Microchemical Journal 158 (2020) 105256

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Analysis of unknowns in recycled LDPE plastic by LC-Orbitrap Tribrid HRMS using MS^3 with an intelligent data acquisition mode

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ARTICLE INFO

Keywords:
Recycled LDPE
Orbitrap Tribrid
NIAS
MS^3
AcquireX
Unknown analysis

ABSTRACT

A new automated approach has been developed for the fast investigation of unknown substances (NIAS/IAS) in recycled LDPE plastic that could be used for food contact material (FCM). The pipeline setup includes an intelligent data acquisition with AcquireX™ linked to LC-Orbitrap Tribrid-HRMS (MS^3) and coupled to Compound Discoverer data processing. Twenty-eight unknown compounds were tentatively identified using very restrictive criteria that includes exact mass, isotopic profile, MS^2 match, retention time and MS^3 match. Most of the identified compounds were additives, mainly plasticizers, used in different plastic applications. To check for the safety of the migration of these compounds for food contact material applications of the LDPE recycled plastic, a risk assessment was performed using mainly the threshold of toxicological concern (TTC) approach. The migration of the identified compounds does not represent a risk.

1. Introduction

Food contact materials (FCM) are designed to fulfill multiple purposes, including the most important of protect the food products from contamination and damage, being plastics one of the most relevant materials. Plastics intended to come into contact with food should be safe, what means that the migration into food of monomers or other starting substances and additives should be authorized before their use (intentioned added substances, IAS) and cannot exceed the specific migration levels established (SML) [1]. Likewise, during the manufacture and use, reaction and degradation products can be formed (non-intentionally added substances, NIAS). Before authorizations of FCM, the potential risk of NIAS should also be assessed [2].

On the other hand, the millions of tons of plastic packaging produced, and the way plastic is used are causing enormous waste generation and environmental problems. According to European Commission, the demand for recycled plastics today accounts for only around 6% of plastics demand in Europe [3], consequently, more efforts need to be done to increase the recycled plastic in the food packaging sector.

Between the different plastic materials, low density polyethylene (LDPE) as great demanded, and is more commonly used for flexible applications such as carrier bags and shrink and stretch film [4]. Post-consumer LDPE plastic waste may contain also residues from previous use, contaminants from misuse and contaminants from non-authorized substances or by substances originating from non-food contact grade plastic. Consequently, in order to ensure the safety of recycled LDPE plastics an exhaustive evaluation of the migrating substances are necessary [5,6].

In order to control the safety of food contact plastic materials [1], appropriate analytical methods are required for both IAS and NIAS. Some significant families of substances have been reported in migration studies from plastic-FCM, such as oligomers [7], bisphenols and related compounds [8], primary aromatic amines [9], photoinitiators [10], phthalates [11], perfluorinated compounds [12]. The main characteristics of the analytical methods employed have been described in several reviews [7,13,14].

For plastics and other food contact materials, the identification of unknown compounds, including NIAS, is an emerging analytical field [15]. For thermally unstable, more polar and non-volatile substances, liquid chromatography coupled to high resolution accurate mass spectrometry (HRAMS) is currently the more employed analytical platform.

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https://doi.org/10.1016/j.microc.2020.105256
Received 18 June 2020; Accepted 4 July 2020
Available online 11 July 2020
0026-265X/

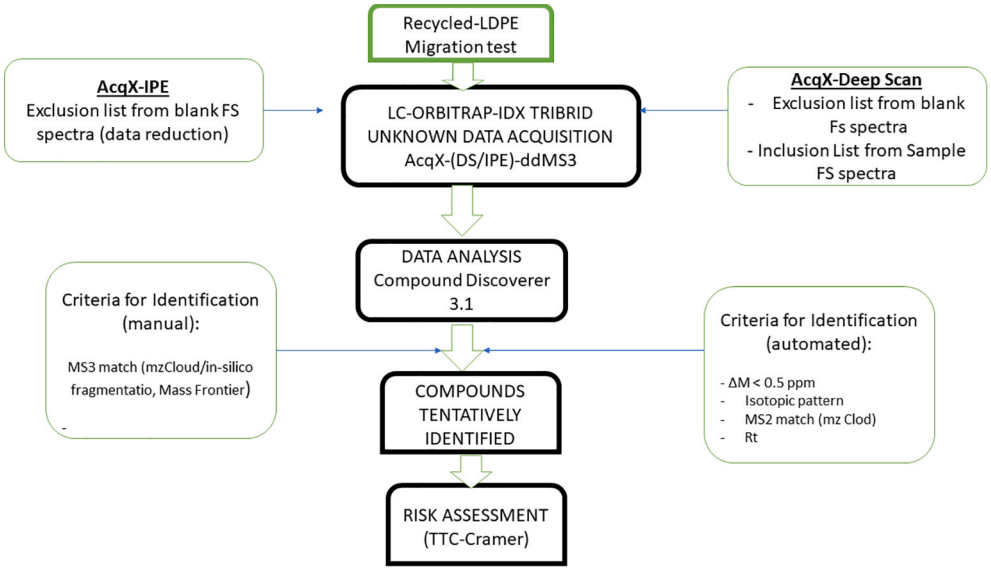


Fig. 1. Scheme of the different stages of the presented study.

