Application note | 002733

Pharma

Improved identification of extractable and leachable substances with the Orbitrap Exploris GC 240 MS

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Keywords

Spectral deconvolution, HRAM spectral libraries, structural elucidation, unknown screening

Goal

The goal of this study is to demonstrate the performance of the Thermo Scientific™ Orbitrap™ Exploris™ GC 240 mass spectrometer in combination with Thermo Scientific™ Compound Discoverer™ software to improve detection and identification of extractable and leachable substances.

Introduction

Analysis of extractable and leachable (E&L) compounds is essential in pharmaceutical product development. Prior to patient administration, toxicological safety must be assessed. This should be performed not only for the drug products being developed, but also for potential impurities introduced through leaching from contact materials during manufacturing and/or storage.

In the assessment of potential leachable impurities, controlled extraction studies of polymetric contact materials are performed to provide a worst-case scenario of the potential profile of leachable substances present in the final product. Potential leachable impurities need to be identified, where mass spectrometry is used to assist with structural elucidation. Liquid chromatography-mass spectrometry (LC-MS) is commonly used for compound identification in E&L workflows. However, chromatographic separation by LC may not be ideal for all substances due to the wide range in physicochemical properties for possible leachable substances (i.e., volatility, polarity).

Addressing these limitations, gas chromatography is an essential and complementary technique to obtain comprehensive compound coverage within E&L workflows. High-resolution accurate-mass (HRAM) GC analysis obtained with Orbitrap technology can provide unsurpassed selectivity (up to 240,000 mass resolution full width at half maximum (FWHM) at *m/z* 200) and mass accuracy(<1 ppm). This greatly enhances the analysts' capabilities for reliable and accurate identification/structural elucidation in unknown analysis of E&L substances.

This study demonstrates the benefits of incorporating the Orbitrap Exploris GC 240 mass spectrometer for unknown substance identification of sample extracts within an E&L workflow. Statistical differences between samples extracted under different conditions are easily visualized using Compound Discoverer software with simplified identification using both the established NIST and in-house built HRAM libraries. The NeverVent™ technology allows for rapid switching between electron impact (EI) and positive chemical ionization (PCI) for molecular ion confirmation/identification and elemental composition determination with structural elucidation being demonstrated for detected unknown substances.

Experimental

Standard and sample preparation

A screening standard of E&L substances for GC was purchased from Sigma-Aldrich (Germany) to generate HRAM spectra and retention time references for known E&L substances (caprolactam, 2-mercatobenzothiazole, bisphenol A, 2,4-di-*tert*-butylphenol, 2,6-di-*tert*-butylphenol, oleamide, palmitic acid (hexadecanoic acid), stearic acid (octadecanoic acid), bis(2-ethylhexyl) phthalate, erucamide, tris(2,4-di-*tert*-buylphenol) phosphate, 1,3-di-*tert*-butylbenzene, 2,6-di-*tert*-butyl-4-methylphenol). A 1 µg·mL^{-1} concentration in dichloromethane (DCM) was injected to prescreen compounds for identification/structural elucidation. A retention index was established through the analysis of a 1 µg·mL^{-1} C7-C40 alkane mixture (Sigma-Aldrich, Germany).

Sample preparation involved the extraction of commercially available rubber stoppers for pharmaceutical applications using isopropanol (IPA) and dichloromethane (DCM) at 50 °C for 72 hours according to the method described in the ISO 10993-12 guideline.[1]

Instrument and method setup

Injection and chromatographic conditions for the Thermo Scientific™ TRACE™ 1610 GC equipped with a Thermo Scientific™ TraceGOLD™ TG-5SilMS (30 m × 0.25 mm i.d. × 0.25 µm film) capillary column (P/N 26096-1420) are summarized in Table 1. Automated liquid injection was performed using a Thermo Scientific™ TriPlus™ RSH SMART autosampler. Data acquisition was carried out with full scan analysis using EI and PCI with the Orbitrap Exploris GC 240 mass spectrometer. Additional MS method parameters are summarized in Tables 2 and 3. External mass calibration was performed prior to analysis, while characteristic ions representing column bleed were used as lock masses when scanning in EI to perform internal mass calibration. Sample acquisition and qualitative processing was performed using Thermo Scientific™ Chromeleon™ version 7.3.2 Chromatography Data System (CDS) software. Unknown analysis and identification performed using Compound Discoverer version 3.3 SP2 software.

Table 1. GC injection and column conditions

Trace 1610 GC system parameters	
Injector	Thermo Scientific™ HeSaver-H_2Safer™ kit for iConnect™ SSL
Injection volume (µL)	1
Liner	Thermo Scientific™ LinerGOLD™ liner single taper with quartz wool (P/N 453A1925-UI)
Injection mode	Splitless (split flow 50 mL·min^{-1} after 2 min)
Split flow (mL·min^{-1})	87
Injector temperature (°C)	280
Carrier gas, (mL·min^{-1})	1.2
Oven temperature program	
Initial temperature (°C)	40
Hold time (min)	1
Rate 1 (°C·min^{-1})	20
Temperature 1 (°C)	150
Hold time 1 (min)	0
Rate 2 (°C·min^{-1})	10
Temperature 2 (°C)	320
Hold time 2 (min)	5
Total run time (min)	29

Table 2. EI source and mass spectrometer conditions

Orbitrap Exploris GC-MS parameters	
Transfer line (°C)	290
Thermo Scientific™ ExtractaBrite™ ion source temperature (°C)	320
Electron energy (eV)	70
Acquisition mode and scan range (*m/z*)	Full scan, 40–650
Resolving power (at 200 *m/z*)	120,000
Emission current (µA)	50
C-Trap offset (V)	0
Mass accuracy on lock mass	5 ppm
Internal lock mass calibration (column bleed, *m/z*)	207.02235, 281.05114, 355.06993

