Potencial de l'espectrometria de masses per a l'anàlisi de contaminants ambientals i alimentaris

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POTENCIAL DE L'ESPECTROMETRIA DE MASSES PER A L'ANÀLISI DE CONTAMINANTS AMBIENTALS I ALIMENTARIS

Presentada per

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Directors: Dra. Maria Teresa Galceran i Dra. Encarnación Moyano

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La Dra. Maria Teresa Galceran i Huguet, catedràtica de Química Analítica de la Universitat de Barcelona, i la Dra. Encarnación Moyano Morcillo, professora titular del mateix departament,

FAN CONSTAR,

Que la present memòria titulada *"Potencial de l'espectrometria de masses per a l'anàlisi de contaminants ambientals i alimentaris",* ha estat realitzada sota la nostra direcció pel Sr. Hèctor Gallart Ayala en el Departament de Química Analítica de la Universitat de Barcelona i que tots els resultats presentats són fruit de les experiències realitzades pel citat doctorant.

I per a que així es faci constar, expedim i firmem el present certificat.

Barcelona, Octubre 2010

Dra. Maria Teresa Galceran i Huguet Dra. Encarnación Moyano Morcillo

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En primer lloc voldria donar les gràcies a la Dra. Maria Teresa Galceran i la Dra. Encarnación Moyano, la direcció d'aquesta Tesi i la confiança dipositada des del primer moment així com pels coneixements rebuts durant tots aquests anys.

Vull donar les gràcies també al Dr. Francesc Ventura i la Sra. Maria Huerta d'Aigües de Barcelona per les mostres d'aigua subministrades. Així com als Serveis Científic Tècnics de la Universitat de Barcelona.

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ÍNDEX

OBJECTIUS I ESTRUCTURA

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L'**objectiu** d'aquesta Tesi ha estat el desenvolupament de metodologia analítica basada en la cromatografia de líquids acoblada a l'espectrometria de masses en tàndem (LC-MS/MS) per a l'anàlisi de compostos àmpliament utilitzats en la fabricació d'envasos per a la industria alimentària com són el bisfenol A i compostos relacionats que s'utilitzen com a material de partida en la producció de plàstics i resines epoxi i els fotoiniciadors addicionats les tintes UV utilitzades en la impressió dels envasos. Aquest objectiu general es pot desglossar en una sèrie d'objectius concrets que són els següents :

- Establir les rutes de fragmentació en espectrometria de masses dels compostos estudiats en aquesta memòria i d'identificar les transicions més característiques i abundants de cadascuna de les famílies.

- Avaluar la capacitat d'un instrument de triple quadrupol equipat amb quadrupols hiperbòlics de treballar a una relativa alta resolució, tant en la selecció dels ions precursors com dels ions producte, així com de dur a terme mesures de massa exacta.

- Utilitzar rebliments i fases estacionaries de cromatografia de líquids de nou disseny i recent desenvolupament per establir mètodes de LC-MS/MS ràpida per a l'anàlisi de bisfenol A, altres bisfenols relacionats, el bisfenol A diglicidil èter (BADGE), el bisfenol F diglicidil èter (BFDGE), així com els seus derivats hidrolitzats i clorohidrolitzats, a més de fotoiniciadors emprats en les tites UV d'impressió.

- Desenvolupar mètodes d' extracció en fase sòlida en línia amb sistemes de cromatografia de líquids ràpida acoblada a l'espectrometria de masses en tàndem (SPE-LC-MS/MS) per a l'anàlisi d'aigua i begudes que permetin minimitzar la manipulació de les mostres i evitar d'aquesta manera la introducció de contaminacions i interferències.

Aquest treball s'ha **estructurat** en quatre apartats:

- Una *introducció* que inclou informació sobre el BPA, els diglicidil èters (BADGEs i BFDGEs), altres bisfenols i fotoiniciadors utilitzats en les tintes UV d'impressió dels *tetra briks*. En aquesta introducció es comenta la metodologia analítica utilitzada per analitzar aquests compostos emfatitzant especialment la part d'espectrometria de masses. S'inclou un treball de revisió bibliogràfia sobre els compostos bisfenòlics intitulat "*Recent advances in mass spectrometry analysis of phenolic endocrine disruptors and related compounds"* publicat a la revista *Mass Spectrometry Reviews* 2010, 29, 779-805.

- El capítol 2 està dedicat a l'estudi exhaustiu de la fragmentació dels compostos estudiats mitjançant l'espectrometria de masses en tàndem en dos analitzadors, un de trampa d'ions i l'altre de triple quadrupol. A més, s'inclouen les dades obtingudes en avaluar la utilització d'un instrument de triple quadrupol equipat amb quadrupols hiperbòlics per realitzar mesures de massa exacta. Els resultats es recullen en dos articles científics intitulats *"Liquid Chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives"* publicat a la revista *Rapid Communications in Mass Spectrometry 21 (2007) 4039-4048* i *"Multiple-stage mass spectrometry analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives"* publicat a la revista *Rapid Communications in Mass Spectrometry (In press)*.

- En el capítol 3 s'han posat a punt mètodes mitjançant l'acoblament de cromatografia de líquids ràpida amb l'espectrometria de masses en tàndem (LC-MS/MS) per a l'anàlisi dels diglicidil èters tant del bisfenol A com del bisfenol F, així com dels seus derivats hidrolitzats, a més de la determinació dels fotoiniciadors. La primera part del capítol es dedica a comentar els paràmetres cromatogràfics optimitzats. A la segona part es mostra l'acoblament LC-MS així com el mode de treball per tal d'obtenir una correcte anàlisis de les mostres. Aquests estudis es recullen en tres articles científics titulats: "*Fast liquid chromatography-tandem mass spectrometry*

for the analysis of bisphenol A- and bisphenol F-diglycidyl ether derivatives in canned food and soft-drinks" enviat a publicar a la revista *Journal of Chromatography A*, "*Liquid chromatography/tandem mass spectrometry (highly selective selected reaction monitoring) for the analysis of isopropylthioxanthone in packaged food"* publicat a *Journal of Chromatography A, 1208 (2008) 182-188* i *"Analysis of UV ink photoinitiators in packaged food by fast liquid chromatography at sub-ambient temperature coupled to tandem mass spectrometry"* enviat a publicar a la revista *Journal of Chromatography A.*

- A la primera part del capítol 4 es comenten els problemes i dificultats existents en l'anàlisi de bisfenol A (BPA) i que es recullen en un treball de revisió intitulat *"Pitfalls in the analysis of Bisphenol A: Sources and Solutions"* publicat al llibre *Bisphenol A and Phthalates: Uses, Health Effects and Environmental Risks* de la editorial *Nova Science Publishers, Inc 2010.* A la segona part del capítol es desenvolupa un mètode de preconcentració en línia amb cromatografia de líquids ràpida acoblada a l'espectrometria de masses en tàndem (SPE-LC-MS/MS) per a l'anàlisi de BPA i els seus derivats halogenats en mostres d'aigua que es recull a l'article intitulat *"On-line solid phase extraction fast liquid chromatography-tandem mass spectrometry for the analysis of Bisphenol A and its chlorinated derivatives in water samples"* publicat a la revista *Journal of Chromatography A, 1217 (2010) 3511-3518*. També s'inclou l'aplicació d'aquest mètode per analitzar BPA i d'altres bisfenols en begudes refrescants, article intitulat "*Analysis of bisphenols in soft-drinks by on-line solid phase extraction liquid chromatography-tandem mass spectrometry"* enviat a publicar a la revista *Analitica Chimica Acta*.

- Finalment s'inclouen les conclusions generals obtingudes en el treball realitzat en aquesta tesis, així com la bibliografia corresponent.

INTRODUCCIÓ

La presència de contaminants en el medi ambient i en els aliments és un tema d'important interès social que ha adquirit rellevància en els últims anys. Entre els compostos que han suscitat més interès cal citar els que poden interferir amb el sistema endocrí dels éssers vius i que es coneixen amb el nom d'alteradors endocrins (*endocrine disruptors,* EDs). El motiu de preocupació es deu a l'observació d'efectes adversos en els éssers vius com són l'augment en la incidència de certes malalties en humans i alteracions en el sistema reproductor d'animals. Entre aquests compostos hom pot citar entre d'altres alguns pesticides i herbicides, els productes de degradació dels alquilfenols polietoxilats, certs productes farmacèutics i plastificants. La majoria d'aquests contaminants no estan encara regulats però són considerats contaminants emergents candidats a formar part en el futur de les llistes de compostos legislats. La seva inclusió en aquestes llistes depèn dels resultats dels estudis toxicològics i del seu potencial efecte sobre la salut. D'entre els alteradors endocrins en aquesta memòria s'ha estudiat el Bisfenol A (BPA) compost àmpliament utilitzat a la industria plàstica. L'elevada producció i utilització d'aquest compost fa que es trobi en el medi ambient i de fet, ha estat un compost força estudiat en els darrers anys. Atès que avui dia aquest compost comença a ser substituït per altres bisfenols (BPs) d'estructures similars és important desenvolupar metodologia analítica per a l'anàlisi d'aquests nous compostos.

Un aspecte de la contaminació alimentària a la qual s'ha prestat relativament poca atenció és a la presència de contaminants que provenen de materials susceptibles d'entrar en contacte amb els aliments, com són els envasos, els utensilis de cuina, la maquinària emprada a la industria alimentària o els contenidors industrials. A la industria alimentaria s'utilitzen un seguit de materials com plàstics, cel·lulosa regenerada, paper i cartró (P&C), vidre, materials ceràmics, elastòmers, metalls, fusta, ceres, etc, i encara que aquests materials es formulen de manera que presentin unes propietats físico-químiques adequades per protegir els aliments de canvis ambientals, químics i físics, petites quantitats dels constituents d'aquests materials poden migrar als aliments degut al contacte directe. Exemples de compostos que poden migrar cap als aliments són el propi bisfenol A així com els altres bisfenols i compostos relacionats emprats en la síntesi de les resines epoxi utilitzades per exemple en els recobriments de les llaunes de conserva. A més de la migració de contaminants deguda al contacte directe entre

l'aliment i el material que els conté també es poden trobar als aliments altres components dels materials. Entre aquests compostos es poden citar els utilitzats en les tintes d'impressió dels empaquetaments. En aquesta memòria s'estudien els fotoiniciadors utilitzats en les tintes UV.

1.1. ELS COMPOSTOS ESTUDIATS

1.1.1. BISFENOL A I COMPOSTOS BISFENÒLICS

Els bisfenols (BPs) i entre ells el bisfenol A (2,2-bis-(4-hidroxifenil)propà, BPA) (Taula 1.1) són una família de compostos utilitzats en la síntesis de plàstics policarbonats i de resines epoxi, materials de gran aplicació industrial. El bisfenol A va ser sintetitzat per primera vegada l'any 1905 per Thomas Zincke mitjançant la reacció entre el fenol i l'acetona (Zincke, T., 1905). Zincke va determinar les propietats físiques del BPA, així com la composició molecular i la solubilitat en diferents solvents però no va proposar cap aplicació o ús del compost sintetitzat. No va ser fins l'any 1953 que el Dr. Hermann Schnell de Bayer a Alemanya i el Dr. Dan Fox de General Electric als Estat Units van desenvolupar de manera independent la fabricació d'un nou material, el policarbonat, emprant BPA com a compost de partida. El policarbonat l'estructura del qual es mostra a la Figura 1.1 és un material que presenta una sèrie de propietats com són transparència, durabilitat, seguretat, versatilitat i resistència al calor i a la ruptura que el fan adient per a moltes aplicacions, per exemple per a la fabricació de lents o de biberons. A més, també és àmpliament utilitzat en suports digitals, tals com CDs i DVDs, electrodomèstics, ampolles, equipament elèctric i electrònic, equipament mèdic i una infinitat d'aplicacions en la industria de la construcció i automobilística. La comercialització d'aquest material va començar el 1957 als EUA i l'any següent a Europa.

La seva àmplia utilització en molts dels materials que ens envolten, fa preveure que la producció mundial de BPA superarà els 5.5 milions de tones l'any 2011 (http://www.sriconsulting.com/CEH/Public/Reports/619.5000/). Avui dia el 70% del BPA produït mundialment és emprat per a la fabricació de policarbonat (www.bisphenol-a-europe.org i www.bisphenol-a.org) el qual s'obté per polimerització de BPA i fosgen en medi bàsic (Figura 1.1A). L'altre 30% de la

producció mundial és emprat per a la síntesis de resines epoxi que s'obtenen per condensació del BPA i la epiclorohidrina (Figura 1.1B). La primera síntesi d'aquestes resines va ser duta a terme per Pierre Castan i S.O. Greenlee a l'any 1936. El producte resultant d'aquesta reacció és el bisfenol A diglicidil èter (BADGE) el qual posteriorment és polimeritzat per tal d'obtenir les resines. Segons les condicions de reacció s'obtenen productes de diferent pes molecular. Convertir la resina epoxi en un producte insoluble i termostable requereix la reacció amb agents de curació entre els quals els més emprats són compostos aminats. Avui dia les resines epoxi són àmpliament emprades a la industria alimentaria en els recobriments de les llaunes de conserva per evitar el contacte directe entre l'aliment i la part metàl·lica de la llauna. Les propietats protectores contra la corrosió, l'estabilitat tèrmica i la resistència mecànica d'aquestes resines les fan també molt adequades per altres aplicacions industrials com per exemple en recobriments protectors en la industria automobilística i nàutica.

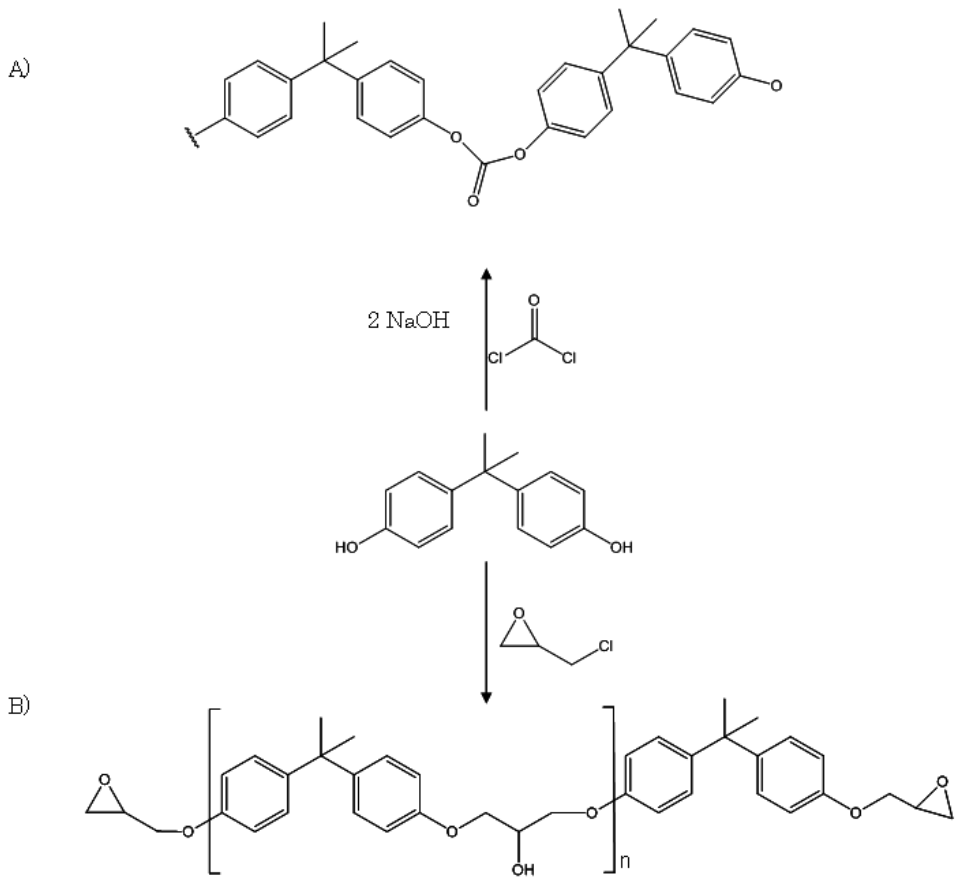
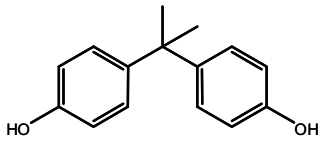
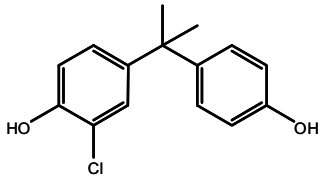
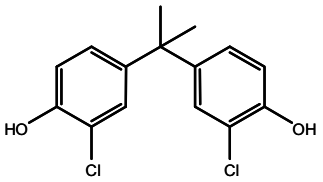
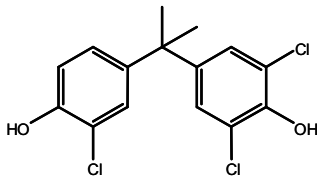
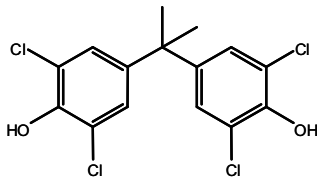
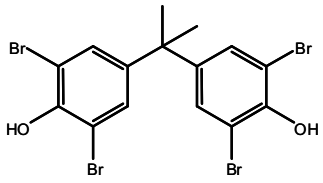
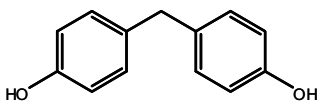
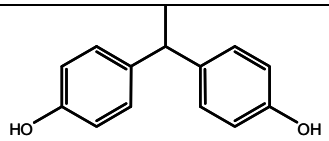
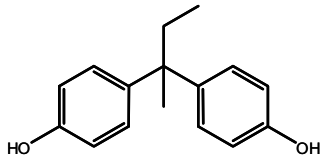
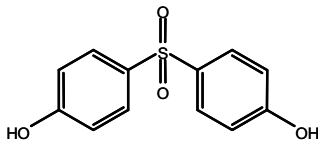


Figura 1.1. Síntesis de: A) Policarbonat i B) Resina epoxi.

Taula 1.1. Bisfenols, Número CAS i propietats físico-químiques

Compost	Número CAS	pK_a	Log P	Solubilitat
 Bisfenol A (BPA)	80-05-7	9.73	3,43	0.089 g/L
 Monoclorobisfenol A (MCBPA)	—	—	—	—
 Diclorobisfenol A (DCBPA)	79-98-1	7.02	4.54	0.02 g/L
 Triclorobisfenol A (TCBPA)	40346-55-2	6.84	5.11	7.6e-3 g/L
 Tetraclorobisfenol A (TeCBPA)	79-95-8	6.42	5.68	2.5e-3 g/L
 Tetrabromobisfenol A (TBBPA)	89-98-1	6.33	7.29	3.6e-3 g/L
 Bisfenol F (BPF)	2467-02-9	9.81	2.73	0.2 g/L

 Bisfenol E (BPE)	2081-08-5	9,77	3,08	0.12 g/L
 Bisfenol B (BPB)	77-40-7	9,71	3,96	0.053 g/L
 Bisfenol S (BPS)	80-09-1	7,80	1,83	0.5 g/L

Avui dia existeixen altres bisfenols que presenten similituds estructurals amb el BPA com són el bisfenol F (BPF), el bisfenol E (BPE), el bisfenol B (BPB) i el bisfenol S (BPS) (Taula 1.1) que s'empren per a substituir el BPA en algunes de les aplicacions industrials comentades anteriorment. La substitució del BPA pel BPF proporciona resines amb un major grau d'entrecreuament i en conseqüència un millor comportament mecànic, químic i tèrmic, sobretot si la curació de la resina té lloc amb amines aromàtiques o anhídrids. Per altra banda el BPS proporciona a les resines una excel·lent estabilitat a altes temperatures i resistència a la llum per la qual cosa en els darrers anys s'ha vist incrementada la seva producció. L'augment de la utilització d'aquestes noves resines comporta la introducció de BPs diferents del bisfenol A tant al medi ambient com als aliments.

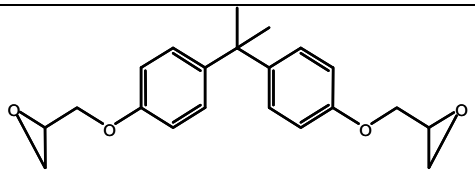
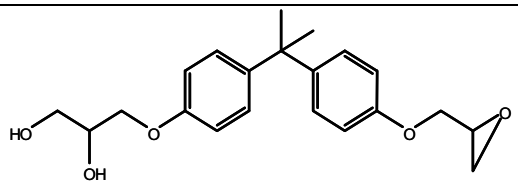
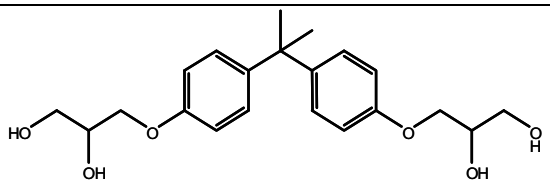
En aquest context cal indicar que també es troben al mercat resines epoxi bromades obtingudes per reacció del tetrabromobisfenol A (TBBPA) i epiclorohidrina les quals presenten característiques ignífugues gràcies a la presència dels quatre àtoms de brom. El TBBPA (Taula 1.1) és un dels retardants de flama bromats (BFRs) més àmpliament utilitzats (Bromine Science and Environmental Forum, BSEF, 2010). Aquest compost és produït als Estats Units,

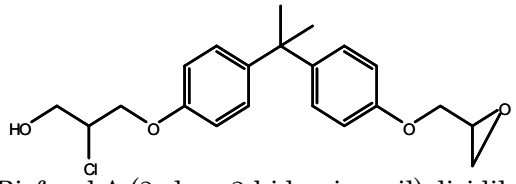
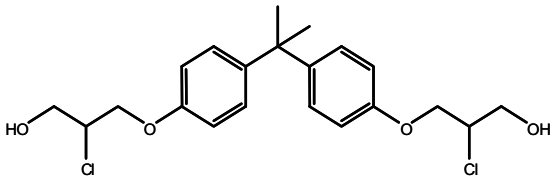
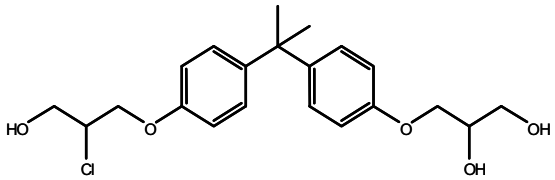
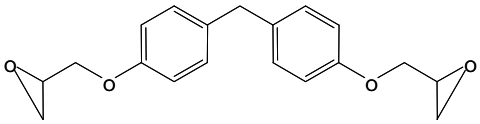
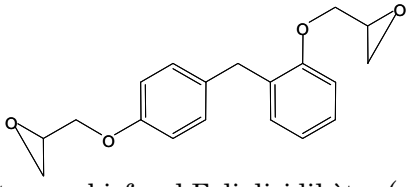
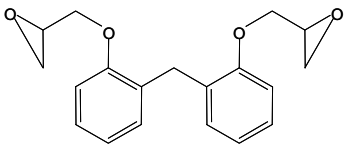
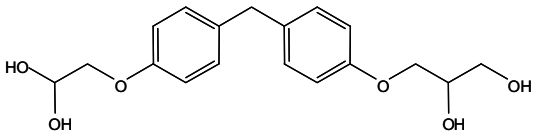
Israel i el Japó però no es produeix a la Unió Europea. El 58% del TBBPA es fa servir com a retardant de flama en les resines epoxi i fenòliques així com en els plàstics policarbonats emprats en circuits elèctrics i en aquests casos s'enllaça covalentment al polímer i passa a formar part integral del material. Un 21% és emprat en l'obtenció de derivats de TBBPA i oligòmers mentre que l'altre 21% s'utilitza com additiu en la fabricació de resines d'acrilonitril-butadiè-estirè (ABS) o en el poliestirè d'alt impacte (HIPS). El mecanisme d'inhibició de la combustió és el mateix independentment de la naturalesa del retardant de flama utilitzat i consisteix essencialment en la seva descomposició abans que la matriu del polímer lo que permet inhibir el procés de combustió. En el cas del TBBPA l'elevada proporció d'àtoms de brom és la responsable de l'activitat inhibidora de la combustió, ja que el brom interfereix en el mecanisme radicalari que té lloc a la fase gas durant la combustió. A Europa el TBBPA s'empra com alternativa a l'octabromodifenilèter (OctaBDE) ja que la UE no permet la utilització d'aquest últim compost. Quan el TBBPA és utilitzat com additiu aquest no reacciona químicament amb els components del polímer, i per tant, pot lixiviar del polímer després de la seva incorporació i ser introduït al medi ambient. Mentre que quan es enllaçat químicament al polímer la seva introducció al medi ambient tan sols pot venir donada pel TBBPA que queda sense reaccionar durant el procés de producció. Un altre derivat halogenat del BPA el tetraclorobisfenol A (TeCBPA) (Taula 1.1), és també emprat com a retardant de flama en les mateixes aplicacions que el TBBPA però en molt menor proporció.

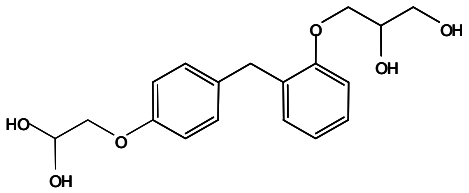
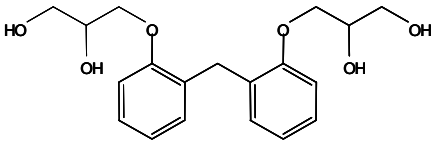
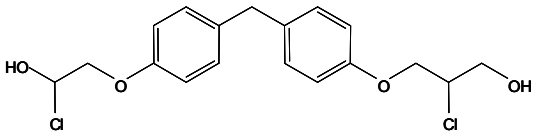
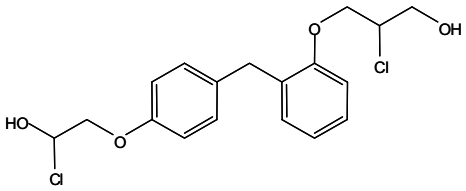
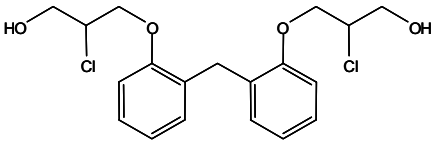
Una de les aplicacions importants del BPA així com dels compostos relacionats amb ell, els altres bisfenols i els derivats diglicidil èters, és a la industria alimentària. Per exemple, el plàstic policarbonat es emprat en recipients per contenir aliments com ampolles i biberons mentre que les resines epoxi s'utilitzen en els revestiments de les llaunes de conserva per tal d'evitar el contacte dels aliments amb la part metàl·lica. En conseqüència és possible la migració d'aquests compostos cap als aliments o begudes amb els que es troben en contacte. A més aquests compostos en contacte amb els aliments poden reaccionar donant lloc a la formació d'altres compostos els quals també són considerats com a no desitjables. Per exemple, tan el BADGE com el BFDGE presenten dos grups epòxid a la seva estructura (Taula 1.2) que poden reaccionar amb la matriu donant lloc als derivats hidrolitzats, BADGE·H_2O i BADGE·$2H_2O$ pel BADGE i BFDGE·H_2O i

BFDGE·2H_2O pel BFDGE. A més, la presència de clorur de sodi i les condicions lleugerament àcides de la matriu afavoreixen les reaccions de hidrocloració donant lloc a la formació de BADGE·HCl, BADGE·2HCl i BFDGE·2HCl. Aquests compostos també es poden trobar en els polímers degut a la reacció del BADGE i/o del BFDGE amb el HCl excedent durant la síntesis dels organosols vinílics (PVC). A més dels derivats esmentats, a les mostres d'aliments també s'hi poden trobar adductes del BADGE i del BFDGE amb els components majoritaris de la matriu. Aquesta tendència a interaccionar amb la matriu ha estat estudiada per diversos autors que han observant una elevada reactivitat del BADGE amb els amino àcids de les proteïnes, especialment la metionina i la cisteïna (Richard i cols. 1999, Petersen i cols. 2008 i Coulier i cols., 2010).

Taula 1.2. Bisfenol A- i bisfenol F-diglicidil èters, Número CAS i propietats físico-químiques.