The use of hybrid instruments provides information on the accurate mass (full scan) and the fragmentation pattern (MS^2), that are relevant for the tentative identification of the unknown compounds. To date, two types of HRAM spectrometers are in use for identification of unknown compounds in FCM: quadrupole-time-of-flight (Q-TOF) and quadrupole-Orbitrap (Q-Orbitrap) [16]. More recently, Orbitrap tribrid mass spectrometers, that include a quadrupole, an orbitrap analyzer and a linear ion trap have increased the mass resolving power (450,000 FWHM at *m/z* 200), scan speed (30 Hz) and permit to perform MS^n for better structural elucidation [17,18]. These instruments have been used for metabolomic and environmental applications, but to our knowledge, have not been employed in the FCM field.

The acquisition methods for non-target screening (unknown) are performed using two main approaches: data independent acquisition (DIA) and data dependent acquisition (DDA). In DIA mode, all ions within a selected *m/z* range are fragmented and the entire MS^2 mass range monitored [19]. On the other hand, in DDA the instrument acquires a full MS scan followed by a set of data-dependent scans with fragmentation (MS/MS). The fragmentation events are triggered by different parameters previously selected (e.g., intensity threshold of the precursor, inclusion list, dynamic exclusion list [20,21]).

The success on performing comprehensive characterization of unknown compounds depends on the exhaustive acquisition of full scan and fragmentation data (MS^2, MS^n) and of the interpretation of these data to identify unknown compounds. This is challenging because of the large number of features both in the samples and background (blanks and/or matrix) and, in general, requires replicates injections, background exclusion, and iterative method creation, as well as library search. All this process is enormously laborious and time consuming.

In order to automate these arduous steps new acquisition tools are developed for background removing, exhaustive precursor selection (inclusion list) and creation of dynamic and real-time exclusion list [22]. Likewise, an automated library search has been improved through the data processing tool Compound Discoverer™ [23].

After the migrant substances (NIAS, IAS) have been detected and identified, a risk assessment is necessary to ensure the safety of recycled plastics. For substances with no toxicological data available the threshold of toxicological concern (TTC) concept, based on the Cramer rules that estimates the theoretical toxicity of compounds linked to their molecular structure [24,25].

In this study, a new approach for the determination of unknown substances in recycled LDPE that could be used as food contact material has been investigated. In a first step we evaluated the capacity for exhaustive extraction of the spectral data from the sample of an innovative intelligent acquisition mode using DDA-MS^3. In the second stage the automatic search on compound databases and spectral libraries was employed to tentative identification of unknown compounds, and finally we assessed the risk of the migrating substances detected.

2. Materials and methods

2.1. Chemicals

Certified commercial standards were of high purity, Terfenadine and Val-Try-Val were purchased from Sigma Life Science (St Louis, MO, USA), Triallyl phosphite was obtained from Alfa Aesar Thermo Fisher (Kandel, Germany) and Sulfaguanidine, Sulfadimethoxine, Reserpine, Caffeine and Acetaminophen were purchased from Sigma Aldrich (St Louis, MO, USA). Standards solutions were prepared in H_2O: MeOH (70:30, v/v) in a concentration of 10 ng mL^{-1}.

2.2. Samples

Post-consumer recycled LDPE plastic film was used. The recycled plastic was obtained in a process including grinding, washing drying and extrusion. In order to remove contaminants a technology that employs surfactants was applied [26]. The recycled was provided by the company CADEL based on Sant Vicent dels Raspeigs, Alicante (Spain).

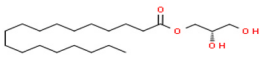
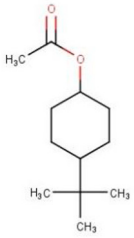
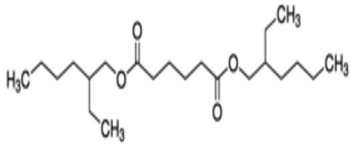
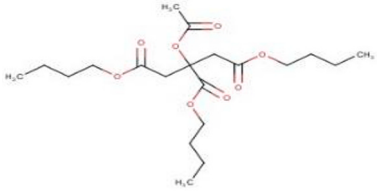
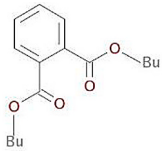
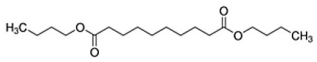
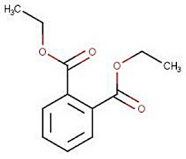
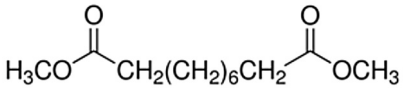
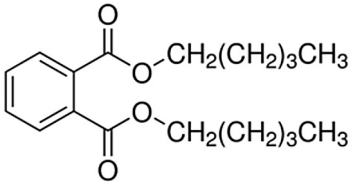
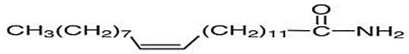
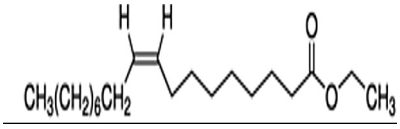
2.3. Migration test. Sample preparation

Migration tests were performed according to the European Regulation (EU) No 10/2011 [1]. The food simulants used were simulant A that consist of 10% of ethanol in water (v/v). The standardized test conditions were the following: contact time 10 days and temperature 40 °C. We used a square of 2.5 cm × 2.5 cm (6.25 cm^2) of the recycled LDPE film and introduced it on a 50 mL beaker with 20 mL of the simulant and keep it in the incubator for ten days. The pool of seven standards were also added. According with the Regulation 10/2011, 6 dm^2 are equivalent to 1 Kg of food.

