Identification of polymer additives extracted from single-use bioreactor bags

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Goal

To explore the performance of accelerated solvent extraction (ASE) as a technique for characterization of extractables from single-use bioreactor (SUBs) bags

Introduction

Single-use technologies (SUTs), in particular single-use bioreactors (SUBs), represent an important improvement in biopharmaceutical manufacturing due to reduced requirements for cleaning and sterilization while providing increased sterility assurance, reduced manufacturing turnaround times, and the elimination of cleaning validation and its associated costs.[1-2] SUTs also increase facility flexibility and productivity when compared with traditional stainless steel.[1-2] Other advantages include reduction of manual handling operations and the ability to create totally closed operating systems, reducing bioburden and contamination risk to the process stream.[3] Despite these advantages, SUTs create new challenges in bioprocessing, as various fluids with distinct chemical and physical properties interact with single-use components, producing extractables (organic and/or inorganic chemical entities) from the plastic material that migrate out of the system and accumulate as leachables.[4] These substances could jeopardize bioprocess performance and cause cell growth inhibition, loss of expensive cell lines, and reduced yields.[5]

Accelerated solvent extraction is a powerful technique that can be reliably used to perform the recommended extractions required for material characterization of plastics. The use of ASE has increased since its acceptance as an official U.S. Environmental Protection Agency (U.S. EPA) method for organic pollutants in environmental samples.[6] ASE is also recommended by the Product Quality Research Institute (PQRI),[7] and proposed in the United States Pharmacopeia's General Chapter <1663>[8] as one of the analytical techniques for extractable testing. ASE has become widely used for trace analysis of environmental organic contaminants,[9] as well as plasticizers and additives from polymers within the pharmaceutical and food industries.[10-11] ASE can work with solvents with different polarities and offers the versatility of working under different extraction conditions, e.g., modifying temperature and/or pressure, so that a wide range of compounds from different chemical families can be extracted.[12]

The present work explored the performance of ASE as an extraction technique for characterization of the plastic films (inner layers) from single-use bioreactor bags. Three different ASE methods were evaluated. The resulting extractable compounds were confidently identified by liquid chromatography and Orbitrap-based high-resolution mass spectrometry (LC-HRMS).

Experimental

Equipment and consumables

Extraction

- Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extractor (P/N 083114 for 120 V or P/N 083146 for 240 V)
  - Stainless Steel Extraction Cells 10 mL (P/N 060070)
  - Dionex ASE 350 Collection Vials, Clear, 60 mL (P/N 048784)
  - Glass Fiber Filters, 27 mm, Type D28 (P/N 068092)
- Thermo Scientific™ Rocket Synergy™ 2 Evaporator System (P/N C0960-01-00085 for 120 V or P/N C0960-01-00086 for 240 V)

Liquid chromatography

- Thermo Scientific™ Vanquish™ Flex UHPLC system including:
  - Thermo Scientific™ Vanquish™ Quaternary Pump F (P/N VF-P20-A) with 200 µL static mixer (P/N 6044.5110)
  - Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VF-A10-A)
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A) with 2-position/6-port valve (P/N 6036.1560)
  - Thermo Scientific™ Vanquish™ Diode Array Detector FG (P/N VF-D11-A) with 2.5 µL titanium flow cell (P/N 6083.0550)
  - Thermo Scientific™ Vanquish™ System Base F (P/N VF-S01-A)

Mass spectrometer

- Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer
- Peak Scientific™ Genius™ 1022 nitrogen generator (P/N 10-6022 (230v))

Software

Data acquisition

- Thermo Scientific™ Xcalibur™ 4.2 software with SII 1.5 for Xcalibur software

Data processing

- Thermo Scientific™ FreeStyle™ Version 1.6 software
- Thermo Scientific™ Compound Discoverer™ 3.2 software
- Thermo Scientific™ Mass Frontier™ 8.0 software

Reagents and chemicals

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better filtered through a 0.2 µm filter immediately before use
- Acetonitrile, Optima™ LC/MS Grade (Fisher Scientific™, P/N A955-1)
- Hexanes, HPLC grade (Fisher Scientific™, P/N H302-4)
- Cyclohexane, HPLC grade (Fisher Scientific™, P/N C620SK-4)
- Thermo Scientific™ Pierce™ LTQ Velos ESI Positive Ion Calibration Solution (P/N 88323)
- Thermo Scientific™ Pierce™ Negative Ion Calibration Solution (P/N 88324)
- Ottawa Sand (Fisher Scientific™, P/N S23-3)
- Ammonium formate, Optima™ LC/MS (Fisher Scientific™, P/N A11550)

