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Extractables & leachables

A high-efficiency workflow for monitoring antioxidant additives within single-use systems

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Abstract

Purpose: Detection and monitoring of antioxidants and their derivatives from SUS components via accelerated solvent extraction (ASE) technique for extraction, followed by LC/HRAM-MS for chemical identification.

Methods: Analysis samples were extracted from representative single-use component polymers and characterized via LC/HRAM-MS to identify the extracted components by searching against multiple databases in parallel, including curated spectral and compound libraries.

Results: The ASE technique enables high efficiency extraction of SUS polymeric materials. HRAM data acquisition by the LC/MS system allowed both targeted screening of known antioxidants and their confirmation, as well as the identification of unknown additives.

Introduction

Single-use Systems (SUS) are increasingly used in the biopharmaceutical industry. Despite their advantages, these polymeric assemblies receive attention from end-users as well as regulatory agencies because they are a potential source of contamination due to extractable and leachable (E&L) compounds. One primary source of E&L compounds encountered in SUS usage are antioxidants and their derivatives, which can migrate into the processing liquid. These substances could potentially jeopardize bioprocessing performance, cause cell growth inhibition, loss of expensive cell lines, and reduce yields. For this reason, their monitoring and characterization is essential. Here we describe a complete workflow from sample preparation to data interpretation for the monitoring of such compounds based on LC-MS detection.

Figure 1. (A)(B) Examples of SUS bioreactors made of various polymer materials; and (C) the resin material used to produce the single-use films

Materials and methods

Testing samples

The following single-use component resin materials were studied as part of this work:

Table 1. Test samples used in the additive characterization

Resin	Material	Resin	Material
1	HDPE	5	PP
2	MDPE	6	PVC
3	LDPE	7	PC
4	PEI	8	TPE

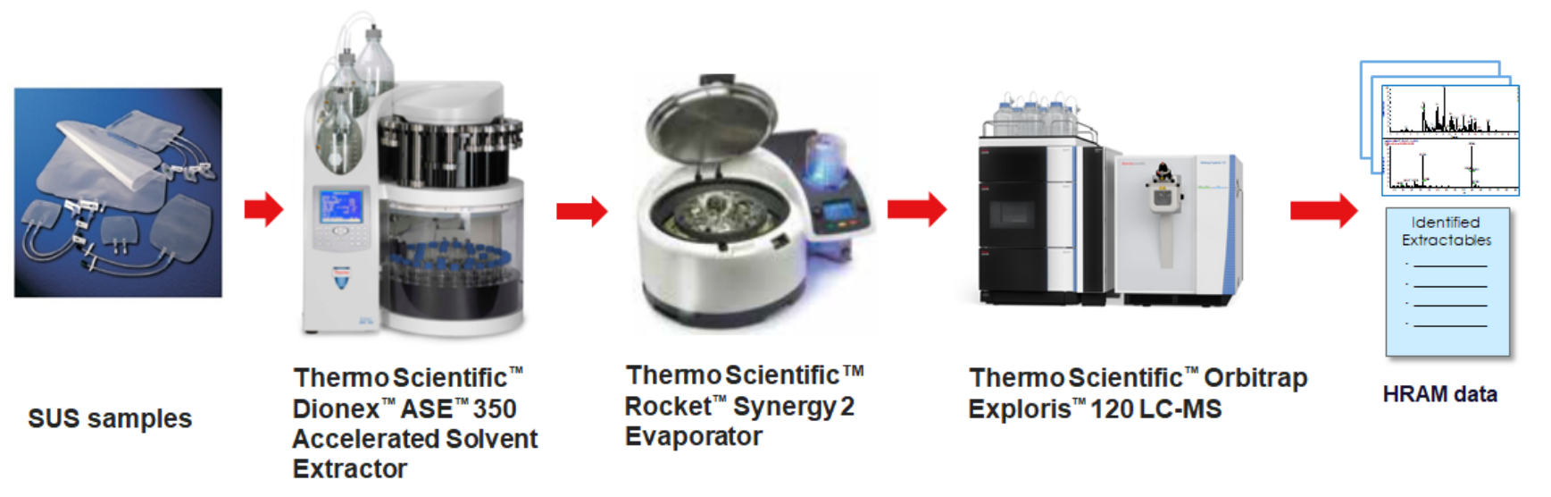


Figure 2. Overview of workflow for antioxidant determination

Extraction method

Samples were extracted in triplicates using a ASE 350 accelerated solvent extractor system. In brief, 0.5 g of the respective resin was placed in 5 mL extraction cells, with diatomaceous earth used to reduce dead volume. Extraction solvent was selected based on the solubility of polymers predicted using the Hildebrand parameter (Table 2). In addition, extraction temperature, static time as well as other extraction parameters were optimized to achieve maximum extraction efficiency (data not shown).