2.4. LC-HRAMS analysis

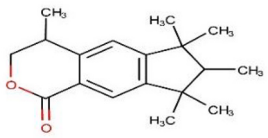
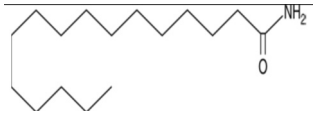
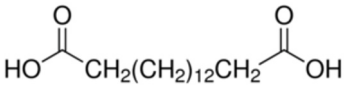
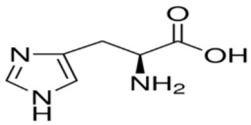
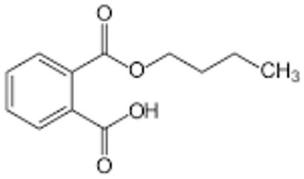
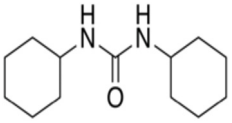
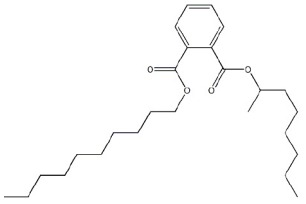
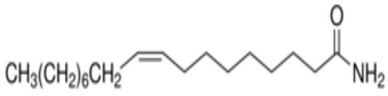
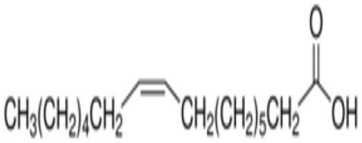
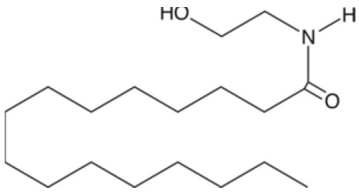
The liquid chromatographic separations were performed on a

Table 1
Identified compounds and spectrometric and chromatographic criteria.

Compound[1,2] (CAS number)	Structure	Δ Mass (ppm)	Isotopic pattern (SFit %)	*m/z* Cloud Match (MS^2 match)	$Rt_{exp\ (min)}$	Rt_{theo} (min)	MS[3] Match [3]	Description [Reference]
1-Stearoylglycerol[a] (123–94-4)		−0.02	69	87.6	22.11	19.36	(1) MF	Emulsifier [29]
4-*tert*-Butylcyclohexyl acetate[a] (32210–23-4)		0.03	85	72.1	20.47	12.23	(1)(2)	Fragrance [30]
Bis(2-ethylhexyl)adipate[a] 103–23-1		0.03	74	84.6	22.04	19.81	mzCloud	Plasticizer [31]
Citroflex A-4 [a] (77–90-7)		−0.02	85	91.4	19.34	16.94	(1)(2)CD	Plasticizer [32]
Dibutyl phthalate [a,b] (84–74-2)		−0.02	68	66.6	11.55	13.41	MzCloud	Plasticizer [32]
Dibutyl sebacate[a] (109–43-3)		0.03	76	95	20.89	15.84	(1)MF	Plasticizer [32]
Diethyl phthalate[a] (84–66-2)		−0.02	68	66.6	11.55	8.94	(1)MF	Plasticizer [29]
Dimethyl sebacate[a,b] (106–79-6)		0.24	94	75.6	16.89	9.41	(1)(2)CD	Plasticizer [32]
Dipentyl phthalate[a] (131–18-0)		0.08	93	70.9	21.00	15.67	(1)MF	Plasticizer [33]
Erucamide[a] (112–84-5)		0.3	96	93.2	22.93	23.79	(3)CD	Slip promoter [32,34]
Ethyl Oleate[a] (111–62-6)		−0.01	86	80.3	20.25	21.58	MzCloud	Plasticizer [35]

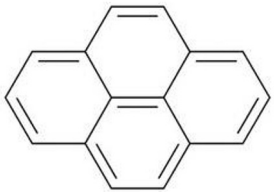
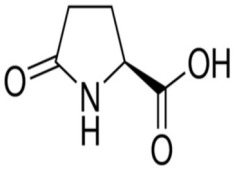
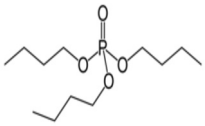
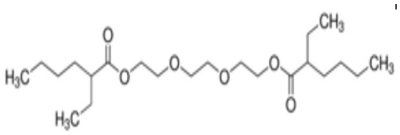
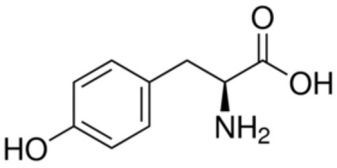
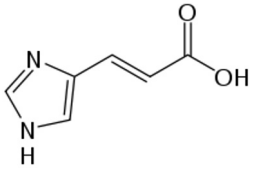
(*continued on next page*)

Table 1 (*continued*)