Samples

- Plastic materials from SUBs provided by internal customers

Sample preparation and ASE procedure

Square samples for polymeric film were cut into 0.25 cm^2 squares using scissors. The sample pieces were dispersed in clean Ottawa sand to prevent coalescence during extraction, and loaded into a 10 mL stainless steel extraction cell with a glass fiber filter in the bottom cap. Then, the remaining cell volume was filled with clean Ottawa sand and closed with the cell cap. The cell was placed on a Dionex ASE 350 Accelerated Solvent Extractor, which was programmed as indicated in the accelerated solvent extraction conditions table. With help of the sequence editor, multiple methods with different extraction conditions were accomplished in one run. The extracts were evaporated using the Rocket Synergy 2 Evaporator system. One milliliter of acetonitrile was carefully added to reconstitute the dried sample and then it was centrifuged for 30 min at 13,000 × g. The supernatant was placed in a vial for analysis.

Accelerated solvent extraction conditions			
Extraction cell size	10 mL		
Preheating	5 min		
Pressure	1,500 psi		
Static time	12 min		
Number of cycles	2		
Flush volume	100%		
Purge time	120 s		
Solvent	Hexane		
	Method I	*Method II*	*Method III*
Cyclohexane (%)	0	0	5
Temperature (°C)	90	100	90

UHPLC-HRMS conditions	
UHPLC system	Vanquish Flex system
MS detector	Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer
Column	Thermo Scientific™ Accucore™ C18 column, 2.1 × 100 mm, 2.6 µm particle size
Mobile phase	A: 0.1% formic acid, 5 mM ammonium formate, pH 3 B: 5 mM ammonium formate in 90:10 (v/v) acetonitrile/water
Gradient	5% B (0–1.2 min) 5–95% B (1.2–18 min) 95% B (18–26 min) 5–95% B (26.1–29 min) 5% B (29.1–32 min)
Flow rate	0.4 mL/min
Injection volume	5 µL
Column temperature	50 °C, still air mode
Run time	32 min
Mass spectrometric detection	
Ion source	Electrospray ionization (ESI), negative mode
HESI source	Sheath gas flow rate: 50 Aux gas flow rate: 10 Sweep gas flow rate: 0 Spray voltage (kV): 3.5 Capillary temp. (°C): 300 S-lens RF level: 60 Aux gas heater temp (°C): 430
Experiments[13]	Full MS/ddMS² with inclusion list (Tables 1 and 2)

Table 1. Full MS/ddMS² inclusion list

Mass [*m/z*]	Formula [M]	Species	CS [z]	Polarity
621.31045	$C_{33}H_{50}O_7P_2$	+ H	1	Positive
647.45876	$C_{42}H_{63}O_3P$	+ H	1	Positive
663.45367	$C_{42}H_{63}O_4P$	+ H	1	Positive
205.15979	$C_{14}H_{22}O$	- H	1	Negative
475.29717	$C_{28}H_{43}O_4P$	+ H	1	Positive
114.09134	$C_6H_{11}NO$	+ H	1	Positive
277.12818	$C_{12}H_{20}O_7$	+ H	1	Positive
267.17197	$C_{12}H_{27}O_4P$	+ H	1	Positive
192.15942	$C_9H_{21}NO_3$	+ H	1	Positive
338.34174	$C_{22}H_{43}NO$	+ H	1	Positive
431.17878	$C_{26}H_{26}N_2O_2S$	+ H	1	Positive
225.19614	$C_{13}H_{24}N_2O$	+ H	1	Positive
250.11862	$C_{12}H_{15}N_3O_3$	+ H	1	Positive
277.17982	$C_{17}H_{24}O_3$	+ H	1	Positive
219.17434	$C_{15}H_{22}O$	+ H	1	Positive
784.52591	$C_{48}H_{69}N_3O_6$	+ H	1	Positive
531.47717	$C_{35}H_{62}O_3$	+ H	1	Positive

Note: Formula [M] stands for the composition of the active compound, and CS [z] for the charge state of the ion to be fragmented

Table 2. Properties of the Full MS/ddMS² method

Global settings	
User role	Standard
Use lock masses	Best
Chrom. peak width (FWHM)	15 s
Method duration	32 min
Properties of Two Scan Events (one for positive, the other for negative)	
Full MS	
Resolution	120,000
AGC target	3e6
Maximum IT	100 ms
Scan range	150 to 1500 *m/z*
dd-MS²/dd-SIM	
Resolution	60,000
Loop count	3
TopN	3
Isolation windows	4.0 *m/z*
Fixed first mass	-
(N)CE/stepped (N)CE	nce: 30, 60, 80