Table 3. CI ion source and mass spectrometer conditions

Orbitrap Exploris GC-MS parameters	
Transfer line (°C)	290
ExtractaBrite ion source temperature (°C)	200
Reagent gas and flow (mL·min^{-1})	Methane; 1
Ionization mode	Positive
Acquisition mode and scan range (*m/z*)	Full scan; 100–700
Resolving power (at 200 *m/z*)	120,000
Emission current (µA)	50

Results and discussion

Enhanced detection of E&L substances using GC-MS

An overview of compounds detected at a concentration of 1 µg·mL^{-1} in E&L screening standards using GC and LC with various modes of ionization/detection is provided in Table 4. Due to the differences in analyte responses among various detection techniques with LC, a multi-detector approach is ideal for a comprehensive untargeted overview for E&L substances.[2] Despite four different modes of ionization/detection using LC, 1,3-di-*tert*-butylbenzene and 2,6-di-*tert*-butyl-4-methylphenol were not detected. However, excellent response for these compounds is observed when using GC-MS as an analytical technique.

In E&L workflows, compound detection and identification with multiple instrumental techniques provides greater confidence to analysts. Such comparison can be easily facilitated using the new dual sequence evaluation feature within the Chromeleon CDS 7.3.2. An example of this shown in Figure 1, where the response and mass spectrum of 2,4-di-*tert*-buytlphenol at 1 µg·mL^{-1} is directly compared between sequence data collected using both (A) GC-HRAM and (B) LC-HRAM techniques. Enhanced sensitivity for the molecular ion is clearly demonstrated when using GC-HRAM, with a response that is 127 times greater than that achieved by LC-HRAM. 2,6-di-*tert*-butyl-4-methylphenol, which was not detected by LC-MS, elutes just after 2,4-di-*tert*-butylphenol (Figure 1). Fragmentation of 2,6-di-*tert*-butyl-4-methylphenol in the EI source produces the M^+ ion of 2,4-di-*tert*-butylphenol, highlighting the importance of chromatographic separation. However, greater sensitivity and selectivity can be achieved using the $[M\text{-}CH_3]^+$ ($C_{13}H_{19}O$) fragment ion where no interference is observed due to the high mass resolving power and accuracy of the Orbitrap Exploris GC 240 mass spectrometer.

Spectral deconvolution and identification with E&L HRAM spectral libraries

Results from the spectra deconvolution analysis with Compound Discoverer software of a rubber stopper sample extracted using both DCM and IPA are shown in Figure 2. Out of a total of 929 compounds detected, 695 unknown E&L substances were identified after background correction from blank analysis using the Mark Background Compounds node within Compound Discoverer software. On visual inspection, greater complexity can be observed in a rubber stopper sample extracted using DCM. Development of HRAM spectra libraries for E&L substances can greatly reduce sample complexity to simplify unknown identification, with new compounds being easily incorporated into the ever-growing E&L HRAM spectral library database. Chromatographic analysis of the rubber stopper analysis extracted using DCM and IPA can be seen in Figure 3. As an example, this figure shows how two compounds present in the E&L screening standard, hexandecanioc acid (palmitic acid) and octadecanoic acid (steric acid) were clearly identified within the rubber stopper processed under extractable conditions with total scores >97 and search index cores of >880 (Figure 3).

High selectivity and mass accuracy providing confident identification

Although several peaks can be clearly identified in the sample chromatograms shown in Figure 3, response factors can vary significantly across different compounds due to differences in structural characteristics and fragmentation pattern. This poses challenges to analysts, particularly in complex sample matrices where a given compound's response may not be sufficient to be detected over the background sample matrix response. The GC-HRAM solution highlighted in this application note can assist in addressing these challenges.

Table 4. Comparison of compounds in E&L screening standards by GC and LC with various detection techniques. Data for LC analysis was previously reported in Thermo Scientific Application Note 001401.[2]

Compound	Retention time (min)		Detected by LC-ESI(+)	Detected by LC-ESI(–)	Detected by LC-CAD	Detected by LC-UV (200–680 nm)	Detected by GC EI(+)
	LC	GC					
Caprolactam	3.7	7.5	X*				X
2-Mercaptobenzothiazole	8.3	11.7	X	X	X	X	X*
Bisphenol A	10.1	15.9		X*	X	X	X*
2,4-Di-*tert*-butylphenol	15.9	9.5	X			X	X
Oleamide	17.0	17.3	X		X		X*
Palmitic acid	17.8	13.8	X	X	X		X
Stearic acid	19.0	15.6	X	X	X		X
Bis(2-ethylhexyl) phthalate	19.1	18.8	X		X		X
Erucamide	19.4	20.5	X		X		X*
Tris(2,4-di-*tert*-butylphenyl) phosphate	22.0	26.2	X	X	X		X
1,3-Di-*tert*-butylbenzene	ND	7.3					X
2,6-Di-*tert*-butyl-4-methylphenol	ND	9.6					X

ND – Not detected
X – Compound detected at 1 ppm concentration
X* – Compound detected at 10 ppm concentration
ESI(+) – Electrospray ionization positive mode
ESI(-) – Electrospray ionization negative mode
CAD – Charged aerosol detector
UV – Ultraviolet detector
EI(+) – Electron impact ionization