Compound[1,2] (CAS number)	Structure	Δ Mass (ppm)	Isotopic pattern (SFit %)	*m/z* Cloud Match (MS^2 match)	$Rt_{exp\ (min)}$	Rt_{theo} (min)	MS^3 Match [3]	Description [Reference]
Galaxolidone[a] (507442–49-1)		0.19	89	72.9	19.96	14.85	(1)MF	Metabolite of galaxolide, a polycyclic musk [36]
Hexadecanamide[a,b] (629–54-9)		−0.04	94	86.7	21.48	17.68	(3)	Slip agent [34]
Hexadecanedioic acid[a,b] (505–54-4)		−0.75	71	90.3	19.15	15.26	(1)CD	Used in the production of plastics
L-Histidine[a,b] (71–00-1)		0.09	79	93.4	0.78	0.58	(3)	Used in adhesives, and plasters
Monobutyl phthalate[a] (131–70-4)		0.12	67	81.2	19.53	8.98	(1)MF	Plasticizer Metabolite of DBPh
N,N'-Dicyclohexylurea[b] (2387–23-7)		−0.02	96	62	14.79	9.58	(3)CD	
Octyl decyl phthalate[a,b] (119–07-3)		−0.18	91	79.6	22.39	22.94	mzCloud	Platicizer [29]
Oleamide[a] (301–02-0)		−0.06	88	86.7	21.56	18.99	mzCloud	Slip agent [1,34]
Palmitoleic acid[a,b] (373–49-9)		0.23	88	68.4	21.51	18.13	(1)(2)CD	
Palmitoyl ethanolamide[a,b] (544–31-0)		0.03	81	71.7	21.32	17.27	mzCloud	
PEG n5[a,b] (225–341-4)		0.08	73	81.7	3.08	0.57	(1)(2)CD	Surfactants and emulsifying agent [37]

(*continued on next page*)

Table 1 (*continued*)

Compound[1,2] (CAS number)	Structure	Δ Mass (ppm)	Isotopic pattern (SFit %)	*m/z* Cloud Match (MS^2 match)	$Rt_{exp\ (min)}$	Rt_{theo} (min)	MS^3 Match [3]	Description [Reference]
Pyrene[a,b] (129–00-0)		−0.01	98	82.6	12.08	14.15	(1)(2)CD	Photoinitiator [32]
L-Pyroglutamic acid[a,b] (98–79-3)		−0.1	88	89.1	0.935	1.50	–	
Tributyl phosphate[a,b] (126–73-8)		0.27	73	88.2	19.86	12.23	(1)MF	Solvent and plasticizer [32,38]
Triethyleneglycol bis(2-ethylhexanoate)[a] (94–28-0)		0.28	73	86.9	21.66	15.00	–	Plasticizer [32]
L-Tyrosine[a,b] (60–18-4)		0.16	92	90.2	0.97	0.54	mzCloud	
Urocanic acid[b] (104–98-3)		−0.02	66	86.5	1.294	2.95	(3)CD	Histidine metabolite

Isotopic pattern (SFit %): match between the experimental isotopic pattern and the theoretical one; *m/z* Cloud Match: % match of the experimental MS^2 spectra and those included in mzCloud library; Rt_{exp}: experimental retention time.
Rt_{theo}: Theoretical time bases on log P.
MF: MS^3 experimental spectra match with Mass Frontier fragmentation.
mzCloud: MS^3 experimental spectra match with mzCloud spectra.
CD: match between the in-silico fragmented with the CD and the experimental MS^3.
(1) mzCloud does not contain a MS^3 spectra of this compound.
(2) The experimental spectra do not match with MF fragmentation.
(3) The experimental MS^3experimental do not match with that stored in mzCloud or the in-silico fragmentation by MF.
[a] Detected with AcqX_DS mode.
[b] Detected with AcqX IPE mode.
[1] All compounds present a full match for a Predicted composition (molecular predicted composition from libraries from the experimental exact) mass; Δ Mass (ppm): mass accuracy.
[2] All compounds have acquires MS^2 and MS^3 spectra.
[3] Match between the experimental MS^3 and the database MS^3 spectra (mzCloud) or the in-silico fragmentation (Mass FrontierF/CD).

Thermo Ultimate 3000 UHPLC system equipped with a UHPLC reversed-phase column Hypersil Gold, 1.9 μm particle size, 100 × 2.1 mm (Thermo Scientific) interfaced with an Orbitrap ID-X Tribrid mass spectrometer (Thermo Fisher Scientific, USA). The injection volume was of 5 μL, and the column was eluted with a flow rate of 300 μL/min. The initial condition of the mobile phase were 90% phase A (water) and 10% B (methanol). The gradient employed was: From 0 to 18 min. linear gradient to 70% B; from 18 to 21.5 min. linear gradient to 98 %B; maintained at 98% B from 21.5 to 25 min; linear decrease to initial conditions from 25 to 26 min, and maintained until 30 min.

The mass spectrometer used a heated electrospray interface (H-ESI) operating in positive/negative ionization. The H-ESI parameters in positive polarity were the following: electrospray voltage of 3.5 kV positive (3.2 Kw negative); sheath gas of 25 arbitrary units (a.u); and auxiliary gas of 5 a.u. The ion transfer tube operated at 270 °C and the vaporizer temperature at 180 °C. The full scan was acquired with resolving power of 120.000 FWHM for parental ions and mass range of