Results and discussion

ASE extraction

The selection of the extraction solvent is crucial to optimize factors governing a successful extraction, such as solubility and mass transfer.[6] Solvents recommended for Soxhlet extractions are often also used for ASE.[6] However, as higher temperatures and pressure are used in ASE, care must be taken to avoid polymer dissolution.[14] The ideal situation is one where the extraction is performed at a temperature that causes the maximum swelling without dissolving the polymer.[15] The solubility of polymers can be broadly predicted using solubility parameters, i.e., Hildebrandt parameter,[16] (Table 3) wherein the closer the solubility parameters between the polymer and solvent, the more polymer will dissolve in a solvent at lower temperatures than another solvent with a different solubility parameter.

Table 3. Hildebrandt solubility coefficients for the common solvents and polymers[17–19]

Solvent	δ (MPa½)	Polymer	δ (MPa½)
2-propanol	23.8	EVOH (32mol%)	38.9
Ethyl acetate	18.6	Nylon	28.8
Cyclohexane	16.8	PET (Polyester)	20.5
n-hexane	14.9	EVA	17.2
		PE	16.7

Addition of a swelling agent to improve solubility and accelerate the diffusion of the components from the inside of the polymer particles to their surface was also investigated.[20] The most widely used swelling solvent for this type of application is cyclohexane.[16, 21] Previous reports suggest that cyclohexane results in a small shift in the solubility parameter but a large shift in extraction rate,[16] potentially due to cyclohexane being selectively absorbed into the plastic, causing significant swelling, even at low concentrations. Using cyclohexane as a swelling agent may be especially advantageous for thermolabile analytes when no suitable solvent is available with the necessary properties to extract rapidly at lower temperature.

In this study, temperature and cyclohexane proportion were evaluated for extraction efficiency. Three ASE methods with different combinations were evaluated. The optimum temperature for our study using *n*-hexane was 90 °C, and the effect of cyclohexane addition at temperatures ≥90 °C is negative, meaning no addition is required. An increase in extraction temperature also had a negative effect. The peak intensity of most common additives decreased with higher temperature and cyclohexane addition (Table 4).

ASE method repeatability was evaluated with three independent extractions using ASE Method I. Figure 1 shows base peak chromatograms of the extractable profile from plastic films using ASE Method I in triplicate. The extracts from three independent experiments gave almost identical extractable profiles, which indicates good consistency of the accelerated solvent extraction technique. All three ASE methods showed good extractable profiles (Figure 2).