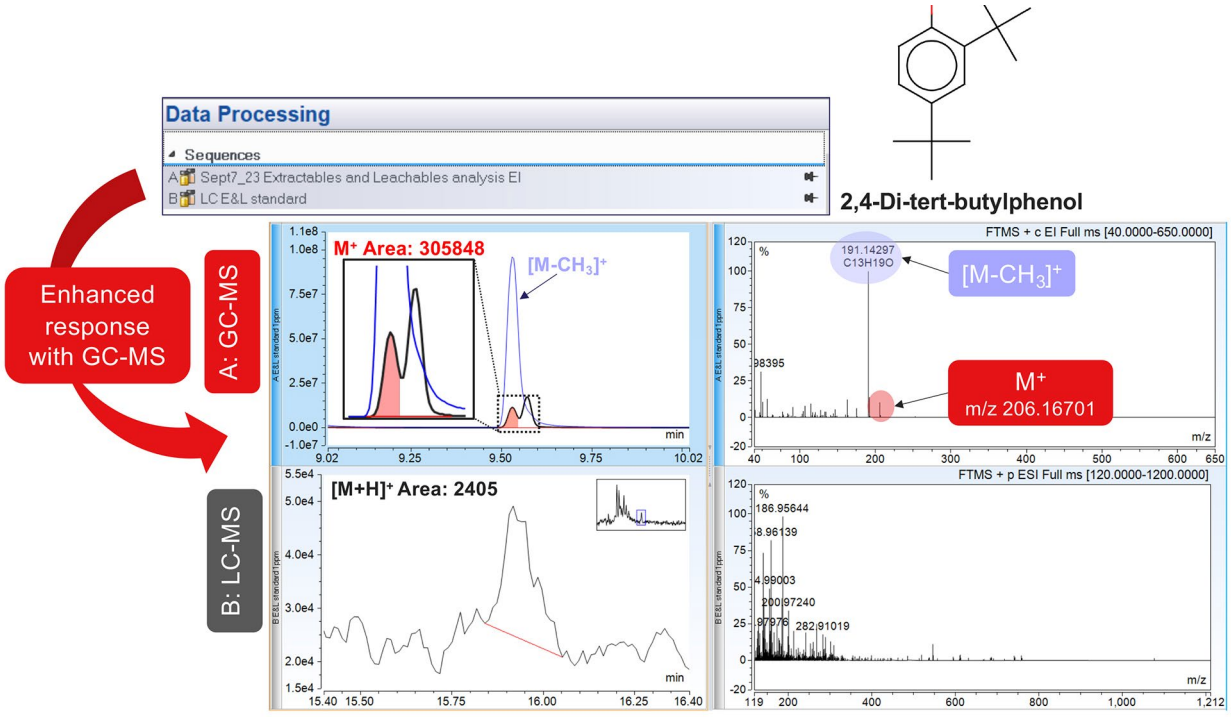


Figure 1. Dual sequence comparison of analyte response and mass spectrum of 2,4-di-*tert*-buytlphenol at 1 µg·mL^{-1} collected by GC-MS (sequence A) and LC-MS (sequence B) in Chromeleon CDS 7.3.2

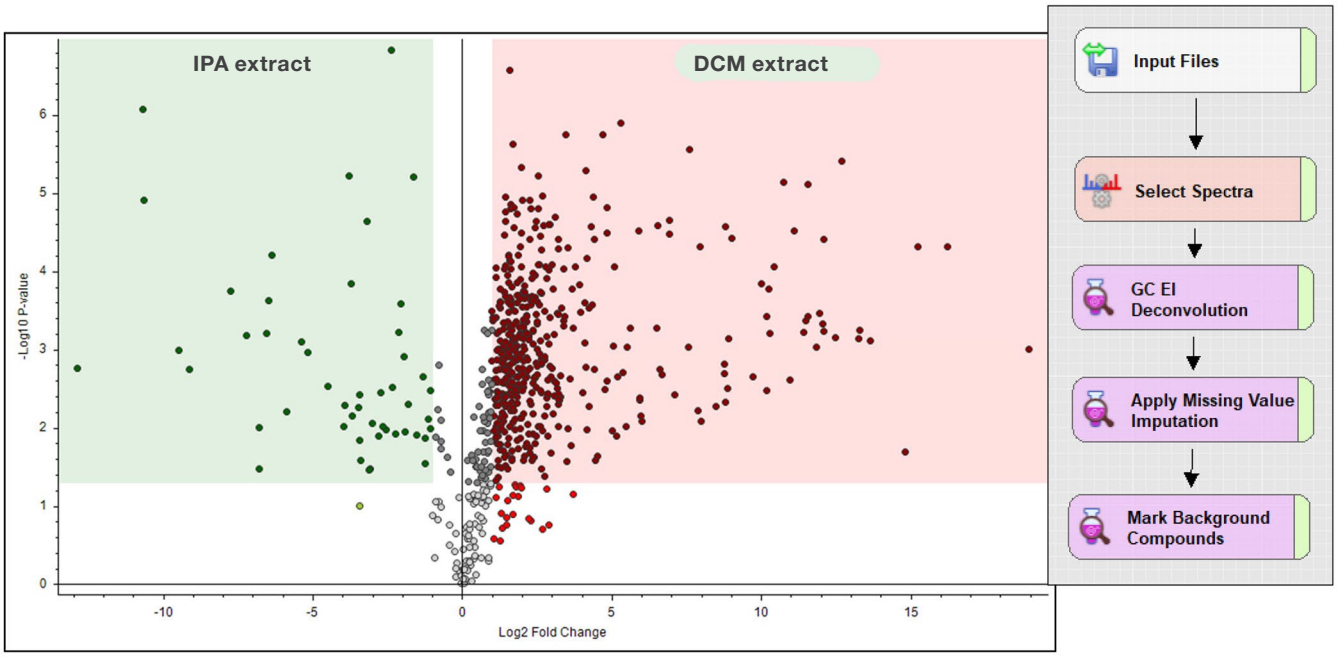


Figure 2. Volcano plot of the unknown compounds (695) identified after spectral deconvolution and background compound removal with the Compound Discoverer software workflow. The X axis represents the difference in response observed by a factor of $\log_2$n change. The Y axis represents the -log *p*-value based on the statistical analysis between the two sample groups. Shaded regions represent compounds whose response differs greater than 2-fold and are significantly different at the 95% confidence interval ($p \leq 0.05$).