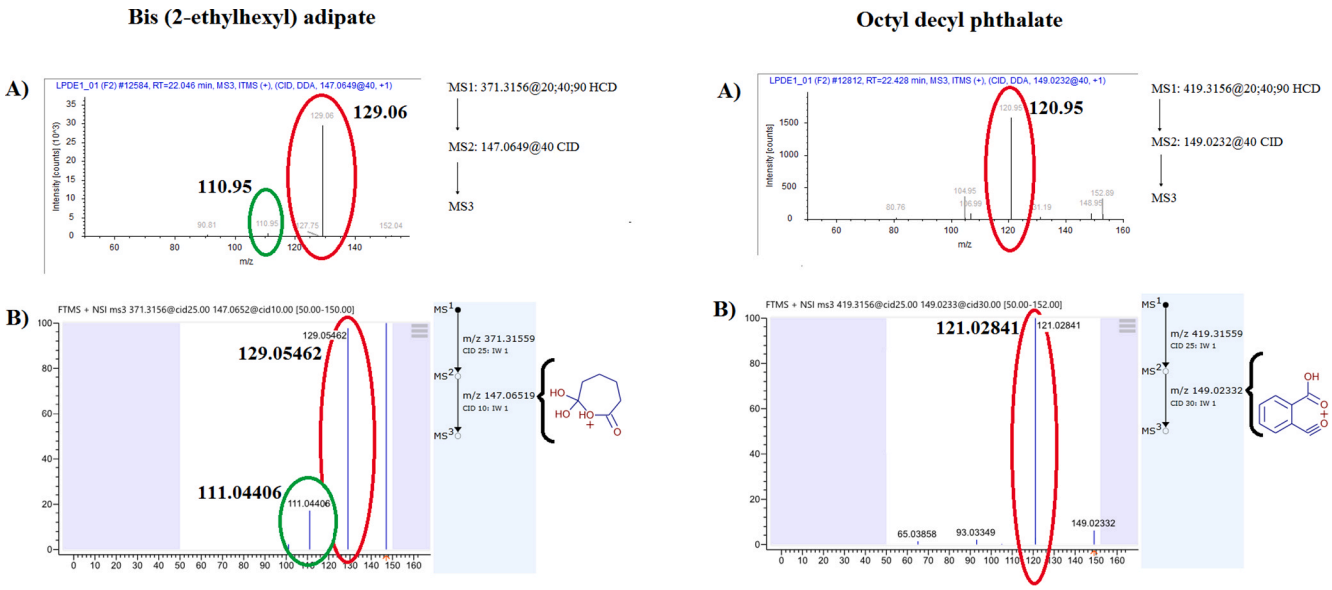


Fig. 2. A) Experimental MS^3 spectra and B) mzCloud MS^3 spectra for bis (2-ethylhexyl) adipate and octyl decyl phthalate.

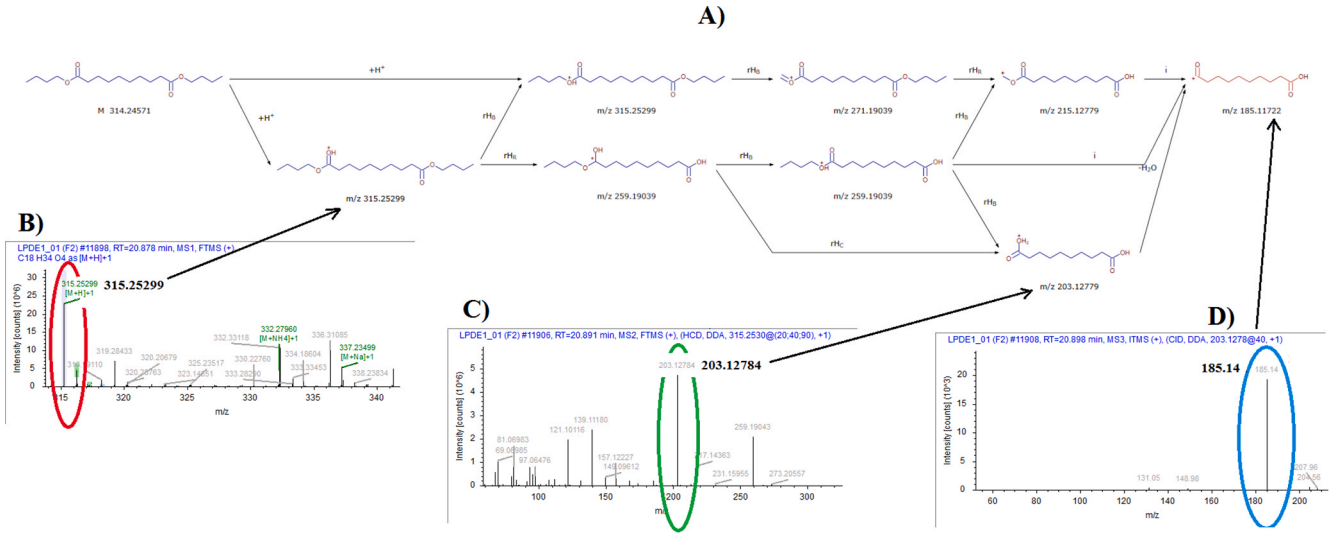


Fig. 3. A) In-silico mass fragmentation using MassFrontier for dibutyl sebacate; B) Experimental MS^1 spectra; C) Experimental MS^2 spectra; D) Experimental MS^3 spectra. rH_B (α,β-charge-site rearrangement); rH_C (γ-charge-site rearrangement); rH_R (charge remote rearrangement); i (inductive cleavage).

100–900 *m/z*. The MS^2 were acquired in the Orbitrap analyser at a resolving power of 15.000 FWHM, with a precursor mass range of 125–900 *m/z* range. The MS^3 fragmentation was carried out and analysed in the Linear Ion Trap (LIT).