Based on antioxidant levels in the extracts, ethyl acetate demonstrated the highest extraction efficiency in PE sample extraction, as shown in Figure 3.

Table 2. Hildebrand parameters of common organic solvents and polymers

Solvent	δ (MPa½)	Resin	δ (MPa½)
n-Hexane	14.9	HDPE	16.7
Methanol	36.2	MDPE	16.7
Cyclohexane	16.8	LDPE	16.7
Xylene	18.2	PEI	28.9
Ethyl acetate	18.2	PP	16.2
Benzene	18.7	PVC	19.6
Acetone	19.7	PP	19.6
Pyridine	21.7	Nylon	28.8
Diethyl ester	15.4	EVA	17.2
Water	48.0	PET	20.5

After completion, extracts were evaporated using a Rocket Synergy 2 Evaporator and reconstituted in 1 mL of methanol. The solutions were then centrifuged before analysis to remove any potentially insoluble materials.

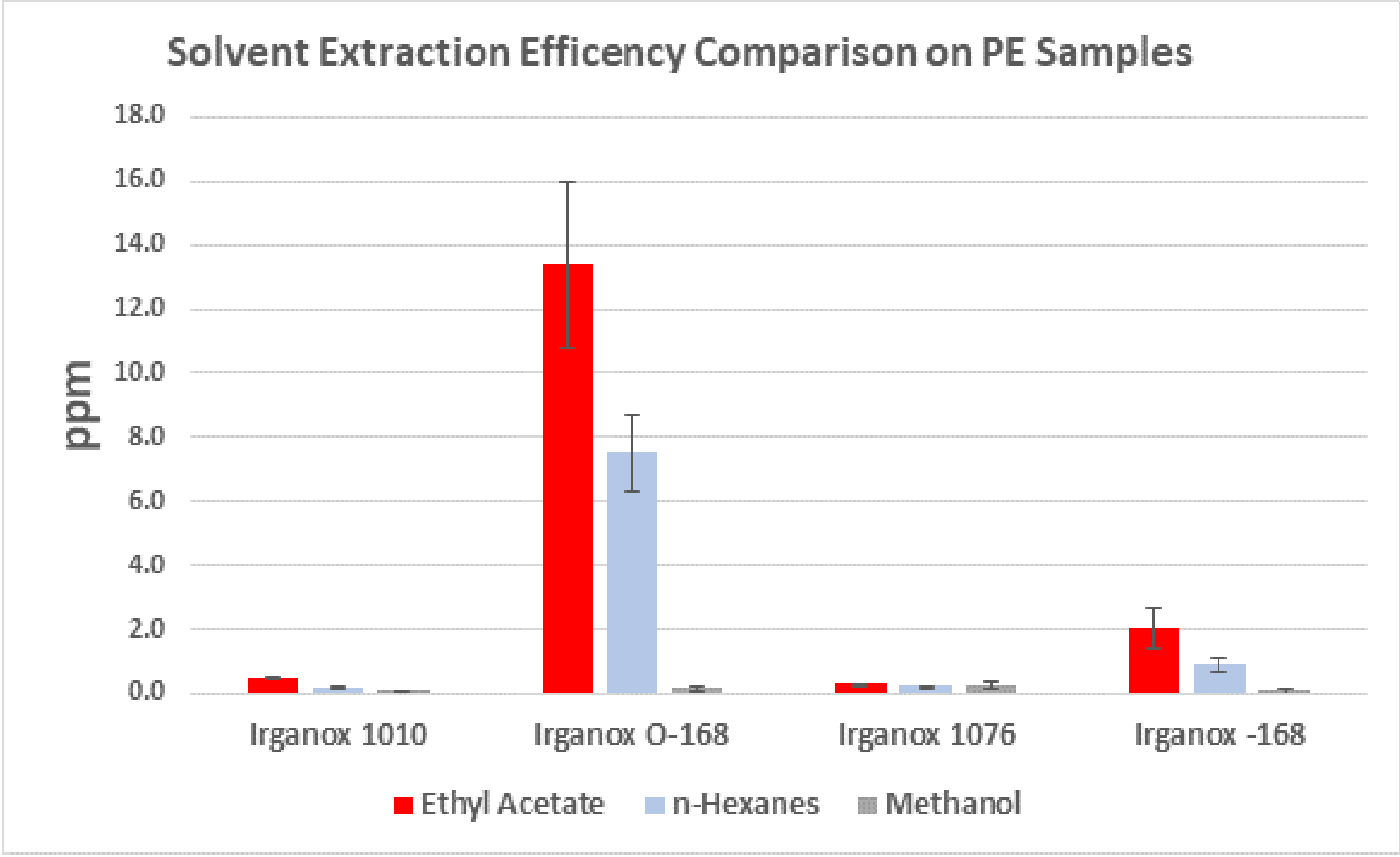


Figure 3. Extraction solvent optimization on PE samples

Test method

The extracts of SUS polymeric materials were analyzed in duplicate using an LC/MS system comprised of a Thermo Scientific™ Vanquish™ Horizon UHPLC system with binary gradient pump and diode array detector, connecting to a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer for high-resolution accurate mass (HRAM) measurement and fragmentation data collection for compound identification. The sample analysis was performed using a reverse-phase separation and heated electrospray ionization. The relevant LC and MS method parameters are summarized in Table 3.

Data analysis

The acquired raw data from duplicate injections of each sample were processed together with the blank extraction for background filtering using Thermo Scientific™ Compound Discoverer™ 3.3 SP1. The data processing for unbiased component detection, peak filtering, compound identification and advanced statistics was based on the preloaded workflow template 'E and L w/ Stats Unknown ID w/ Online and Local Database Searches'. Automated compound annotation was based on consensus from multiple data sources, comprising both online and offline validated spectral libraries (mzCloud and NIST MS/MS HR), local and online compound databases (E&L Mass List, ChemSpider), as well as custom local libraries generated from previous studies.[5]