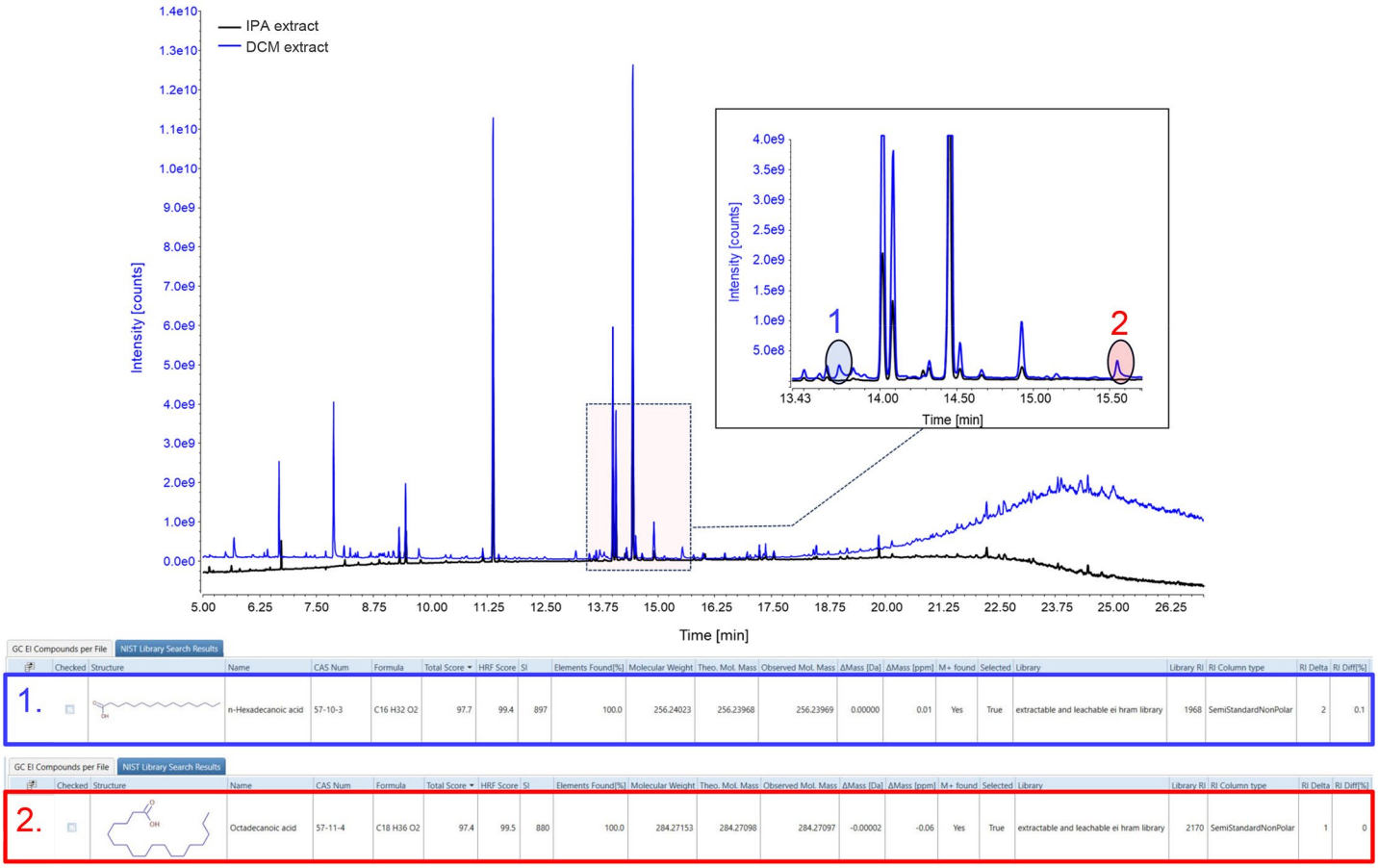


Figure 3. Total ion chromatogram of rubber stopper sample extracted with DCM (blue line) and IPA (black line) with hexadecanoic acid (peak 1) and octadecanoic acid (peak 2) identified based on the E&L HRAM spectral library hit

An example of this can be seen in Figure 4, where the observed sample mass spectra at 12.07 min matched the E&L HRAM library spectra of 3,5-di-*tert*-butyl-4- hydroxybenzaldehyde. Despite not being visible from the TIC of the sample extract, the mass spectra of this compound could be easily distinguished from the surrounding background using the deconvolution capability of Compound Discoverer software. Extraction of the exact mass of the molecular ion ([M-e]$^+$: 234.16144 *m/z*) provides a clean extracted ion chromatogram of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde free of background noise/sample matrix interferences, highlighting the high mass resolving power of the Orbitrap Exploris GC 240 mass spectrometer.

In addition to high mass resolving power, sub-ppm mass accuracy provides identification confidence. For example, in the case of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, the observed molecular ion showed a mass error of 0.03 ppm from the theoretical mass (Figure 5). Based on the number of possible elemental compositions, only $C_{15}H_{22}O_2$ can be associated with this mass at <1 ppm mass error.