Compost	Número CAS	pK_a	Log P	Solubilitat
 Bisfenol A diglicidil èter (BADGE)	1675-54-3	—	3.95	4.8e-3 g/L
 Bisfenol A (2,3-dihidroxipropil)glicidil èter (BADGE·H_2O)	76002-91-0	13.46	2.96	0.035 g/L
 Bisfenol A bis(2,3-dihidroxipropil)glicidil èter (BADGE·2H_2O)	5581-32-8	13.23	1.86	0.18 g/L

 Bisfenol A (3-cloro-2-hidroxipropil)glicidil èter (BADGE·HCl)	13836-48-1	13.13	3.98	9.0e-3 g/L
 Bisfenol A bis(3-cloro-2-hidroxipropil) glicidil èter (BADGE·2HCl)	4809-35-2	12.82	4.01	9.9e-3 g/L
 Bisfenol A (3-cloro-2-hidroxipropil)(2,3-dihidroxipropil)glicidil èter (BADGE·HCl·H_2O)	227947-06-0	13.13	2.98	0.04e-3 g/L
 *para,para*-bisfenol F diglicidil èter (*p,p*-BFDGE)	2095-03-6	—	3.26	4.8e-3 g/L
 *orto,para*-bisfenol F diglicidil èter (*o,p*-BFDGE)	—	—	—	—
 *orto,orto*-bisfenol F diglicidil èter (*o,o*-BFDGE)	—	—	—	—
 *para,para*-bisfenol F bis(2,3-dihidroxipropil)glicidil èter (*p,p*-BFDGE·2H_2O)	72406-26-9	—	—	—

 *orto,para*-bisfenol F bis(2,3-dihidroxipropil)glicidil èter (*o,p*-BFDGE·2H_2O)	—	—	—	—
 *orto,orto*-bisfenol F bis(2,3-dihidroxipropil)glicidil èter (*o,o*-BFDGE·2H_2O)	—	—	—	—
 *para,para*-bisfenol F bis(3-chloro-2-dihidroxipropil)glicidil èter (*p,p*-BFDGE·2HCl)	235741-59-0	12.82	3.31	0.023 g/L
 *orto,para*-bisfenol F bis(3-chloro-2-dihidroxipropil)glicidil èter (*o,p*-BFDGE·2HCl)	338974-97-3	12.81	3.31	0.024 g/L
 *orto,orto*-bisfenol F bis(3-chloro-2-dihidroxipropil)glicidil èter (*o,o*-BFDGE·2HCl)	338974-98-4	12.81	3.31	0.026 g/L

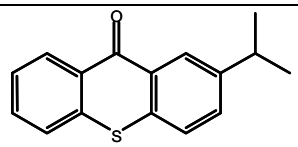
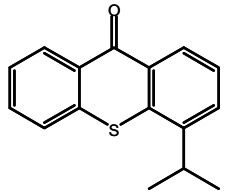
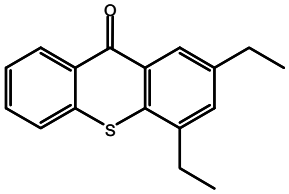
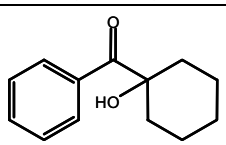
1.1.2. FOTOINICIADORS

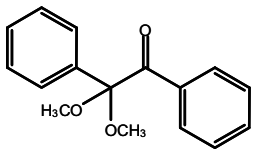
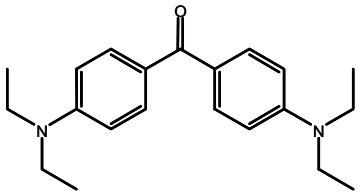
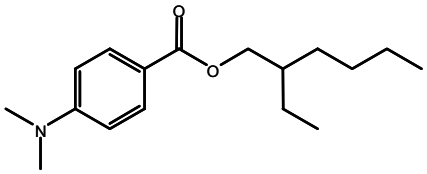
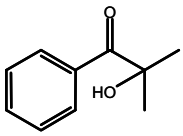
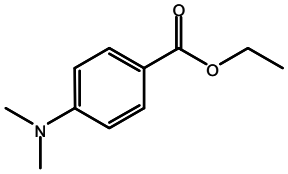
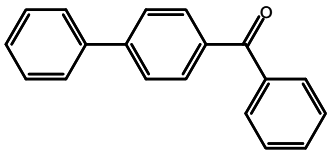
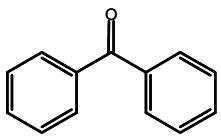
En aquesta memòria també s'han estudiat diferents fotoiniciadors que s'addicionen a les tintes utilitzades en materials d'empaquetatge d'aliments com el cartró i el plàstic. Les tintes utilitzades tradicionalment en els materials emprats per empaquetar aliments es curen tèrmicament. Actualment i degut a l'elevada demanda industrial d'aquests productes es fan servir cada vegada més processos de producció curts i ràpids utilitzant tintes-UV la curació de les quals té lloc mitjançant processos de radiació que aprofiten els grups fotosensibles d'aquests compostos. Ara bé, els fotoiniciadors han de ser eliminats de forma efectiva després del procés d'impressió per evitar que es trobin en els aliments envasats. Avui dia existeix una àmplia quantitat de compostos emprats com a fotoiniciadors en aquestes tintes, entre els quals cal citar la benzofenona (BP) (Taula 1.3) que és un dels més utilitzats degut a la seva efectivitat i preu. Altres fotoiniciadors emprats en els processos industrials de fixació de les tintes s'indiquen a la Taula 1.3 on es pot observar que en general, es tracta de compostos orgànics que presenten diferents grups funcionals i al menys un anell aromàtic a la seva estructura. Segons els grups funcionals els compostos es poden agrupar en quatre grans grups, els que tenen un grup tioxantè, les fenones, les amines i les fenil cetones. Generalment les tintes i laques utilitzades contenen entre un 5 i un 10% de fotoiniciador.

L'interès per l'anàlisi dels fotoiniciadors en aliments va sorgir l'any 2005 quan les autoritats italianes de control dels aliments van detectar la presència de 2-isopropil-9-H-tioxanté-9-ona (2-ITX), compost emprat com a fotoiniciador de les tintes UV en llets infantils (Morlock i Schwach 2006, Sun i cols., 2007) (Taula 1.3). Com a resultat de la detecció de l'ITX es van haver de retirar del mercat més de 30 milions de litres de llet a França, Itàlia, Espanya i Portugal al novembre de 2005 (Nestlé Company 2005). Aquest episodi va portar a que l'Autoritat Europea de Seguretat Alimentaria (EFSA) advertís de la presència d'aquest compost i es decidís que s'havia d'estudiar la seva toxicitat i el risc per a la població. A més del 2-ITX, també s'han detectat residus d'altres fotoionitzadors com el 2-etilhexil-4-dimetilaminobenzoat (EHDAB) en aliments com sucs de fruites i llet (Gil-Vergara i cols., 2007, Sagratini i cols., 2008 i Shen i cols., 2009). La contaminació dels aliments amb aquests compostos prové del contacte entre les cares internes i

externes del material d'empaquetatge durant el procés de fabricació de les planxes i bobines. La cara externa del material tractada amb el fotoiniciador contamina la cara interna la qual es trobarà posteriorment en contacte amb l'aliment (Sagratini i cols., 2006). A més, si no es fan servir materials impermeables en les capes internes dels materials (envasos multicapes) aquests contaminants també poden migrar de forma directa cap als aliments. Aquesta última via és poc probable ja que avui dia la majoria d'envasos emprats a la industria alimentària utilitzen sistemes de protecció multicapes. Cal dir també que aquests compostos poden trobar-se en els aliments si l'envàs prové de reciclatge de paper i cartró en el cas que el procés de reciclatge no s'hagin eliminat completament.

Taula 1.3. Fotoiniciadors, Número CAS i propietats físico químiques.

Compost	Número CAS	pK_a	Log P	Solubilitat
 2-isopropil-9-H-tioxanté-9-ona (2-ITX)	5495-84-1	—	5.33	5.1e-4 g/L
 4-isopropil-9-H-tioxanté-9-ona (4-ITX)	83846-86-0	—	5.33	5.1e-4 g/L
 2,4-dietil-9-H-tioxanté-9-ona (DETX)	82799-44-8	—	5.97	2.4e-4 g/L
 1-hidroxifenilciclohexil fenil cetona (HCPK)	947-19-3	13,22	2.34	0.49 g/L

 2,2-dimetoxi-2-fenilacetofenona (DMPA)	24650	—	4.76	0.062 g/L
 4,4'-bis(dietilamino)-benzofenona (DEAB)	90-93-7	4,14	5.99	9.1e-4 g/L
 2-etilhexil-4-(dimetilamino)benzoat (EHDAB)	21245-02-3	2,39	6.15	2.1e-3 g/L
 2-hidroxi-2-metilpropiofenona (HMPP)	7473-98-5	13,22	1.14	6.4 g/L
 Etil 4-dimetilaminobenzoat (EDMAB)	10287-53-3	2,56	3.14	0.21 g/L
 4-benzoilfenil (PBZ)	2128-93-0	—	4.83	4.9e-3 g/L
 Benzofenona (BP)	119-61-9	—	3.18	0.13 g/L

1.2. TOXICITAT I LEGISLACIÓ

La toxicitat del BPA ha estat estudiada extensament durant les últimes dècades. Aquests estudis han posat de manifest una baixa toxicitat i han permès confirmar la dèbil estrogenicitat del BPA in vitro (Gaido i cols., 1997). Ara bé, recentment diversos autors coincideixen en indicar que l'exposició al BPA fins i tot a dosis extremadament baixes pot provocar efectes adversos sobre la salut dels humans, incloent alteracions en el funcionament hormonal normal. En aquesta línia alguns autors han avaluat l'estrogenicitat del BPA in vitro (Kim i cols., 2001; Matthews i cols., 2001) demostrant que pot interaccionar amb els receptors α i β-estròg ens (Kuiper i cols., 1997). Pel BPA aquesta "teoria de dosis baixes" ha estat exhaustivament estudiada per la industria, per organismes governamentals i per centres de recerca i sembla que no hi ha evidencies d'efectes adversos a concentracions baixes. Tot i això, Vom Saal i Welshons l'any 2006 van publicar una revisió bibliogràfica en la que exposen que a la literatura científica existeixen més de 100 publicacions en les quals es posa de manifest que el BPA a dosis baixes (< 50 μg/kg/dia) presenta efectes adversos i aquest és el nivell que estableix la US-FDA i la US-EPA com a nivell de seguretat. De fet, cada vegada hi ha més publicacions on es mostren efectes del BPA sobre la salut a nivells de concentració molt inferiors al que estableix la EPA com a "dosis sense efecte" (en animals 5 mg/kg/dia i en humans 0.05 mg/kg/dia). Pel que fa al BPF, aquest també presenta activitat com a disruptor endocrí i genotoxicitat (Yamasaki i col., 2002; Cabaton i col., 2009). Un aspecte important de la toxicitat del BPA és que hi ha algunes dades que indiquen que a dosis extremadament baixes, de poques ppt, causa proliferació de les cèl·lules cancerígenes prostàtiques (Wetherill i cols., 2002). Cal dir però que en un treball recent Goodman i cols., 2009 després de fer una revisió dels efectes del BPA a dosis baixes, conclouen que existeix una elevada controvèrsia entre els diferents treballs i que per tant el tema encara està obert.

Respecte als derivats clorats del BPA, s'han fet alguns estudis per tal d'avaluar la seva activitat estrogènica i en general s'ha vist que presenten una activitat estrogènica major que la del propi BPA (Fukazawa i cols., 2001; Kim i cols., 2001; Matthews i cols., 2001; Kuruto-Niwa i cols., 2002; Takemura i cols., 2005). Una comparació dels valors de EC50 del BPA i els seus derivats clorats posa de manifest que els derivats clorats presenten una activitat estrogènica superior a la del BPA excepte el TeCBPA. El MCBPA i els dos isòmers del DCBPA són els que presenten els màxims efectes

estrogènics a concentracions baixes. El BPA generalment es metabolitza en el fetge per donar majoritàriament el monoglucurònid que és excretat per l'orina (Pottenger i cols., 2000), en canvi els compostos organoclorats es degraden poc i s'acumulen a través de la cadena alimentaria. Aquest fet fa pensar que els derivats clorats del BPA podrien tenir un patró de bioacumulació diferent al BPA i per tant una exposició crònica als derivats clorats del BPA a baixa concentració podria causar un efecte major que el propi BPA.

Pel que fa referència al TBBPA es disposa de poques dades sobre la seva toxicitat, però estudis in vitro indiquen que no és irritant i no provoca reacció dèrmica en animals encara que podria tenir alguns efectes adversos en el sistema immunològic i hormonal. El LD-50 en rates, ratolins i conills per via oral és inferior a 5-10 g/Kg, i l' obtingut per a conills per via dèrmica és inferior a 2 g/Kg (Environmental Health Criteria 1995), la qual cosa indica una relativa baixa toxicitat. Per altra banda, alguns estudis indiquen que el TBBPA pot afectar al sistema immunològic causant una reducció de les defenses contra infeccions i tumors (Pullen i cols., 2003). A més, en estudis in vitro s'ha observat que el TBBPA competeix fortament amb l'hormona tiroidea (tiroxina o T4), per enllaçar-se amb la proteïna d'enllaç en el sèrum (transtiretina) degut a la semblança estructural entre en TBBPA i les hormones T3 i T4 (Figura 1.2) encara que estudis *in vivo* indiquen que aquest efecte sembla ser relativament baix (Meerts i cols., 1999).

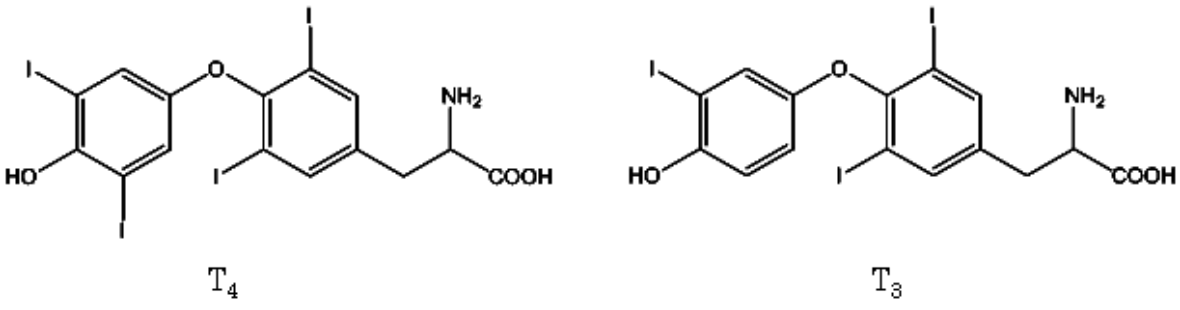


Figura 1.2. Estructura de les hormones tiroideas 3,3',5,5'-tetraiodo-L-tironina (tiroxina o T4) i la congènere 5'-desiodonitzada 3,3',5-triiodotironina (T3).

Per tal d'avaluar si els diglicidil èters tant del BPA com de BPF (BADGE i BFDGE) presenten activitat estrogènica s'han dut a terme estudis toxicològics en els quals s'ha posat de manifest que la presència de l'enllaç èter fa perdre la seva activitat estrogènica (Nakazawa i cols., 2002). De totes maneres molts dels estudis toxicològics duts a terme per a aquests compostos conclouen que fan falta més

estudis sobre la seva activitat estrogènica. Per altra banda Satoh i col., 2004 van observar que els derivats hidroclorats el BADGE·2HCl i el BFDGE·2HCl, presenten activitat en els andròg ens i que el BADGE i el BFDGE indueixen la mort de les cèl·lules o apoptosi que és una forma de mort cel·lular regulada genèticament. A més, tant els BADGEs com els BFDGEs presenten efectes citotòxics, genotòxics i mutagènics (Suarez i cols., 2000; Sueiro i cols., 2006, 2003 & 2000; Cabaton i cols., 2009).

Tot i els innumerables estudis que s'han realitzat que demostren tant la presència de EDCs en el medi ambient com el perill per a la salut que pot suposar la seva exposició, cap estat de la UE ha establert una legislació específica que reguli la presència d'aquest tipus de substàncies en els diferents compartiments ambientals, encara que en alguns països s'han creat comitès d'experts per a estudiar aquest tema. La Comissió Europea ha posat en marxa una Estratègia Comunitària en matèria d'alteradors endocrins i l'EPA ha desenvolupat un Programa d'Investigació pels alteradors endocrins (EDSP-Endocrine Disruptor Screening Program). Una de les iniciatives que s'ha portat a terme en aquest context, ha estat l'elaboració de llistes de substàncies de les quals es sospita que interfereixen en el sistema hormonal i entre aquestes substàncies es troba el BPA. Per als altres compostos bisfenólics estudiats en aquesta tesis únicament el BPB ha estat classificat per la UE com a substància amb evidència o potencial evidència d'alterar el sistema endocrí. Per als derivats clorats del BPA (MCBPA, DCBPA, TCBPA i TeCBPA) tampoc existeix avui dia cap regulació dels nivells en el medi ambient. El TBBPA apareix a la llista de substàncies químiques prioritàries (EU Regulation No. 793/93) de la Unió Europea encara que actualment no existeixen restriccions en la seva producció ni en la dels seus derivats. L'any 2003 la Directiva Europea sobre el tractament de residus d'equips elèctrics i electrònics (WEE) va establir la necessitat de dur a terme un tractament especial dels plàstics que contenen retardants de flama bromats (BFRs). Per altra banda la UE ha avaluat els riscos d'aquest compost concloent que no s'han identificat riscos sobre la salut humana quan s'utilitza com a retardant de flama, com és el cas de les resines epoxi emprades en els circuits elèctrics. En canvi, les valoracions de riscs medi ambientals a la UE confirmen que en alguns escenaris com aigües superficials, sediments i sòls existeixen riscs quan el TBBPA s'utilitza com additiu en els plàstics ABS (acrilonitril-butadiè-estirè). L'avaluació de riscs posa de manifest

també que existeix risc si els fangs que contenen TBBPA són utilitzats en l'agricultura. Aquest informe també conclou que es necessiten més estudis i/o informació al respecte ja que és possible la degradació del TBBPA a BPA en el tractament de fangs en condicions aeròbiques, així com en condicions anaeròbiques en els sediments d'aigua dolça i marina, encara que actualment, el TBBPA no es troba a la llista de la Directiva Europea de qualitat de l'aigua. Als Estats Units la legislació s'ha focalitzat en les restriccions dels difenil èters polibromats (PBDEs) i s'ha prestat poca atenció al TBBPA. En canvi, en altres països com Canada i Austràlia aquest compost ha estat inclòs a les llistes de compostos prioritaris per tal de ésser avaluat en un futur.

La nova política Europea de substàncies i preparats químics, denominada REACH (**R**egistration, **E**valuation, **A**uthorisation and Restriction of **Ch**emical substances) va entrar en vigor l'1 de Juny del 2007. Un dels principals objectius del REACH és assegurar que hi hagi suficient informació disponible per a poder avaluar els riscos potencials de les substàncies químiques. Els polímers com el policarbonat i les resines epoxi estan exemptes d'aquest procés de registre, però en canvi el BPA que és la unitat monomèrica a partir de la qual es sintetitzen aquests polímers si que ha d'estar registrada. La data de registre de cadascun dels compostos químics depèn del tonelatge de fabricació o importació. Així, el BPA degut al seu elevat tonelatge de fabricació ha d'estar en registre abans del dia 1 de Desembre del 2010. El TBBPA degut al seu elevat volum de producció també ha de ser registrada.

Pel que fa a la legislació en matèria alimentaria si que s'especifiquen límits de migració en els aliments d'alguns dels compostos estudiats en aquesta tesis. Al Gener del 2007, l'Autoritat Europea de Seguretat Alimentaria (EFSA) va emetre la seva opinió sobre la seguretat del BPA en aplicacions que poden entrar en contacte amb els aliments. Després de realitzar uns 200 estudis es va establir la ingesta diària tolerable (TDI) en 50 $\mu g\ kg^{-1}$ de pes corporal al dia. Aquest valor representa la quantitat de BPA que un consumidor, incloent nadons i nens, pot ingerir amb seguretat sense que s'observin efectes. Els nivells típics de migració de BPA en materials en contacte amb aliments normalment son inferiors a 10 $\mu g\ kg^{-1}$ valor que es troba molt per sota de la concentració de migració específica establerta per al BPA en aliments. Cal dir però que al mes de Març de 2010 la EFSA ha rebut una petició urgent per par de la Comissió per tal de que es dugui a terme una revisió

dels arguments científics sobre el BPA per tal de prohibir el seu ús en materials que es troben en contacte amb aliments per infants (de 0 a 3 anys) basant-se en els estudis realitzats per Stump i cols., 2010 que posen de manifest la posibilitat de que aquest compost presenti efectes perjudicials per al desenvolupament neuronal (http://www.efsa.europa.eu/en/ceftopics/topics/bisphenol.htm).

Per al BADGE la EFSA ha establert un TDI de 150 μg kg^{-1} de pes corporal al dia. Degut a que el BADGE és ràpidament i extensament metabolitzat *in vivo* donant el derivats hidrolitzats, BADGE·H_2O i BADGE·$2H_2O$, les autoritats europees van incloure aquests compostos en el valor de TDI recomanat. Pe que fa al límit de migració específica la Directiva 2004/19/CE estableix un valor de 150 μg kg^{-1} mentre que per als derivats hidro-clorats, BADGE·HCl, BADGE·2HCl i BADGE·HCl·H2O, degut a la baixa genotoxicitat in vivo el valor és de 1000 μg kg^{-1}. Per al BFDGE i els seus derivats la UE desaconsella el seu ús degut a les insuficients dades toxicològiques de què es disposen avui dia (Directiva 2002/16/CE).

Pel que fa referència als fotioniciadors, la Unió Europea (EU) encara no ha establert ni definit una legislació específica per aquesta família de compostos en els materials emprats en l'empaquetatge d'aliments. Únicament la benzofenona te fixat un límit de migració específic (SML) de 600 μg kg^{-1} d'acord amb la directiva 2002/72/EC. Ara bé, la Comissió Europea (EC) en la regulació No. 1935/2004 referent a materials i articles susceptibles d'entrar en contacte amb els aliments estableix que no han de provocar efectes adversos sobre la salut dels consumidors, produir canvis en la composició dels aliments, ni alterar les propietats organolèptiques d'aquests. Per altra banda la Regulació de la Comissió Europea (EC) 2023/2006 estableix que els materials susceptibles d'entrar en contacte amb aliments no poden modificar les propietats físiques i químiques d'aquests. Tot i que la presència de 2-ITX en els aliments no és desitjable, el Grup Científic que treballa sobre Additius Alimentaris, Aromes i Materials en Contacte amb els Aliments de l'EFSA va determinar que el 2-ITX i el 2-etil 4-(dimetilamino)benzoat (EHDAB) no suposen un problema de salut en les quantitats considerades en l'avaluació de riscos. Cal dir però que s'han de continuar estudiant per tal de disposar de més dades toxicològiques.

1.3. PRESÈNCIA EN ELS ALIMENTS I EL MEDI AMBIENT

1.3.1. ALIMENTS

Tal i com hem comentat anteriorment el BPA és utilitzat a la industria alimentaria tant en els recobriments de llaunes de conserva com per a l'obtenció de plàstics policarbonats. Degut a la seva àmplia utilització és més que probable trobar aquests compost en els aliments i de fet a la literatura existeixen moltes dades sobre nivells en diferents tipus d'aliments, com per exemple en aliments enllaunats, com verdures, fruites, peix i carn, begudes refrescants i aliments per a infants. Recentment Ballesteros-Gómez i cols., 2009 han publicat un treball de revisió sobre l'anàlisi d'aquest compost en mostres d'aliments. A la Taula 1.4 s'inclouen les concentracions de BPA, extretes d'aquest treball de revisió complementada amb dades de nivells de concentració publicades amb posterioritat a l'esmenta't treball de revisió. El BPA es troba generalment a concentracions de μg/kg o μg/L (ppb). Especial interès presenten les mostres d'aliments per a infants tant enllaunats en els quals s'han trobat concentracions entre 0.48 i 11.0 ng g^{-1} (Ackerman i col., 2010; Cao i col., 2008), com continguts en pots de vidre amb tapes metàl·liques en les quals les concentracions són força similars, 0.27 - 7.2 ng g^{-1} (Cao i col., 2009a).

Pel que fa referència als altres bisfenols (BPs) estudiats en aquesta tesis, BPF, BPE, BPB i BPS, a la literatura existeixen molt poques dades. El bisfenol S (BPS) ha estat analitzat recentment en mostres d'aliments enllaunats de base aquosa com vegetals i fruites (Taula 1.4) trobant-se a concentracions de 11.5 – 175 ng g^{-1} (Viñas i col., 2010). En aquest treball també es va analitzar el BPA que es va trobar a les mateixes mostres a concentracions més elevades, entre 11.7 – 317 ng g^{-1}. Un altre bisfenol que també ha estat analitzat en mostres d'aliments és el bisfenol B (BPB) el qual s'ha trobat en mostres de tomàquet enllaunat a concentracions entre 27 μg kg^{-1} i 86 μg kg^{-1} (Grumetto i col., 2008) del mateix ordre que les del BPA (21 – 115 μg kg^{-1}). Convé comentar que alguns autors han dut a terme l'anàlisi simultània de BPA i BPF en mostres d'aliments enllaunats i en cap ha estat detectat el BPF (Goodson i cols., 2002 i Inoue i cols., 2003a).

Pel que fa referència al BADGE i al BFDGE utilitzats com ja s'ha comentat en els recobriments de les llaunes de conserva, els resultats de les dades de la literatura posen de manifest (Taula 1.4) que és freqüent trobar BADGE i alguns

dels derivats hidrolitzats com el BADGE·2H_2O, BADGE·2HCl i BADGE·HCl·H_2O a les mostres. Ara bé, cal indicar que últimament s'ha detectat una disminució dels nivells de concentració d'aquests compostos a les mostres d'aliments enllaunats les quals han passat dels mg/kg abans de l'any 2002 als μg/kg d'avui dia. Probablement això és degut a les millores produïdes tant en el procés de síntesis de les resines epoxi com en la fixació d'aquestes resines a les llaunes de conserva que fa que el BADGE i el BFDGE no migrin amb tanta facilitat cap als aliments. Els compostos detectats amb més freqüència d'aquesta família són el BADGE, el BADGE·2H_2O, BADGE·HCl·H_2O i el BADGE·2HCl. Per exemple, en un treball recent publicat per Yonekubo i cols., 2008 en el que es va realitzar l'anàlisi de 38 mostres d'aliments enllaunats entre els que hi havia mostres de verdures, peix, diferents salses i altres mostres com llet de coco i ous es va observar que els compostos detectats en un major nombre de mostres van ser el BADGE·HCl·H_2O (100% de les mostres), BADGE·2H_2O (92% de les mostres), mentre que el BADGE va ser detectat en el 68% de les mostres, el BADGE·2HCl en el 58% i els altres derivats hidrolitzats en menys del 20%. També cal destacar que sobre el BFDGE i dels seus derivats hidrolitzats hi ha molta menys informació a la literatura, de fet hi ha dades a finals dels anys 90 i principis del 2000 i després no hi ha informació fins molt recentment que en un treball de Zhang i cols., 2010 s'indica que aquests compost ha estat detectat en dos mostres de peix enllaunat una de tonyina i l'altre de la família dels ciprínids, a concentracions de 40.57 ng/g i 77.64 ng/g, respectivament.

Pel que fa als fotoiniciadors ja hem comentat anteriorment que han estat trobats a partir de l'any 2005 quan es va començar a controlar i analitzar la presència de ITX i altres fotoiniciadors en mostres d'aliments empaquetats. A la Taula 1.4 es recullen les publicacions científiques que fan referència a l'anàlisi d'aquesta família de compostos en mostres d'aliments. D'entre els fotoinitzadors estudiats en aquesta tesis el ITX és el més analitzat en mostres com sucs de fruita, llet i altres begudes envasades en cartró. La majoria de les dades sobre nivells de ITX fan referència a la suma de concentracions dels dos possibles isòmers (2- i 4-ITX) atès que la seva separació no és fàcil. Tan sols Bagnati i col., (2007) han desenvolupat un mètode per analitzar els dos isòmers per separat la qual cosa els va permetre posar de manifest la presència predominant del 2-ITX en mostres de llet a concentracions entre 173 μg L^{-1} i 439 μg L^{-1} mentre que l'altre isòmer, el 4-

ITX, hi és present en molt menor concentració (< 6 μg L^{-1} – 25 μg L^{-1}). La benzofenona (BP) per altra banda ha estat àmpliament analitzada i s'ha trobat en mostres de llet a concentracions entre 2.84 – 217 μg kg^{-1} (Sagratini i cols., 2008; Shen i cols., 2009). Dels altres fotoiniciadors tractats en aquesta tesis només la 1-hidroxifenilciclohexilfenilcetona (HCPK) i el 4-(dimetilamino)benzoat de 2-etilhexil (EHDAB) han estat detectats a concentracions de 0.1 – 0.8 μg L^{-1} i a 1.2 μg L^{-1}, respectivament en mostres d'aliments empaquetats (Sagratini i cols., 2008; Shen i cols., 2009). Els fotoiniciadors també han estat analitzats en els envasos on les concentracions de ITX, BP,EHDA i HCPK són de 0.01 – 1.5 μg dm^{-2}, 0.2 – 387 μg dm^{-2}, 0.01 – 3.8 μg dm^{-2} i 0.13 – 0.5 μg dm^{-2}, respectivament (Sagratini i cols., 2008; Shen i cols., 2009).