Table 3. LC/MS method details for the antioxidant monitoring

Method Parameters	
UHPLC Column	Thermo Scientific™ Hypersil GOLD™ C18, 1.9 µm, 100 × 2.1 mm
Column Temp.	45ºC
Flow Rate	0.4 mL/min
Injection Volume	2 µL
Sampler Temp.	15 ºC
Mobile Phase A	5 mM ammonium formate + 0.05% formic acid in water
Mobile Phase B	0.05% formic acid in methanol
Gradient	Time (min) / %B: 0.0 / 5 1.0 / 5 15.0 / 100 25.0 / 100 25.1 / 5 30.0 / 5
UV Detector	Diode Array 3D Field, 200 – 600 nm, 10 Hz
Divert Valve Setup	Flow to waste 0 – 0.5 min and 25.0 – 30.0 min
ESI Spray Voltage	+3500 / –2800 V
Sheath / Aux / Sweep Gas setting	40 / 10 / 1 a.u.
Ion Transfer Tube/ Vaporizer Temp.	300 ºC / 350 ºC
Sprayer Position	M/H, 1.5, center
Acquisition Mode	ESI Polarity-switching FullScan-ddMS2 (Top3)
Resolution	60,000 / 15,000 (MS1 / MS2)
Scan Range	120 - 1200
Collision Energy	Stepped HCD 20,40,80%
Acquisition Settings	• Easy-IC "scan-to-scan" for MS1 • Background exclusion list containing 3000+ background ions above 1e^7((positive mode) and 2e^6 (negative) peak intensity, generated by AcquireX Background Exclusion workflow • Dynamic Exclusion – 6 s • MS2 maximum injection time 50 ms

Results

Resin extract data overview

Prior to the sample analysis, the instrument performance was verified using a QC standard mixture injection, confirming elution profile, peak shape, mass accuracy and sensitivity.

The acquisition of the mass spectral data in an unbiased fashion using a full scan data dependent MS2 method allowed the screening for known antioxidant compounds, such as those listed in Table 4, by extracting their precursor accurate mass and confirming their elemental composition based on isotopic peak fine structure and comparison of their fragmentation spectra against the large mzCloud™ spectral library and local libraries. These processes are automated in Compound Discoverer 3.3 software, as shown in Figure 6, on right.