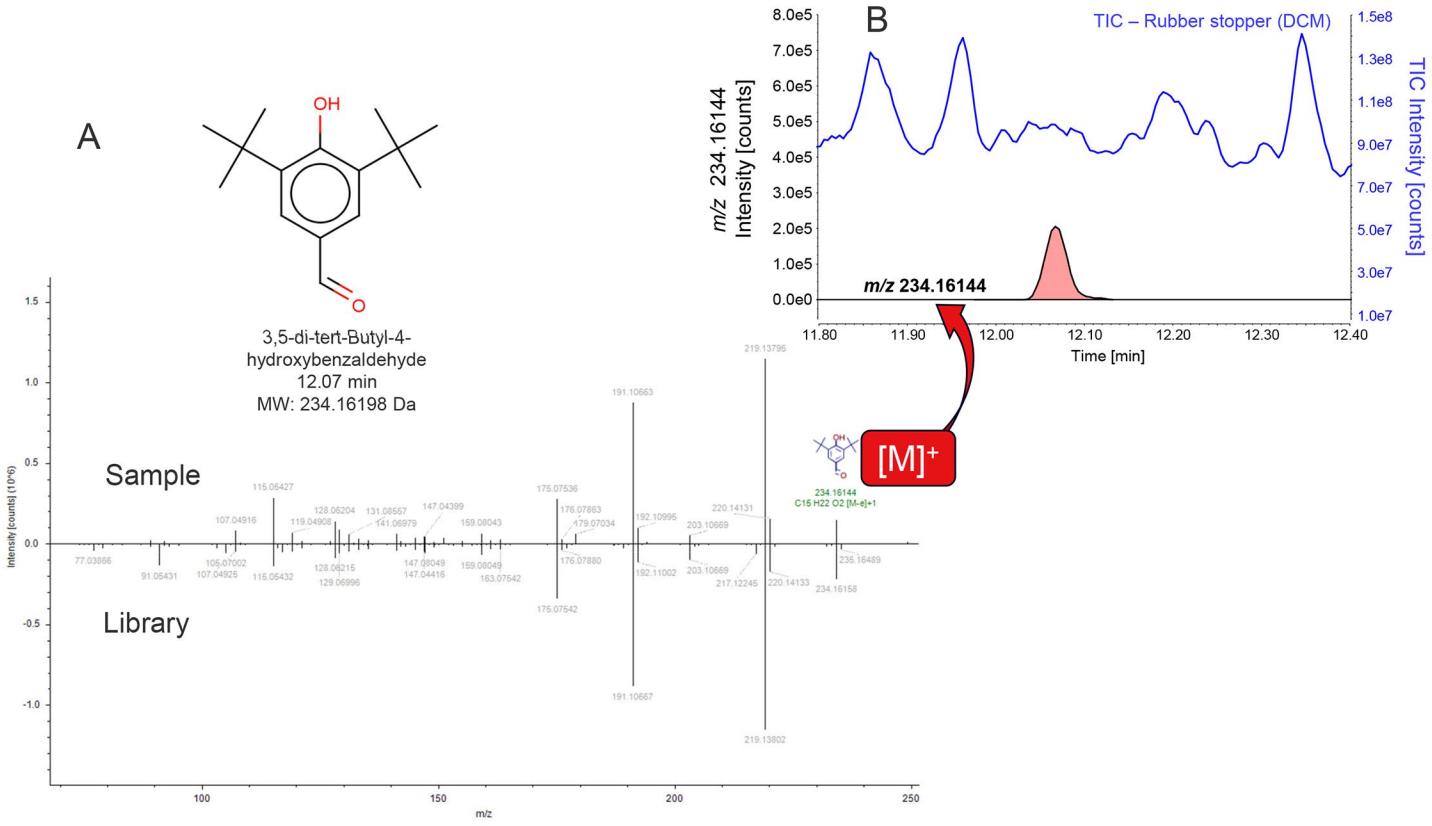


Figure 4. (A) Observed sample mass spectra and matching HRAM library hit of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde and (B) total ion chromatogram (TIC) and extracted exact mass of the molecular ion ([M]$^+$: *m/z* 234.16144) of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde

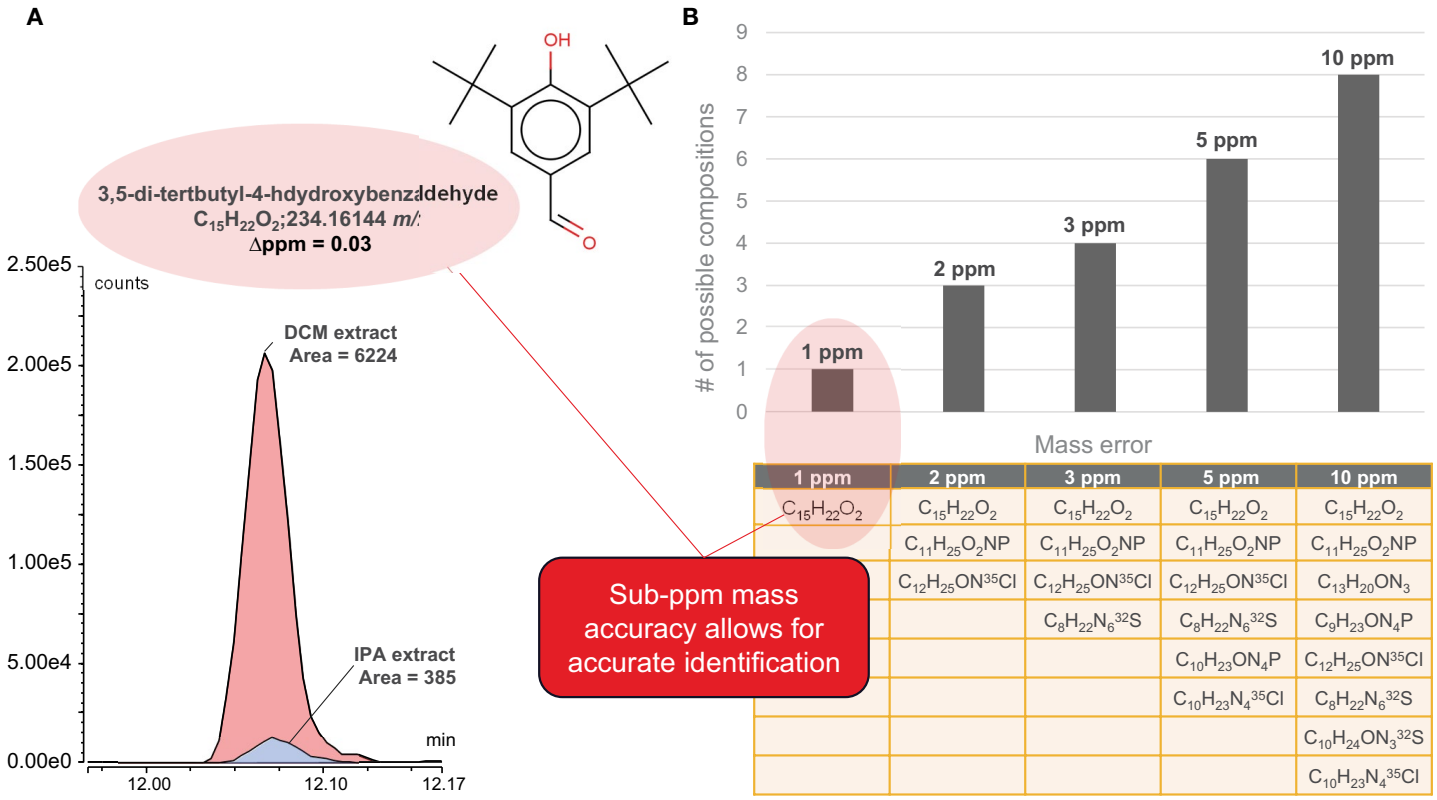


Figure 5. (A) Extracted ion chromatogram and response of molecular ion ([M-e]⁺: 234.16144 *m/z*) of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde in extractable and leachable sample extracts, (B) Number of possible elemental compositions for 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde molecular in relation to mass error

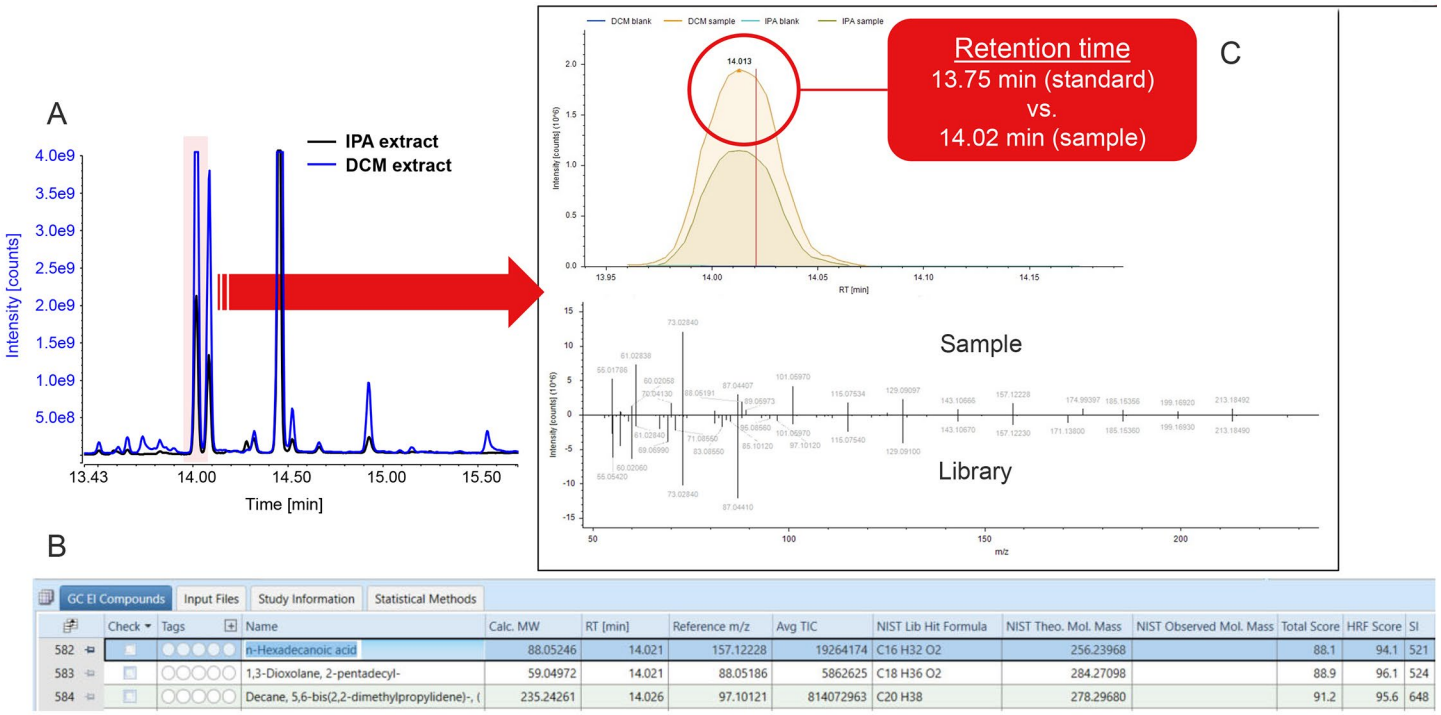
Molecular ion confirmation and identification using chemical ionization with E&L HRAM mass lists

Although over 695 unknown substances were identified from spectral deconvolution after background compound removal, analysts must prioritize the identification of substances that present the greatest risk to human health. This is often achieved by prioritizing compounds that were detected over an experimentally determined analytical evaluation threshold (AET). For this study, the response of 2,4-di-*tert*-butylphenol at 1 µg·mL^{-1} served as an arbitrary threshold to prioritize compounds for identification. Several compounds detected had responses that exceeded this threshold. To demonstrate the unknown workflow within Compound Discoverer software, the compound eluting at 14.02 min was selected for identification (Figure 6A).

After the deconvolution process and library comparison, the compound eluting at 14.02 min was initially identified as hexadecanoic acid. Despite having a hit within the library search, the search index (SI) score obtained was very poor (521). This is likely attributed to co-elution of several additional compounds (Figure 6B). In addition, the retention time does not match the time for hexadecanoic acid (13.75 min) within the E&L HRAM library (Figure 6C), contributing further to the poor score.

