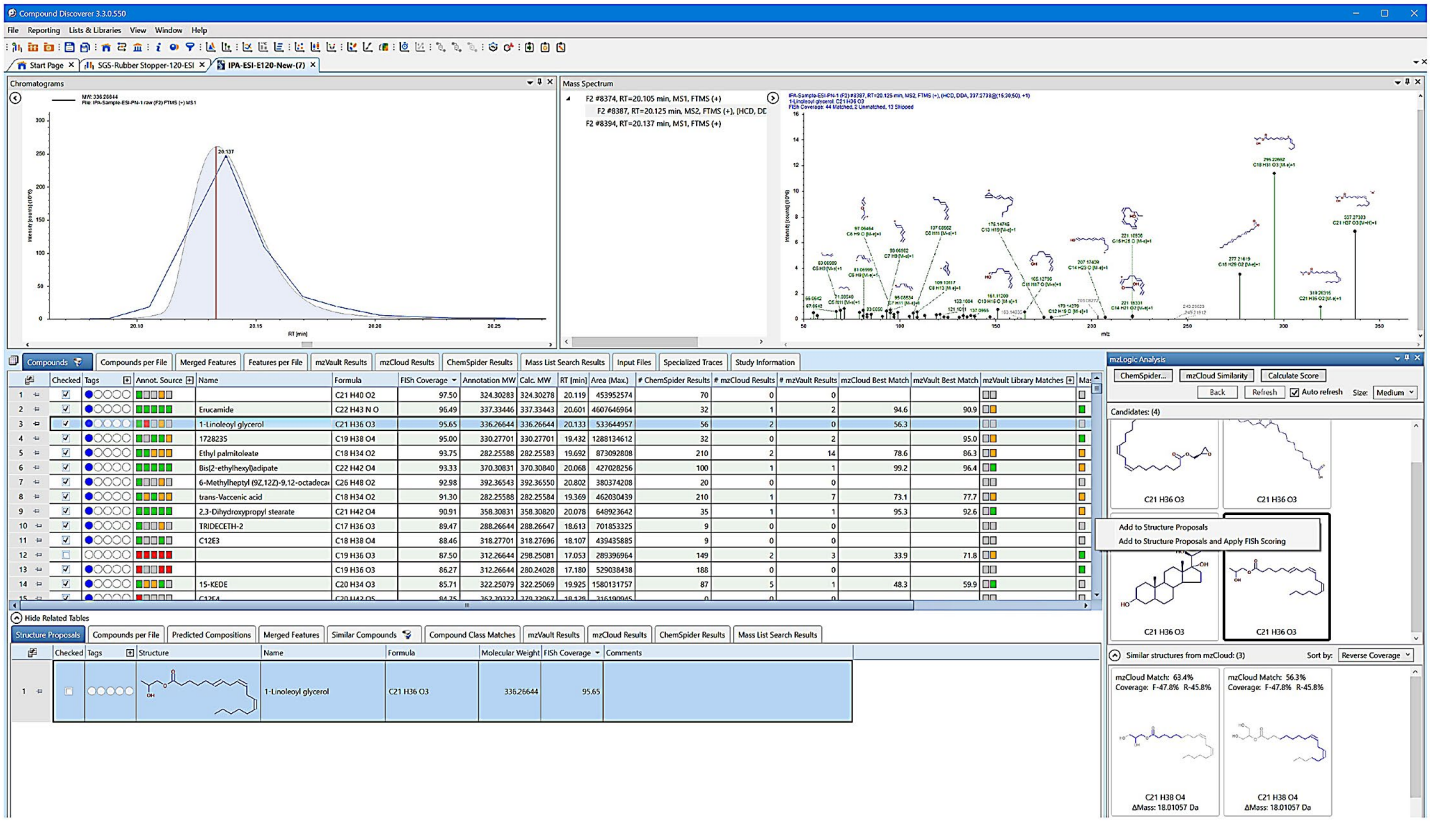

Figure 10. mzLogic Analysis for structure determination from ChemSpider candidates