Taula 1.4. Anàlisis de BPs, BADGEs, BFDGEs i fotoiniciadors en mostres d'aliments.

	Compostos	**Nivells**	**Ref.**
Bisfenol A i compostos bisfenòlics:aliments enllaunats			
Fruites i verdures			
	BPA	5 — 317 ng/g	Ballesteros-Gómez i cols., 2009; Viñas i cols., 2010
	BPB	27.1 — 85.7 ng/g	Grumetto i cols., 2008
	BPS	11.5 — 175 ng/g	Viñas i cols., 2010
	BADGE	0.1 — 106.4 ng/g	Yonekubo i cols., 2009
	BADGE·HCl	1.3 ng/g	
	BADGE·$2H_2O$	1.2 — 860 ng/g	Yonekubo i cols., 2008; Berger i cols., 2001
	BADGE·HCl·H_2O	0.8 — 480 ng/g	
	BADGE·2HCl	0.8 — 140 ng/g	
	BFDGE·$2H_2O$	n.d. — 420 ng/g	Berger i cols., 2001
	BFDGE·2HCl	0.15 — 0.70 ng/g	
Peix			
	BPA	2.1 — 109 ng/g	Ballesteros-Gómez i cols., 2009
	BADGE	0.1 — 11800 ng/g	Zhang i cols., 2010; Yonekubo i cols., 2009; Simoneau i cols., 1999
	BADGE·$2H_2O$	0.6 — 142 ng/g	Yonekubo i cols., 2008
	BADGE·HCl·H_2O	0.2 — 133.8 ng/g	Yonekubo i cols., 2008; Zhang i cols., 2010
	BADGE·2HCl	1.2 — 155.2 ng/g	
	BADGE·HCl	0.3 — 68.8 ng/g	
	BFDGE	20 — 4200 ng/g	Zhang i cols., 2010; Theobald i cols., 2000; Biedermann i cols., 1998
	BFDGE·$2H_2O$	n.d. — 1060 ng/g	Berger i cols., 2001
	BFDGE·2HCl	1120 ng/g	Lintschinger i cols., 2000
Carn			
	BPA	9.6 — 98 ng/g	Ballesteros-Gómez i cols., 2009
	BADGE	25 — 113 ng/g	Petersen i cols., 2003; Zhang i cols., 2010
	BADGE·HCl·H_2O	20.47 — 1085 ng/g	
	BADGE·HCl	74.42 — 477 ng/g	
	BADGE·$2H_2O$	458 — 590 ng/g	Petersen i cols., 2003
	BADGE·2HCl	476 — 751 ng/g	

Aliments infantils			
	BPA	0.48 — 11.0 ng/g	Cao i cols., 2009a; Ackerman i cols., 2010
	BPA (pots de vidre amb tapa metàl·lica)	0.27 — 7.2 ng/g	Cao i cols., 2009b
Begudes refrescants			
	BPA	0.032 — 4.5 ng/mL	Ballesteros-Gómez i cols., 2009; Cao i cols., 2009a;
Salses			
	BPA	0.9 — 235.4 ng/g	Ballesteros-Gómez i cols., 2009
	BADGE	0.1 — 3.4 ng/g	Yonekubo i cols., 2008
	BADGE·2H_2O	1.2 — 106.4 ng/g	
	BADGE·HCl·H_2O	0.8 — 28.2 ng/g	
	BADGE·2HCl	0.8 — 13.7 ng/g	
	BADGE·HCl	1.3 n/g	
Altres aliments			
Mel	BPA	3 — 33 ng/g	Inoue i cols., 2003a
Ous	BPA	0.5 — 31.0 ng/g	Ballesteros-Gómez i cols., 2009; Yonekubo i cols., 2008
Llet	BPA	7.11 — 27.0 ng/g	Ballesteros-Gómez i cols., 2009; Yonekubo i cols., 2008
Cafè	BADGE·2HCl	n.d. — 0.07 mg/kg	Uematsu i cols., 2001
	BADGE·HCl·H_2O	0.36 mg/kg	
	BADGE·2H_2O	4.03 mg/kg	
Fotoiniciadors:aliments envasats amb cartró			
Llet			
	BP	2.84 — 39 ng/g	Shen i cols., 2009; Sagratini i cols., 2008
	ITX	0.81 — 439 ng/g	Shen i cols., 2009; Gil-Vergara i cols., 2007; Sun i cols., 2007; Bagnati i cols., 2007
	EHDAB	0.13 — 120 ng/g	Shen i cols., 2009; Gil-Vergara i cols., 2007; Sagratini i cols., 2008
Sucs de fruites			
	BP	5 — 90 ng/mL	Sagratini i cols., 2008
	EHDAB	0.14 — 0.8 ng/mL	
	ITX	0.05 — 80.90 ng/mL	Sagratini i cols., 2008; Sun i cols., 2007
Vi			
	BP	4.73 — 217 ng/mL	Sagratini i cols., 2008
	ITX	0.2 — 0.24 ng/mL	
	HCPK	1.2 ng/mL	

Per tal d'entendre millor el comportament dels fotoiniciadors en els envasos alguns autors han dut a terme estudis de migració. Així Johns i cols., van estudiar la migració d'alguns d'aquests compostos del cartró cap a l'aliment emmagatzemant les mostres a -20ºC, i van observar que fins i tot a aquestes baixes temperatures la BP migra cap a l'aliment. Per altra banda Rodriguez-Bernaldo i cols., 2009 van realitzar estudis de migració de la BP en aliments secs per tal de poder relacionar la migració dels fotoiniciadors amb la seva pressió de vapor. En

aquests estudis es va posar de manifest que els compostos que presenten una major pressió de vapor migren amb més facilitat i a més, que la major concentració de fotoiniciadors es detecta en els aliments amb major contingut de greix. Per altra banda també es va trobar una relació entre la migració i la porositat de l'aliment. Aquests estudis de migració també s'han dut a terme per al BPA i els seus compostos relacionats com el BADGE i el BFDGE. Per aquests últims s'ha observat que la migració dels anàlits es veu incrementada en els aliments amb un elevat contingut lipídic (Cabado i cols., 2008). A més, en el cas del BPA també s'ha observat un increment de la migració per efecte de la cafeïna tal i com es demostra en el treball realitzat per Kang i cols., 2002. La migració d'aquests compostos en les llaunes de conserva així com la del BPA del plàstic policarbonat es veu incrementada per factors com la temperatura — tant durant el procés d'obtenció dels materials com durant l'emmagatzemament dels aliments —, i el temps d'emmagatzemament (Cao i cols., 2009a & b; Simoneau i cols., 2002; Cabado i cols., 2008; De Coensel i cols., 2009). En els estudis de migració de BPA en el plàstic policarbonat s'ha observat una relació directa entre la migració i la neteja dels recipients la qual cosa probablement és deguda a degradacions del polímer en el procés de neteja (Brede i cols., 2003; Maia i cols., 2009; Biedermann-Brem i cols., 2009a & b).

1.3.2. MEDI AMBIENT

El Bisfenol A ha estat àmpliament estudiat i analitzat en el medi ambient des de finals del segle XX fins avui dia i en conseqüència a la literatura existeix una gran quantitat de dades de nivells i distribució d'aquest compost en els diferents compartiments ambientals com aigua, aire, sediments i fangs. Per exemple, si fem una cerca al *SciFinder Scholar* entrant per *Bisphenol A* i a més *Environmental* i prenent en consideració tan sols les publicacions científiques escrites en anglès apareixen 2149 referències bibliogràfiques, les quals tal i com es mostra a la Figura 1.3 corresponen majoritàriament als últims 10 anys (1999 – 2009).

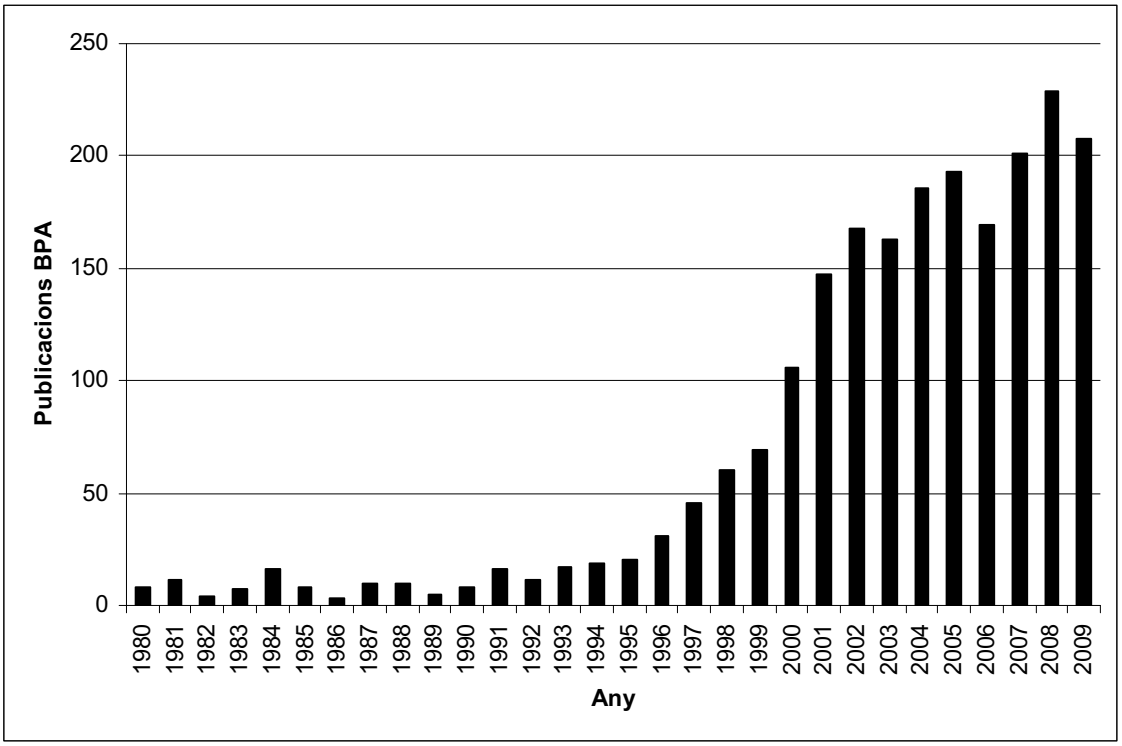


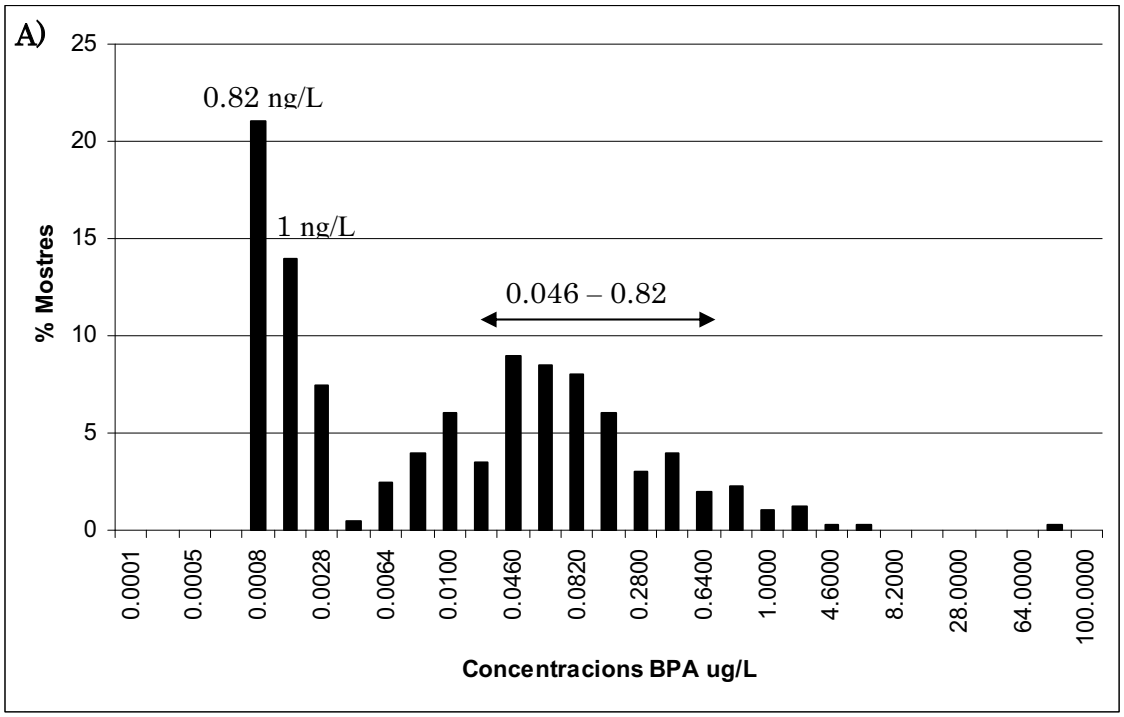
Figura 1.3. Publicacions sobre la presència de BPA en el medi ambient en els darrers 20 anys.

Recentment Klecka i cols., 2009 han publicat un treball de revisió sobre els nivells de BPA en mostres d'aigües superficials i sediments de Nord Amèrica (Canada i USA) i Europa (Austria. Bèlgica, República Txeca, Dinamarca, França, Alemanya, Itàlia, Holanda, Noruega, Portugal, Espanya, Suècia, Suïssa i Regne Unit) que recull les publicacions compreses entre l'any 1991 i el 2007. A la Figura 1.4 es representa, a partir de les dades extretes d'aquests treball de revisió i en forma de diagrama de barres, la distribució de concentracions de BPA en aigües superficials tant a Europa (Figura 1.4A) com a Nord Amèrica (Figura 1.4B). Es pot observar que a Europa les concentracions són en general inferiors, per exemple un percentatge significatiu de les mostres analitzades presenta concentracions per sota dels 2.8 ng L^{-1} (42.5%), mentre que a Nord Amèrica la major part de les mostres analitzades presenten concentracions de BPA al voltant de 0,25 μg L^{-1}.

Pel que fa referència a les concentracions de BPA en aigües superficials d'Espanya (Rodríguez-Mozaz i cols., 2004; Gómez i cols., 2006), cal dir que es troben entre els valors comentats anteriorment a Europa. Diversos estudis sobre la presència d'alteradors endocrins en aigües de riu han posat de manifest la presència del BPA en diferents rius catalans on s'han trobat concentracions de

BPA d'entre 90 i 2970 ng/L i entre 70 i 1510 ng/L a diferents punts del Riu Llobregat i el Riu Ter, respectivament (Céspedes i cols., 2005 & 2006) mentre que al Riu Ebre s'han trobat concentracions de BPA entre 10 i 20 ng/L (Brossa i cols., 2005).

Altres estudis que s'han dut a terme a Catalunya posen de manifest que el BPA és parcialment eliminat durant el procés de tractament d'aigües residuals. Així Céspedes i cols., van detectar la presència de BPA a concentracions entre 90-6980 ng/L a l'entrada d'una planta de tractament d'aigües residuals (WWTP) mentre que a la sortida es van trobar concentracions molt més baixes, entre 90 i 340 ng/L. En altres països com el Japó, Itàlia i França també s'han realitzat estudis sobre la presència del BPA a l'entrada i sortida de plantes de tractament d'aigües residuals, però les concentracions trobades han estat més baixes en tots els casos essent de l'ordre de 335 ng/L a l'entrada de la planta i entre 13 ng/L i 36 ng/L a la sortida (Laganà i cols., 2004; Watabe i cols., 2005; Jeannot i cols., 2002; Liu i cols., 2004; Kawaguchi i cols., 2004). Per altra banda, també hi ha informació sobre el comportament del BPA en una planta potabilitzadora d'aigua que feia un tractament de filtració amb sorra,una etapa d'ozonòlisi i filtració amb carbó actiu on es va posar de manifest una important reducció de les concentracions de BPA, dels 120-271 ng/L en l'aigua d'entrada a 0 — 6 ng/L a l'aigua de sortida de la planta (aigua de distribució) (Rodríguez-Mozaz i cols., 2004).



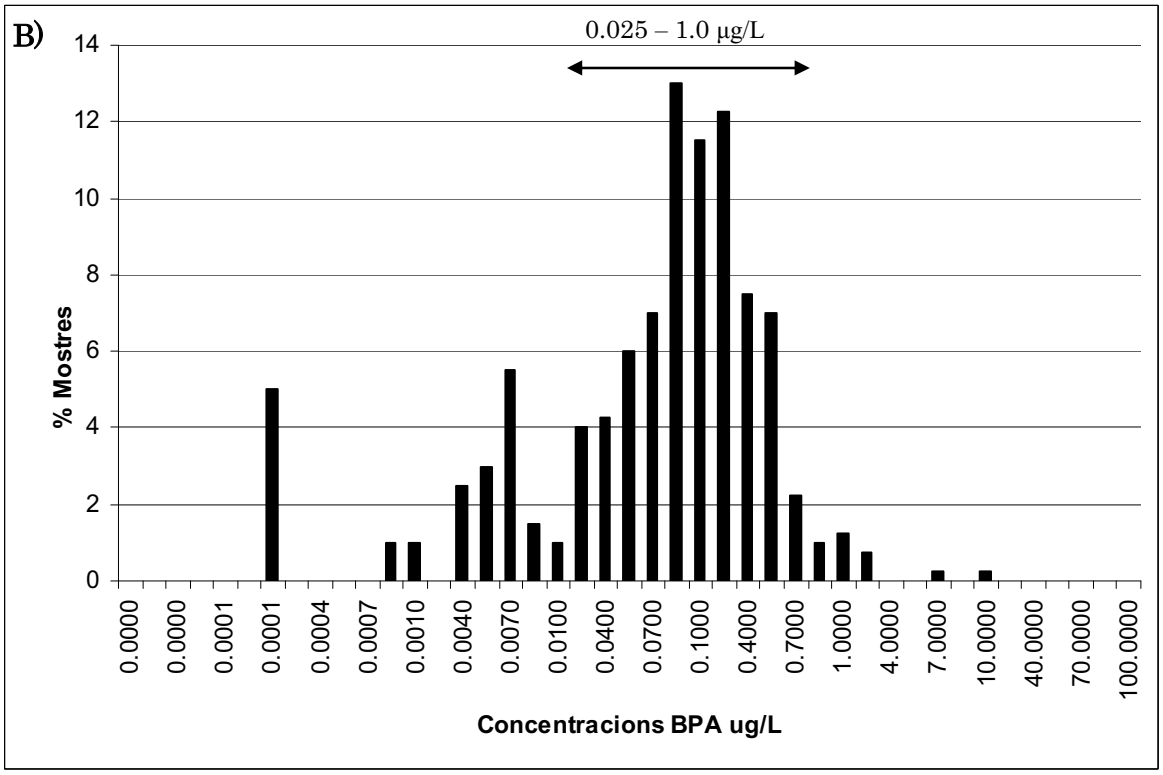


Figura 1.4. Concentracions de BPA en aigües superficials. A) Europa, B) Amèrica del Nord.

Tant en els tractaments d'aigües residuals com en la potabilització es duen a terme etapes de cloració per desinfectar que poden donar lloc a la formació de subproductes de cloració. La cloració del BPA és relativament fàcil atesa la seva estructura bisfenòlica. El clor ataca les posicions orto dels dos anells aromàtics, generant els derivats clorats monocloro- (MCBPA), dicloro- (DCBPA), tricloro- (TCBPA) i tetraclorobisfenol A (TeCBPA). En el cas del DCBPA es poden obtenir dos isòmers, el 3,3'-diclorobisfenol A i el 3,5-diclorobisfenol A, segons que tingui lloc la doble cloració en un mateix anell o en cadascun dels anells. Aquests derivats clorats del BPA també es poden trobar a les aigües residuals provinents de la industria paperera on el BPA s'utilitza principalment en tints per a paper tèrmic i degut a la utilització de clor com agent blanquejant (Fukazawa i cols., 2001 & 2002). A la literatura només es troben dos treballs on s'indica que s'han trobat derivats clorats del bisfenol A encara que concentracions baixes, 2.0 µg/L, de fet molt inferiors a la concentració de BPA que pot arribar a ser de 370 µg/L (Fukazawa i cols., 2001 & 2002).

El BPA també s'ha determinat en l'aigua de mar, encara que a la literatura hi ha relativament pocs estudis en aquest tipus de mostres degut a que en aigua de mar el contingut de BPA es molt baix i fins fa poc no es disposava de metodologia analítica suficientment sensible per a detectar aquest nivells. Per exemple en aigua de la zona costanera del Mar Bàltic les concentracions de BPA que s'han trobat han estat 0.22-5.4 ng/L (Beck i cols., 2005), encara que a la costa de Singapur les concentracions son molt més elevades, 10-2.470 ng/L (Basheer i cols., 2004) probablement degut a l'insuficient tractament de les aigües residuals i a l'elevada població a la zona de Singapur. Aquesta elevada concentració de BPA comporta que s'hagin trobat alts nivells de BPA de 27 — 213 ng/g en crustacis, cefalòpodes i peixos d'aquestes zones. Recentment els estudis realitzats per Saido i el seu grup de treball (Saido i cols., 2010) han posat de manifest que els plàstics durs, a diferència del que es pensava, es biodegraden en el mar alliberant grans quantitats de BPA i han detectat la presència de BPA a concentracions d'entre 0.01 i 50 mg L^{-1} en mostres d'aigua de 200 llocs de 20 països, principalment del Sud-est Asiàtic i Amèrica del Nord.

En altres compartiments ambientals també ha estat detectada la presència de BPA. Per exemple hi ha alguns estudis de sòls, sediments i fangs de depuradora on s'han trobat concentracions entre 3.78 i 74.38 ng/g (Chu i cols., 2005). A la Figura 1.5 es mostra la distribució de les concentracions en mostres de sediments de Nord Amèrica i Europa recollides entre l'any 1991 i 2007 (Klecka i cols., 2009) on es posa de manifest que les concentracions de BPA als sediments d'Europa (el 84% de les mostres es troben concentracions entre 2.5 i 750 ng/g) (Figura 1.5A) són superiors que a Nord Amèrica (al 90% de les mostres es troben concentracions entre 0.5 i 5 ng/g) (Figura 1.5B). Aquests resultats són contraris als observats en les mostres d'aigües superficials la qual cosa es pot deure al menor nombre de mostres de sediments analitzades (427 mostres) en front de les 2682 en el cas de les aigües i a que les mostres de sediments corresponen tan sols a mostres contaminades la qual cosa no permet comparar aquestes dades amb les de les aigües (Klecka i cols., 2009). El BPA també ha estat analitzat en mostres d'aire. Per exemple en mostres de l'aire interior d'una fàbrica de material electrònic s'ha trobat a concentracions entre 5.7 i 13.9 ng/m^3 (Sabatini i cols., 2005), mentre que en mostres d'aire d'interiors domèstics s'han detectat concentracions menors entre <0.1 ng/m^3 i 3.6 ng/m^3 (Inoue i cols., 2006).

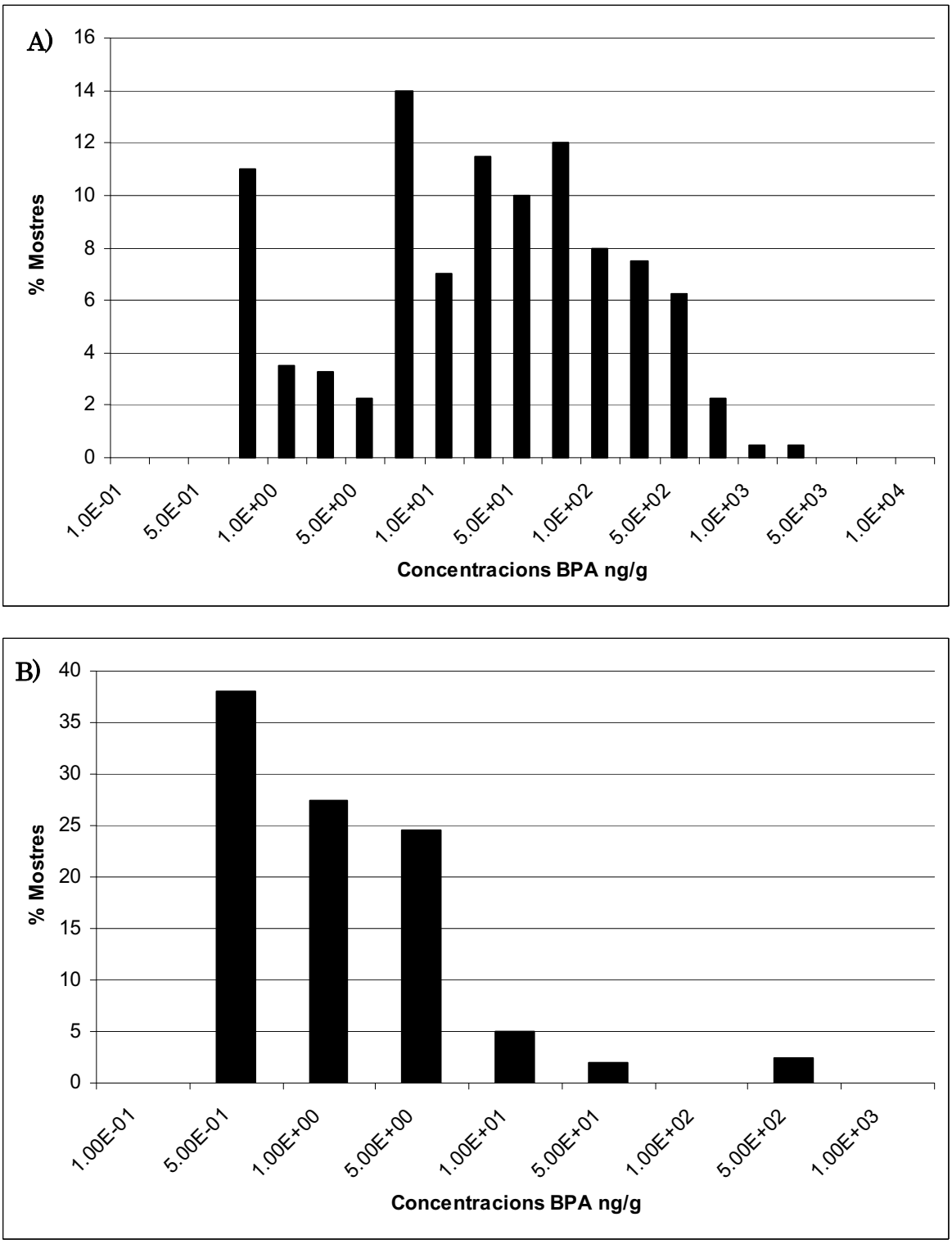


Figura 1.5. Concentracions de BPA en sediments. A) Europa, B) Amèrica del Nord

Pel que fa referència al TBBPA Covaci i cols., 2009 han realitzat recentment un treball de revisió en el que es mostren els nivells de concentració d'aquest compost en els diferents compartiments ambientals. Els nivells en fangs de plantes de tractament d'aigües residuals el TBBPA són de 2 ng/g fins a 510 ng/g. En sediments les concentracions trobades han estat inferiors, de 0.04 ng/g a 25 ng/g (en pes sec), mentre que tal i com era d'esperar en una zona pròxima a una empresa d'obtenció de retardants de flama les concentracions foren molt més altes (9.8 µg/g). Els valors trobats en estudis recents són del mateix ordre. Per exemple, Guerra i cols., 2010 han trobat concentracions d'entre 2.8 i 15 ng/g i 132 i 945 ng/g en mostres de sediments i fangs del Riu Ebre respectivament, mentre que les concentracions en mostres de sediments del Riu Sena (París) són d'entre 65 i 280 pg/g (Labadie i cols., 2010). El TBBPA també ha estat analitzat en sòls de la Xina on les concentracions són d'entre 0.12 ng/g i 1800 ng/g, les màximes concentracions corresponen a mostres recollides en un abocador d'escombreries. Pel que fa referència a mostres de sòls a Espanya, Sánchez-Brunete i cols., 2009 van trobar concentracions de 0.3 ng/g en sòls agrícoles i de 3.4 ng/g fins a 32 ng/g en sòls d'origen industrial de la zona de Madrid. Degut a la baixa solubilitat del TBBPA és poc probable detectar la seva presència en mostres d'aigua, encara que a la literatura existeixen algunes dades que donen concentracions entre 0.3 ng/L i 620 ng/L (Covaci i cols., 2009). Recentment Labadie i cols., 2010 han detectat la presència de TBBPA en mostres d'aigua del Sena encara que a concentracions molt inferiors de l'ordre de 50 – 64 pg/L.