2.5. Acquisition workflow for unknown analysis

The Orbitrap-tribrid-HRAMS instrument permits a great variety of different acquisition workflows. In the present study the AcquireX© Data Acquisition Technology (ThermoFisher Scientific) was employed, in the modes Deep Scan (DS) and Interative Processing Exclusion (IPE). The acquisition workflows for DS and IPE modes in data dependent (DD-MS^3) are depicted in Figs. SI-1–2.

In Deep Scan mode exclusion and inclusion lists are automatically created after the injection of a blank and a sample in full scan, respectively. Iterative injections (three to six) of the LPDE sample automatically update both list (inclusion/exclusion) which permits an exhaustive acquisition of the relevant features, excluding those present in the blank. For IPE mode, only an exclusion list is automatically created from the blank injection and updated after each injection of samples. The sequence designs of the two AcquireX© modes are depicted in Figs. SI-3–4.

2.6. Data processing

Post-acquisition data processing was performed for annotation and structural characterization of chemicals. Raw files were processed by Compound Discoverer™ 3.1 (CD) (Thermo Fisher Scientific). The workflow employed is shown in Fig. SI-5, and a description of each node is described in Table SI-1. Taking the description of this workflow provided by the supplier, this workflow "performs retention time alignment, unknown compound detection, and compound grouping across all samples. Predicts elemental compositions for all compounds, and hides chemical background (using Blank samples). Identifies compounds using mzCloud (ddMS2), ChemSpider (exact mass or formula) and local database search against Mass Lists (in the present study the Extractable and Leachable database containing 1741 compounds with exact mass without RT). Performs spectral similarity search

Table 2
Risk assessment of the identified compounds.

Substance	Migration concentration (mg/Kg food)	SML (mg/Kg food)	EDI (mg/person/day)	Cramer class	TDI_{Cramer} (mg person^{-1} day^{-1})	TDI (mg person^{-1} day^{-1})	HQ (EDI/TDI)
1-Stearoylglycerol	1.86		0.67	1	1.8		0.37
4-*tert*-Butylcyclohexyl acetate	0.59		0.21	2	0.54		0.39
Bis(2-ethylhexyl)adipate	0.22	18	0.08				
Citroflex A-4	0.04	60*	0.02				
Dibutyl phthalate	0.04		0.02			0.6 [1]	0.03
Dibutyl sebacate	0.52		0.19	1	1.8		0.1
Diethyl phthalate	0.04		0.02			49.2[2]	0.0004
Dimethyl sebacate	0.12		0.04	1	1.8		0.02
Dipentyl phthalate	0.06		0.02	1	1.8		0.01
Erucamide	0.05		0.02	3	0.09		0.20
Ethyl Oleate	0.63		0.23	1	1.8		0.13
Galaxolidone	0.04		0.01	3	0.09		0.14
Hexadecanamide	0.05		0.02	3	0.09		0.21
Hexadecanedioic acid	0.03		0.01	1	1.8		0.01
L-Histidine	0.01		0.004	1	1.8		0.002
Monobutyl phathalate	0.11		0.04	1	1.8		0.02
N,N'-Dicyclohexylurea	0.05		0.02	3	0.09		0.20
Octyl decyl phthalate	0.08		0.03	1	1.8		0.02
Oleamide	0.02		0.01	3	0.09		0.07
Palmitoleic acid	0.08		0.03	1	1.8		0.02
Palmitoyl ethanolamide	0.03		0.01	3	0.09		0.11
PEG n5	0.06		0.02	1	1.8		0.01
Pyrene	0.03		0.01	3	0.09		0.11
L-Pyroglutamic acid	0.04		0.01	1	1.8		0.01
Tributyl phosphate	0.21		0.08	3	0.09		0.85
Triethyleneglycol bis(2-ethylhexanoate)	0.21		0.08	1	1.8		0.04
L-Tyrosine	0.01		0.003	1	1.8		0.002
Urocanic acid	0.03		0.01	3	0.09		0.11

(*) Group restriction (sum of substances group restriction 32). Regulation 10/2011.
[1] EFSA TDI 0.01 (mg kg-bw^{-1} day^{-1}) [42]. Calculation of TDI in mg person^{-1} day^{-1} considering a person weight of 60 kg.
[2] US EPA TDI 0.8 (mg kg-bw^{-1} day^{-1}) [43]. Calculation of TDI in mg person^{-1} day^{-1} considering a person weight of 60 kg.

against mzCloud for compounds with ddMS2. Applies mzLogic to rank order structures from ChemSpider and mass list search results. Applies spectral distance scoring to mass list and ChemSpider matches

The criteria for tentative identification of compounds were the following

Parameter*	Criteria	Observations
Molecular formula (-Predicted composition)	Full match	Molecular formula predicted from compound libraries (Chem Spider,…) taking into account the exact mass and the isotopic pattern
Δ Mass (ppm)	< 0.5 ppm	From Chem Spider/…
Isotopic profile (SFit %)	> 70%	From databases (Chem Spider, mz Cloud…)
m/z Cloud Match	> 70%	Match of MS^1/MS^2 spectra
MS^2 Data	Yes	The spectra compound is included in a spectra library (mz Cloud)
Rt (min)	Consistent with their log P	Comparing with the Rt of a set of standards with a wide range of lop P
Uses**	Yes	It is possible their presence in plastic because their use, degradation or contamination

*Parameters automatically provided by Compound Discoverer.
**Literature search.