Detection of the molecular ion will greatly assist in compound identification. However, this can often prove challenging for some compounds due to extensive fragmentation of the molecular ion occurring in EI. However, with the NeverVent technology, switching ion source configuration from EI to CI is straightforward without needing to vent the mass spectrometer, allowing for fast molecular ion confirmation. In positive chemical ionization using methane as a reagent gas, Compound Discoverer software automatically identified the $[M+H]^+$ (*m/z* 285.27881) and methane adduct ion of the molecular ion $[M+C_2H_5]^+$ confirming the molecular weight of this unknown to be 284.27153 Da (Figure 7).

Using the E&L HRAM mass list within Compound Discoverer software, several compounds matching this molecular weight were identified with mass errors less than 0.01 ppm (Figure 8A). With further investigation of the proposed compound list, several compounds can be eliminated. For example, octadecanoic acid cannot be a candidate for this compound as the retention time (14.02 min) does not align with the E&L HRAM library (15.56 min). 2-Ethylhexadecanoic acid is also an unlikely candidate as the sample mass spectra identify several ions that correspond to specific fragments of ethyl palmitate (Figure 8B), providing additional confirmation on the identify of this compound. A tentative list of additional unknown compounds detected can be found in Appendix Table A1.



	Check	Tags	Name	Calc. MW	RT [min]	Reference m/z	Avg TIC	NIST Lib Hit Formula	NIST Theo. Mol. Mass	NIST Observed Mol. Mass	Total Score	HRF Score	SI
582			n-Hexadecanoic acid	88.05246	14.021	157.12228	19264174	C16 H32 O2	256.23968		88.1	94.1	521
583			1,3-Dioxolane, 2-pentadecyl-	59.04972	14.021	88.05186	5862625	C18 H36 O2	284.27098		88.9	96.1	524
584			Decane, 5,6-bis(2,2-dimethylpropylidene)-, (	235.24261	14.026	97.10121	814072963	C20 H38	278.29680		91.2	95.6	648

Figure 6. Total ion chromatogram overlay of IPA and DCM sample extracts between 13.4 and 15.7 min (A), proposed identification based on library match search (B), and deconvoluted spectrum with head to tail mass spectrum comparison between sample and library match for peak at 14.02 min (C)

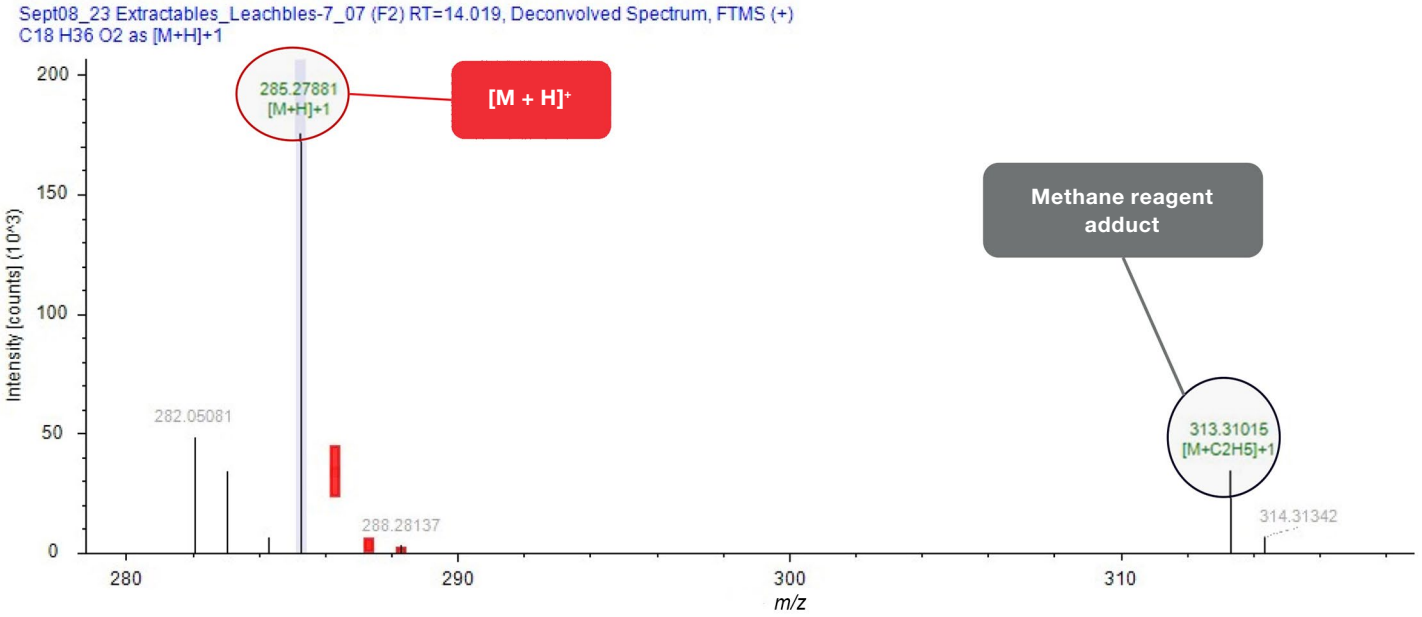


Figure 7. PCI mass spectra of compound eluting at 14.02 min with molecular ion ($[M+H]^+$) and methane reagent adduct ($[M+C_2H_5]^+$) detected for molecular weight confirmation