In addition to the targeted screening, the untargeted acquisition approach allowed the detection and identification of additional unknown additives present in the different samples, some of which are listed in Table 5. For this, Compound Discoverer software uses multi-consensus matching to online and offline spectral libraries and compound databases, as illustrated in Figure 4.

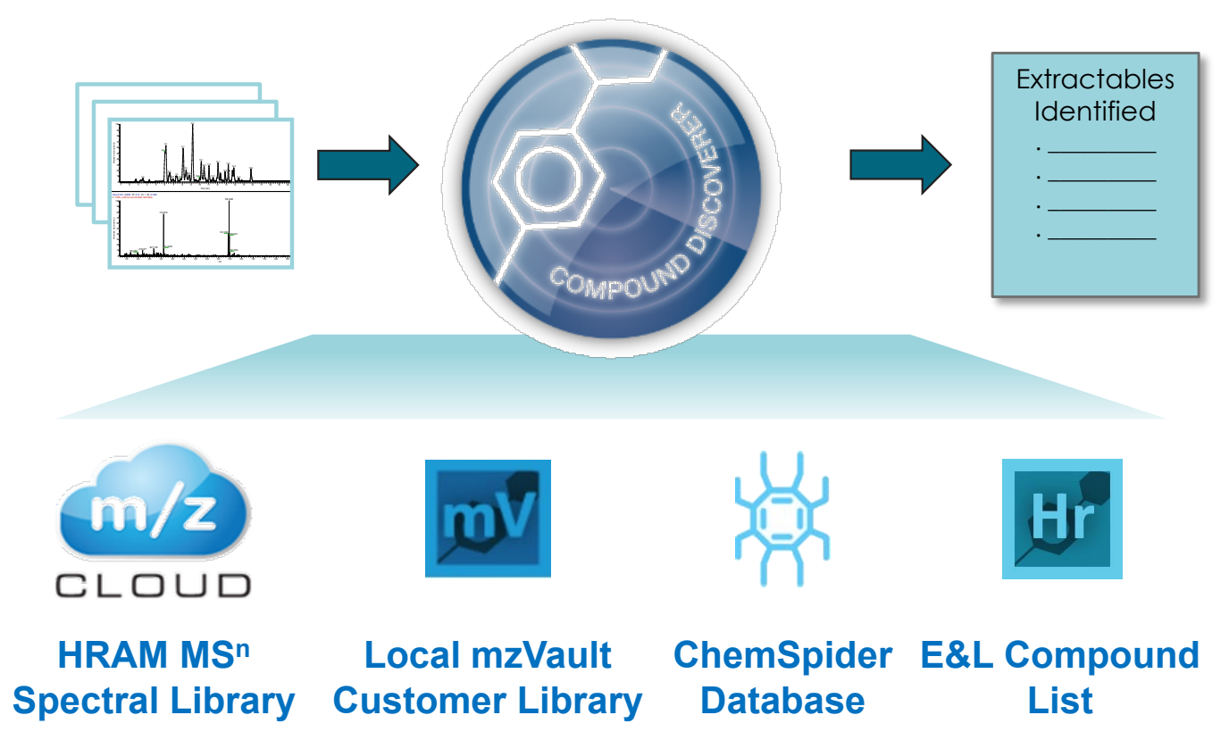


Figure 4. Annotation sources in Compound Discoverer 3.3 software including the mzCloud spectral library, local mzVault library and public or user-created compound databases

In addition to those compounds that could be confidently annotated based on mzCloud or NIST library matches of their fragmentation spectra, several compounds originating from epoxidized soybean oil (ESBO) could be detected in PVC, HDPE and LDPE that matched against a custom library generated in previous work.[5] ESBO is a common plasticizer found in plastics, but is a plant-derived mixture of compounds, primarily containing triglycerides. Figure 7 shows the example of a compound with MW 932.6954 @ 16.07 min having a match score of 85.0 to the MS2 library spectrum of TG(52:5-e), a triglyceride with 5 sites of epoxidation.

Based on the known antioxidants from Table 4, principal component analysis (PCA) of the resin samples could be performed to visualize the similarity between the materials, as shown in Figure 5A. From this, Resins 6 and 8 can be seen to differ from the other resins in terms of the antioxidant additives used. This contrasts with the PCA plot calculated from all detected compounds, shown in Figure 5B, where resins 6 and 7 cluster separate from the bulk of the resins.