El TBBPA també s'ha determinat en mostres d'aire (material particulat) de zones de treball on hi ha material informàtic i altres equips electrònics. Les concentracions en aquestes mostres són de l'ordre dels 0,5 ng/m^3 (Inoue i cols., 2003b) mentre que les dades corresponents a plantes de reciclatge de material electrònic a Suècia són molt més altes, de 13,8 ng/m^3 a 150ng/m^3 (Tollback i cols., 2006). La preocupació per l'exposició de treballadors a aquesta substància ha portat a la determinació de TBBPA en mostres de plasma i sèrum de treballadors on s'ha trobant a concentracions de l'ordre de 6,2 a 8,7 pg/g (Wehler i cols., 1997; Hagmar i cols., 2000; Hayama i cols., 2004).

Encara que els altres compostos estudiats en aquesta memòria, bisfenol A-diglicidil èters, bisfenol F-diglicidil èters i fotoiniciadors, són àmpliament utilitzats

a la industria alimentària fins avui no es disposa de dades de nivells d'aquests compostos en el medi ambient.

1.4. MÈTODES D'ANÀLISI

En aquest apartat es comenten el mètodes de tractament de mostra emprats per a l'extracció i purificació de mostres ambientals i alimentàries dels compostos estudiats en aquesta memòria, així com els mètodes de determinació utilitzats en els últims anys i que estan basats majoritàriament en tècniques de separació acoblades a l'espectrometria de masses.

1.4.1. MÈTODES DE TRACTAMENT DE MOSTRA

Es conegut que el tipus de mostra a analitzar condiciona el tractament de mostra a realitzar. A més, el grau de purificació necessari que depèn del procediment de determinació i de la complexitat de la mostra fa que aquesta etapa sigui més o menys complexa. Generalment el procés de tractament de mostra es pot dividir en quatre etapes. En primer lloc, es requereix una homogeneïtzació de la mostra, per tal de que sigui representativa. A continuació, s'ha de realitzar una extracció dels anàlits de la matriu i una vegada extrets s'ha de dur a terme la purificació de l'extracte obtingut per tal d'eliminar les possibles interferències i una preconcentració per tal d'obtenir un extracte net i a una concentració adequada per a ésser analitzat.

Els procediments descrits a la literatura per al cas concret de l'anàlisi dels compostos estudiats en aquesta memòria es comenten a continuació segons la naturalesa de les mostres a analitzar.

Mostres d'aliments

El BPA i els seus compostos relacionats així com també els fotoiniciadors es poden trobar en una gran varietat de mostres d'aliments, tant en mostres sòlides com en mostres líquides enllaunades o empaquetades. L'anàlisi d'aquests compostos en aquestes matrius generalment requereix un adequat tractament de

mostra abans de dur a terme la seva anàlisi. Les mostres sòlides s'homogeneïtzen mentre que les líquides són filtrades o centrifugades, encara que algunes mostres requereixen tractaments especials com per exemple, desgasificar les mostres de begudes carbonatades o eliminar les proteïnes de les mostres que en contenen un elevat percentatge per tal que no interfereixin en l'anàlisi. Pel que fa referència a l'anàlisi de BPA en aliments els mètodes de tractament de mostra en aliments es troben àmpliament descrits en el treball de revisió publicat per Ballesteros-Gómez i cols., 2009.

L'extracció en fase sòlida (SPE) és la tècnica d'extracció més emprada en l'anàlisi d'aquesta família de compostos, mentre que l'extracció líquid-líquid (LLE) encara que és una bona tècnica sobretot per a mostres líquides és emprada en menor proporció. L'acetonitril (ACN) és el solvent orgànic més utilitzat encara que altres solvents com l'acetona, el metanol (MeOH), l'acetat d'etil (AcEt) i l'etanol (EtOH) també permeten una extracció eficaç d'aquests compostos. En canvi, les mostres líquides són generalment extretes amb solvents menys polars com acetat d'etil, cloroform o diclorometà. Els mètodes d'extracció publicats a la literatura normalment empren volums elevats de dissolvent orgànic, entre 40-100 mL, i els temps d'extracció estan compresos entre els 10 min i les 2 hores amb agitació mecànica o mitjançant l'ajuda d'un bany d'ultrasons.

Degut a la baixa selectivitat dels mètodes d'extracció emprats normalment cal realitzar una etapa de purificació dels extractes obtinguts. A més, les mostres que contenen un elevat contingut lipídic i/o proteic necessiten una etapa addicional per tal d'eliminar les proteïnes i greixos. En cromatografia de líquids (LC) la presència de lípids i/o proteïnes afecta a la retenció dels anàlits, a la resolució i a més escurça la vida de la columna. En cromatografia de gasos acoblada a l'espectrometria de masses (GC-MS), els lípids es poden acumular a l' injector, a la columna i a la font de ionització. Generalment els greixos són eliminats per extracció líquid-líquid amb solvents orgànics apolars com heptà, trimetilpentà i hexà o mitjançant la congelació de les mostres a –24ºC durant uns 40 minuts i posterior filtració. L'extracció en fase sòlida és la tècnica més emprada per a la purificació dels extractes obtinguts, i entre els sorbents més utilitzats cal citar els de fase invertida C18, i els polimèrics de polidivinil-benzè/*N*-vinilpirrolidina (HLB). Ara bé, són aquests últims els més emprats avui dia degut a que presenten avantatges davant els sorbents de base sílice com són per exemple una major àrea

específica, la possibilitat d'assecar-los sense que es vegi afectada la retenció i com totes les resines polimèriques l'elevada estabilitat en un ampli interval de pH, entre 2 i 10.

Recentment s'ha proposat un sistema de tractament de mostra que s'està imposant en l'anàlisi d'aliments i productes agraris, que s'anomena QuEChERS (de l'anglès **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged i **S**afe) (Majors 2007). Aquest mètode ofereix unes bones recuperacions, resultats prou precisos i rapidesa en el tractament de la mostra i a més, minimitza l'ús de solvents orgànics. Aquest mètode es duu a terme en dues etapes, una primera en la que té lloc l'extracció dels anàlits i una segona etapa de neteja. Generalment la mostra es extreta amb solvents miscibles en aigua com acetonitril o acetat d'etil en presència d'elevades quantitats de sals, que poden ser clorur de sodi i/o sulfat de magnesi, aquest últim s'utilitza per tal l'eliminar el contingut d'aigua de la mostra, a més també se solen emprar solucions reguladores per tal d'induir la separació de fases i estabilitzar les especies àcides o bàsiques dels compostos d'interès. Després d'agitar i centrifugar la mostra, una alíquota es sotmet al procés de neteja emprant extracció en fase sòlida dispersiva mitjançant l'addició de petites quantitats de sorbents sòlids, d'entre els quals el PSA (amina primària/secundaria) és un dels més emprats. Finalment l'extracte obtingut es centrifuga i el sobrenedant o bé s'injecta directament en el sistema cromatogràfic o es realitza una etapa de preconcentració mitjançant l'evaporació del solvent orgànic en funció de la sensibilitat del mètode.

Altres tècniques d'extracció menys emprades en l'anàlisi d'aquests compostos en mostres d'aliments són la microextracció en fase sòlida (SPME) que s'utilitza quan la determinació es realitza per GC-MS o l'extracció amb líquids pressuritzats (PLE) en la qual l'extracció amb solvents orgànics té lloc a una elevada temperatura (40-200 ºC) i pressió (1000-2500 psi). En aquest últim cas normalment es realitza una etapa de neteja dels anàlits per SPE, ja que aquesta extracció és poc selectiva.

Mostres ambientals

En mostres ambientals tant sols hi ha informació sobre procediments d'anàlisis per al BPA i els seus derivats halogenats els quals són diferents segons es tracti de mostres líquides (aigües) o sòlides (sediments, sòls i fangs).

En l'anàlisi de mostres d'aigua la primera etapa consisteix en la separació de la matèria particulada que es duu a terme per filtració, amb filtres generalment de 0.45 μm de mida de porus. Posteriorment s'utilitza extracció en fase sòlida (SPE) per extreure i purificar els anàlits. En general, degut a les baixes concentracions d'aquests contaminants en les mostres aquoses, es requereix la preconcentració de la mostra, per exemple en mostres d'aigua superficial els volums de mostra emprats es troben entre els 500 mL i els 2 L mentre que en mostres d'aigües residuals són menors, 100-500 mL. Els sorbents més emprats igual que en el cas de mostres d'aliments són els copolimers de polidivinil-benzè/*N*-vinilpirrolidina (HLB) els quals ofereixen bones recuperacions, generalment entre el 75 i el 100%. Altres sorbents com els de fase invertida de base sílice (C18) i els Strata X que són polimèrics també han estat emprats per a l'anàlisi d'aquests compostos en mostres d'aigua. En alguns casos i per tal d'augmentar el factor de preconcentració i millorar la sensibilitat del mètode es requereix preconcentrar volums de mostra grans i en aquests casos per disminuir el temps de percolació de la mostra alguns autors empren discos d'extracció de fase invertida. Aquests tipus de sorbents també són utilitzats en mètodes d'extracció, preconcentració i neteja que es realitzen en línia amb la separació cromatogràfica. Aquests mètodes en línia permeten reduir el temps d'anàlisi, el volum de mostra a tractar així com la seva manipulació. Aquest últim aspecte tal i com veurem més detalladament en el Capítol 4 d'aquesta memòria és especialment crític en l'anàlisi de BPA degut a que la seva ubiqüitat en el medi i la seva presència en algun dels materials emprats en el laboratori possibilita la contaminació de les mostres a analitzar. Per a la purificació i preconcentració d'aquests compostos en mostres aquoses també s'ha emprat la microextracció en fase sòlida (SPME) prèvia a la determinació per GC-MS.

En l'anàlisi de mostres sòlides com sediments o fangs es requereix una etapa d'assecat de les mostres prèvia a l'extracció. Així, es recomana assecar les mostres sòlides a 30ºC durant més de 16h o bé liofilitzar-les per tal d'eliminar l'aigua. Pel que fa referència a les tècniques d'extracció més utilitzades cal esmentar l'extracció mitjançant ultrasons i l'extracció Soxhlet la qual requereix un elevat temps d'extracció i un elevat consum de solvent orgànic, raons que expliquen que avui dia estigui essent substituïda per l'extracció amb líquids pressuritzats (PLE). L'extracció del BPA i compostos relacionats es realitza generalment amb solvents polars com poden ser MeOH o ACN la qual cosa comporta a la coextracció

d'impureses de les mostres que normalment obliga a realitzar una posterior etapa de purificació mitjançant SPE.

1.4.2. TÈCNIQUES DE SEPARACIÓ ACOBLADES A L'ESPECTROMETRIA DE MASSES

Ateses les baixes concentracions dels contaminants orgànics tant al medi ambient con en els aliments es requereixen metodologies analítiques prou sensibles i alhora selectives per analitzar aquests compostos en les diferents matrius. Les tècniques més emprades avui dia per a l'anàlisi d'aquests compostos són la cromatografia de gasos i la cromatografia de líquids acoblades a l'espectrometria de masses.

1.4.2.1. COMPOSTOS FENÒLICS

Les referències bibliogràfiques relacionades amb l'anàlisi de compostos fenòlics i compostos relacionats per tècniques cromatogràfiques acoblades a l'espectrometria de masses estan recollides en un treball de revisió que s'inclou en aquest apartat de la memòria (Article I: *Recent advances in the mass spectrometry analysis of phenolic endocrine disruptors and related compounds*). En aquest article i després d'una breu introducció que explica la problemàtica de l'anàlisi dels compostos fenòlics per tècniques cromatogràfiques acoblades a l'espectrometria de masses, es recullen els treballs científics més significatius publicats fent especial incidència en els mètodes basats en l'acoblament de la cromatografia de gasos i la cromatografia de líquids a l'espectrometria de masses (LC-MS i GC-MS). La caracterització d'aquesta família de compostos per espectrometria de masses així com les estratègies seguides per a la quantificació i confirmació d'aquests compostos està discutida en aquest article on es posen de manifest els problemes existents en l'anàlisi, com per exemple la falta d'estàndards analítics comercials per alguns dels derivats clorats del BPA així com els problemes de supressió iònica en cromatografia de líquids acoblada a espectrometria de masses.

Amb posterioritat a la realització d'aquest treball de revisió no s'han publicat treballs que aportin res de nou des del punt de vista de l'acoblament de la cromatografia a l'espectrometria de masses.

ARTICLE CIENTÍFIC I.

Recent advances in mass spectrometry analysis of phenolic endocrine disruptors and related compounds

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RECENT ADVANCES IN MASS SPECTROMETRY ANALYSIS OF PHENOLIC ENDOCRINE DISRUPTORS AND RELATED COMPOUNDS

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This article reviews recent literature on current methodologies based on chromatography coupled to mass spectrometry to analyze phenolic compounds with endocrine-disrupting capabilities. For this review we chose alkylphenol ethoxylates, bisphenol A, bisphenol F, and their degradation products and halogenated derivatives, which are considered important environmental contaminants. Additionally, some related compounds such as bisphenol diglycidylethers were included. Growing attention has been paid to the mass spectrometric characterization of these compounds and the instrumentation and strategies used for their quantification and confirmation. The current use of gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) methodologies with different mass spectrometers and ionization and monitoring modes is discussed. Practical aspects with regards to the use of these analytical techniques, such as derivatizing reagents in GC–MS, ion suppression in LC–MS, and the most problematic aspects of quantification, are included in the discussion. © 2009 Wiley Periodicals, Inc., Mass Spec Rev 29:776–805, 2010

Keywords: *mass spectrometry; LC-MS; GC-MS endocrine disruptor; alkylphenolic compounds; bisphenol A*

I. INTRODUCTION

The development of new, more sensitive analytical methods to detect chemicals has increased the number of compounds of potential environmental concern, and brought substances under study that had not previously been considered. Some of these chemicals, known as endocrine-disrupting compounds (EDCs), affect the reproductive function of living organisms by interfering with the endocrine system. They might alter, compete with, or displace the natural estrogens in living species to change the functions of these natural hormones. Interest in EDCs is increasing, because they show biological activity at very low concentrations. Various groups of man-made chemicals that are reported or alleged to have endocrine-disrupting activity are of environmental relevance. These chemicals include synthetic estrogens, pesticides, dioxins, coplanar polychlorinated biphenyls, and plasticizers such as bisphenol A or alkylphenol ethoxylate surfactants. Exposure to these compounds might have deleterious health effects, and cause reproductive abnormalities and hormonal changes in humans and wildlife species. EDCs can reach the aquatic environment via the effluents of sewage treatment plants. Consequently, fish and wildlife might be exposed to them. Moreover, humans can become exposed through the entry of these waters into drinking-water treatment plants.

This article covers some phenolic compounds such as alkylphenol ethoxylates; their degradation products; bisphenol A; bisphenol F; and the halogenated derivatives of the bisphenols, whose endocrine-disrupting capabilities are well-documented. The estrogenic activity of these compounds is mainly related to the alkylphenol structure that they share with the native hormone 17β-estradiol. In addition, bisphenol diglycidylethers, which are used as precursors of epoxy-based coatings of food cans, have also been included. A list of compounds and acronyms appears in Table 1. Alkylphenol polyethoxylates (AP*n*EOs, *n*: number of ethoxy units) are effective nonionic surfactants that are widely used in industrial formulations (textiles, tannery, paper industries, and metal-working fluids) as pesticide adjuvants, paint ingredients, and personal care products. Nonionic surfactants account for approximately 40% of the surfactant market worldwide. Among them, AP*n*EOs belong to the most widely used surfactant class. Approximately 80% of AP*n*EOs are nonylphenol ethoxylates (NP*n*EOs), whereas the remaining 20% are mainly from octylphenol (OP*n*EOs). Commercial blends of AP*n*EOs are polydisperse mixtures of isomers (with different branching on the alkyl moiety) and oligomers (with different numbers of ethylene oxide units). Environmental (bio)degradation of the parent compound, by progressive shortening of the ethoxylate chain under aerobic conditions, leads mainly to the formation of alkylphenol mono- and diethoxylates (short-chain AP*n*EOs), whereas under anaerobic conditions, fully deethoxylated alkylphenols are also produced (alkylphenols) (Giger, Brunner, & Schaffner, 1984; Ahel & Giger, 1985; Ahel, Giger, & Koch, 1994; Ahel, Giger, & Schaffner, 1994; Lu et al., 2008). Further transformation, via oxidation of the ethoxylate chain, can produce carboxylic derivatives. For instance, it is accepted that nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC) are aerobic metabolites of NP*n*EOs generated by ω-carboxylation of the ethoxylate chain (Di Corcia et al., 2000; Jonkers, Knepper, & de Voogt, 2001; Ying, Williams, & Kookana, 2002; Langford et al., 2005). The analysis of AP*n*EOs is, therefore, dominated by the complexity of

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TABLE 1. List of target compounds and acronyms

Compounds	Acronyms
Alkylphenolic compounds	
Alkylphenol polyethoxylates	AP*n*EO
Alkylphenols	APs
Halogenated (chlorinated and brominated) alkylphenols	XAP (ClAP, BrAP)
Alkylphenol polyethoxycarboxylates	AP*n*EC (ClAP*n*EC, BrAP*n*EC)
Halogenated (chlorinated and brominated) polyethoxycarboxylates	XAP*n*EC
Nonylphenol	NP
Nonylphenol polyethoxylate	NP*n*EO
Nonylphenol polyethoxycarboxylate	NP*n*EC
Octylphenol	OP
Octylphenol polyethoxylate	OP*n*EO
Octylphenol polyethoxycarboxylate	OP*n*EC
Halogenated (chlrorinated, brominated) alkylphenols polyethoxylated and analogs	XAP*n*EO (ClAP*n*EO, BrAP*n*EO)
Bisphenol A and related compounds	
Bisphenol A	BPA
Monochlorobisphenol A	MCBPA
Dichlorobisphenol A	DCBPA
Trichlorobisphenol A	TCBPA
Tetrachlorobisphenol A	TeCBPA
MonobromoBisphenol A	MBBPA
Dibromobisphenol A	DBBPA
Tribromobisphenol A	TriBBPA
Tetrabromobiphenol A	TBBPA
Bisphenol F	BPF
Bisphenol A diglycidyl ether	BADGE
Bisphenol A (2,3-dihydroxypropyl) glycidyl ether	BADGE·H_2O
Bisphenol A bis(2,3-dihydroxypropyl) ether	BADGE·$2H_2O$
Bisphenol A (3-chloro-2-hydroxypropy) glycidyl ether	BADGE·HCl
Bisphenol A bis(3-chloro-2-hydroxypropyl) ether	BADGE·2HCl
Bisphenol A (3-chloro-2-hydroxypropyl)(2,3-dihydroxypropyl) ether	BADGE·HCl·H_2O
Bisphenol F diglycidyl ether	BFDGE (mixture of 3 isomers)
Bisphenol F bis(2,3-dihydroxypropyl) ether	BFDGE·$2H_2O$ (mixture of 3 isomers)
Bisphenol F bis(3-chloro-2-hydroxypropyl) ether	BFDGE·2HCl (mixture of 3 isomers)

n is the number of ethoxy groups.

the mixtures. Even in the form of matrix-free standards, their detection, identification, and quantification present considerable challenges.

Bisphenol A (BPA) (2,2-bis[4-hydroxyphenyl] propane) is an industrially important chemical that is widely used as raw material in the production of polycarbonate plastics and epoxy resins, which have a variety of applications, such as plastic food containers and epoxy food-can coatings. Additional applications of BPA include printed circuit boards, composites, adhesives, and tooling. Bisphenol F (BPF), which is a mixture of three isomers (2,2′-, 2,4′-, and 4,4′-dihydroxydiphenylmethane), is also used in the manufacture of epoxy resins. Due to the widespread use and manufacture of BPA, a large amount of BPA is discharged into the environment. In addition, some halogenated derivatives of BPA, such as tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TeCBPA), are commonly used as flame retardants in polymers. TBBPA is the primary flame retardant in electronic circuits. Other brominated derivatives of BPA have been found in the environment, due to the biodegradation of TBBPA to less-brominated analog under aerobic and anaerobic conditions in soil and sediments (Hakk et al., 2000; Hakk & Letcher, 2003; Gerecke et al., 2006). Chlorinated derivatives of BPA can also be found in the environment, because BPA is easily chlorinated when it reacts with the residual chlorine used as a disinfectant in water treatment plants and as a bleaching agent in paper recycling plants (Fukazawa et al., 2001).

Two derivatives of BPA, bisphenol A diglycidyl ether (BADGE) and bisphenol F diglycidyl ether (BFDGE), are also of industrial importance. These compounds are used as starters in the manufacture of epoxy resins and epoxy-based polymers, and as additives in poly(vinyl chloride) (PVC) organosols, as heat stabilizers and HCl scavengers. These polymeric materials are mainly used as interior coatings for food and drink containers to prevent corrosion and migration of metals into the food during heat stabilization and storage. Both coating types contain residual monomers. In addition, PVC aerosols contain chlorinated derivatives of BADGE and BFDGE, formed by the reaction with surplus hydrochloric acid generated during the production process. These chlorinated compounds could migrate into fat-containing food products during autoclaving. Moreover, several

reactions can take place in foodstuffs. For instance, the epoxy groups might be hydrolyzed when they come into contact with aqueous and acidic food during storage to generate the corresponding hydrolyzed derivatives.

Owing to the high production volumes of BPA, its toxicity has been intensively studied in the last 20 years. The results show that it is not genotoxic or carcinogenic, but it can be considered as slightly toxic (Dekant & Voelkel, 2008). Moreover, the toxicity of halogenated derivatives of BPA, such as TBBPA and TCBPA, is greater than that of BPA. This suggests that halogen atoms participate in the toxicity (Nakagawa et al., 2007). Parent APnEOs are also classified as slightly toxic. Thus, the environmental harm caused by these compounds is mainly a result of the generation of persistent biodegradation products, such as alkylphenols and short-chained AP*n*EOs, which are toxic to aquatic organisms. Moreover, potentially mutagenic ring-halogenated derivatives can be produced during chlorination in wastewater or drinking water treatment plants (Reinhard, Goodman, & Mortelmans, 1982). For all these compounds, the main environmental concern is not usually their toxicity, but rather their estrogenic potential, as confirmed by numerous studies *in vitro* and *in vivo* (Jobling et al., 1996; Sonnenschein & Soto, 1998; vom Saal & Hughes, 2005; vom Saal et al., 2005; Richter et al., 2007). The estrogenic activity of chlorinated derivatives is stronger than that of their non-halogenated parent compounds (Fukazawa et al., 2002). Furthermore, it has been reported that there are indications of the potential toxicology of TBBPA as an endocrine disruptor (Van der Ven et al., 2008). Regarding BADGE, BFDGE and their derivatives, their toxicology is not completely elucidated. However, it has been reported that BADGE and BFDGE show some cytotoxic effects (Ramilo et al., 2006).

As a result of these findings, some regulations on the use of these compounds have been established. BPA and TBBPA are both candidates to be among the first substances to go through Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) EU registration (EU Regulation (EC) No. 1907/2006). AP*n*EOs are banned or restricted in Europe. Nevertheless, they are still being used in substantial amounts, mainly due to their low production costs. Some of their degradation products, such as nonylphenol (NP) and octylphenol (OP), are included in the priority list of the EU's Water Framework Directive (Decision 2455/2001/CE), in which they are identified as priority hazardous substances. Moreover, the presence of these compounds in surface waters has been regulated in the EU (Directive 2000/60/CE). Specific migration limits in food have been established for BADGE and its derivatives. However, the use of BFDGE is prohibited, due to the lack of toxicological information (Directive 2002/16/CE).

The polarity of these compounds and their low concentrations in environmental, food, or biological samples makes it difficult to devise appropriate analytical methods. Generally, extraction techniques such as liquid–liquid extraction (LLE), solid phase extraction (SPE), or solid phase microextraction (SPME) are used to isolate and concentrate these compounds. Subsequently, highly efficient separation techniques, such as gas chromatography (GC) and liquid chromatography (LC), mainly coupled to mass spectrometry (MS), are used to identify and quantify them. The method of choice basically depends on the polarity characteristics of the compounds. GC is appropriate for volatile non-polar and moderately polar compounds, whereas LC is the technique of choice for non-volatile and more-polar compounds, although GC is also used after derivatization.

Gas chromatography coupled to mass spectrometry (GC–MS) is the technique most frequently used in the analysis of most the contaminants studied in this review, even those with low volatility. In such cases, a derivatization step is added to increase the volatility and to improve the sensitivity of MS. However, different derivatizing reagents must be used for different target analytes. In addition, derivatization requires additional manipulation of the sample, which increases the analysis time and reduces reproducibility. Therefore, the number of methods based on liquid chromatography coupled to mass spectrometry (LC–MS) has increased in recent years. Atmospheric pressure ionization (API) sources, combined with analyzers such as single quadrupole (Q), triple quadrupole (QqQ), ion trap (IT), and time-of-flight (TOF), offer a wide range of instruments for use in the LC–MS and LC–MS/MS analysis of most of the compounds included in this review.

This article reviews the GC–MS and LC–MS methods to analyze some phenolic compounds, with special emphasis on the MS behavior of the analyzed compounds, and on strategies to improve selectivity and sensitivity. The large amount of literature on the analysis of phenol-related compounds led to the selection of the information included in this review. The main literature sources are reviews from recent years (de Voogt, de Beer, & van der Wielen, 1997; Petrovic & Barcelo, 2001; Petrovic et al., 2002a; Lopez de Alda et al., 2003; Petrovic, Schroeder, & Barcelo, 2003c; Eljarrat & Barcelo, 2004; González, Barceló, & Petrovic, 2007; Ballesteros-Gómez, Rubio, & Pérez-Bendito, 2009; Covaci et al., 2009) and research articles that reported new analytical developments, published in the period 2000–2008. However when required, literature published before this time has also been used. The review is divided into two main sections. The first is devoted to GC–MS analysis, and includes a short discussion on derivatizing reagents, the most commonly employed ionization sources and analyzers, and a thorough description of the most characteristic ions used for identification and quantitation purposes. The second section reviews the LC–MS methods proposed to identifying and characterize several classes of phenolic compounds. Several items, such as ion-suppression effects, the most characteristic ions generated in the API sources, and the use of tandem mass spectrometry for identification and quantitation, are also examined in detail. Finally, a short section is devoted to quantification procedures, and some future perspectives are discussed.

II. GAS CHROMATOGRAPHY–MASS SPECTROMETRY

A. Chromatography

For years, gas chromatography coupled to low-resolution mass spectrometry (LRMS), with electron ionization (EI) or negative ion chemical ionization (NICI), has been the most popular technique to analyze the compounds included in this review, mainly due to its high separation capabilities and sensitivity. An

overview of selected analysis methods, including information on derivatization, gas chromatography and mass spectrometry procedures, as well as quantitation method and detection limits, is given in Table 2.

Gas chromatography–mass spectrometry (GC–MS), can be directly applied to the analysis of alkylphenol ethoxylates (AP*n*EOs) with a low number of ethylene oxide groups, and to some metabolites such as NP and OP. For instance, GC–MS has been used to determine short-chain lipophilic metabolites of NP*n*EOs, such as NP, NP1EO, and NP2EO in environmental samples (Planas et al., 2002) and to analyze NP and OP in baby foods (Li, Cheng, & Ding, 2008). Few studies have been published on the determination BADGE, BFDGE, and related compounds with GC–MS. However, direct analysis without derivatization has often been performed. For the separation of all these underivatized compounds, a 5% phenyl–methyl polysiloxane column is usually used. For instance, some authors (Brede et al., 2002) analyzed BFDGE in food with this column and achieved enough resolution to separate the three isomers of BFDGE (*o,o′*-BFDGE, *o,p′*-BFDGE, and *p,p′*-BFDGE). In some cases, moderated polar columns are recommended for better performance. As an example, a 50% phenyl–methyl polysiloxane column has been proposed for the direct analysis of BPA and 4-*n*-OP (Morales-Muñoz et al., 2005).