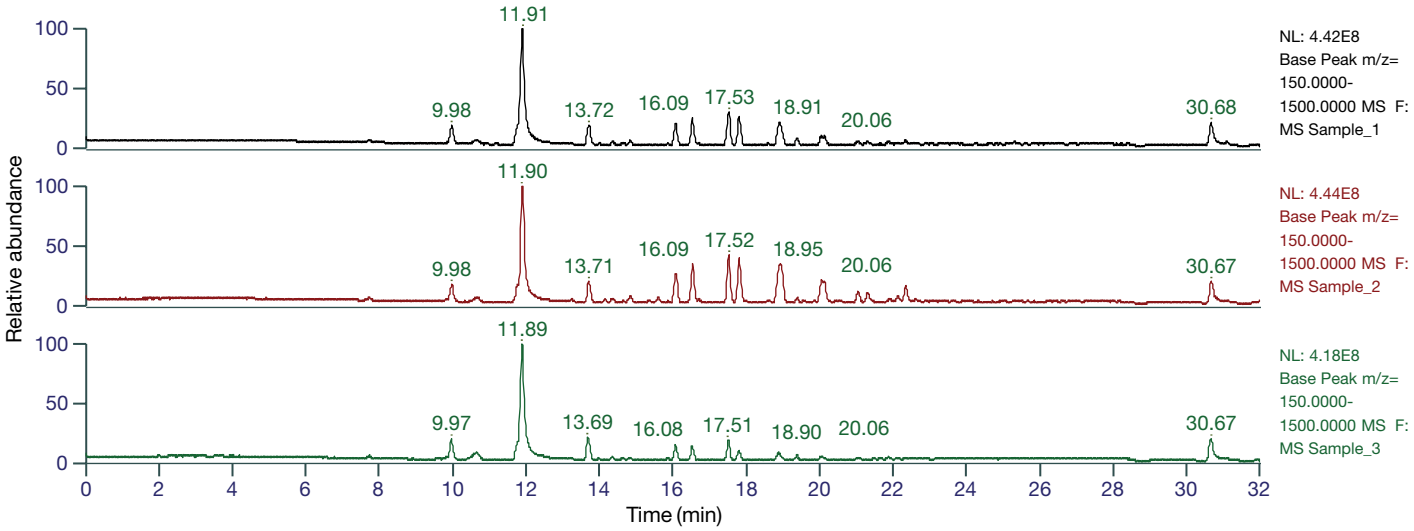

Figure 1. Base peak chromatograms for extractable profile of plastic films with ASE Method I in triplicate