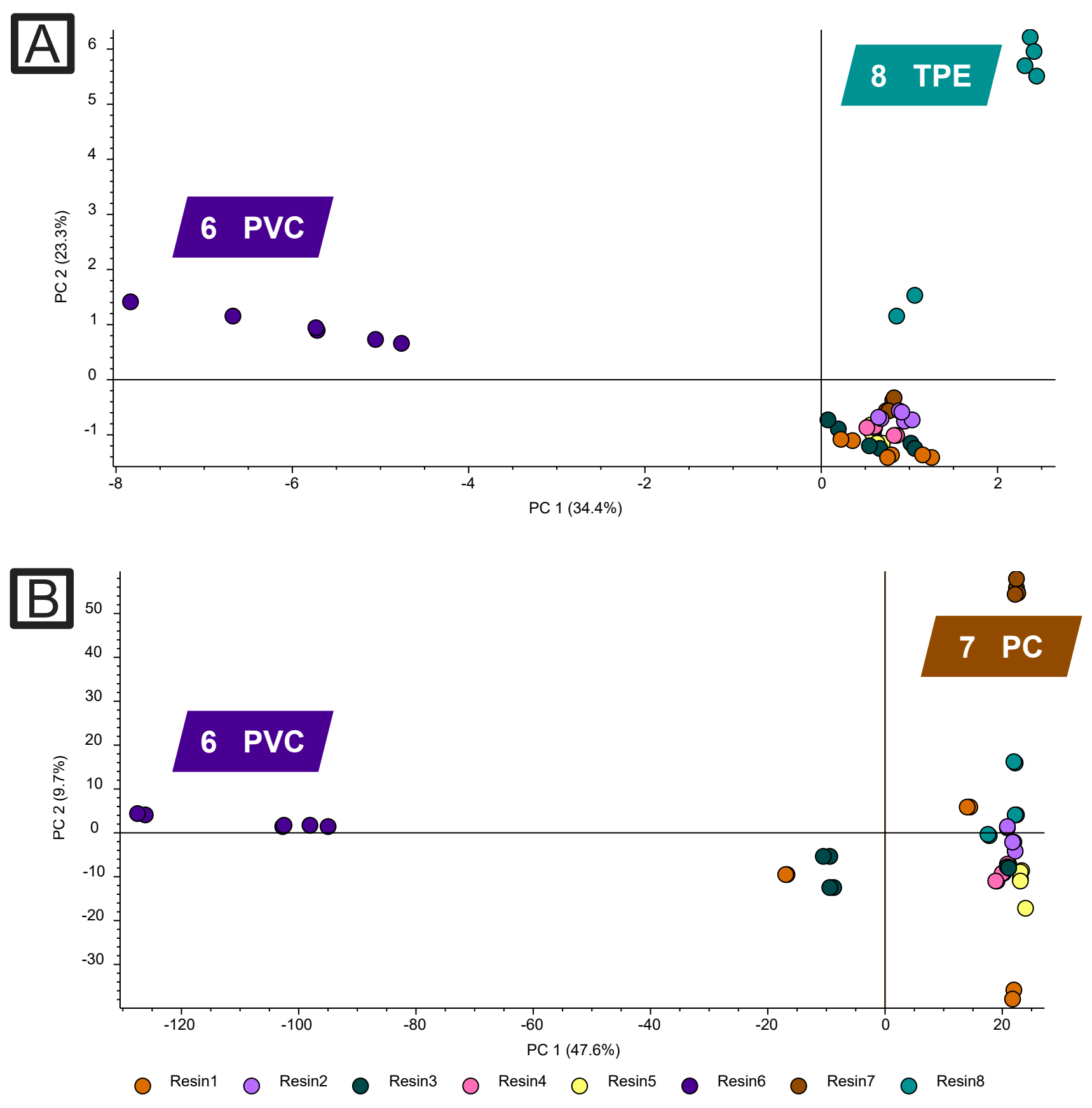