Compounds

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Structure	Name	RT [min]	Formula	Calc. MW	Group Areas
	Bis(2-ethylhexyl)adipate	20.07	C22 H42 O4	370.30840	4.27e8

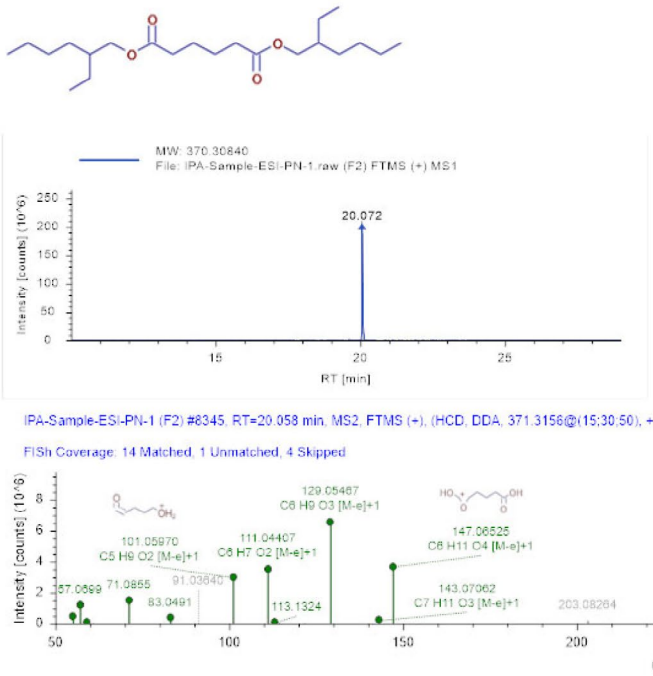

Figure 11. CD 3.2 data report

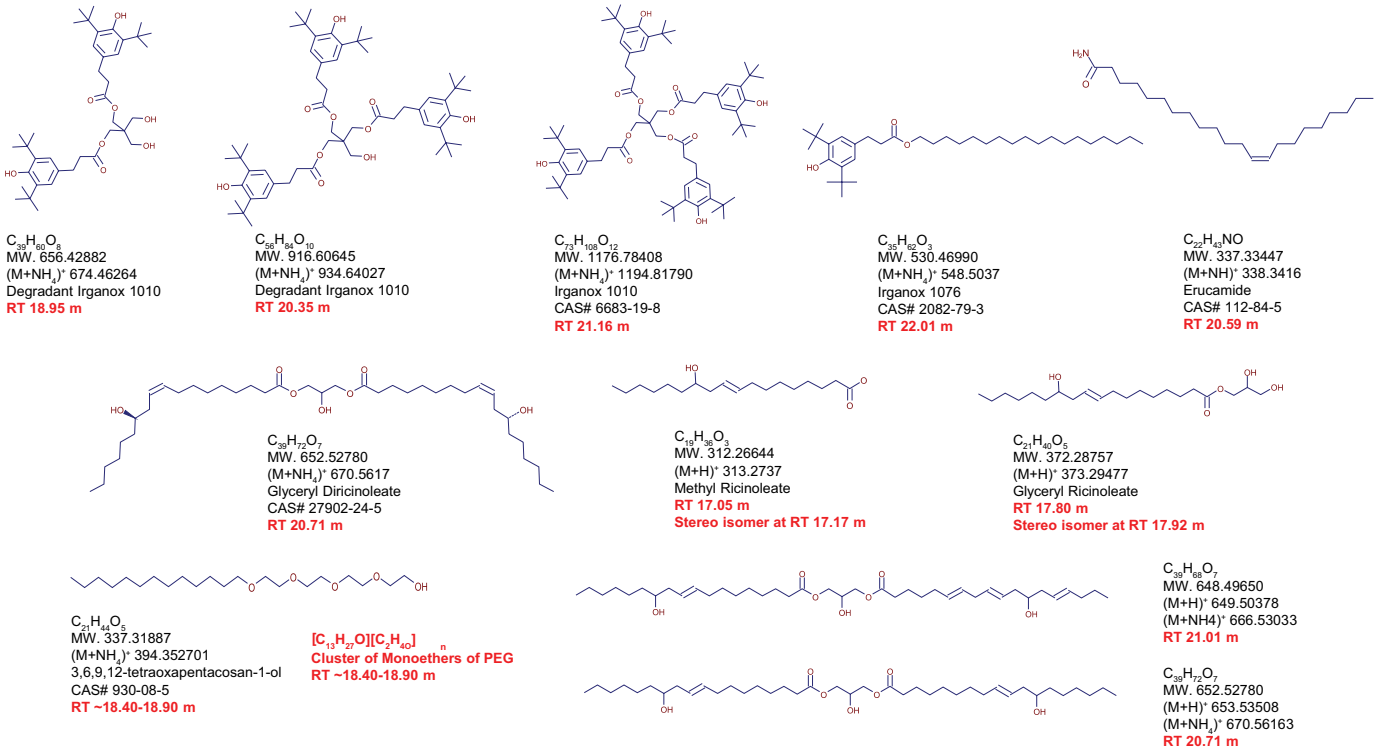

Figure 12. Proposed structures of extractables in IPA extract (partial list)

Table 1. Compounds identified in IPA and DCM extracts (partial list)