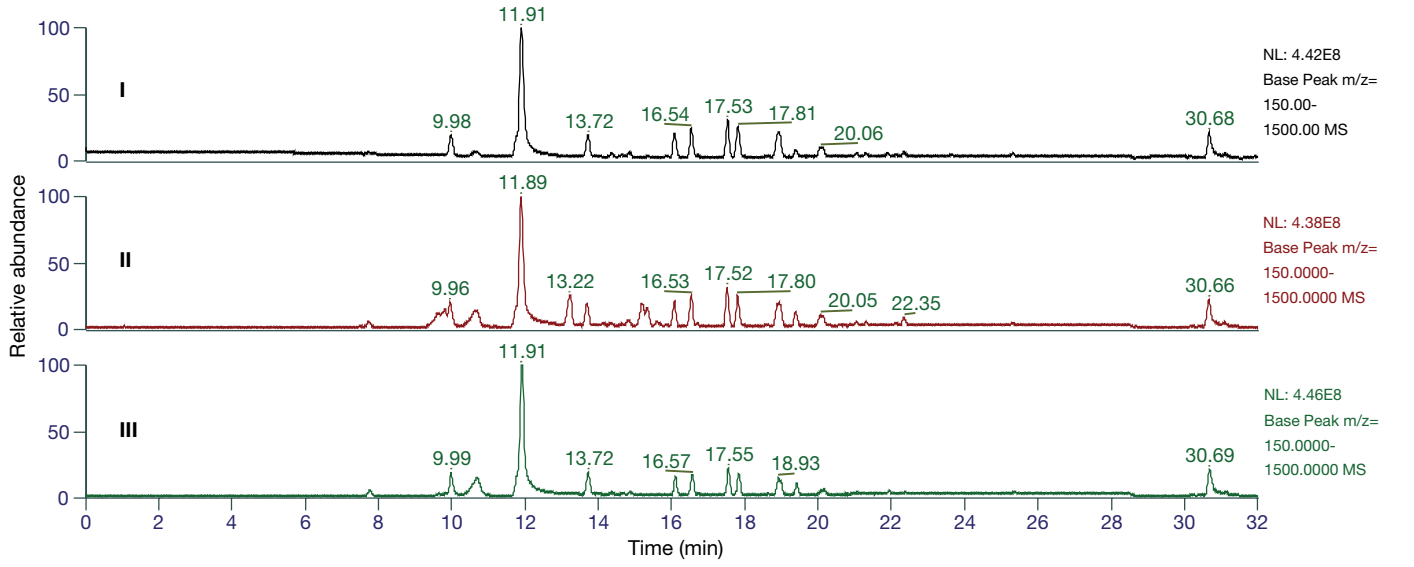

Figure 2. Base peak chromatograms for extractable profiles of three ASE methods

Table 4. Some identified common additives in the plastic films

	Formula	Retention time (min)	Exact mass $[M+H]^+$	Mass error (ppm)	Name	CAS annotation	ASE Method (*e7 counts) I	II	III
1	$C_{33}H_{50}O_7P_2$	22.37	621.3104	-0.08	Oxidised Irgafos 126	-----	1.344	1.322	0.9
6	$C_{12}H_{20}O_7$	9.12	277.1282	0	2-hydroxy-1,2,3-propanetricarboxylic acid, triethyl ester (Citroflex® 2)	77-93-0	0.21	0.19	0.17
7	$C_{12}H_{27}O_4P$	14.69	267.1720	0	Tributyl phosphate	126-73-8	2.47	2.31	2.00
8	$C_{22}H_{43}NO$	21.89	338.3413	-1.18	*cis*-13-docosenoic amide (Erucamide)	112-84-5	24.3	10.3	14.2
9	$C_{26}H_{26}N_2O_2S$	22.37	431.1787	-0.23	2,5-bis(5-*tert*-butylbenzoxazol-2-yl)thiophene (Uvitex® OB)	7128-64-5	0.099	0.15	0.11
10	$C_{12}H_{15}N_3O_3$	12.53	250.1186	0	2,4,6-triallyloxy-1,3,5-triazine(Triallyl cyanurate)	101-37-1	1.35	1.22	1.07
11	$C_{17}H_{24}O_3$	15.29	277.1797	-0.36	7,9-di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (Irganox 1076 degradation product)	82304-66-3	0.67	0.77	0.59
12	$C_{15}H_{22}O$	14.03	219.1745	0.91	3,5-di-*tert*-butylbenzaldehyde (Degradation product from BHT)	17610-00-3	0.46	0.46	0.42
					Irgafos 168 and derivatives				
4	$C_{14}H_{22}O$	15.97	205.1592 $[M-H]^-$	-2.88	2,4-di-*tert*-butylphenol (DtBP)	96-76-4	0.032	0.011	0.039
5	$C_{28}H_{43}O_4P$	15.92	475.2973	0.21	bis(2,4-di-*tert*-butylphenyl)phosphate (bDtBPP)	69284-93-1	0.52	0.59	0.27
3	$C_{42}H_{63}O_4P$	27.06	663.4540	0.45	Tris(2,4-di-*tert*-butylphenyl)phosphate (Oxidised Irgafos 168)	95906-11-9	95.4	15.6	15.8
2	$C_{42}H_{63}O_3P$	27.33	647.4593	0.77	Tris(2,4-di-*tert*-butylphenyl) phosphite (Irgafos 168)	31570-04-4	1.29	15.2	12.3

Identification of compounds

One hundred thirty compounds were identified from the plastic films by library identity searches using Compound Discoverer 3.2 software. Figure 3 shows representative base peak chromatograms, as well as extracted ion chromatograms (XICs), for some commonly used additives that were identified (Table 4), demonstrating the performance and confidence of the applied analytical method for the detection and identification of the extracted compounds.

For some additives, the extracted EIC can contain multiple compounds with similar *m/z* values within a mass-extracted window of 5 ppm, but different retention times. To further confirm their identities, Mass Frontier 8.0 software is used for the MS/MS data to interpret the fragmentation spectra. Bis(2,4-di-*tert*-butylphenyl)phosphate (bDtBPP), a degradation product of Irgafos® 168, has been shown to be highly toxic to mammalian cells even at concentrations below the parts-per-million level.[22-24] The theoretical *m/z* 475.2972 of bDtBPP was calculated by its $[M+H]^+$ adduct in positive mode ESI. Two peaks were extracted from the total ion chromatogram using *m/z* 475.2972, one peak at 15.92 min, the other one at 25.35 min. Figure 4 demonstrates our identification of bDtBPP through fragmentation at 15.92 min. Confident structure elucidation also helped us confirm the identity of oxidized Irgafos 168 (Figure 5).

Three different ASE methods provided excellent results for screening purposes. As shown in Figure 6A, a total of 130 compounds were extracted by three methods—58 compounds (44.6%) were commonly extracted by three methods, 44 compounds (33.8%) were selectively extracted by one method, and 28 compounds (21.5%) were extracted by two out of three methods. The highest number of compounds (122) were identified by ASE Method I (Figure 6B). ASE Method I also showed the highest number of compounds (36) that were extracted only by this method (Table 5).