Although good chromatographic performance and peak shape can be obtained for bisphenol A and its derivatives (Hernando et al., 2004; Stuart et al., 2005), some authors recommend the derivatization of these compounds to improve GC–MS sensitivity (Stuart et al., 2005). In contrast, in the analysis of long-chain alkylethoxylates and their polar metabolites, a derivatization step must be included to improve peak shape and volatility. A wide range of procedures has been used (e.g., methylation, acetylation, and silylation) to analyze the compounds included in this article. Silylation is the method most commonly applied because the reaction is fast and quantitative, and yields thermally stable and highly volatile derivatives. *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is the most popular silylation reagent for most of the applications. For instance, BSTFA has been used to analyze 4-NP, 4-*t*-OP, and BPA in tap water and reservoir water (Basheer & Lee, 2004); to determine BPA, its chlorinated derivatives, OP and NP in wastewater (Ballesteros et al., 2006); and to determine 4-*n*-NP, 4-*n*-OP, and 4-*n*-OP in fish bile (Jonsson et al., 2008). Methylation has been also used to analyze alkylphenolic compounds. For instance, the analysis of estrogenic short ethoxy chain nonylphenols and their acidic metabolites (nonylphenolcarboxylates, NP*n*ECs) in water with in-sample derivatization with dimethyl sulfate (DMS) to form methoxy (for NP*n*EOs) and methyl ester (for NP*n*ECs) derivatives has been reported (Diaz, Ventura, & Galceran, 2002a). For example, Figure 1 shows the chromatogram of a river-water sample in which NP, NP*n*EOs, and NP*n*ECs were detected. For the analysis of TBBPA in different biological samples, such as plasma and serum, methylation with diazomethane is the most commonly used procedure (Thomsen, Lundanes, & Becher, 2001; Thomsen, Liane, & Becher, 2007).

Pentafluoro-derivatizing reagents are also used to analyze this family of compounds. The main advantage of pentafluoro reagents over the aforementioned ones is that they convert turn the analytes into highly electrophilic compounds by introducing 5 or 10 fluorine atoms to improve sensitivity and selectivity in mass spectrometry. Lerch and Zinn (2003) examined different fluorine-containing compounds to be used as derivatization agents for the simultaneous analysis of some EDCs, including 4-*t*-OP, NPs, and BPA. They concluded that pentafluorobenzoyl bromide (PFBBr) was selective to phenolic hydroxyl groups, but that the final solution contains many by-products. However, PFBBr has been used to simultaneously determine OPs, NPs, and BPA in water (Boitsov et al., 2004; Boitsov, Mjos, & Meier, 2007) and in biological samples such as biota (Meier et al., 2005), urine, and human serum (Yoshimura et al., 2002; Kuklenyik et al., 2003). Other pentafluoro reagents such as pentafluoropropionic anhydride (PFPA) have been used to derivatize nonylphenol isomers and BPA to determine them in sediments (Peng et al., 2006) and to determine BPA and TBBPA in human serum (Dirtu et al., 2008).

All these derivatization procedures are time-consuming and frequently lead to the formation of by-products during in-sample derivatization (Li, Park, & Oh, 2001; Kojima, Tsunoi, & Tanaka, 2003; Kojima et al., 2005). To minimize any interferences and to improve sensitivity, clean-up after derivatization (Kojima et al., 2005; Jonsson et al., 2008) has been used. To reduce the analysis time, Li, Park, and Oh (2001) developed an on-column derivatization kit, based on a florisil layer that was previously conditioned with BSTFA for simple and fast solid-phase silylation of alkylphenols and BPA in seawater at room temperature. Kojima et al. (2005) used pentafluoropyridine for the solid-phase derivatization of 4-*t*-OP and 4-NP on a C_{18} SPE cartridge. Methods that combine SPME with derivatization have been also reported. Most use in-sample derivatization combined with headspace-SPME: for example, the method proposed to analyze NPEOs and NPECs in water (Diaz, Ventura, & Galceran, 2002b) and that to simultaneously determine TBBPA and TeCBPA in water samples by acetylation–HS–SPME–GC–MS (Polo et al., 2006). Stir bar sorptive extraction (SBSE) followed by *in tube* silylation has been also used to analyze alkylphenols and BPA in environmental samples (Kawaguchi et al., 2005b).

In spite of the high resolution obtained with GC capillary columns, co-elution of different compounds can occur to limit the applicability of the GC method and to make it difficult to interpret mass spectra when GC–MS and even GC–MS/MS are used. In this context, comprehensive two-dimensional gas chromatography (GCxGC) might help to solve the demanding separation of NP isomers when analyzing technical 4-nonylphenols and their (bio)degradation products (Moeder et al., 2006) or the separation of TBBPA from other brominated flame retardants, such as polybrominated diphenyl ethers (Korytár et al., 2005).

B. Mass Spectrometry

Two complementary ionization techniques have been used in GC–MS analysis of the phenolic compounds. EI is the most common ionization source used for quantitation and identification, although NICI (Table 2) has also been applied, mainly to enhance sensitivity, particularly when pentafluoro derivatives are analyzed. In contrast, positive chemical ionization (PCI) has hardly been used to analyze these compounds. Table 3 summarizes the most characteristic ions currently used to

TABLE 2. GC–MS methods

Compound	Sample	Derivatization	Column	Ionization technique	Mass Analyzer	Acquisition mode	Quantitation method	LODs	Ref.
NP, NP1EO, NP2EO	Sludges River sediments Water	_	5% phenyl-methyl polysiloxane	EI	Q	Full-scan	Isotope dilution	4-2122 ng L^{-1}	Planas et al., 2002
NP, NPnEOs, NPnECs,	River water Tap water	DMS	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	0.02-1.5 μg L^{-1}	Díaz et al., 2002b
NPnECs	Standard solutions	BF3/methanol	5% phenyl-methyl polysiloxane	EI NICI (isobutane)	IT	SIM	External calibration	_	Hao et al., 2000
NP, NPnEOs, , BrNP, BrNPnEOs,	Water	_	5% phenyl-methyl polysiloxane	EI	Q	SIM	_	30-150 ng L^{-1}	Díaz et al., 2002a
APs	Fish bile	BSTFA	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	10 ng g^{-1}	Jonson et al., 2008
APs	Water	PFBC	5% phenyl-methyl polysiloxane	NICI (methane)	Q	SIM	Internal standard	1 μg L^{-1} (water) 0.1 μg kg^{-1} (Cod)	Boitsov et al. 2004 ; Meier et al. 2005
APs, BPA	Seawater Sediment	BSTFA	100% dimethyl polysiloxane	EI	Q	SIM	Internal standard	1 μg L^{-1}(LOQ)	Li et al., 2001; Li et al., 2003
APs, BPA	Wine Beer	EOC/PFP	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	1.7-3.2 μg L^{-1}	Paik et al. 2006
APs, BPA	Urine	PFBBr	5% phenyl-methyl polysiloxane	NICI (methane)	Q	SIM	Isotope dilution	0.1-0.7 μg L^{-1}	Kuklenyik et al., 2003
NP , OP, BPA	Surface water Drinking water	PFBCl	5% phenyl-methyl polysiloxane	NICI (methane)	Q	SIM	Isotope dilution internal standard	0.04-0.05 ng L^{-1}	Kuch et al., 2001
NP, BPA	Sewage sludge	Acetic anhydride	5% phenyl-methyl polysiloxane	EI	IT	SIM	Internal standard	0.125 ng kg^{-1}	Meesters et al. 2002
NP, BPA	Sediments	PFPA	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	0.1 μg kg^{-1} (BPA) 0.2 μg kg^{-1} (NP)	Peng et al., 2006
NPs	Septic Soils Grownwater	BSTFA	100% dimethyl polysiloxane	EI	IT	Full-scan SRM	Isotope dillution	500 ng L^{-1} (water) 50 ng g^{-1} (soil)	Standford et al., 2008
NPs and biodegration products	Technical mixture	_	GCxGC 5% phenyl (30m) cyanopropil (1.6 m)	EI	TOF	Full-scan	Internal standard	1 μg L^{-1}	Moeder et al., 2006
OP, BPA	Wastewater	BSTFA	5% phenyl-methyl polysiloxane	EI	IT	Product ion scan	Internal standard (matrix-matched)	20 ng L^{-1}	Hernando et al., 2004
OP, NP	Sediments	BSTFA	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	0.1 μg kg^{-1}	Fiedler et al., 2007
OP, NP, BPA	Water Urbam solid	_	5% phenyl-methyl polysiloxane	EI	TOF	Full-scan	Internal standard	0.05 μg L^{-1} (LOQ)	Hernandez et al., 2007
OP, NP, BPA,	Sludge Wastewater	BSTFA	5% phenyl-methyl polysiloxane	EI	IT	SIM SRM	Internal standard	0.15-0.33 ng L^{-1}	Jeannot et al., 2002
OP, NP, BPA, Cln-BPA	Wastewater	BSTFA	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	4-60 ng L^{-1} (CCα)	Ballesteros et al. 2006

(*Continued*)

TABLE 2. (*Continued*)

OP, NP, NP1EO	Air	BSTFA	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	2-8 pg m^{-3}(vapour) 0.6-5 pg m^{-3} (particle)	Xie et al., 2006
OP, NP, OP1EO, NP1EO	Fruits Vegetables Baby food	–	5% phenyl-methyl polysiloxane	EI	Q	SIM	Internal standard	0.2 µg kg^{-1} (LOQ)	Yang et al., 2005 Li et al., 2008
BPA, 4-n-OP	Marine sediments	-	5% phenyl-methyl polysiloxane	EI	IT	SIM SRM	-	0.16-1.0 ng g^{-1}	Morales-Muñoz et al., 2005
BPA	Infant forlmula powders	BSTFA	5% phenyl-methyl polysiloxane	EI	Q	SIM	Surrogate	0,3 ng g^{-1}	Kuo et al., 2004
BPA ClnBPAs	Wastewater	BSTFA	5% phenyl-methyl polysiloxane	EI	Q	SIM	-	-	Fukazawa et al., 2001
Chlorinated BPA	Human plasma	BSTFA	100% dimethyl polysiloxane	EI	Q	SIM	Internal standard	0.5-3.0 µg L^{-1} (ccα)	del Olmo et al., 2005
BPA, TBBPA	Human serum	PFPA	5% phenyl-methyl polisiloxane	NICI (methane)	Q	SIM	–	280-50 ng L^{-1}	Dirtu et al., 2008
TBBPA	Bilogical samples	MSTFA	5% phenyl-methyl polisiloxane	EI	BE	SIM	Isotope dilution	0.06-1.2 ng kg^{-1}	Cariou et al., 2005
TBBPA	Human plasma Serum	Diazomethane	5% phenyl-methyl polysiloxane	NICI (methane)	Q	SIM	Internal standard	0.12-1.1 pg g^{-1}	Thomsen et al., 2001 & 2007
TBBPA	Eggs	methyl chloroformate (MCF)	5% phenyl-methyl polysiloxane	EI	EBE	SIM	Internal standard	1 pg g^{-1} (GC-IIRMS) 10 pg g^{-1} (GC-LRMS) 20 pg g^{-1} (LC-TOF MS)	Berger et al., 2004
BPF, BPA, BADGEs, BFDGEs	Wastewater	BSTFA	100% dimethyl polysiloxane	EI	Q	SIM	Internal standard	0.006-0.13 µg L^{-1}	Vílchez et al., 2001
BADGE and BFDGE	Vegetable oil	-	5% phenyl-methyl polysiloxane	EI	Q	SIM	External calibration	0.015 mg kg^{-1}	Brede et al., 2002

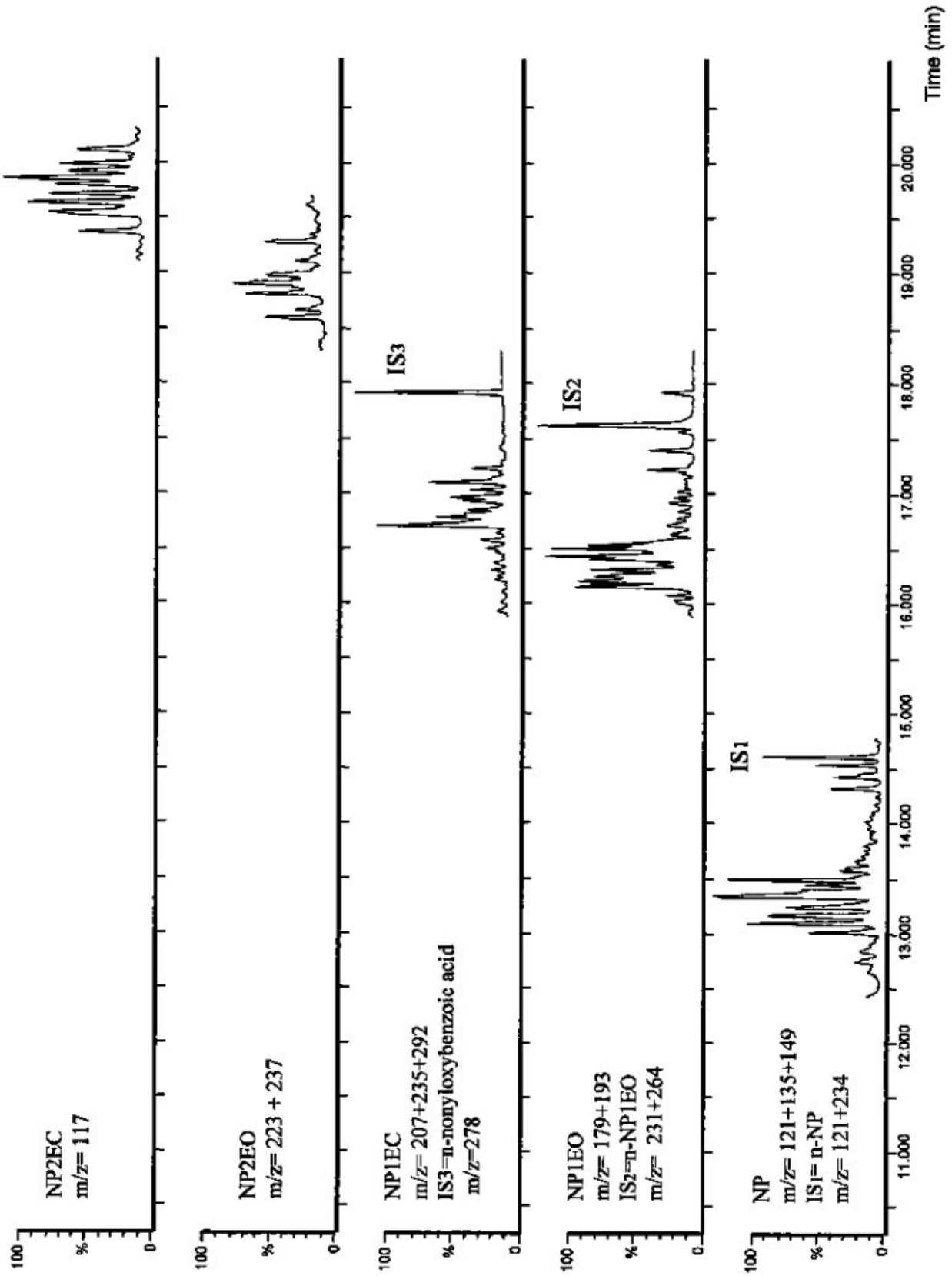


FIGURE 1. HS-SPME-GC/MS of methyl derivatives of NP, NP*n*EOs and NP*n*ECs in river water. Reproduced from the publication of Diaz et al. (2002), with permission from the American Chemical Society, Copyright 2002.

TABLE 3. GC–MS characteristic ions

Compound	EI		PCI		NICI	
	Ion assignment	*m/z*	*Reagent gas:* Ion assignment	*m/z*	*Reagent gas:* Ion assignment	*m/z*
APs	$[M]^{+\bullet}$	*	–		–	
BrAPs	$[M\text{-}COH]^{+}$ (APs and BrAPs)	*				
BrAP*n*EOs	$[M\text{-}C_nH_{2n+1}]^{+}$	*				
(underivatized)	$[C_7H_7]^{+}$	91				
	$[C_6H_5]^{+}$	77				
BrAPs and BrAP*n*EOs	$[M\text{-}C_5H_{11}]^{+}$	*	–		–	
(underivatized)	$[M\text{-}C_6H_{13}]^{+}$	*				
Alkyl, silyl-APs derivatives	$[M]^{+\bullet}$	*	*Methane:*		–	
	$[M\text{-}C_nH_{2n+1}]^{+}$	*	$[M+H]^{+}$	*		
	$[TMS]^{+}$	73	$[M+C_2H_5]^{+}$	*		
Acetyl-APs	$[M\text{-}C_nH_{2n+1}]^{+}$	*	*Methane:*		*Water:*	
			$[M\text{-}C_nH_{2n+1}]^{+}$	*	$[M-H]^{-}$	*
HFB-APs and AP*n*EOs	$[C_3F_3]^{+}$	93	*Methane:*		*Methane:*	
derivatives			$[M\text{-}C_nH_{2n+1}]^{+}$	*	$[COC_3F_6]^{-}$	178
					$[OCOC_3F_6]^{-}$	174
Pentafluorobenzyl-APs and	$[M]^{+\bullet}$	*	–		*Methane:*	
AP*n*EOs derivatives	$[COC_6F_5]^{+}$	195			$[M]^{-}$	*
					$[M\text{-}HF]^{-}$	*
Pentafluorobenzoyl-APs and	–		–		*Methane:*	
AP*n*EOs derivatives					$[M\text{-}CH_2C_6F_5]^{-}$	*
AP*n*EOs	$[M]^{+\bullet}$	*	–		–	
(underivatized)	$[M\text{-}C_nH_{2n+1}]^{-}$	*				
	$[M\text{-}C_6H_{13}\text{-}C_2H_4O]^{+}$	*				
	$[M\text{-}C_5H_{11}\text{-}C_2H_4O]^{+}$	*				
Me-AP*n*EOs derivatives	$[M\text{-}C_nH_{2n+1}]^{-}$	*	*Methane:*		–	
			$[M+H]^{-}$	*		
			$[M+C_2H_5]^{+}$	*		
Me-AP*n*ECs derivatives	$[M]^{+\bullet}$	*	*Methane:*		–	
	$[M\text{-}C_nH_{2n+1}]^{-}$	*	$[M+H]^{-}$	*		
	$[C_2H_4O\text{–}CH_2\text{–}COOCH_3]^{+}$	117	*Amonia:*			
	$[C_7H_7]^{-}$	91	$M+NH_4]^{+}$	*		
	$[C_6H_5]^{-}$	77	*Isobutane:*			
			$[M-H]^{+\bullet}$	*		
			$[M\text{-}C_nH_{2n+1}]^{+}$	*		
			$[C_2H_4O\text{–}CH_2\text{–}COOCH_2]^{+}$	116		
			$[C_7H_7]^{+}$	91		
			$[C_6H_5]^{+}$	77		
But-AP*n*ECs	$[M]^{+\bullet}$	*	–		–	
	$[M\text{-}C_nH_{2n+1}]^{-}$	*				
BPA	$[M]^{+\bullet}$	228	–		–	
(underivatized)	$[M\text{-}CH_3]^{+}$	213				
	$[M\text{-}C_2H_6]^{+}$	198				
	$[M\text{-}C_6H_5O]^{-}$	135				
	$[M\text{-}C_7H_9O]^{-}$	119				
Sylil, acetyl-BPA derivatives	$[M]^{+\bullet}$	*	–		–	
	$[M\text{-}CH_3]^{+}$	*				
	$[M\text{-}Der]^{\bullet}$	*				
	$[M\text{-}Der\text{-}CH_3]^{-}$	*				
PFP-BPA	$[M\text{-}CH_3]^{+}$	505	–		*Methane:*	
					$[M-H]^{-}$	373
					$[M\text{-}H\text{-}PFP]^{-}$	226
					$[PFP]^{-}$	147
Pentafluorobenzyl-BPA	–		–		*Methane:*	
					$[M-H]^{-}$	407
Pentafluorobenzoyl-BPA	–		–		*Methane:*	
					$[M-H]^{-}$	616
Sylil-Cl_nBPA derivatives	$[M]^{+\bullet}$	*	–		–	
	$[M\text{-}CH_3]^{+}$	*				
	$[M\text{-}Der\text{-}CH_3(HCl)_n]^{+}$	*				
	$[C_6H_5O]^{+}$	93				
	$[TMS]^{+}$	73				

TABLE 3. (*Continued*)

Compound	EI		PCI		NICI	
	Ion assignment	*m/z*	*Reagent gas:* Ion assignment	*m/z*	*Reagent gas:* Ion assignment	*m/z*
Cl_nBPA (underivatized)	$[M]^{+\bullet}$	*	–		–	
	$[M\text{-}CH_3]^+$	*				
SylIl-TBBPA	$[M]^{+\bullet}$	688	–		*Methane:*	
	$[M\text{-}CH_3]^+$	673/675			$[M\text{-}Br]^-$	607
	$[M\text{-}C_6H_3Br_2OTMS]^+$	365			$[M\text{-}BrTMS]^-$	536
	$[TMS]^+$	73			$[Br]^-$	79/81
Me-TBBPA	–				*Methane:*	
					$[Br]^-$	79/81
PFP-TBBPA	–		–		*Methane:*	
					$[M]^{+\bullet}$	689
					$[M\text{-}H]^-$	607
					$[M\text{-}CH_4Br]^-$	592
					$[M\text{-}Der]^-$	527
					$[C_3F_5O_2]^+$	163
					$[Br]^-$	79/81
BADGE (underivatized)	$[M]^{+\bullet}$	340	–		–	
	$[M\text{-}CH_3]^+$	325				
p,p-BFDGE (Underivatized)	$[M]^{+\bullet}$	312	–		–	

*Depending on the compound.

identify phenolic compounds and their derivatives. The use of the above mentioned ionization techniques is discussed for each of the family of compounds covered in this review.

Mass spectra of AP*n*EOs, and their (bio)degradation products and halogenated derivatives, in EI and chemical ionization have been reported. Generally, the EI spectra of underivatized and derivatized analytes both show a low intensity molecular ion and a large number of fragment ions that provide rich information to identify individual alkylphenolic compounds. In the EI mass spectra of underivatized alkylphenolic compounds, the main ions result from benzylic cleavage and the loss of the longest alkyl chain moiety $[M - C_nH_{2n+1}]^+$. Ions such as $[M - 85]^+$, formed by the loss of a hexyl group (C_6H_{13}), and $[M - 71]^+$, from the loss of a pentyl group (C_5H_{11}), are commonly used as diagnostic ions. They are very useful to determine the number of ethoxylate units in short ethoxy chain AP*n*EOs. Additionally, ions due to the cleavage of the residual alkyl moiety produced by the loss of CH_2 units are also observed. For instance, ions $[M - 85 - 14]^+$ (*m/z* 121) and $[M - 85 - 28]^+$ (*m/z* 107) for NP and $[M - 71 - 14]^+$ (*m/z* 121) and $[M - 71 - 28]^+$ (*m/z* 107) for OP are usually used as diagnostic ions to identify these compounds in environmental water samples (Serodio & Nogueira, 2004; Hernandez et al., 2007). The molecular ion has also been taken into account to confirm the identity of the analyte, despite its low intensity. For derivatized alkylphenols and AP*n*ECs the EI spectra show a rather weak molecular ion. In addition, important fragment ions are formed by the cleavage of the different alkyl groups. For instance, the fragmentation patterns of alkyl, silyl, and acetyl derivatives are in agreement with those obtained for underivatized compounds (Table 3). However, for highly electrophilic derivatives, characteristic fragment ions can be observed, depending on the derivatizing reagent. For instance, for HFBA derivatives of NP and NP*n*EOs that show the ion $[C_3F_7]^+$ as one of the main ions in the EI spectra (Lerch & Zinn, 2003). Another example is the pentafluorobenzoyl derivatives of AP*n*EOs, which mainly fragment by cleavage of the pentafluorobenzoyl group. The ion $[COC_6F_5]^+$ with *m/z* 195 is the most intense in the spectra. Thus, specific determination of the different isomers is hampered, because the $[M - C_nH_{2n+1}]^+$ ions are only observed at low intensity (Wahlberg, Renberg, & Wideqvist, 1990).

Chemical ionization (CI) is less frequently used, and NICI is the ionization mode preferred when electrophilic reagents are used to derivatize alkylphenolic compounds (Stephanou, 1985; Stephanou, Reinhard, & Ball, 1988). PCI with methane as the reagent gas has been used to detect methylated derivatives of both NP*n*EOs and NP*n*ECs (Table 2). The most characteristic ions are the protonated molecule ion, $[M + H]^+$, and the adduct ion $[M + C_2H_5]^+$, although some fragment ions are also present in the spectra (Stephanou, 1984). Other reagent gases have been proposed. For instance, Field and Reed (1996) recommend the use of ammonia to analyze NP*n*ECs, because molecular ion adducts with ammonia, $[M + NH_4]^+$ are produced, which are the base peak of the spectra with little or no secondary fragmentation, that improve sensitivity. In contrast, if isobutane is used as a reagent gas then the spectra are dominated by $[M - H]^+$ ions that originated from hydride ion abstraction, and no molecular or adduct ions are observed (Hao et al., 2000). NICI has also been used in combination with highly electrophilic derivatives to analyze derivatized alkylphenolic compounds. This chemical ionization technique is highly selective and sensitive, because only highly electrophilic compounds can form negative ions. Pentafluorobenzoyl derivatives generate intense molecular ions, whereas pentafluorobenzyl derivatives produce give ions that result from the loss of the derivate moiety $[M - CH_2C_6F_5]^-$ as the main fragment. In contrast, for heptafluorobutyric acid (HFB) derivatives, the dominating ions are $[COC_3F_6]^-$ (*m/z* 178) and $[OCOC_3F_6]^-$ (*m/z* 194), which correspond to the derivatizing agent. Consequently, no specific identification is possible (Wahlberg, Renberg, & Wideqvist, 1990; Kuch & Ballschmiter,

2001; Meier et al., 2005). Methane is the most commonly used reagent gas in NICI, but other gases have also been employed. For instance, water has been proposed as a reagent gas to analyze OP and NP derivatized with fluorinated reagents (Lerch & Zinn, 2003), to provide intense deprotonated molecule ions $[M - H]^-$. However, the technique seems to be less efficient than EI.

Electron ionization (EI) has been used to analyze underivatized BPA, its halogenated derivatives, and BADGE. The EI spectra of these compounds show, as a base peak, the fragment ion that corresponds to the loss of a methyl group $[M - CH_3]^+$ from the molecular ion (Vilchez et al., 2001; Yamamoto & Yasuhara, 2002; Hernando et al., 2004; Morales-Muñoz et al., 2005). In contrast, spectra of BFDGE isomers show higher fragmentation, and each isomer provides a characteristic mass spectrum that allows its selective identification. Isomers *o,o′*-BFDGE and *o,p′*-BFDGE show the fragment ions at *m/z* 181 and *m/z* 197, respectively, as base peak, whereas for *p,p′*-BFDGE the base peak of the MS spectra corresponds to the molecular ion, at *m/z* 312 (Cottier et al., 1997). BPA and its chlorinated derivatives after silylation have also been analyzed with EI. In this case, the spectra show as the base peak the loss of a methyl group from the cleavage of the isopropyl group (Fukazawa et al., 2001; del Olmo et al., 2005; Ballesteros et al., 2006). The molecular ion with a relatively low intensity (Fig. 2) is used for confirmation purposes.

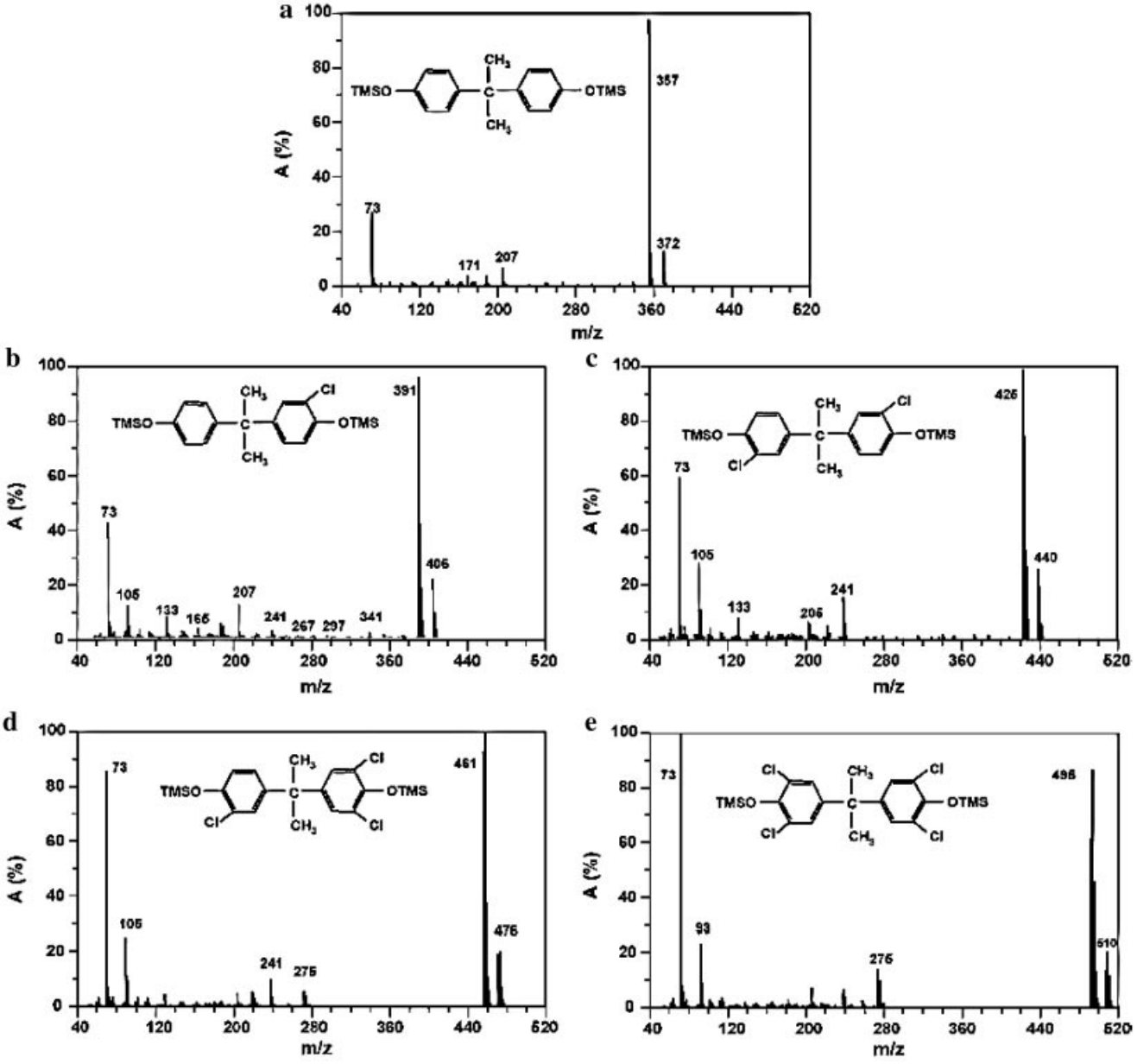


FIGURE 2. Mass spectra of bisphenol and its chlorinated derivatives silylated (**a**) BPA-TMS; (**b**) MCBPA-TMS; (**c**) DCBPA-TMS; (**d**) TCBPA-TMS, and (**e**) TeCBPA-TMS. Reproduced from the publication of Ballesteros et al. (2006), with permission from Elsevier B.V., Copyright 2006.