RT (min)	Measured (singly charged)	Ion type	Formula weight	Elemental composition	Error (ppm)	Name
14.29	313.2373	$(M+H)^+$	312.2301	$C_{18}C_{33}O_4$	-0.21	
14.49	313.2373	$(M+H)^+$	312.2301	$C_{18}C_{33}O_5$	-0.15	
17.06	313.2737	$(M+H)^+$	312.2664	$C_{19}H_{36}O_3$	0.06	
17.17	313.2737	$(M+H)^+$	312.2664	$C_{19}H_{36}O_3$	-0.32	
17.80	373.2948	$(M+H)^+$	372.2876	$C_{21}H_{40}O_5$	-0.22	
17.92	373.2948	$(M+H)^+$	372.2876	$C_{21}H_{40}O_6$	-0.06	
18.26	421.1948	$(M+H)^+$	420.1875	$C_{20}H_{37}O_4Br$	0.05	
18.4-18.9	mixture	$(M+H)^+$		$[C_{13}H_{27}O][C_2H_4O]_n$		Cluster of PEG polymer
18.95	674.4627	$(M+NH_4)^+$	656.4288	$C_{39}H_{60}O_8$	-0.2	Irganox1010 degradent
19.25	327.2892	$(M+H)^+$	326.2821	$C_{20}H_{38}O_3$	-0.58	
19.43	331.2843	$(M+H)^+$	330.2770	$C_{19}H_{38}O_4$	-0.05	
19.53	341.3050	$(M+H)^+$	340.2977	$C_{21}H_{40}O_3$	-0.12	
19.79	682.5253	$(M+NH_4)^+$	664.4909	$C_{39}H_{68}O_8$	0.03	
19.84	698.5203	$(M+NH_4)^+$	680.4863	$C_{39}H_{68}O_9$	0.14	Epoxidized $C_{39}H_{68}O_8$
19.92	323.2580	$(M+H)^+$	322.2508	$C_{20}H_{34}O_3$	-0.3	
20.12	325.3101	$(M+H)^+$	324.3028	$C_{21}H_{40}O_2$	-0.05	
20.12	337.2737	$(M+H)^+$	336.2664	$C_{21}H_{36}O_3$	-1.66	
20.20	764.4668	$(M+H)^+$	746.4332	$C_{46}H_{70}O_3NBr$ (?)		
20.35	934.6403	$(M+H)^+$	916.6065	$C_{56}H_{84}O_{10}$	0.113	Irganox1010 degradent
20.59	338.3416	$(M+H)^+$	337.3345	$C_{22}H_{43}NO$	-0.3	Erucamide
20.71	670.5617	$(M+NH_4)^+$	652.5278	$C_{39}H_{72}O_7$	-0.25	CAS# 27902-24-5
20.76	411.3831	$(M+H)^+$	410.3760	$C_{26}H_{50}O_3$	-0.296	
21.02	666.5305	$(M+NH_4)^+$	648.0000	$C_{39}H_{68}O7$	0.285	
21.16	1194.8179	$(M+NH_4)^+$	1176.7841	$C_{73}H_{108}O_{12}$	-0.02	Irganox1010
21.32	662.4990	$(M+NH_4)^+$	644.0000	$C_{39}H_{64}O_7$	-0.02	
21.40	628.5511	$(M+NH_4)^+$	610.5172	$C_{37}H_{70}O_6$	0.13	
21.58	663.4537	$(M+H)^+$	662.4464	$C_{42}H_{63}O_4P$	0.1	Oxidized Irgafos 168
21.71	656.5822	$(M+NH_4)^+$	638.0000	$C_{39}H_{74}O_6$	-0.23	
21.71	624.5198	$(M+NH_4)^+$	606.0000	$C_{37}H_{66}O_6$	0.02	
22.03	548.5037	$(M+NH_4)^+$	530.4699	$C_{35}H_{62}O_3$	0	Irganox 1076 CAS 2082-79-3
22.07	950.8022	$(M+H)^+$	933.7769	$C_{57}H_{104}O_9$	0.34	