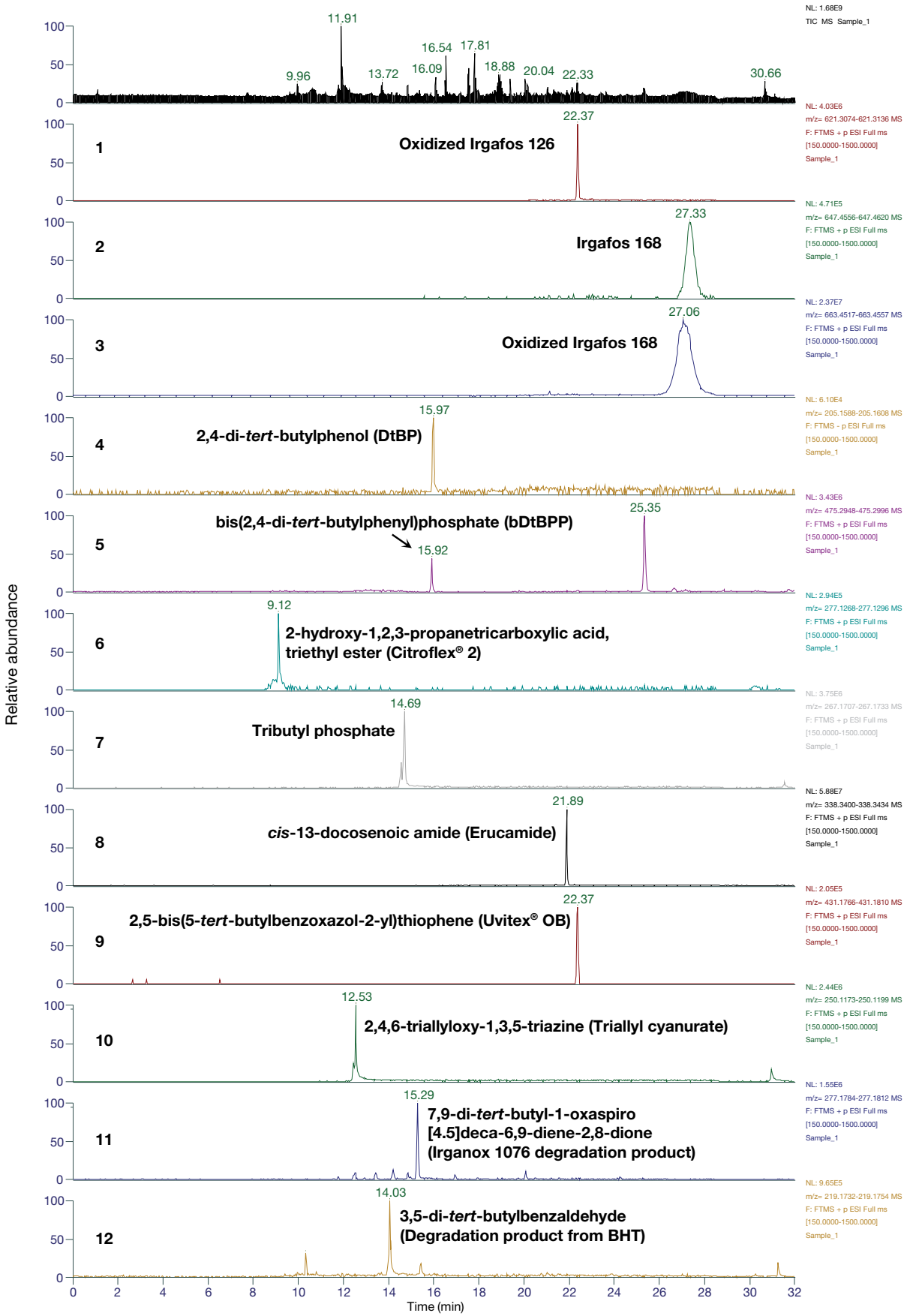

Figure 3. Selected common additives were identified in the plastic film