Moreover, fragments due to the elimination of the derivatized moiety are also observed. For instance, the loss of a trimethylsilyloxyphenyl radical is generally observed for BPA and its halogenated derivatives. Characteristic ions of the silyl derivatizing agent such as those at *m/z* 93/95, related to $[(CH_3)_2SiCl]^+$, or *m/z* 137/139, related to $[(CH_3)_2SiBr]^+$, and that at *m/z* 73 assigned as $[(CH_3)_3Si]^+$ (Fukazawa et al., 2001; Ballesteros et al., 2006; Xie et al., 2007) are also observed.

Negative ion chemical ionization (NICI) has scarcely been used to analyze BPA with GC–MS, but it is generally applied for their fluorinated derivatives. The fragmentation of derivatized BPA under NICI conditions follows different patterns that depend on the derivatization reagent. For instance, the NICI spectrum of the pentafluorobenzyl derivative shows the ion $[M-H]^-$ (*m/z* 407) as a base peak (Yoshimura et al., 2002), whereas that ion that corresponds to the pentafluoropropionic derivative is dominated by a non-specific fragment ion related to the derivatizing reagent $[C_2F_5CO]^-$ at *m/z* 147 (Dirtu et al., 2008). Thus, in the latter case, the low-intensity characteristic ion $[M-H]^-$ (*m/z* 373) is used for quantification purposes to avoid false positives. NICI has been commonly used to analyze TBBPA simultaneously with other flame retardants. Although NICI is a soft-ionization technique, the mass spectra of brominated flame retardants, such as TBBPA, reflects significant fragmentation. The molecular ion is present at very low intensity, and the base peak corresponds to the isotopic cluster of bromide ions at *m/z* 79/81. These ions are currently used to analyze this compound, although they are not specific enough for a selective identification and make quantitation by isotope dilution difficult, because the corresponding labeled compound also provide the same base peaks (*m/z* 79/81). The use of pentafluoropropionic acid as a derivatizing reagent (Dirtu et al., 2008) allows to work with isotope dilution by selecting the molecular ion for quantitation. In this case, the base peak is the ion at *m/z* 163, which corresponds to the derivatizing reagent, whereas $[Br]^-$ ions (*m/z* 79/81) are present at a low intensity.

A survey of the last decade's literature shows that linear quadrupoles are the most-widely used mass analyzers for GC–MS analysis of the compounds reviewed in this article. The low cost, compactness, and simplicity of operation are probably the main reasons for their popularity. Some new developments in quadrupole technology, related to the stability of mass calibration and higher scan-speed and sensitivity, continue to make quadrupoles attractive. These mass analyzers in the full-scan mode, especially with EI sources, are recommended to screen and analyze unknown compounds. The highly fragmented and reproducible spectrum, which is useful for library searching, is a valuable tool for non-target analysis. This procedure is frequently used to analyze technical 4-NP or metabolites of NP*n*EOs (Planas et al., 2002) and brominated derivatives of NP (Diaz, Ventura, & Galceran, 2002a,b) in environmental samples, which require a full-scan spectrum with enough information for unambiguous identification. In contrast, the analysis of target analytes, such as some alkylphenols (4-*t*-OP, 4-*n*-NP) as well as BPA and its halogenated derivatives, at low concentrations in complex matrices demands high sensitivity and selectivity. Therefore, SIM (monitoring at least two or three ions) is the acquisition mode chosen in these cases (Table 2).

Quadrupole ion-trap mass analyzers coupled to GC offer good sensitivity and a relatively high mass range. The high sensitivity in the full-scan mode makes this analyzer attractive for screening. However, the number of articles published on this type of analyzer for phenolic compounds is surprisingly lower than the number of studies that use linear quadrupole analyzers. Nevertheless, the analysis of NPs and BPA (Lerch & Zinn, 2003; Basheer & Lee, 2004; Liu, Zhou, & Wilding, 2004) in solid environmental samples, the analysis of NP*n*EOs and their related metabolites such as NP*n*ECs (Ding & Chen, 1999) in water samples, and the determination of TBBPA, TeCBPA, and other phenolic flame retardants in water samples have been reported (Polo et al., 2006). On the other hand, quadrupole ion-traps offer the ability to manipulate ions during storage to perform multistage mass spectrometry experiments (MS^n). This characteristic was used by Hao et al. to analyze methyl esters of NP1EC and NP2EC by GC–MS^n, and to propose their fragmentation pathway, which is given in Figure 3. They studied the fragmentation up to MS^3 (Hao et al., 2000). Although MS^n is highly selective and sensitive; only a few articles have reported the use of GC–MS/MS using an ion trap. The first was that of Jeannot et al. (2002), who analyzed 4-NP, 4-t-OP, and BPA after derivatization with BSTFA, and found ng/L levels in water. In another article, Hernando et al. (2004) compared GC–MS results of silyl derivatives of 4-*t*-OP and BPA with GC–MS/MS without derivatization. In both cases, ion-trap analyzers were used. The authors concluded, that although both methods are applicable to the analysis of these compounds in wastewater samples, in routine analysis GC–MS/MS represents an easy and fast analytical approach that avoids the derivatization step. In addition, the higher selectivity that contributed to the diminution of matrix interferences in the chromatogram, and the structural information provided by the product ions mass spectra, allows a more reliable confirmation of the target compounds in the samples.

Low-resolution mass spectrometry (LRMS) can successfully solve most of the analytical problems where alkylphenolic compounds, BPA, and its halogenated derivatives are involved. Nevertheless, to achieve low LODs, quadrupole instruments must operate in SIM and ion-trap instruments in the MS/MS mode. Under these circumstances, the determination of a finite number of target compounds can be achieved. However, most of the chemical information on sample composition is lost; these techniques are unfeasible when searching for other analytes (unless additional analyses are performed), because full-scan spectra are required for this aim. Time-of-flight mass spectrometry (TOF-MS) provides a notable amount of chemical information in a single experiment; this technique is very attractive to investigate non-target compounds and to post-target search for analytes. This last approach has only been used in one article for the fast screening and identification of some of the compounds (OPs, NPs, and 4-*t*-OP), among another 60 target organic contaminants in water at low ppb levels (Hernandez et al., 2007). Subsequent investigation of the presence of non-target contaminants in these water samples showed the presence of BPA. High-resolution mass spectrometry (HRMS), using double-focusing electromagnetic instruments, has scarcely been used. Only Cariou et al. and Berger et al. have developed a GC-HRMS method to analyze TBBPA in serum and eggs,

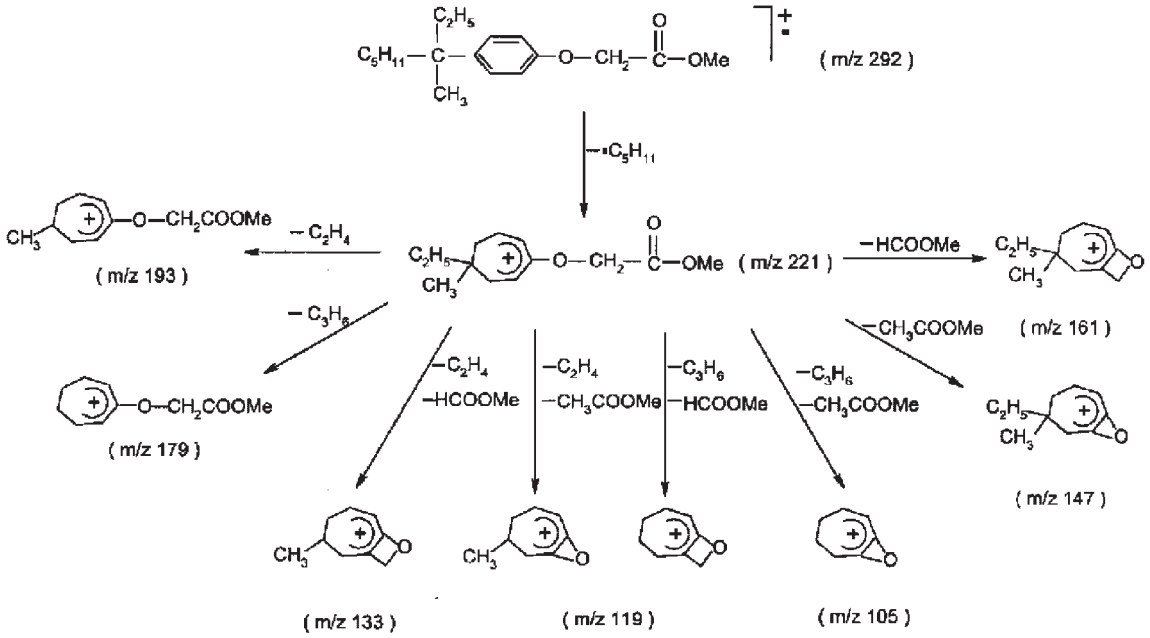


FIGURE 3. Fragmentation pathway of methyl ester NP1EC by GC–MS^n. Reproduced from the publication of Hao et al. (2000), with permission from John Wiley & Sons, Copyright 2000.

respectively (Berger, Herzke, & Sandanger, 2004; Cariou et al., 2005).

One advantage of TOF instruments is their high-speed acquisition, which makes this analyzer perfect for coupling with comprehensive two-dimensional GC (GCxGC). As mentioned above, this technique has been used to improve the separation of 4-nonylphenol isomers and their biodegradation products (Moeder et al., 2006). The very high GCxGC resolution of co-eluting isomers, additionally supported by fast-scanning TOF-MS, provides clear, non-interfered mass spectra of individual isomers. Owing to their structure, 2D chromatograms support the visualization of isomeric and homologous series of compounds to give a detailed insight into highly complex mixtures of NP. Although the identification of new compounds is facilitated by clearer mass spectra with significantly less interference, structure elucidation remains a challenge that requires additional effort, such as derivatization or the use of appropriate reference standards. In contrast, GCxGC-TOF-MS has also been used by Korytár et al. (2005) to analyze brominated flame retardants, including TBBPA.

III. LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

A. Liquid Chromatography

Table 4 summarizes the most significant reported LC–MS methods to analyze the phenolic compounds included in this review. Information on the LC procedure, mass spectrometry sources, analyzers and acquisition modes, quantitation method, and detection limits are included.

Although reversed-phase liquid chromatography (RP-LC) is the most commonly used technique to analyze the phenolic compounds, for alkylphenolic compounds normal-phase LC has been also applied. The advantage of using normal-phase chromatography (silica, amino-bonded or cyano-bonded silica, alumina, etc.) is that alkylphenolic ethoxylate compounds are separated according to the number of ethylene oxide units, whereas oligomers with the same number of ethoxy units but a different alkyl chain (e.g., NP*n*EOs and OP*n*EOs) co-elute (Shang et al., 1999; Wang, Ma, & Wang, 2007). Thus, the information that is provided on the distribution of oligomers in environment samples helps to identify the major contaminating sources. In contrast, the length of the ethylene oxide chain does not influence its behavior in RP chromatography (C8 or C18), where the separation is related to the relation hydrophobicity of alkyl groups (homologue-by-homologue separation). Therefore, oligomers that contain the same alkyl group are not resolved, and are eluted in a single chromatographic peak. Nevertheless, since the last decade, RP chromatography is the most commonly used technique to analyze alkylphenolic ethoxylate compounds. This approach is suitable for routine analysis, because the quantification is easy and the elution of all isomers in a single peak increases sensitivity. Mixed-mode stationary phases, in which both size-exclusion and reversed-phase mechanisms are involved, have also been proposed to analyze NPs and AP*n*EOs to prove a resolution among oligomers similar to that obtained with normal-phase liquid chromatography (Ferguson, Iden, & Brownawell, 2000, 2001) and to allow the chromatographic separation of mass isobaric compounds. These columns have also been used for the simultaneous analysis of OP, NP, AP*n*EOs, and AP*n*ECs in environmental samples (Loyo-Rosales et al., 2003; Lara-Martín, Gomez-Parra, & Gonzalez-Mazo, 2006; Loyo-Rosales, Rice, & Torrents, 2007).

TABLE 4. LC–MS methods

Compounds	Sample type	Liquid Chromatography	Ionization source	Analyzer	Acquisition mode	Quantitation method	LODs	Ref.
APEOs APs, APECs	Sludge Water	Reversed phase: C8 ACN:MeOH:water (NH_4Ac)	APCI (+) ESI (-)	IT	Full-scan	External calibration Internal standard	0.09-0.38 mg kg^{-1} (sludge) 14-193 ng L^{-1} (water)	Cantero et al. 2004 & 2005 & 2006
APEOs, APECs, APs	Wastewater	Reversed phase: C18 (sub 2 μm) ESI(+) MeOH:ACN:NH_4AC/HAc ESI (-) ACN:water	ESI (+) ESI (-)	QTOF	Product ion scan	-	10-100 μg L^{-1}	González et al., 2008
AP*n*EOs	Textiles	Normal phase: ammino n-hexane-isopropanol:isopropanol-water post column addition: NH_4Ac Reversed phase: C18 ACN:water	ESI (+)	Q	SIM	External calibration	0.1-0.4 μg kg^{-1}	Wang et al., 2007
APs AP*n*EOs,	Surface water Sediments	Mixed mode: C18 + size exclusion MeOH:NH_4Ac	ESI (+) ESI (-)	QqQ	SRM	Internal standard	0.004-29 ng L^{-1} (water) 0.02-8.1 ng g^{-1} (sediments)	Loyo-Rosales et al., 2003 & 2007
APs AP*n*EOs	Biota	Mixed mode: C18 + size exclusion MeOH:water (NH_4Ac)	ESI (+) ESI (-)	QqQ	SRM	Internal standard	4-12 ng g^{-1}	Shmitz-Afonso et al., 2003
APs, AP*n*EOs, AP*n*ECs	Water	Reversed phase: C18 MeOH:water (NH_4AC)	ESI (+) ESI (-)	QqQ	SRM	External calibration	0.04-12 ng L^{-1}	Jahnke et al. 2004
APs, AP*n*EOs, AP*n*ECs, XAPs, XAPEOs, XAP*n*ECs, BPA	Sediment Water	Reversed phase: C18 MeOH or ACN:water	ESI (+) ESI (-)	Q	SIM	External calibration	0.5-5 ng g^{-1} (sediments) 20-100 ng L^{-1} (water)	Petrovic et al., 2002a Petrovic et al., 2002b Petrovic et al., 2001
APs, BPA, OP*n*EC, NP*n*EC, OP*n*EO and NP*n*EO; n: 1-3	Wastewater	Reversed phase: C18 ACN:HAc (NP, OP) ACN:water (BPA)	ESI (+) ESI (-)	QqQ	SRM	Internal standard Isotope dilution	1-100 ng L^{-1}	Loos et al., 2007
APs. XAPs, APnEOs	Water Sediments	Reversed phase: C8 MeOH:water	ESI (+) ESI(-)	Q	SIM	Surrogate	0.04-0.92 ng L^{-1}	Ferguson et al., 2000
NP NPnEOs	Sediments	Normal phase: ciano Toluene:toluene-MeOH-water post column addition: toluene-MeOH-water	ESI (+) ESI (-)	Q	SIM	-	2-10 ng g^{-1}	Shang et al., 1999
NP and NPnEOs	Sediments Wastewater	Mixed mode: C18 + size exclusion MeOH:NaAc	ESI (+) ESI (-)	Q	SIM	Surrogate Internal standard	0.78-37.3 ng g^{-1}	Ferguson et al., 2001
NP, XNP NPnEOs, NPnECs, XNPXNPnEOs, XNPnECs,	Water Sludge	Reversed phase: C18 MeOH:water	ESI (+) ESI (-)	QqQ	SIM SRM	External calibration Internal standard	1-20 ng L^{-1} (water) 1-1500 ng g^{-1} (sludge)	Petrovic et al., 2003a Petrovic et al., 2003b
NPnEOs, AP1EC	Sediments Water	Reversed phase: C18 MeOH:HAc + TEA	ESI (+) ESI (-)	IT	Full-scan	-	0.05-0.5 ng ml^{-1} (water) 1-10 ng g^{-1} (sediments)	Lara-Martín et al., 2006
OP, NP, APnEOs	Soils Sewage sludge	Reversed phase: C18 MeOH:water (NH_4Ac)	APCI (+) APCI (-)	Q	SIM	-	0.3-30 μg kg^{-1}	Andreu et al., 2007
BPA, NP, OP	Surface marine Water	Reversed phase: hydro-RP MeOH:water (NH_4Ac)	ESI (-)	QqQ	SRM	Internal standard	0.04-0.14 ng L^{-1}	Beck et al., 2005

(*Continued*)

TABLE 4. (*Continued*)

BPA, OP	Human serum	Reversed phase: C18 MeOH:water	APCI (-)	Q	SIM	External calibration	0.05-0.1 ng mL^{-1}	Liu et al., 2006
BPA, OP, NP	River water	Reversed phase: C18 ACN:NH_4For	ESI (-)	QqQ	SRM	Internal standard	0.5-20 ng L^{-1}	Benijts et al., 2004
BPA APs	Beverages, eggs, milk and meat	Reversed phase: C18 MeOH:water (NH_4OH)	ESI (-)	QqQ	SRM	Internal standard	0.01-0.3 ng L^{-1}	Shao et al., 2005 & 2007a & 2007b
BPA	Human semen	Reversed phase: C18 ACN:HAc	ESI (-)	Q	SIM	Isotope dillution	0.1 ng mL^{-1}	Inoue et al., 2002
BPA	Milk	Reversed phase: C18 MeOH:water	ESI (-)	Q	SIM	Isotope dilution	5 ng g^{-1}	Maragou et al., 2006
BPA	Urine	Reversed phase: C18 ACN:water	ESI (-)	QTOF	Full-scan Product ion scan	-	-	Nielen et al., 2004 Anal. chem
BPA	Air	Reversed phase: C18 ACN:HAc	ESI (-)	QqQ	SRM	Isotope dilution	0.0035 µg/filter	Sabatini et al., 2005
BPA	Wastewater	Reversed phase (RP) MeOH:NH_4Ac	ESI (-)	QqQ	SRM	External calibration	10 ng L^{-1}	Trenholm et al., 2008
BPA, TeCBPA, TBBPA	River water	Reversed phase: C18 ACN:water	ESI (-)	Q	SIM	Surrogate	5-10 pg mL^{-1}	Sambe et al., 2006
BPA XnBPAs	Wastewater	Reversed phase: C18 MeOH:water	ESI (-)	IT	Product ion scan	Standard addition	0.016-0.38 µg L^{-1}	Gallart-Ayala et al., 2007
BPA BrnBPAs	Sediments biota	Reversed phase: C18 MeOH:water	ESI (-)	Q-Trap	SRM EPI	-	-	Guerra et al., 2008
BPA, XnBPAs	Sediments	Reversed phase: C18 MeOH:water	ESI (-)	QqQ	SRM	Surrogate	0.02-0.15 ng g^{-1}	Chu et al., 2005
TBBPA	Marine biota	Reversed phase: C18 ACN:water	ESI (-)	QqQ	SRM	Surrogate	0.11-0.79 ng/g	Frederiksen et al., 2007
TBBPA	Tissues	Reversed phase: C18 MeOH:NH_4Ac	ESI (-)	QqQ	SRM	External calibration	0.01 pg g^{-1}	Johnson-Restrepo et al., 2008
TBBPA	Sediments Sewage sludge	Reversed phase: C18 MeOH:water	ESI (-)	IT	Product ion scan	External calibration	6-18 ng mL^{-1}	Saint-Louis et al., 2004
TBBPA	WEEE*	Reversde phase: C18 MeOH: NH_4Ac	APCI (-)	QqQ	SRM	External calibration	-	Schlummer et al., 2005
TBBPA	Air	Reversed phase: C18 MeOH: NH_4Ac	ESI (-)	Q	SIM	Isotope dilution	-	Tollbäck et al., 2006
BPA; BADGEs	Canned food	Reversed phase: C18 (sub 2 µm) MeOH:NH_4Ac (BADGEs) ΛCN/water (BPΛ analysis (NI))	ESI (+) ESI (-)	QqQ	SRM	External calibration	0.39-0.69 ng g^{-1}	Yonekubo et al., 2008
BADGEs and BFDGEs	Cans	Reversed phase: C18 ACN:water	APCI (+) APCI (-)	Q	SIM	External calibration	0.05-1 mg L^{-1}	Sendón García et al., 2004 & 2005
BADGEs BFDGEs	Topical dosage forms	Reversed phase: C18 MeOH:NH_4For	ESI (+)	QqQ	SRM	Matrix matched	0.3-3.4 µg L^{-1}	Søeborg et al., 2006
BADGEs, BFDGEs	Canned food	Reversed phase: C18 ACN:water	APCI (+)	QqQ	SRM	External calibration	0.25-1 ng g^{-1}	Pardo et al., 2006

For the analysis of BPA and its halogenated derivatives, as well as bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE), and their chlorohydroxy- and hydrolyzed derivatives, RP-LC is always used. The mobile-phase organic modifier has an important effect on the elution of diglycidylethers. For instance, Lintschinger and Rauter (2000) reported a change in the elution order of BADGE·H_2O and BADGE·HCl·H_2O when methanol was used instead of acetonitrile; that difference is probably due to the relative hydrophobicity of both solvents. In methanol, BADGE·H_2O appears before BADGE·HCl·H_2O and BFDGE elutes between these compounds to provide a good resolution among the three compounds. In general, for the analysis of this family of compounds, methanol provides good chromatographic profiles for the different isomers of BFDGE and its derivatives (Lintschinger & Rauter, 2000; Leepipatpiboon, Sae-Khow, & Jayanta, 2005).

Most of the columns used in LC–MS to analyze the compounds included in this review are of small internal diameter (2.1-mm i.d.), with a particle size of 3.5–5 μm. However, the use of a capillary column to determine BPA in air samples has been reported (Sabatini, Barbieri, & Violante, 2005). The low flow rate required by capillary columns provided the maximum ionization efficiency in electrospray ionization (ESI), and consequently higher sensitivity was obtained. Recently, sub-2 μm particle size columns have been used to improve resolution and decrease analysis time, because these columns provide better peak efficiency. As an example, a reversed-phase column with 1.7 μm particle size packaging has been used to analyze BPA, BADGE, and their derivatives in canned foods. The compounds were separated in less than 8 min (Yonekubo, Hayakawa, & Sajiki, 2008). This column has also been used to determine surfactants such as NP*n*EOs, NP, OP, NP1EC, and OP1EC in textile wastewater (González et al., 2008). In addition, Norton and Shamsi (2007) recently proposed the use of electrochromatography (CEC) coupled to mass spectrometry (CEC–MS) to analyze a triton X (TX)-series. The different oligomers OP*n*EOs (*n*: 1–70) were well-resolved with a reversed-phase CEC column. The direct sample injection using flow injection analysis (FIA) coupled to mass spectrometry has also been proposed for the screening of these compounds in water samples (Barco et al., 2003). Though care must be taken to correct ion suppression and isobaric interferences.

B. Mass Spectrometry

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most common ionization sources in LC–MS. Nevertheless, because ESI generally provides better sensitivity than APCI, the ESI source is preferred for the LC–MS analysis. The positive ionization mode is usually used to analyze AP*n*EOs, BADGEs, and BFDGEs, whereas APs, BPA, AP*n*ECs, and their halogenated derivatives are commonly analyzed in the negative mode. However, for the simultaneous analysis of APs and AP*n*EOs, an LC–MS method has been developed that uses a mixed-mode column and switching dual polarity (Loyo-Rosales et al., 2003). This method monitor compounds in a single run (OP and NP in negative-ESI, and AP*n*EOs in positive-ESI) to reduce the analysis time. Other ionization sources have scarcely been used; for instance, only one article has been published (Debrauwer et al., 2005) on the use of atmospheric pressure photoionization (APPI) to identify new polymeric photodegradation compounds of TBBPA that could not be ionized with ESI or APCI.

Ion suppression is one of the major problems in LC–MS with API sources, especially ESI. Suppression occurs because of competition among several ions in the ionization source. Due mainly to the presence of buffer additives, sample matrix components, and poor chromatographic separation of analytes, ion suppression can occur. For instance, it has been reported that the addition of acetic acid and ammonium acetate to the mobile phase strongly decreases the response of NP, OP, and BPA and its halogenated derivatives when negative ESI is used in combination with methanol as organic modifier (Chu, Haffner, & Letcher, 2005; Shao et al., 2005; Gallart-Ayala, Moyano, & Galceran, 2007; Guerra et al., 2008). This phenomenon is more important for BPA than for its halogenated derivatives, because the presence of halogen atoms in the molecule decreases the pK_a value to thus improve ESI. In contrast, some authors reported that, when acetonitrile was used, the addition of acetic acid or ammonia to the mobile phase did not produce any variation in the signal of BPA (Laganà et al., 2004; Shao et al., 2005). However, higher responses are always observed when methanol was used rather than acetonitrile (Chu, Haffner, & Letcher, 2005; Shao et al., 2005; Maragou et al., 2006); that higher responses is probably attributable to the lower surface tension of methanol. For compounds that are analyzed in positive ionization mode, such as AP*n*EOs, BADGE, BFDGE, and their derivatives, the use of additives such as ammonium and sodium salts in the mobile phase to improve chromatographic performance has been recommended, and no decrease in the response has been reported. Moreover, some authors reported the enhancement of the negative-ESI responses of BPA and APs with post-column addition of strong basic solutions. For instance, Laganà et al. (2004) noted a significant increase in the response of NP with the post-column addition of 40 mM ammonia solution and Carabias-Martinez, Rodriguez-Gonzalo, and Revilla-Ruiz (2004) proposed the use of a 2 M 1,8-diazabicyclo-[5.4.0]-7-undecene (DBU)-methanol (1:20) solution, to increase the BPA response. The deprotonation of the compounds is favored under these strong basic conditions. However, the high ionic strength can produce a high electrospray current, which limits the operating voltage. Moreover, the low volatility of some of these additives can produce contamination of some mass spectrometer components. Other post-column approaches have been proposed to improve positive-ESI responses (Shang et al., 1999; Wang, Ma, & Wang, 2007). For instance, the post-column addition of aqueous buffer solutions to non-polar hydro-organic mobile phases has been recommended for the analysis of AP*n*EOs when normal-phase liquid chromatography is used.