22.34	1002.6974	$(M+NH_4)^+$	984.6623	$C_{55}H_{101}O_9Br$	0.72	
22.89	1030.7281	$(M+NH_4)^+$	1012.6936	$C_{57}H_{105}O_9Br$		
22.98	988.7181	$(M+NH_4)^+$	994.6841	$C_{55}H_{103}O_8Br$	0.63	
23.14	908.7918	$(M+NH_4)^+$	890.7569	$C_{55}H_{102}O_8$	0.56	
23.88	904.7604	$(M+NH_4)^+$	886.7256	$C_{55}H_{98}O_8$	0.42	
24.36	988.7179	$(M+H)^+$		$C_{55}H_{107}O_8Br$ (?)		
25.00	866.7810	$(M+NH_4)^+$	848.7464	$C_{53}H_{100}O_7$ (?)	0.32	
26.04	894.8124	$(M+NH_4)^+$	876.7777	$C_{55}H_{104}O_7$	0.45	
26.52	862.7497	$(M+NH_4)^+$	844.7151	$C_{53}H_{96}O_7$	0.3	
27.34	922.8433	$(M+NH_4)^+$	904.8090	$C_{57}H_{108}O_7$	-0.01	
27.98	890.7811	$(M+NH_4)^+$	872.7464	$C_{55}H_{100}O_7$	0.38	

Note: RT 18.4-18.9 are clusters of monoethers of polyethylene glycol PEG-X Dodecyl ether (X=3,4,5... units of ethylene glycol)

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Conclusion

The result of extractable analysis of rubber stoppers for pharmaceutical applications demonstrates an effective, comprehensive workflow for extractable and leachable analysis using the Orbitrap Exploris 120 mass spectrometer, Vanquish Horizon UHPLC system, and Compound Discoverer 3.2 data processing software.

- The Orbitrap Exploris 120 mass spectrometer HRAM Full Scan and HCD ddMS^2 data acquisition with fast polarity switching in a single run increase the confidence and throughput of routine extractable and leachable analysis.
- Compound Discoverer 3.2 data processing software with multiple databases search in parallel, together with Structure Proposal, FISh Scoring, Partially Reprocessing, mzLogic, and other unique features, provides powerful data mining tools for confident known extractable identification and unknown compound structure elucidation.
- This workflow is well suited to high-throughput routine extractables and leachables analysis and other small molecule structure identification applications.

References

1. FDA guideline “Container closure systems for packaging human drugs and biologics” USP <1663>, “Assessment of Extractables Associated with Pharmaceutical.
2. ISO-10993-18 and ISO-10993-12.

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