2.7. Safety assessment of the identified migrants

A theoretical evaluation of the safety of the tentatively identified substances based on the Cramer rules and the Threshold of Toxicological Concern (TTC) was done. The Toxtree software (http://toxtree.sf.net/predict) developed by Ideaconsult ltd., commissioned by JRC Computational Toxicology and Modelling (https://apps.ideaconsult.net/data/ui/toxtree) was used. This software is an open source application that estimates the threshold of toxicological concern (TTC) following Cramer rules by entering the molecular structure/CAS number of the substance. Each of the three Cramer class is allocated to an tolerable daily intake (TDI, mg person^{-1} day^{-1}): Class I, the least toxic class, has a TDI = 1.8 mg person^{-1} day^{-1}; Cramer class II (intermediate) has a TDI = 0.54 mg person^{-1} day^{-1}; and Cramer class III (most toxic) has a TDI = 0.09 mg person^{-1} day^{-1}.

The Estimated Daily Intak (EDI) was calculated according the equation established by the US Food and Drug Administration [27].

EDI (mg/person × day) = migration (mg/kg) × 3 kg (food intake per person and day) × CF.

where Consumer Factor (CF) describes the fraction of the daily diet expected to contact specific contacting material. CF for LDPE is 0.12 [27]. We estimated the migration (mg/Kg) using the average response factor of seven substances used as internal standards [28] (see Table SI-2).

3. Results and discussion

The different steps followed in the present study are shown in Fig. 1. Before proceeding to the data analysis, we checked the automatically updated of the inclusion and exclusion lists performed by the two acquisition workflows used (AcqX-DS-ddMS^3 and AcqX-IPE-ddMS^3). In Tables SI-3,4 are shown the updated run-to-run number of ions in the inclusion/exclusion lists. For these LDPE plastic samples it seems that three injections with DS workflow are enough. However, for IPE workflow and exhaustive checking of the sample requires more injections. Most of the substances were identified in the raw files from the AcqX-DS acquisition mode (see Table 1).

3.1. Identification of unknown substances

All raw data (positive and negative acquisitions) were processed with the CD, using the workflow described in Section 2.6. The selected workflow in the CD automatically annotated a total of 2243 compounds. Out of all annotated compounds, just 28 meet the criteria selected for tentative identification (Table 1). The chosen criteria were very restrictive in order to permit, in a first step, their automated identification, that is mainly based on the presence of these compounds on the spectral libraries (mzCloud) and databases (ChemSpider, Extractables and Leachables HRAM). A more in deep manually examination of the annotated compounds that requires structure elucidation using in silico fragmentation tools, more spectral databases, bibliographic search could identify more compounds [16]. However, in the present study we only discus the automatically identified compounds.

All compounds tentatively identified present a predicted composition (molecular formula) with a mass accuracy (ΔM), in general, better than 0.05 ppm, and an isotopic pattern that fits with the theoretical (SFit% > 75%). Likewise, their MS^2 spectra match well with those included in the spectral libraries (mzCloud match in Table 1).

Complementary to this data, we compare the experimental retention time (Rt_{ex}) of each compound with the theoretical one (Rt_{th}) derived from their LogP. A pool of internal standards to build up the relationship between LogP vs Rt was used (See Fig. SI-6). In general, the experimental Rt (min) fits well with the theoretical one (Table 1). Following the level system for structure identification proposed by Schymanski [39], the tentatively identified compounds present a high level (level 2) of identification confidence.

The most recent studies for non-target screening strategies applied to plastic food packaging materials employ two data acquisition approach, DDA with Q-Orbitrap and DIA with Q-TOF-MS instruments [16]. Both approaches acquire full scan and MS^2 spectra. The tentative identification strategy is carried out manually, using libraries, database and bibliographic search. This is a very labour-intensive activity with, in general, low number of identified compounds. Bignardi et al. [20] performed an untargeted analysis to identify unknown molecules from polycarbonate food-contact plastic using a DDA-MS^2 acquisition approach. The identification of compounds was carried out in a three consecutively-three steps, performed manually: search or a molecular formula from the recorded accurate mass; comparison of the isotopic pattern using a simulator; investigation of the molecular structures in available chemical databases. They claim for the identification of several and polycarbonate degradation products, but no MS^2 data were employed for structural identification. For identification of NIAS coming from biodegradable food packaging, Canellas et al. [19] used a non-target DIA approach with a Q-TOF-MS instrument. They obtained the elemental formula using the isotopic pattern and the mass tolerance (3 ppm). A list of candidates was obtained using the ChemSpider and SciFinder databases, and the accurate masses of fragments (MS^2) were used for identification, considering if the fragments could be generated from the candidates. Three nonvolatile compounds were tentatively identified. Likewise, in a recent study, Martinez-Bueno et al. [40] used a DIA acquisition mode (SWATH) with a Q-TOF-MS for non-target analysis of migrating from a multilayer plastic packaging. The identification strategy consisted in the obtention of a molecular formula with a formula finder tool (mass tolerance > 5 ppm; isotopic pattern) followed by manual process that data reduction and the elucidation of possible structures using MS^2 data interpretation with the aid of database ChemSpider. The theoretical fragmentation pattern (in silico) was compared with the MS^2 experimental spectra.