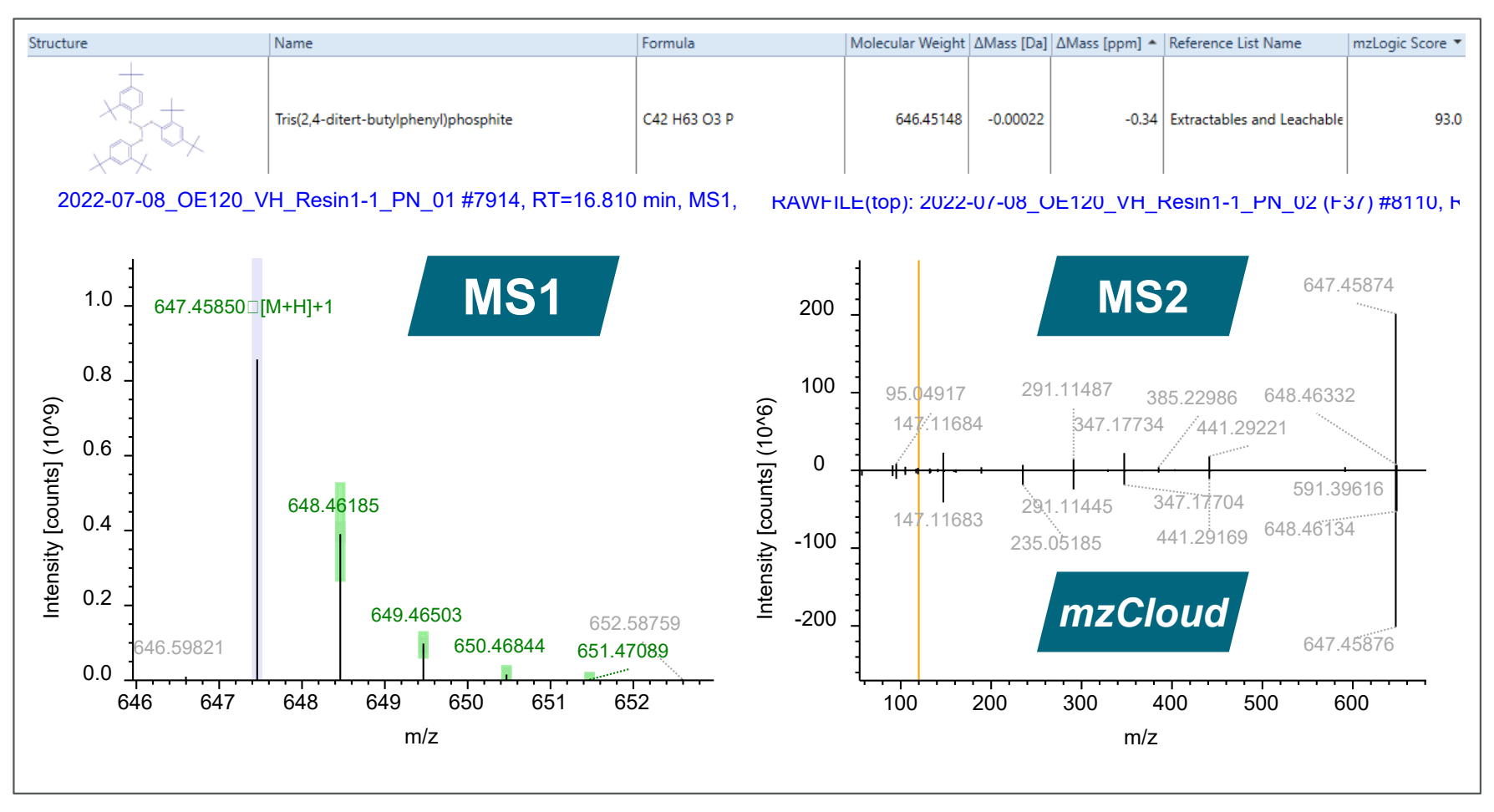