Regarding the ion suppression caused by the matrix effect, the co-elution of matrix components can interfere with the signal of the analytes. In this case, improved sample preparation and increased chromatographic selectivity are the two most-effective ways to circumvent this problem. For instance, when analyzing BPA and its halogenated compounds in surface water, the signals of BPA and MCBPA were improved after modifying the mobile

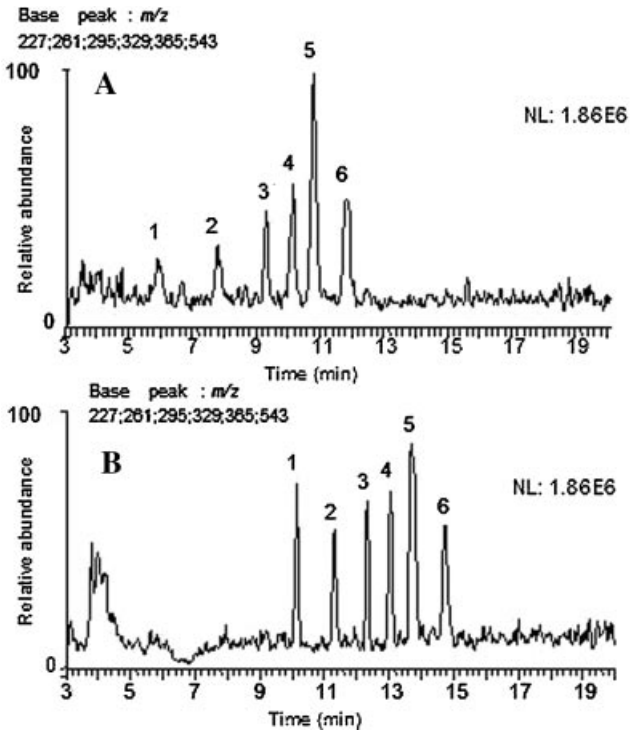


FIGURE 4. Full-scan LC–MS of river water spiked with BPA and its chlorinated derivatives using two gradient elution programs. Peak numbers: 1. BPA, 2. MCBPA, 3. DCBPA, 4. TCBPA, 5. TeCBPA, and 6. TBBPA. Reproduced from the publication of Gallart-Ayala, Moyano, and Galceran (2007), with permission from John Wiley & Sons, Ltd., Copyright 2007.

phase gradient to increase the retention of the analytes and to force their elution into a cleaner chromatographic area to thus avoid co-elution with matrix components in the eluting front (Gallart-Ayala, Moyano, & Galceran, 2007). Figure 4 shows the LC/MS chromatograms that corresponds to the analysis of a water sample, using two different gradient elution programs, where this effect is clearly shown. Nevertheless, it is not always possible to separate the analytes from chromatographic interferences. In such cases, the sample treatment must be improved to overcome matrix effects. For instance, the use of an on-line column-switching system with restricted access material (RAM) has been proposed to remove humic acids and other macromolecular matrix components from sediment extracts when analyze some AP*n*EOs, APs, AP*n*ECs, and their chlorinated and brominated derivatives in sediments (Petrovic, Tavazzi, & Barcelo, 2002c). Moreover, RAM materials have also been proposed for on-line LC–MS determination of BPA and 4-OP in human serum samples with less influence of the matrix (Liu et al., 2006).

Because soft ionization occurs in API techniques, mass spectra with only one cluster ion related to the intact molecule are generally obtained that provide molecular weight information. In general, negative-ESI and negative-APCI spectra of APs, AP*n*ECs, BPA, and their halogenated compounds are dominated by the deprotonated molecule ion $[M-H]^-$, and no further fragmentation is usually observed. Moreover, the overall sensitivity of the halogenated compounds is generally higher than that of the non-halogenated ones. Although negative-ESI is not frequently used for the analysis of BADGEs, the application of this ionization technique for the analysis of BADGE·$2H_2O$ has been reported (Inoue et al., 2001). In this case, the spectrum was dominated by the acetate adduct $[M+CH_3COO]^-$ instead of the deprotonated molecule ion $[M-H]^-$. In negative-APCI, even under mild conditions, BPA and its halogenated compounds suffer in-source fragmentation to yield ions in agreement with those obtained in tandem mass spectrometry (MS/MS). This behavior is more pronounced for the highly halogenated BPAs (TeCBPA and TBBPA) than for BPA itself. Some authors have taken advantage of this in-source fragmentation for confirmatory purposes when single quadrupoles were used as analyzers. For instance, the identity of BPA and its chlorinated derivatives have been confirmed in wastewater samples by monitoring characteristic in-source fragment ions (Zafra-Gomez et al., 2008), and TBBPA has been identified and determined in electronic equipment with the same strategy (Schlummer et al., 2005). Some chlorinated and hydroxylated bisphenol diglycidyl ethers also suffer from significant in-source fragmentation under negative-APCI conditions. Although the base peak of the spectra is generally $[M-H]^-$, other abundant characteristic fragment ions are also present, particularly those from the cleavage of the central alkyl chain (Sendon Garcia & Paseiro Losada, 2004; Sendon Garcia, Perex Lamela, & Paseiro Losda, 2005).

As mentioned above, AP*n*EOs are usually analyzed with ESI and APCI in the positive ion mode. In general, positive-ESI provides better sensitivity and specificity than positive-APCI for these compounds (Petrovic et al., 2001). Nevertheless, several authors (Cantero, Rubio, & Perez-Bendito, 2004; Andreu et al., 2007) recommend the use of APCI for the analysis of these compounds in sewage sludge because it is less sensitive to matrix interferences. Bisphenol diglycidylethers as well as AP*n*EOs tend to form adducts and clusters with positive ionization. For instance, positive-APCI spectra of AP*n*EOs show a high number of adduct ions, $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, and cluster ions such as $[M+(H_2O)_n+H]^+$ originated from buffer, sample components, and also from the LC–MS system. Therefore, with APCI, the ionization of AP*n*EOs is dispersed among many adduct ions with highly variable abundance, to produce a significant decrease in sensitivity and to make quantitation difficult. Acetonitrile cluster ions $[M+CH_3CNH]^+$ of BADGEs and BFDGEs have been used as quantifiers to analyze these compounds in metal packaging, as well as precursor ions when these compounds are determined with LC–MS/MS (Sendon Garcia & Paseiro Losada, 2004; Sendon Garcia, Perex Lamela, & Paseiro Losda, 2005; Pardo et al., 2006). Another effect observed in positive-APCI is that the mass spectra of BADGE·2HCl and BFDGE·2HCl are identical to those obtained for BADGE and BFDGE, respectively. This equivalence can be explained by the loss of chlorine atoms, due to a nucleophilic attack by the hydroxyl group, to thus generate BADGE and BFDGE in the APCI source (Sendon Garcia & Paseiro Losada, 2004; Sendon Garcia, Perex Lamela, & Paseiro Losda, 2005; Pardo et al., 2006).

When ESI is used in combination with aprotic solvents such as acetonitrile (without any additives), AP*n*EOs and their halogenated derivatives show a great affinity for alkali metal

ions, and the mass spectra are always dominated by the $[M+Na]^+$ ions. If protic solvents such as methanol are used, then $[M+Na]^+$ is also the main ion; although the protonated molecule ion $[M+H]^+$ and other ions such as $[M+K]^+$, $[M+NH_4]^+$, $[M+(H_2O)_n+Na]^+$, and $[M+(H_2O)_n+H]^+$ are also present in the spectra. For highly ethoxylated AP*n*EOs, double-charged ammonium or sodium adduct ions, such as $[M+2NH_4]^{2+}$ and $[M+2Na]^{2+}$, have been also described. Mono-charged ions are currently used for quantitation purposes, as occurs, for instance, in the routine analysis of AP*n*EOs in environmental samples, where $[M+Na]^+$ (Petrovic et al., 2002b; Cespedes et al., 2006, 2008) and $[M+NH_4]^+$ (Andreu et al., 2007) have both been monitored. SIM based on these adduct ions is also used to quantify the individual homologs of OP*n*EOs and NP*n*EOs with single-quadrupole mass analyzers. Moreover, information about the ethoxy chains can be obtained by registering the full-scan spectra, because these spectra display a series of masses shifted 44 Da, which corresponded to (CH_2CH_2O). This profile makes it possible to determine the length of the ethoxylate chains and the distribution of the different homologues of AP*n*EOs in a single chromatographic peak (Cespedes et al., 2006; Andreu et al., 2007). Sodium adducts are also frequently observed in the positive-ESI spectrum of BADGEs and BFDGEs. These ions are relatively stable, and generally no further fragmentation is observed.

In LC–MS, single-quadrupoles are not as popular as in GC–MS, because API techniques produce soft ionization, and not many fragments are usually observed. Thus, structural information, that is needed for confirmatory purposes is absent. Nevertheless, SIM based on the main ions (protonated, deprotonated, or adduct ions) and in-source collision-induced dissociation (CID) fragmentation to generate characteristic ions are usually used with these analyzers (Schlummer et al., 2005; Liu et al., 2006; Zafra-Gomez et al., 2008). For instance, for the analysis of AP*n*ECs and their halogenated derivatives in wastewater, it has been proposed to increase the extraction voltage to force the in-source fragmentation by CID (Petrovic & Barcelo, 2001; Petrovic et al., 2001). Under the in-source CID conditions, intense signals are produced due to the loss of the carboxylated (ethoxy) chain, $[M-H-CH_2COO]^-$ for AP1ECs and $[M-H-(CH_2CH_2O)_nCH_2COO]^-$ for AP*n*ECs ($n>1$). The same strategy has been used to obtain characteristic ions for the analysis of BPA and BADGE·H_2O in human plasma (Inoue et al., 2001). For BPA, ions that correspond to the loss of CH_4, $^{\cdot}CH_3$, and C_6H_6O have been obtained, whereas for BADGE·H_2O, the fragment ions are those that correspond to the cleavage of one or two ether bonds (Sendon Garcia & Paseiro Losada, 2004).

Nowadays, the coupling of the TOF analyzer to LC has increased its popularity, but in spite of that, only few articles reported its use for the analysis of alkylphenolic compounds, BPA, and their halogenated derivatives. In these cases, this method was selected to take advantage of its relatively high resolution and high mass accuracy. As an example, LC-TOF-MS has been used to identify new chlorination and bromination products of 4-NP and NP*n*EOs and NP*n*ECs obtained in a halogenation study. The authors recommended the use of a TOF mass analyzer with an average mass accuracy of ~1 ppm to avoid false positives caused by mass isobaric interferences in the analysis of these compounds, because chlorinated derivatives of AP*n*EOs and AP*n*ECs have the same nominal mass, and yield the same ions as brominated compounds with one ethoxy group less (AP($n-1$)EOs and AP($n-1$)ECs) (Thurman, 2008). If low resolution analyzers are used, then chromatographic separation of these two groups of compounds is essential for their quantitative confirmation, because they can only be distinguished by their different isotopic profiles (Petrovic et al., 2001). LC-TOF-MS has been also used to analyze target and non-target compounds in a single run. For instance, BPA degradation products and its chlorinated derivatives (Mezcua et al., 2006) have been identified in samples from a chlorination study.

C. Tandem Mass Spectrometry

Tandem mass spectrometry is the approach used to improve selectivity and sensitivity in the analysis of these compounds in complex matrices at low concentration levels. Triple quadrupole mass analyzers (QqQ) are the most popular instruments, due to their sensitivity and selectivity when operated in selected reaction monitoring (SRM) mode. Nevertheless, other mass analyzers such as iIT, quadrupole-linear ion-trap (Q-Trap) and quadrupole-time-of-flight (Q-TOF) have also been used in LC–MS/MS to analyze the phenolic compounds reviewed in this article (Laganà et al., 2004; Beck et al., 2005; Sabatini, Barbieri, & Violante, 2005; Loos et al., 2006; Frederiksen et al., 2007; Shao et al., 2007b; Johnson-Restrepo, Adams, & Kannan, 2008; Trenholm et al., 2008).

Fragmentation of alkyl phenolic compounds has been exhaustively studied with LC–MS/MS on triple-quadrupole instruments (Loyo-Rosales et al., 2003; Petrovic et al., 2003a; Jahnke, Gandrass, & Ruck, 2004). Surprisingly, ion-traps have scarcely been used for this application (Lara-Martín, Gomez-Parra, & Gonzalez-Mazo, 2006). Precursors and the most characteristic product ions of the phenolic compounds included in this review are given in Table 5. For APs, AP*n*ECs, and their halogenated derivatives, the $[M-H]^-$ ion is always selected as the precursor for tandem mass spectrometry experiments in negative mode. Product ions observed in the MS/MS spectra of APs result from the cleavage of the alkyl chain to generate a series of ions separated 14 Da, due to the sequential loss of CH_2 units. These series go down to *m/z* 93, which corresponds to the characteristic ion, $[C_6H_5O]^-$, of phenolic compounds. The most-abundant product ion of these series depends on the branched alkyl chain, because the fragmentation occurs preferentially on a tertiary carbon. Generally, the ion at *m/z* 133 $[C_9H_9O]^-$, corresponding to $[M-H-C_6H_{14}]^-$ for NP and to $[M-H-C_5H_{12}]^-$ for OP, is the most important fragment in the MS/MS spectra of the commercial mixtures of these compounds. These ions, which come from the neutral losses of hexane (for NP) and pentane (for OP), suggest that the α-carbon of the APs is mainly a tertiary carbon. Therefore, for quantitation purposes, the transitions *m/z* 219 → 133 (for NP) and *m/z* 205 → 133 (for OP) in SRM are used, and a precursor ion scan of *m/z* 133 is generally used for APs screening. Nevertheless, the MS/MS spectra of 4-*n*-OP and 4-*n*-NP show an intense product ion at *m/z* 106, rationalized by the radical loss of the full alkyl chain ($^{\bullet}C_7H_{15}$) and ($^{\bullet}C_8H_{17}$), which yields the radical ions $[M-H-99]^{-\bullet}$ and $[M-H-113]^{-\bullet}$, respectively. The transitions *m/z* 205 → 106

TABLE 5. LC–MS and LC–MS/MS characteristic ions

Compound	Mass spectrometry		Tandem Mass spectrometry	
	Assignment	*m/z*	Assignment	*m/z*
APnEOs	$[M+Na]^+$	*		
	$[M+NH_4]^+$	*	$[M+NH_4\text{-}NH_3]^+$	*
			$[M+NH_4\text{-}NH_4\text{-}C_8H_9O_2]^+$	*
			$[C_6H_6O(C_2H_4O)_nC_2H_5O]^+$	*
			$[C_6H_5\text{–}(OCH_2CH_2)_X\text{–}OH+H]^+$	*
			$[C(CH_3)_3]^+$	57
			$[C_5H_{11}C(CH_3)_2]^+$	113
			$[C(CH_3)_3CH_2]^+$	71
			$[C_6H_{13}C(CH_3)_2]^+$	127
			$[C_3H_7C(CH_3)_2]^+$	85
ClAPnEOs; BrAPnEOs	$[M+Na]^+$	*		
APnECs; (Cl or Br)APnECs	$[M\text{-}H]^-$	*	$[M\text{-}H\text{-}CH_2COO]^-$	*
			$[M\text{-}H\text{-}(CH_2CH_2O)_nCH_2COO]^-$	*
			$[M\text{-}H\text{-}(CH_2CH_2O)_nCH_2COO\text{-}C_6H_{14}]^-$	*
			$[M\text{-}H\text{-}(CH_2CH_2O)_nCH_2COO\text{-}C_5H_{12}]^-$	*
	$[M\text{-}H\text{-}(CH_2CH_2O)_nCH_2COO]^-$	*	$[M\text{-}H\text{-}(CH_2CH_2O)_nCH2COO\text{-}C_6H_{14}]^-$	*
BrAPnECs	$[M\text{-}H\text{-}CH_2COO]^-$	*	$[Br]^-$	79/81
	$[M\text{-}H\text{-}(CH_2CH_2O)_nCH_2COO]^-$	*		
NP	$[M\text{-}H]^-$	219	$[M\text{-}H\text{-}C_5H_{12}]^-$	147
			$[C_9H_9O]^-$	133
			$[M\text{-}H\text{-}C_7H_{16}]^-$	119
			$[C_6H_5O]^-$	93
n-NP	$[M\text{-}H]^-$	219	$[M\text{-}H\text{-}C_8H_{17}]^{-\bullet}$	106
OP	$[M\text{-}H]^-$	205	$[C_9H_9O]^-$	133
			$[M\text{-}H\text{-}C_7H_{15}]^{-\bullet}$	106
			$[C_6H_5O]^-$	93
n-OP	$[M\text{-}H]^-$	205	$[M\text{-}H\text{-}C_7H_{15}]^{-\bullet}$	106
ClNP	$[M\text{-}H]^-$	253	$[M\text{-}H\text{-}C_6H_{14}]^-$	167
			$[M\text{-}H\text{-}C_5H_{12}]$-	181
BrNP	$[M\text{-}H]^-$	297	$[M\text{-}H\text{-}C_6H_{14}]^-$	211
			$[Br]^-$	79/81
BPA	$[M\text{-}H]^-$	227	$[M\text{-}H\text{-}CH_3]^-$	212
			$[M\text{-}H\text{-}CH_4]^-$	211
			$[C_9H_9O]^-$	133
			$[C_6H_5O]^-$	93
Cl_nBPA	$[M\text{-}H]^-$	*	$[M\text{-}H\text{-}CH_3]^-$	*
			$[M\text{-}H\text{-}CH_4Cl]^-$	*
			$[M\text{-}H\text{-}C_2H_4OCl]^-$	*
			$[M\text{-}H\text{-}C_6H_5OCl]^-$	*
Br_nBPA	$[M\text{-}H]^-$	*	$[M\text{-}H\text{-}CH_3]^-$	*
			$[M\text{-}H\text{-}CH_4Br]^-$	*
			$[M\text{-}H\text{-}C_2H_4OBr]^-$	*
			$[M\text{-}H\text{-}C_6H_5OBr]^-$	*
			$[Br]^-$	79/81
BADGE	$[M+CH_3CNH]^+$	382	$[M+CH_3CNH\text{-}CH_3CN\text{-}C_9H_{10}O_2]^+$	191
BADGE·2HCl	$[M+CH_3CNH\text{-}2HCl]^+$	382	$[M+CH_3CNH\text{-}CH_3CN\text{-}C_9H_{10}O_2]^+$	191
BFDGE·2HCl	$[M+CH_3CNH\text{-}2HCl]^+$	354		
BADGE	$[M+NH_4]^+$	358	$[C_{12}H_{15}O_2]^+$	191
			$[C_9H_{11}O]^+$	135
BADGE·H_2O	$[M+NH_4]^+$	*	$[C_9H_{11}O]^+$	135
BADGE·$2H_2O$				

TABLE 5. (*Continued*)

Compound	Mass spectrometry		Tandem Mass spectrometry	
	Assignment	*m/z*	Assignment	*m/z*
BADGE·HCl BADGE·2HCl BADGE·HCl·H_2O	$[M+NH_4]^+$	*	$[C_9H_{11}O]^+$	135
BFDGE	$[M+NH_4]^+$	330	$[C_{10}H_{11}O_2]^+$ $[C_{10}H_{11}O_2\text{-}C_3H_4O]^+$	163 107
BFDGE·2HCl	$[M+NH_4]^+$	402		
BADGE·H_2O·HCl	$[M\text{-}H]^-$	393	$[M\text{-}H\text{-}C_3H_7O_2Cl]^-$ $[M\text{-}H\text{-}C_6H_{11}O_3Cl]^-$	283 227
BADGE·$2H_2O$	$[M\text{-}H]^-$ $[M+CH_3COO]^-$	* 	$[C_{18}H_{21}O_4]^-$ $[C_{15}H_{15}O_2]^-$	301 227

*Depending on the compound.

and *m/z* 219 → 106 have been monitored for the analysis of 4-*n*-OP and 4-*n*-NP in water samples (Benijts, Lambert, & De Leenheer, 2004; Shao et al., 2005, 2007a,b; Loos et al., 2006). Tandem mass spectrometry fragmentation of AP*n*ECs occurred by the loss of the carboxylated (ethoxy) chain, to provide intense product ions that correspond to $[M-H-CH_2COO]^-$ for AP1ECs (*m/z* 219 for NP1EC and *m/z* 205 for OP1EC) and $[M-H-(CH_2CH_2O)_nCH_2COO]^-$ for AP*n*ECs ($n>1$). Moreover, additional fragments such as *m/z* 133 $[M-H-CH_2COO-C_6H_{14}]^-$ and *m/z* 147 $[M-H-CH_2COO-C_5H_{12}]^-$ were also observed, due to the cleavage of the alkyl chain, as described above for APs. For halogenated derivatives of APs and AP*n*ECs, the deprotonated molecule ion cluster yielded doublet signals characteristic of bromine and chlorine isotopes, and their MS/MS fragmentation follow the same pattern as the non-halogenated compounds. However, the chloride ion $[Cl]^-$, which is only present at low intensity when high collision energy is applied, is sometimes monitored to identify chlorinated compounds but with low specificity (Petrovic et al., 2003a). In contrast, the MS/MS spectra of brominated compounds, such as BrNP and BrNP*n*ECs, are dominated by the non-selective bromide ion $[Br]^-$, even at low collision energy. In this case, product ions from the cleavage of the alkyl chain are usually present at low intensity. To increase selectivity, some of the characteristic in-source CID fragment ions are used as precursor ions in tandem mass spectrometry. For instance, to determine ClNP2EC (356 Da) and BrNP2EC (400 Da) in water and sludge samples, ions at *m/z* 253 and 297 $[M-H-(CH_2CH_2O)CH_2COO]^-$ have been monitored for ClNP2EC and BrNP2EC, respectively (Petrovic et al., 2001, 2003a,b).

As mentioned above, positive ESI is the most convenient ionization source for AP*n*EOs, and the most abundant ions generated are ammonium and sodium adducts that depend on the composition of the mobile phase. Nevertheless, sodium adducts $[M+Na]^+$ are more stable than ammonium ones, and no fragmentation in the collision reaction cell is observed, even at high collision energy. Sometimes, ammonium salts are added to the mobile phase to force the formation of ammonium adducts $[M+NH_4]^+$ when MS/MS analysis is required, because these adducts are easy to fragment. Generally, AP*n*EOs loses ammonia to produce the protonated molecule ion, which is fragmented by the alkyl chain-aromatic side bond. For instance, the MS/MS spectra of AP1EOs are dominated by the corresponding alkyl carbocation, $[C_5H_{11}C(CH_3)_2]^+$ (*m/z* 113) for OP1EOs and $[C_6H_{13}C(CH_3)_2]^+$ (*m/z* 127) for NP1EOs. The ion is $[M+H]^+$ intense enough to be used for confirmation (Loyo-Rosales et al., 2003). Other fragment ions probably originated from the cleavage of the alkyl chain ($C(CH_3)_3^+$ at *m/z* 57 and $C(CH_3)_3CH_2^+$ at *m/z* 71) were observed at low intensity. As the ethoxylate chain length increases, the stability of the ammonium adducts also increases, and the fragmentation pattern changes. For instance, for AP*n*EOs with $n=2-7$, the main fragment corresponds to the phenol-ethoxylated moiety $[C_6H_5-(OCH_2CH_2)_n-OH+H]^+$, and for $n>7$, the loss of ammonia from $[M+NH_4]^+$ is the most abundant product ion (Loyo-Rosales, Rice, & Torrents, 2007). Finally, the ion at *m/z* 85 corresponds to another tertiary carbocation, $C_3H_7C(CH_3)_2^+$, which is used to differentiate between NP*n*EOs and OP*n*EOs derivatives, because this ion only appears in the MS/MS spectrum of NP*n*EOs.

Fragmentation of the compounds of the bisphenol family (Table 5) is simpler than that of the alkylphenolic compounds and has been studied with MS^n on IT. However, triple quadrupole instruments are commonly used for quantitation at low ppb levels. For BPA and its halogenated derivatives (MCBPA, DCBPA, TCBPA, TeCBPA, and TBBPA) as well as for BADGEs and BFDGEs, fragmentation pathways have been proposed (Berger & Oehme, 2000; Gallart-Ayala, Moyano, & Galceran, 2007). BPA produces an intense product ion that results from the cleavage of the hydroxy-benzyl group $[M-H-C_6H_5OH]^-$ (*m/z* 133). The ion at *m/z* 93, characteristic of phenolic compounds, is also observed at low intensity. Moreover, the elimination of one aromatic ring in tandem mass spectrometry is another important fragmentation observed for the halogenated derivatives; it provides information on the number of halogen substitutions in each phenolic group. As an example, Figure 5 shows the fragmentation pathway proposed for BPA compounds. As can be seen, the main fragmentation of these compounds starts by the

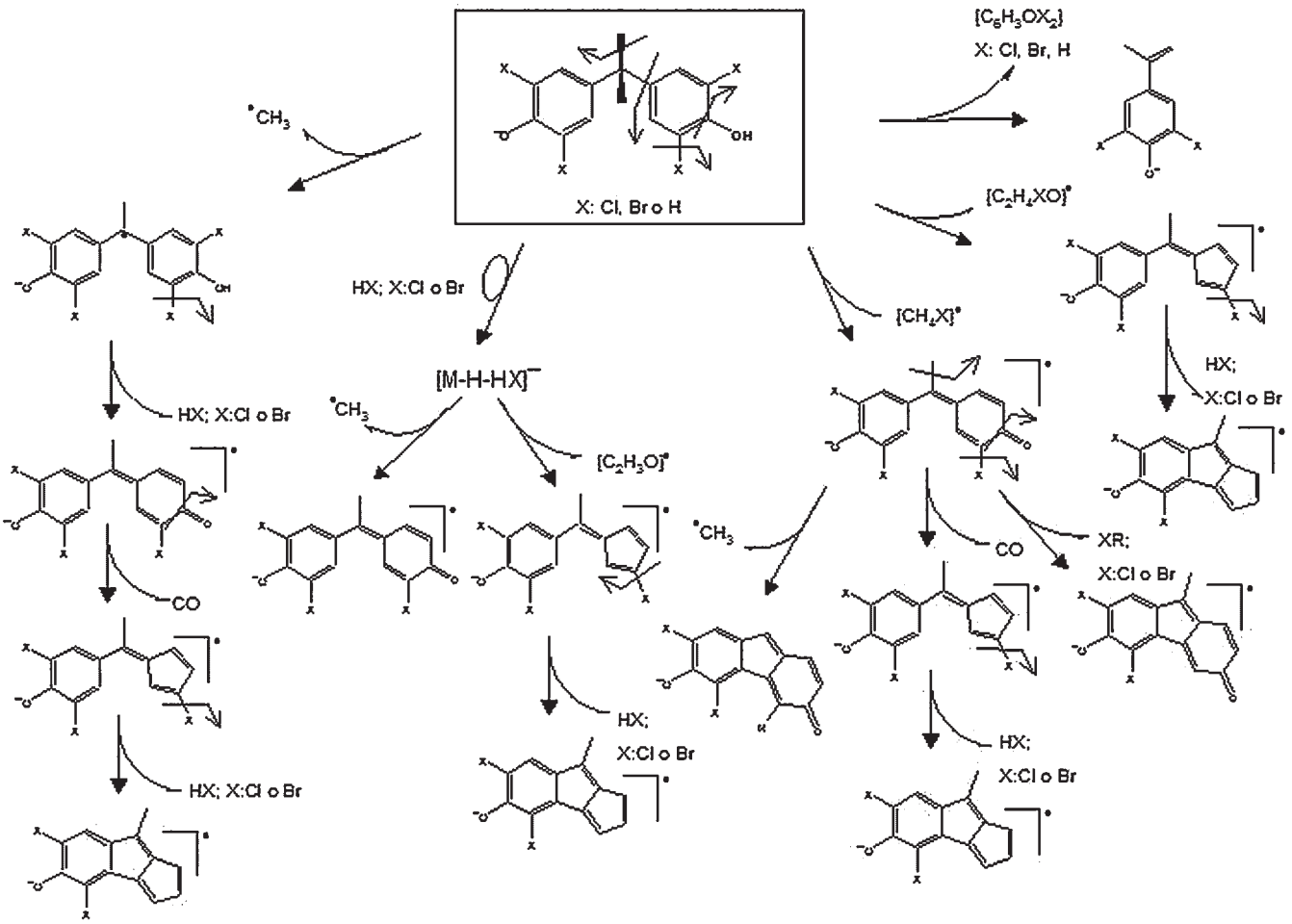


FIGURE 5. Fragmentation pathway of BPA chlorinated derivatives by LC–MS^n using and Ion Trap mass analyzer. Reproduced from the publication of Gallart-Ayala, Moyano and Galceran (2007), with permission from John Wiley & Sons, Ltd., Copyright 2007.

loss of a methyl radical ($^{\bullet}CH_3$) and/or a neutral loss of HX (X=Cl or Br). Later, the 3rd- or 4th-generation product ions yield the characteristic fragmentation of phenolic compounds (neutral loss of CO). The fragmentation of BADGEs and BFDGEs is slightly more complex. These compounds first fragment by the cleavage of the alkyl chain central carbon atom-aromatic ring bond. Then, the consecutive loss of an epoxide group (C_3H_4O) in the MS^3 stage takes place.