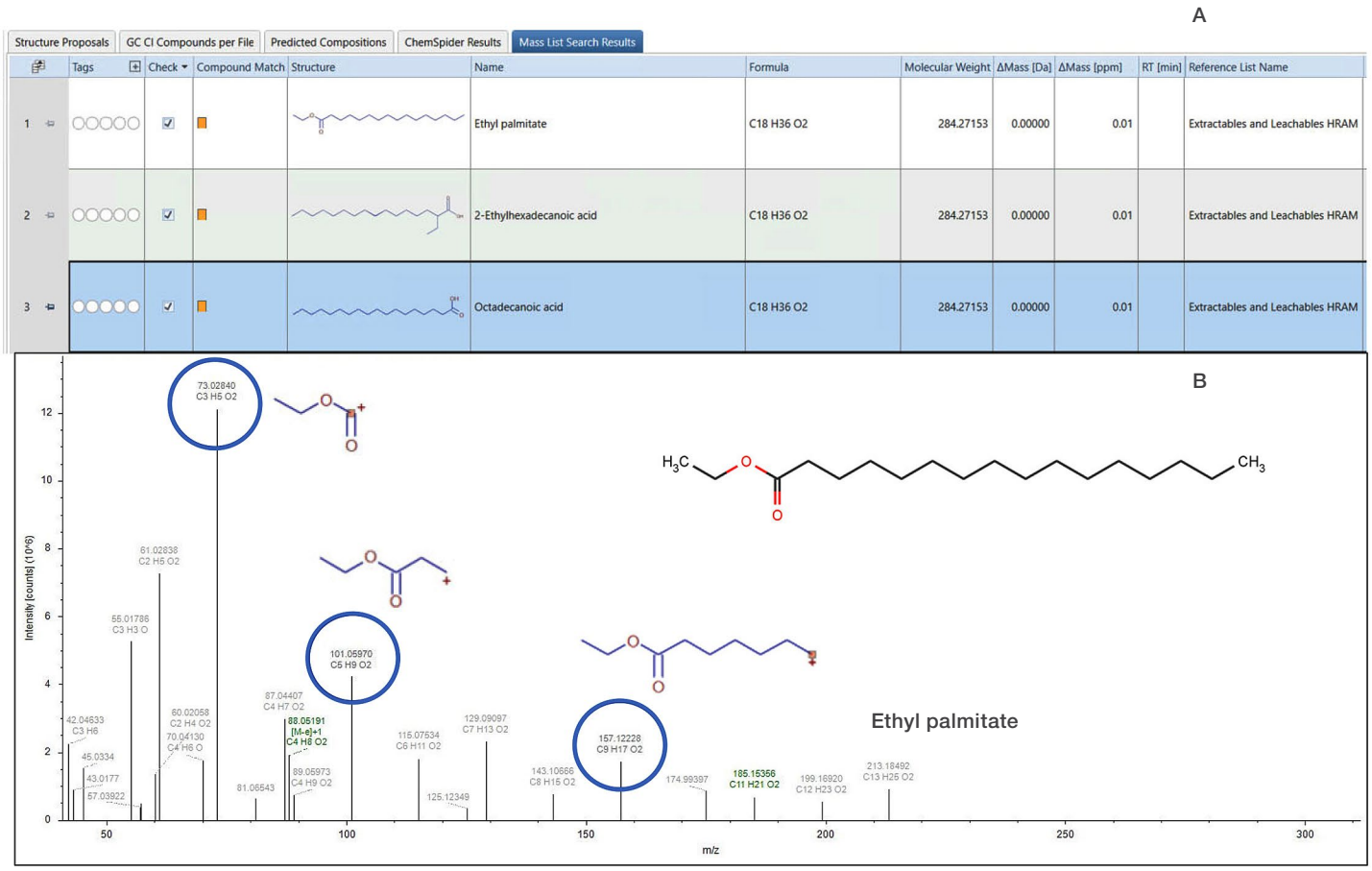


Figure 8. Compound Discoverer software proposed candidates from E&L HRAM mass list (A) and EI mass spectrum of unknown compound eluting at 14.02 min (B)

Conclusion

Due to the large spectrum of potential E&L substances with varying physical/chemical properties, both GC- and LC-based techniques are needed to provide analysts with a comprehensive overview. Combining the high mass resolving and accuracy power of the Orbitrap Exploris GC 240 mass spectrometer with LC-based techniques along with intuitive software solutions provides several key advantages:

- GC combined with HRAM provides enhanced sensitivity for detection of E&L substances with poor response using LC-HRAM techniques.
- Dual sequence evaluation with Chromeleon CDS 7.3.2 facilitates data set comparison between different instrumental techniques to verify compound detection and identification.
- Spectral deconvolution with Compound Discoverer software combined with dedicated E&L HRAM spectra libraries simplifies identification, providing confidence in unknown identification.
- High selectivity from mass resolving power helps facilitate compound detection within complex sample matrix with sub-ppm mass accuracy, providing confident compound identification.
- NeverVent technology provides streamlined switching from EI to PCI analysis for ion confirmation with dedicated E&L mass lists to assist in unknown identification.

References

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2. Pinedo-Gonzalez, P.; Lui, C.; Hower, D.; Lu, D.; Hackbusch, H.; Lamb, A.; Bardsley, J.; Multi-detector platform for comprehensive identification and quantitation of extractables and leachables. Thermo Scientific Application Note 001401, 2022. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-001401-pb-extractables-leachables-impurity-testing-an001401-na-en.pdf

Appendix

Table A1. Tentative unknown identification list

Compound name	Retention time (min)
Furan, 2 pentyl-	5.32
2,5-Furandione, 3-(1,1-dimethylethyl)-	6.15
4-methoxy-2,6-di(propan-2yl)phenol	9.08
1,3-Bis(2(-methyl-2-propanyl)-1,3-cyclopentadiene	9.32
Hexathiane	9.74
$C_{19}H_{34}$ rubber oligomer	11.39
3,5-di-*tert*-buytl-4-hydroxybenzaldehyde	12.07
7,9-di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	13.51
Hexadecanoic acid (palmitic acid)	13.74
Ethyl palmitate	14.20
Carbamazepine 10,11-epoxide	14.54
Cyclic octaatomic sulfur	14.94
Octadecanoic acid (steric acid)	15.55
Cetyl acetate	16.04
Bis(2-ethylhexyl)adipate	17.54

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