Figure 5. PCA Plot of the resin materials based on (A) the compounds from Table 4 and (B) all detected compounds.

Table 4. Some common antioxidants found in the different resin samples with annotation source and relative intensities denoted

#	Calc. MW (Da)	RT (min)	Compound Name	Annotation Source	Relative Area Count (%) in Resin: HDPE	MDPE	LDPE	PEI	PP	PVC	PC	TPE
1	530.4698	16.51	Irganox 1076	mzCloud	3	4	**100**	0	0	0	0	2
2	688.4983	17.87	Tris(nonylphenyl)phosphite	E&L Mass List	0	0	0	0	0	**100**	0	0
3	1176.784	15.87	Irganox 1010	mzVault	0	0	1	0	0	0	0	**100**
4	783.5186	15.40	Irganox 3114	mzVault	0	0	0	0	0	0	0	**100**
5	430.381	15.94	Vitamin E	E&L Mass List	16	7	**27**	4	1	**100**	4	5
6	682.5911	16.50	Dioctadecyl 3,3'-thiodipropanoate	E&L Mass List	15	4	25	1	1	18	**100**	4
7	480.3931	8.80	Bis(3,3,5,5-tetramethyl-4-piperidinyl) sebacate	E&L Mass List	15	14	11	14	7	6	**25**	**100**
8	514.4055	16.32	Dilauryl 3,3'-thiodipropionate	mzVault	**45**	1	20	1	1	22	**100**	51
9	351.2311	15.72	2-(2H-Benzotriazol-2-yl)-4,6-di-tertpentylphenol	mzCloud	2	0	6	0	0	20	0	**100**
10	369.2878	11.26	Methyl 1,2,2,6,6-pentamethyl-4-piperidyl sebacate	E&L Mass List	4	1	11	1	2	**100**	0	1
11	266.1647	12.74	Tributyl phosphate	mzCloud	13	9	11	7	7	**100**	10	6
12	1160.813	14.37	Pentaerythrityl tetra(beta-laurylthiopropionate)	E&L Mass List	17	**48**	**45**	2	2	**29**	**100**	**40**
13	263.2249	9.42	2,4-Di-tert-butyl-6-[(dimethylamino)methyl]phenol	mzVault	0	0	0	0	0	0	0	**100**
14	646.4513	16.82	Irgafos 168	mzCloud	**100**	11	3	10	10	1	10	17
15	212.0685	13.76	Propyl gallate	E&L Mass List	5	1	22	1	2	**100**	1	4
16	337.3344	15.17	Erucamide	E&L Mass List	**36**	12	**47**	8	3	**100**	7	8
17	239.1422	6.54	1,3-Di-o-tolylguanidine	mzCloud	3	2	2	2	**100**	2	2	5


Structure	Name	Formula	Molecular Weight	ΔMass [Da]	ΔMass [ppm]	Reference List Name	mzLogic Score
	Tris(2,4-ditert-butylphenyl)phosphite	C42 H63 O3 P	646.45148	-0.00022	-0.34	Extractables and Leachable	93.0

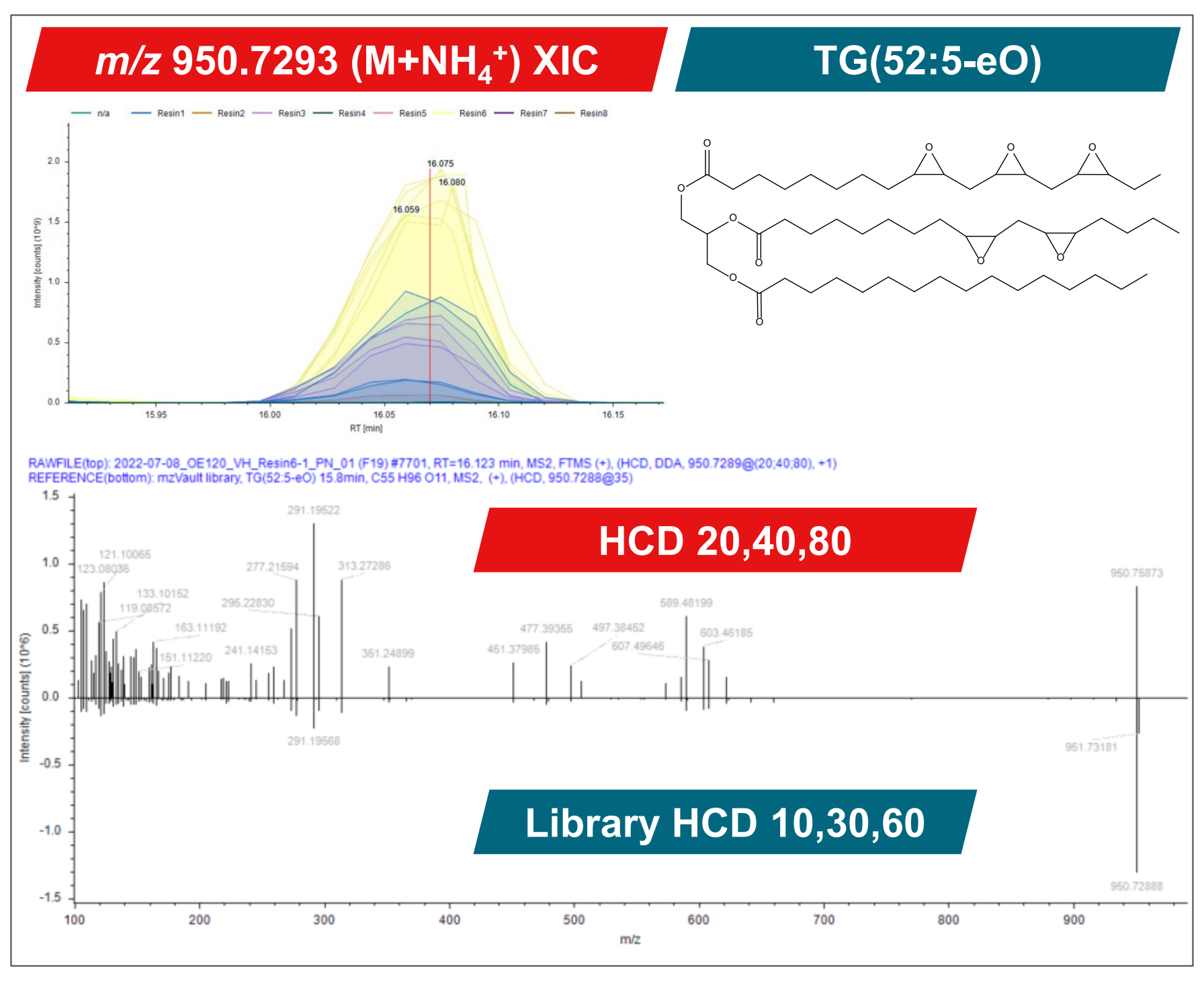
2022-07-08_OE120_VH_Resin1-1_PN_01 #7914, RT=16.810 min, MS1,