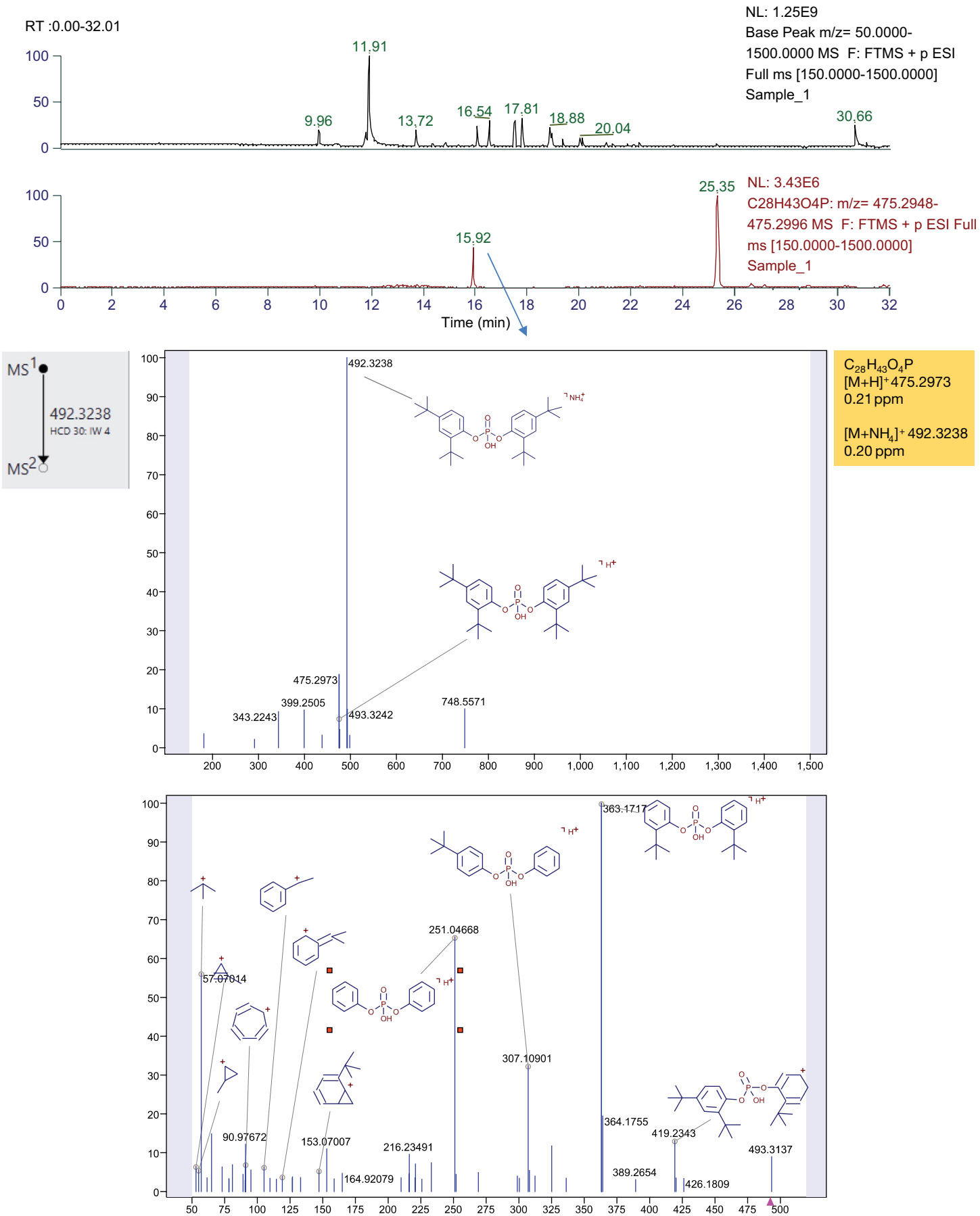

Figure 4. Identification of bDtBPP from the plastic film with Full MS and MS/MS spectra