3.1.1. MS^3 spectra

The tentatively identification was automatically provided by acquisition workflow (AcqX-ddMS^3) hyphenated with the data processing workflow (Compound Discoverer). However, this pipeline for unknown identification only use the MS^1 and MS^2 data. An in deep identification could be performed using the added structural data provided by the MS^3 spectra performed in the Linear Trap of the Orbitrap instrument.

As can be seen in Table SI-5, almost all compounds identified have their MS^3 acquired spectrum. However, the CD does not compare automatically the acquired MS^3 spectra of identified compounds with the MS^3 spectra of the spectra libraries (e.g. mzCloud), which means that does not use this useful information for structure elucidation and identification.

We compare manually the experimental MS^3 spectra (see Table 1) with that present on the mzCloud library. For those compounds that do not have MS^3 spectra in mzCloud, we fragmented them in-silico using CD and Mass Frontier [41].

Out of 28 identified compounds, 7 had an acquired MS^3 spectra that matched well with that stored in mzCloud for the same compound. Fig. 2 shows, as an example, the spectra of bis (2-ethylhexyl) adipate and octyl decyl phthalate. As can be seen in both MS^3 spectra the precursor ion (*m/z* 147.0649; *m/z* 149.0232, respectively) matches with that of the mzCloud, as well as the main fragment (*m/z* 129.06 and *m/z* 120.95, respectively). It is necessary to keep in mind that the MS^3 was acquired by the ion trap detector in low resolution.

Contrarily, the MS^3 spectra of 5 substances not matched (fail) with those of the library. In some cases because the mzCloud spectra were acquired from a different MS^2 precursor ion (see SI-4). For example, the Hexadecanamide was acquired in our sample by fragmentation of the MS^2 ion 173.4440, while the library spectrum was that derived of the fragmentation of the *m/z* 88.07569, 102.09134 and 116.10699. On the other hand, fourteen compounds do not have MS^3 spectra in mzCloud. We fragmented in-silico these substances using CD and Mass Frontier, and check for coherence (match) between the experimental spectra and the in-silico fragmentation (see Table 1). With Mass Frontier, we found concordance between experimental MS^n spectra and in-silico fragmentation routes in eight substances. As an example, Fig. 3 shows the experimental spectra (MS^1/MS^2/MS^3) of the dibutyl sebacate and the in-silico fragmentation route. In the MS^3 spectrum, both the precursor and one of the fragments are provided by the theoretical fragmentation. In Figs. SI-7 to SI-13 we depicted the MS^1/MS^2/MS^3 spectra and the in-silico fragmentation other seven substances. When we fragmented in-silico with CD, the same criteria (MS^2 precursor and one fragment) was considered for matching with the experimental MS^3. These MS^3 data provide more evidences for the correct identification of the unknown substances

Apart from the analytical tentative identification of substances, we study the nature and uses of the identified compounds to justify their presence in the recycled LDPE plastic. Table 1 shows a shorten description of the substances. Many of them are plasticizers and slip agents used in plastics.

3.2. Safety assessment of the identified migrants

It is important for FCM to ensure the safety of their use regarding the migration of IAS and NIAS. For recycled plastic it is still more relevant because we use as raw material post-consumer plastic waste, which may contain residues, contaminants or non-authorised substances. For risk assessment we distinguish three categories of substances: i) IAS with SML established in Regulation 10/2011, ii) Substances with toxicological reference values; iii) Compounds for which a TTC (Cramer rules) have been calculated because no toxicological references are established. We applied the methodology described in the section 2.7 to assess the risk of the substances tentatively identified.

As can be seen in Table 2, the IAS 2,2,4-Trimethyl-1,3-pentadienol diisobutyrate; Bis(2-ethylhexyl) adipate and Citroflex A-4 present a migration concentration lower of the established SML, therefore their exposures are not of concern. For all substances for which a Cramer-TDI or a TDI was estimated, present a HQ < 1, consequently the migration of these substances does not represent a risk.

4. Conclusions

The new and automated approach for investigations of unknown substances in recycled LDPE-plastic has permitted the fast identification of 28 compounds, mainly plastic additives. The use AcquireX in modes Deep Scan and IPE has permitted a fast and exhaustive data acquisition from the recycled LDPE samples. Both modes are complementary and require 3 and 6 injections, respectively. The introduction of a MS^3 node in the classic DDA mode, provides more structural information that is very useful for more accurate identification of unknown compounds.

For examination of databases and spectra libraries to identify unknown compounds, the use of an automatized software is essential to avoid labour intensive and time-consuming searches. However, current MS^n libraries, although are in continuous enlargement, still not cover many substances of interest, such as NIAS in plastic materials. This automated search should include also MS^3 match. It is important to define specific criteria for tentatively identified substances, because from the hundreds or thousands of annotated features, only few could be considered has tentatively identified. Guidelines for this interpretation of results may be useful for researchers in different fields.

No concern on the safety of the migrant identified substances could be expressed after risk assessment. However, more in deep investigation of the hundreds of non-identified features need to be done before use this recycled LDPE for food applications

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was developed in the framework of a collaboration agreement between FISABIO and Cadel Deinking S.L. Study co-funded by the European Union through the European Regional Development Fund Operational Programme (ERDF) of the Valencia Region (2014-2020). Pablo Dualde acknowledges his “Ayudas para la contratación PTA” (PTA2018-016320-I) from “Ministerio de Ciencia e Innovación” (Spain).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105256.

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