RAWFILE(top): 2022-07-08_OE120_VH_Resin1-1_PN_02 (F37) #8110, F

Figure 6. Automated annotation of Irgafos 168 based on mass list match and elemental composition determination from full scan data, confirmed by spectral match of the MS2 to the reference standard in the mzCloud spectral library

Table 5. Selection of other high abundant compounds found in samples – including compounds originating from ESBO

Calc. MW (Da)	RT (min)	Compound Annotation	Source	Found in Resin
402.2252	13.29	Citroflex A-4	mzCloud	**6**,**3**,**1**,8,*5*,*7*,*2*,*4*
974.7059	15.57	TG(54:6-eO) Iso. A	*mzVault*	**6**,**3**,**1**,8
988.6851	15.19	TG(54:7-eO)	*mzVault*	**6**,3,1,8
960.7266	15.97	TG(54:5-eO)	*mzVault*	**6**,**3**,**1**,8
426.3707	15.72	Didecyl adipate	E&L masslist	**6**,**3**,1,2,4
918.7160	16.39	TG(52:4-eO)	*mzVault*	**6**,**3**,**1**,7,8
974.7058	15.67	TG(54:6-eO) Iso. B	*mzVault*	**6**,**3**,**1**,8,2,*5*,*7*,*4*
342.2042	13.76	Tributyl aconitate	E&L masslist	
904.7367	16.87	TG(52:3-eO)	*mzVault*	**6**,3,1
360.2147	12.88	Citroflex 4	mzCloud	**6**,**3**,1,8,*5*
932.6954	16.07	TG(52:5-eO)	*mzVault*	**6**,3,1
414.2042	11.56	Bis(ethylbenzylidene) sorbitol	mzCloud	**5**,1,*8*,*2*,*7*



RAWFILE(top): 2022-07-08_OE120_VH_Resin6-1_PN_01 (F19) #7701, RT=16.123 min, MS2, FTMS (+), (HCD, DDA, 950.7289@(20,40,80), +1)
REFERENCE(bottom): mzVault library, TG(52:5-eO) 15.8min, C55 H96 O11, MS2, (+), (HCD, 950.7288@35)

Figure 7. MS2 spectral match of MW 932.6954 @ 16.07 min to TG(52:5-eO) and the structure of a representative isomer

Conclusions

- A complete workflow based on the ASE technique and LC/HRAM-MS was developed to screen antioxidants and their derivatives in SUS polymeric materials.
- ASE enables high throughput, high efficiency, and reproducible extraction of antioxidants from SUS polymeric materials.
- Acquisition of the HRAM data on an Orbitrap LC/MS system allowed both targeted screening of known antioxidants, as well as the detection and identification of unknown or unexpected additives, including derivatives or degradants.

References

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