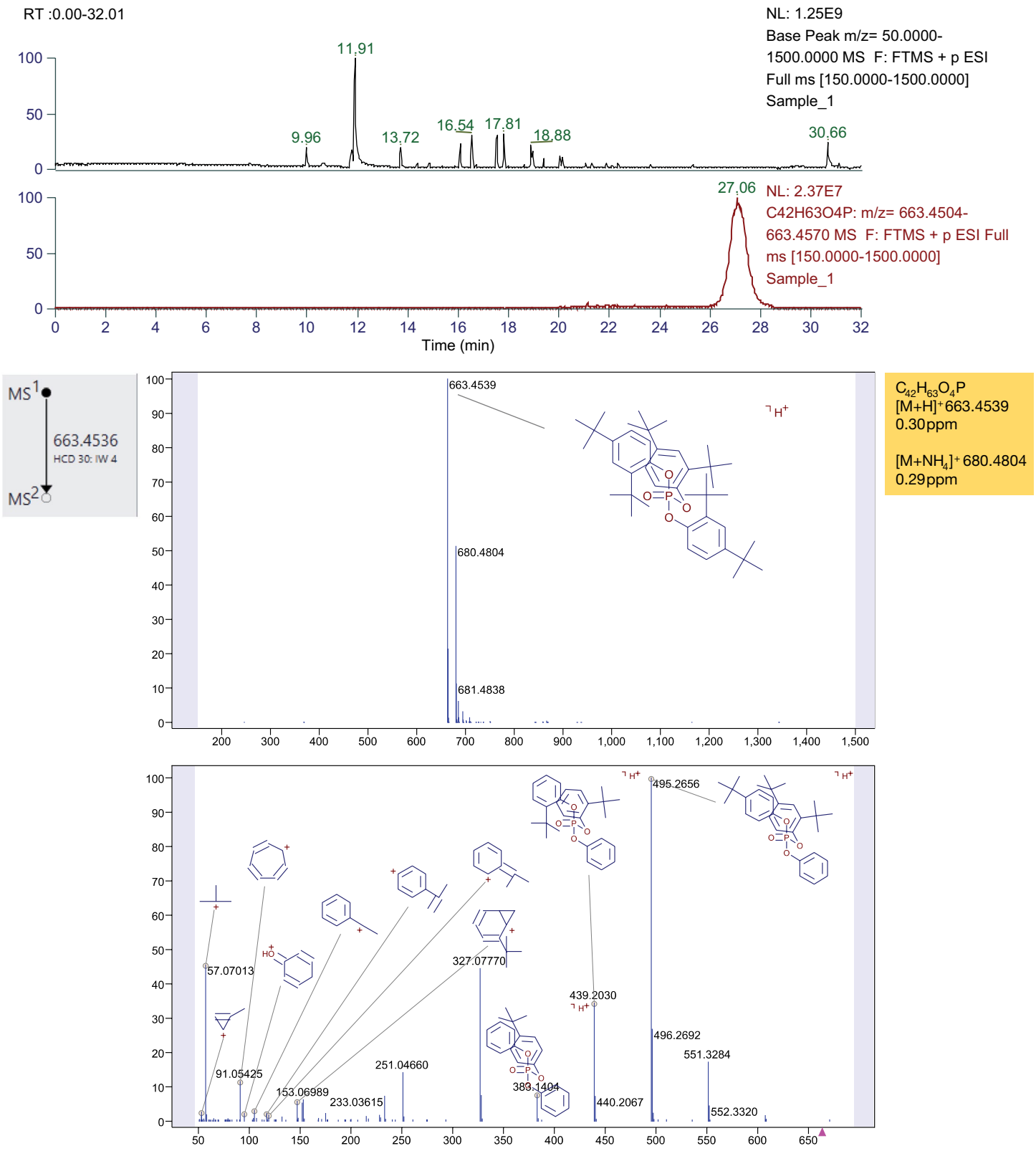

Figure 5. Identification of oxidized Irgafos 168 from the plastic film with Full MS and MS/MS spectra

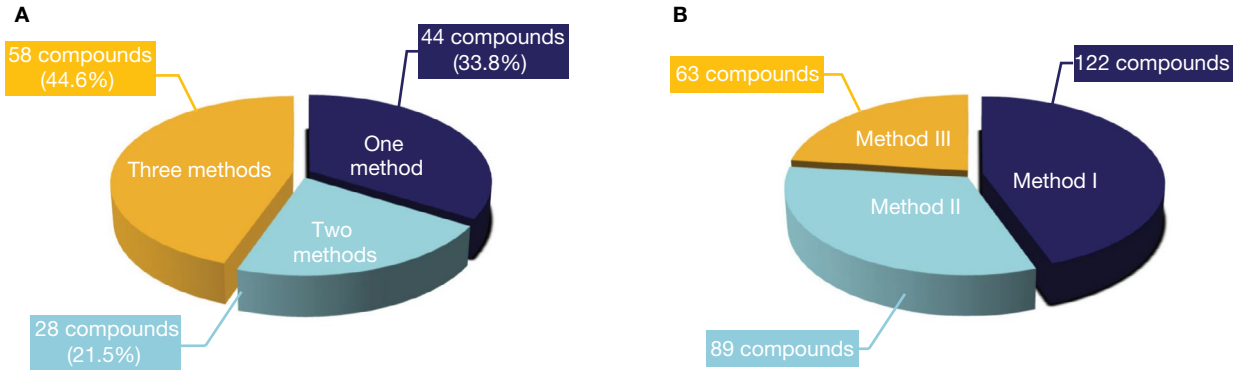

Figure 6. Distribution of identified compounds per number of ASE methods, (A); and per type of ASE method, (B)

Table 5. Classification of identified compounds using the three ASE methods

	Total	Method I	Method II	Method III
	130	122	89	63
Selectively extracted	**44**	36	5	3
		Method I and II	**Method I and III**	**Method II and III**
Selectively extracted	**28**	26	2	0
		Three methods		
Commonly extracted	**58**	58		

Conclusion

This application note evaluated the capability and performance of an automated Dionex ASE 350 system to extract additives in the plastic film from single-used bags. Temperature and swelling agent proportion as selected variables were assessed for ASE extraction. More than 100 additives and degradation products were confidently identified by liquid chromatography and Orbitrap-based high-resolution accurate mass (HRAM) mass analysis.

This analytical method, composed of an efficient and versatile extraction method, along with the excellent performance of Orbitrap HRMS, has all the necessary features to be used for raw material evaluation of bags during manufacturing process development, and it should be readily extended to other single-use components.

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