It must be mentioned that the MS/MS spectrum of TBBPA obtained in triple quadrupole instruments is quite different from that obtained in an ion trap. The first is dominated by the bromide ion (*m/z* 79/81), whereas other ions are only present at very low intensities. The most intense transition $[M-H]^-$ (*m/z* 543, $^{79}Br_2{}^{81}Br_2) \rightarrow [^{79}Br]^-$ is generally selected as a quantifier, whereas for confirmatory purposes $[^{81}Br]^-$ is monitored. In ion-traps, the limitation on the lowest *m/z* to be registered depends on the *m/z* of the precursor ion. Thus, it is not possible to observe the product ion $[Br]^-$ in the MS/MS spectrum of TBBPA (precursor ion *m/z* 543). In this case, other fragments, such as $[M-H-CH_3]^{-\bullet}$ and $[M-H-CH_3-HBr]^{-\bullet}$, are the main ions of the spectra and are monitored for quantification, although they are originated with less efficiency. These ions are also recommended with triple quadrupoles to increase selectivity for brominated alkyl phenolic compounds, although sensitivity is sacrificed. One characteristic of ion traps is that ion–molecule reactions can occur inside the trap. For instance, the MS/MS spectra of BPA and BPA-d_{16} show ions at *m/z* values higher than the precursor ions (Gallart-Ayala, Moyano, & Galceran, 2007). These ions could be explained by an ion–molecule reaction between the fragment ion, initiated by the loss of the methyl radical and the methanol (mobile phase), remains inside the trap.

Hybrid mass spectrometers have introduced new acquisition modes to increase sensitivity and selectivity, and to provide increased confirmation capabilities. For instance, the Q-Trap has been used to determine BPA and its brominated derivatives in sediments and biota matrices with LC–MS/MS (Guerra et al., 2008). In this work, enhanced product-ion scan (EPI)-precursor ions selected in the quadrupole, and product ions analyzed in the linear ion-trap in combination with information-dependent

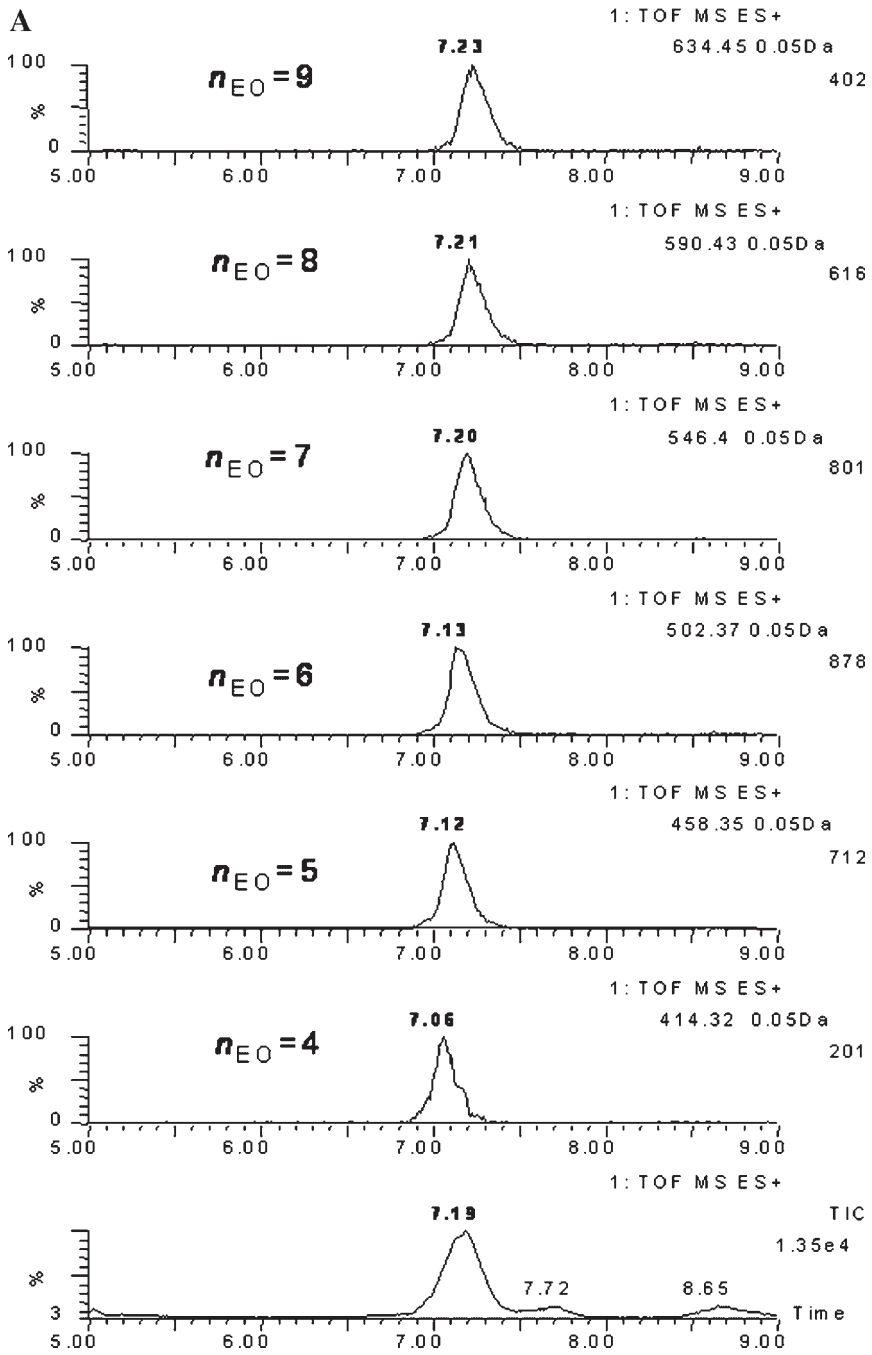
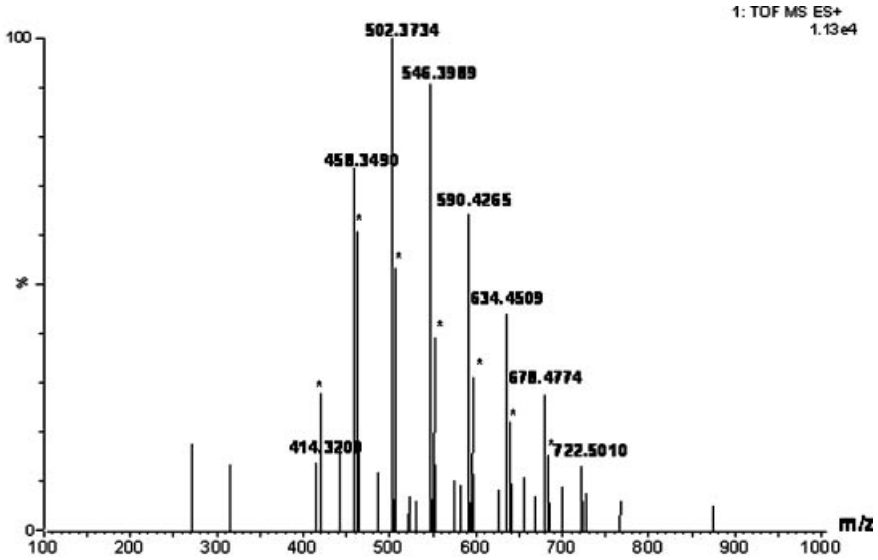


FIGURE 6. Confirmation of NP*n*EOs in textile effluent. (**A**) extracted chromatogram; (**B**) TOF-MS spectrum of NP*n*EOs showing the ammonium and sodium adducts; (**C**) accurate mass measurements. Reproduced from the publication of González et al. (2008) with the permission from John Wiley & Sons, Ltd., Copyright 2008.

acquisition (IDA) were used to develop an LC–MS/MS method that provides better sensitivity (4- to 10-times) than the conventional SRM mode, with the additional advantage that valuable structural information is provided by the product-ion spectra.

Despite advances in LC–MS technologies, problems can still occur in the analysis of complex mixtures. Such problems can be solved with HRMS, which provides additional selectivity. As an example, an LC–MS/MS method has been developed by González et al. (2008) to screen and confirm surfactants with a

B



C

Compound	Elemental composition	Theoretical mass (*m/z*)	Experimental mass (*m/z*)	Error	
				mDa	ppm
$NPEO_4$	$C_{23}H_{44}NO_5$	414.3219	414.3200	-1.9	-4.7
$NPEO_5$	$C_{25}H_{48}NO_6$	458.3481	458.3490	-0.9	-1.1
$NPEO_6$	$C_{27}H_{52}NO_7$	502.3744	502.3734	-1.0	-1.9
$NPEO_7$	$C_{29}H_{56}NO_8$	546.4006	546.3989	-1.7	-3.1
$NPEO_8$	$C_{31}H_{60}NO_9$	590.4268	590.4265	-0.3	-0.5
$NPEO_9$	$C_{33}H_{64}NO_{10}$	634.4530	634.4509	-2.1	-3.3
$NPEO_{10}$	$C_{35}H_{68}NO_{11}$	678.4792	678.4774	-1.8	-2.7
$NPEO_{11}$	$C_{37}H_{72}NO_{12}$	722.5055	722.5010	-4.5	-6.2

FIGURE 6. (*Continued*)

Q-TOF instrument. This method permits reliable quantification, with narrow *m/z* windows for the extracted chromatograms, and high precision in the confirmation, based on accurate mass measurements. In fact, this technology helps to minimize false positives when very complex matrices must be analyzed. It has been used to identify AP*n*EOs, APs, and AP*n*ECs in effluent textile wastewater samples. As an example, Figure 6 shows the extracted chromatograms and the TOF-MS spectrum of NP*n*EOs detected in one of these effluents.

IV. QUANTIFICATION

The phenolic compounds reviewed in this article are very heterogeneous due to differences in chemical structures, polarity, and hydrophobic character. This heterogeneity sometimes forces researchers to tailor specific analytical protocols for their determination. Nowadays, LC–MS methods are generally preferred to analyze these compounds, because generic and fast methods for screening purposes can be developed, and the derivatization steps needed for a proper GC–MS analysis can be omitted. Although GC–MS and LC–MS have been successfully used for quantitative and qualitative analysis of these compounds, some difficulties have been found and some precautions must be taken into account to obtain a precise analysis.

One key prerequisite for accurate analysis is the availability pure synthetic reference compounds for the calibration. The lack of standards is a major problem, especially when dealing with metabolites and/or transformation products. Although tandem mass spectrometry, accurate mass measurement, and library searches can be used to identify compounds, authentic standards are still needed for the final and definitive confirmation and quantitation. Thus, the synthesis of non-available standards has been performed in most cases. For instance, chlorinated and brominated derivatives of BPA (Fukazawa et al., 2001; Eriksson et al., 2004; Kondo et al., 2004; Kubo et al., 2005; Gallart-Ayala, Moyano, & Galceran, 2007) have been synthesized, because only TeCBPA and TBBPA are commercially available. For alkylphenolic compounds analysis, standards are only available for a

small number of isomers (4-*n*-NP, 4-*n*-OP, 4-*t*-OP, and 4-*t*-OP1EO) and some oligomers (NP, NP1EO, and NP2EO). However, for oligomers each standard is composed of several isomers with different hydrocarbon-chain branching. For metabolites and transformation products of alkylphenolic standards must be synthesized for quantitative analysis. For instance, short ethoxy chain AP*n*EOs, AP*n*ECs, and their brominated derivatives have been synthesized for determination in water samples (Reinhard, Goodman, & Mortelmans, 1982; Diaz, Ventura, & Galceran, 2002a,b). Nevertheless, accurate quantitation of the alkylphenol family is still a difficult challenge. Commercial oligomeric mixtures are usually used for calibration, but environmental samples often contain mixtures with a composition that is different from that of the available standards. Quantitation based on the response factors of the available individual standards or the commercial oligomeric mixtures is a common practice. Nevertheless, the response in MS is generally structurally dependent, and the accuracy of the determination of AP*n*EOs is highly dependent on the similarity between the oligomer composition of the reference material and the analyzed sample. For instance, when using an NPEO mixture with an average of 10 ethoxy units as a calibration standard solution for the analysis of NPEO in sewage treatment plant effluents with reversed-phase LC–MS, researchers obtained underestimated concentrations (1.2- to 2.5-times) (Crescenzi et al., 1995). This underestimated means that different environmental samples cannot be quantified with the same oligomeric mixture because the standard and the results obtained with different calibrants vary substantially (Petrovic & Barcelo, 2001). This means that quantitative data are generally accompanied by a relatively high and usually unknown uncertainty. To overcome these problems, the oligomeric distribution of each sample in full-scan mode should be estimated. On the other hand, in GC–MS the high chromatographic resolution provides a series of peaks for each oligomer, which are related to the different isomers. The quantitation is generally performed with the total area of peak isomers, whereas in RP–LC–MS the quantitation seems to be easier, because all the oligomers that contain the same hydrophobic moiety elute in a single peak. This has the advantage of increasing the peak intensity and, therefore, increasing the sensitivity of the determination. However, interference of isobaric doubly charged ions of highly ethoxylated AP*n*EOs with singly charged ions of less ethoxylated APEOs can occur, and an error of up to 40% in the quantification of less ethoxylated NPEOs has been reported (Shang et al., 1999; Jahnke, Gandrass, & Ruck, 2004). With regard to bisphenol diglycidylethers, individual standards are available except for the isomers of BFDGE and their derivatives. For compounds related to BFDGE, standards are isomeric mixtures, and these compounds are always quantified as the isomer mixture.

Some authors recommend the addition of salts to the mobile phase or the use of a specific solvent to ensure reproducible adduct formation, because most of these compounds form adduct ions with sodium, ammonium, and/or acetonitrile. Relative abundances of adduct ions are sensitive to experimental variables that are sometimes difficult to control, such as levels of Na^+, K^+, and/or NH_4^+, solvent impurities, or contamination in the ionization source and in the solvent transfer lines. To overcome these difficulties and to improve reproducibility, the amount of additives must be controlled. For instance, Shang et al. (1999) proposed the addition of NaOAc to force the formation of reproducible sodium adduct ions in an analysis of AP*n*EOs in marine sediments with normal-phase LC–MS, and Pardo et al. (2006) proposed the use of acetonitrile as an organic modifier to guarantee acetonitrile adduct formation $[M + CH_3CNH]^+$ in the analysis of BADGEs and BFDGEs in canned food.

To overcome matrix effects and problems with recoveries in sample treatment, the use of surrogates and quantitation by isotope dilution are generally used if labeled target compounds are available. This approach allows matrix effects and recoveries to be corrected, because labeled and native compounds will both show the same behavior. For instance, BPA-d_{16} has been used as a labeled compound for the analysis of BPA in air samples (Sabatini, Barbieri, & Violante, 2005) and in milk (Maragou et al., 2006) with LC–MS. Additionally, BPA-$^{13}C_{12}$ has been proposed as a surrogate in the LC–MS analysis of water samples (Kawaguchi et al., 2005a). Another compound that is frequently analyzed by isotope dilution is TBBPA. In this case, the labeled standard is TBBPA-$^{13}C_{12}$ (ring), which has been used to LC–MS analyze TBBPA in sediment (Guerra et al., 2008) and marine biota (Frederiksen et al., 2007) samples. However, the isotope-dilution method is less common for alkylphenolic compounds, because isotope-labeled standards remain scarce. Even though several authors have reported the use of isotope-labeled standards. For instance Planas et al. (2002) used isotope dilution for the GC–MS analysis of NP, NP1EO, and NP2EO with $^{13}C_6$-NP*n*EO ($n = 0–2$) as internal standards to quantify these compounds in water samples from a sewage-treatment plant. Kuklenyik et al. (2003) determined OP and NP in human urine with GC–MS with isotope dilution, with labeled compounds 4-*n*-OP-d_{17} to quantify 4-*t*-OP and 4-*n*-OP and 4-*n*-NP-$^{13}C_6$ to quantify 4-*n*-NP and the isomeric mixture of the technical-grade NP.

If labeled standards are not available, then other compounds similar to the target analytes are generally used as the internal standard for quantitative analysis. For instance, 4-heptylphenol has been used to determine NP*n*ECs with LC–MS in environmental samples. Moreover, to overcome matrix effects and recovery problems in quantitation when no labeled standards are available, matrix-matched calibration is a good approach if blank samples are available. This method was used, for instance, by Søeborg, Hansen, and Halling-Sorensen (2006) to LC–MS/MS determine BADGE, BFDGE, and their derivatives in cream samples. Finally, it must be mentioned that standard addition is the most appropriate alternative to correct matrix effects and recoveries. However, this quantification method is not very convenient when a high number of samples must be analyzed, and, therefore, it is not frequently used. As an example, the standard-addition method has been used to analyze BPA and its halogenated derivatives in water (Gallart-Ayala, Moyano, & Galceran, 2007) with LC–MS/MS and to analyze BPA and other ECDs in cereals (Carabias-Martinez, Rodriguez-Gonzalo, & Revilla-Ruiz, 2006) with LC–MS. External calibration seems to be the least suitable quantification method, because errors due to recoveries and MS response are not controlled. However, this method is frequently used to analyze these compounds in different matrices, mainly when a great number of compounds and samples must be analyzed.

The analysis of blank control samples to verify the absence of contamination from the analytical procedure is a general practice, but in some cases it is difficult to perform, because contamination from the laboratory environment can be easily produced. Several compounds can be found in the laboratory material; for instance, phenol and *para*-substituted alkylphenols (*p*-cresol, 4-*tert*-butylphenol and 4-nonylphenol) are monomers in epoxyacrylic polymers that are intensively used in the plastic industry and BPA is used in the production of polycarbonate plastics and epoxy resins. Nonylphenols are found in PVC wrapping films, gloves, and rubber products in considerable concentrations (530–5,550 ppm), and have been identified as one of the major potential migrants from plastics and rubber. As an example, in the analysis of alkylphenols in food samples, a significant concentration of NP from plastic and rubber products used in the laboratory (vinyl gloves, rubber stoppers for glass funnels, and plastic tubes used for the nitrogen evaporation) has been reported (Meier et al., 2005). A similar problem has been observed for BPA, because contamination from the experimental process itself has often been encountered (Inoue et al., 2000; Yoshiyuki et al., 2004; Carabias-Martinez, Rodriguez-Gonzalo, & Revilla-Ruiz, 2006; Shao et al., 2007a).

V. CONCLUSIONS AND FUTURE TRENDS

The different chemical characteristics of phenolic compounds with endocrine-disrupting capabilities included in this review shows that the analytical methods and techniques are tailor-made and that universal procedures have not been established for their determination. Most of the methods reviewed here involve the analysis of a limited number of target analytes mainly of the same family, in a single run. However, the number of compounds under scrutiny probably would be extended much further in the near future because it is expected that the analysis of additional transformation products and metabolites of the parent compounds would be emphasized in future research.

Traditionally GC–MS has been the instrumental technique of choice. However, LC–MS has already reached the status of a routine analysis technique for monitoring and screening purposes, and is nowadays preferred for the analysis of compounds of relatively high polarity with the additional advantage that any derivatization previous to determination is not required. Even though, for alkyl phenolic compounds, GC–MS is still a good choice because this technique provides in addition to high sensitivity, the best resolution between oligomers and isomers. Nevertheless, the high separation capacity of GC is not sufficient to completely separate alkylphenolic isomer mixtures and to analyze complex environmental matrices. In these cases, GCxGC is a good option, and an increase of the number of studies directed towards the application of this technique can be expected in next years. For the rest of the compounds included in this review, the technique of choice is LC–MS/MS, because the selectivity of reversed phase columns is generally enough for their analysis. In relation to alkyl phenolic compounds, because normal phase and reversed phase both did not provide enough chromatographic selectivity, the development of new stationary phases such as the mix-mode ones and even the use of LCxLC methods will offer to the scientific community extra separation capabilities. Moreover, the proved good performance of the new sub-2 μm particle columns will probably direct the LC methods towards fast chromatographic separations such as ultra-performance liquid chromatography (UPLC) because high throughput analysis can profit from the high-resolution and high-efficiency of fast-chromatographic LC methods.

With regard to structural information of the compounds reviewed in this article, alkyl phenolic compounds, BPA, and chlorinated derivatives of BPA are well-documented. The fragmentation studies available with GC–MS and LC–MS provide enough information to establish the ionization and fragmentation behavior of these compounds that can help to identify new related compounds in complex matrices. Nevertheless, the fragmentation behavior of diglycidyl ethers needs more research because the fragmentation pattern of these compounds is not established yet. Moreover, more structural studies are necessary for the characterization of new transformation products and metabolites and their identification and confirmation in complex samples to avoid false positives.

With the recent advances in mass spectrometry, mainly high-resolution instruments with tandem mass spectrometry capabilities, new powerful identification tools have became available-although their environmental applications are still scarce. High resolution has been a challenge for LC–MS, and now the good performance of the new instruments will make possible the introduction of this mass spectrometry technique as a routine in analytical laboratories to provide extra confirmation capabilities. New hybrid high-resolution mass analyzers, such as QTOF, hyperbolic triple quadrupole, Orbitrap, and LIT-FT will enable high mass accuracy to identify of target and non-target compounds in complex matrices and avoid false positives, especially in studies that involve new transformation products and metabolites. With regards to GC–MS advances, the TOF analyzer is not only going to fulfill the accurate confirmation objectives but also the fast acquisition required with GCxGC.

On the other hand, matrix effects are a serious matter of concern for quantitative analysis in LC–MS, and demand sufficient attention during method development. Real sample extracts must be used at an early stage of method development, because the matrix effects might have serious impact on the choice of the most appropriate sample treatment, mass spectrometry ionization source and data acquisition mode. The best way to eliminate the influence of matrix effects on quantitation is the use of stable-isotopically labeled internal standards. Nevertheless, limited availability of such standards excludes the wide and general application of an isotope dilution method. As a result, matrix effects continue to be a challenge in developing reliable quantitation methods, although the use of new ionization sources that are less sensitive to matrix effects is expected to improve this picture in the next future. In addition to the lack of labeled standards, there is also a need for pure alkylphenolic individual standards. The complexity of the mixtures makes difficult a precise quantitation of environmental samples because the response factors are not always identical for the different oligomers, and isomers introduce uncertainty in the quantitative results. Moreover, the samples taken from the

environment usually contain mixtures with a composition different from that of the available standards. These last comments show that the lack of standards (native and labeled) is a crucial problem in quantitative analysis of the compounds included in this review.

VI. ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photoionization
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
C18	octadecyl bonded silica
C8	octyl bonded silica
CEC	capillary electrochromatography
CEC–MS	capillary electrochromatography–mass spectrometry
CI	chemical ionization
CID	collision-induced dissociation
DBU	1,8-diazabicyclo-[5.4.0]-7-undecene
ECDs	endocrine chemical disruptors
EDCs	endocrine-disrupting compounds
EI	electron ionization
EPI	enhanced product ion
ESI	electrospray ionization
FIA	flow injection analysis
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
GC–MS/MS	gas chromatography tandem mass spectrometry
GCxGC	two-dimensional gas chromatography
GCxGC-TOF-MS	two-dimensional gas chromatography time-of-flight mass spectrometry
HFBA	heptafluorobutyric acid
HRMS	high-resolution mass spectrometry
IDA	information-dependent acquisition
ILODs	instrumental limits of detection
IT	ion-trap
LC	liquid chromatography
LC–MS	liquid chromatography–mass spectrometry
LC–MS/MS	liquid chromatography tandem mass spectrometry
LC-TOF-MS	liquid chromatography time-of-flight-mass spectrometry
LODs	limits of detection
LRMS	low-resolution mass spectrometry
LLE	liquid–liquid extraction
MLODs	method limits of detection
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multiple-stage mass spectrometry
NI	negative ionization
NICI	negative ion chemical ionization
PCI	positive chemical ionization
PFBBr	pentafluorobenzoyl bromide
PFPA	pentafluoropropionic anhydride
PI	positive ionization
PVC	poly(vinyl chloride)
Q	quadrupole
QqQ	triple quadrupole
QTOF	quadrupole time-of-flight
Q-Trap	quadrupole-linear ion-trap
RAM	restricted access material
REACH	Registration, Evaluation, Authorization and Restrictions of Chemicals
SBSE	stir bar sorptive extraction
SIM	selected ion monitoring
SPE	solid phase extraction
SPME	solid phase microextraction
SRM	selected reaction monitoring
TOF	time-of-flight
TOF-MS	time-of-flight-mass spectrometry

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1.4.2.2. FOTOINICIADORS

Pel que fa referència a la determinació de fotoionitzadors en mostres alimentàries mitjançant cromatografia de gasos i cromatografia de líquids acoblades a l'espectrometria de masses a la Taula 1.5 es recullen els mètodes d'espectrometria de masses emprats, on s'inclou el mètode cromatogràfic, així com la font d'ionització, l'analitzador, el mode d'adquisició i els ions o transicions monitoritzades en cada cas. També s'indica el mètode de quantificació utilitzat.

Degut a la complexitat de les mostres alimentàries i a les baixes concentracions d'aquests compostos es requereix una etapa prèvia d'extracció, preconcentració i purificació. Per a l'extracció generalment s'utilitzen solvents orgànics com l'acetonitril o solucions hidro-orgàniques MeOH:aigua o ACN:aigua. Alguns autors com Benetti i cols., 2008 i Bagnati i cols,. 2007 proposen l'anàlisi directe una vegada realitzada l'extracció, mentre que d'altres, Allergrone i cols,.2008 i Sun i cols,. 2007, proposen realitzar una etapa de purificació amb SPE per tal de disminuir les possibles interferències de la matriu. Per altra banda, Gil-Vergara i cols,. 2007 i Sagratini i cols,. 2006 van utilitzar líquids pressuritzats (PLE) per a l'extracció de l'ITX emprant acetat d'etil i una mescla d'acetona/hexà respectivament.

Per a l'anàlisi dels fotoiniciadors s'ha utilitzat tant la cromatografia de líquids (LC) com la cromatografia de gasos totes dues acoblades a l'espectrometria de masses. En cromatografia de líquids la majoria dels treballs publicats empren columnes de fase invertida, C8 o C18 que permeten una bona separació dels diferents fotoiniciadors. Ara bé, amb aquestes fases estacionares els dos isòmers de l'ITX (2- i 4-ITX) coelueixen i són per tant, determinats conjuntament. Tant sols, Bagnati i cols,. 2007 aconsegueixen separar aquests dos isòmers amb una columna de zirconi modificada amb cadenes entrecreuades de poliestirè, la qual cosa els hi va permetre determinar cadascun dels isòmers per separat. En tots els mètodes d'anàlisi mitjançant LC-MS es fa servir l'electroesprai en mode positiu (ESI +) com a font d'ionització, i s'obté com a ió molecular la molècula protonada $[M+H]^+$, encara que alguns d'aquests compostos com la benzofenona (BP), la 1-hidroxifenilciclohexilfenilcetona (HCPK), la isopropil-9-H-tioxanté-9-ona (ITX), l'etil-4-dimetilaminobenzoat (EDAB) i el 2-etilhexil-4-(dimetilamino)benzoat

(EHDAB), presenten una elevada tendència a formar adductes amb els metalls alcalins com el Na, $[M+Na]^+$. Aquesta tendència a formar adductes amb el Na és especialment important en el cas del HCPK que arriba a presentar una resposta per a l'ió $[M+Na]^+$ fins a 5 vegades superior a la del corresponent $[M+H]^+$ (Shen i cols., 2009). Ara bé aquest ió, $[M+Na]^+$, no proporciona una bona fragmentació la qual cosa impossibilita la seva utilització per a l'anàlisi per MS/MS. Per contra la selecció del $[M+H]^+$ com a ió precursor dóna lloc a dos ions producte *m/z* 187 i 77 amb una bona resposta que permeten confirmar la presència d'aquest compost. En general la major part dels treballs utilitzen com analitzador en triple quadrupol (QqQ) (Shen i cols., 2009, Bagnati i cols,. 2007 i Sagratini i cols,. 2006) encara que Sun i cols,. 2007 empren un Q-Trap. En ambdós casos es monitoritzen dues transicions selectives (SRM) que permeten la confirmació de l'anàlit. De totes maneres Benetti i col,. 2008 analitzen l'ITX mitjançant LC-MS emprant com a analitzador un quadrupol (Q) i treballant en monitorització selectiva d'ions (SIM). Per confirmar proposen utilitzar fragmentació a la font monitoritzant la molècula protonada i l'ió producte a *m/z* 213 corresponent a la pèrdua del grup isopropil.

Alguns autors utilitzen GC-MS per a l'anàlisi. En general s'empra una columna de 5% difenil-95% dimetil polisiloxà per a la separació cromatogràfica i ionització electrònica (EI) tant en una trampa d'ions (IT) com en un quadrupol. La ionització electrònica proporciona una elevada fragmentació amb suficient informació estructural. En aquests casos es monitoritzen els ions més abundants obtinguts en l'espectre de masses, per exemple Sagratini i col., 2008 monitoritza l'ió molecular a *m/z* 254 $[M]^{+\cdot}$ i l'io a *m/z* 239 corresponent a la pèrdua de metil, $[M-CH_3]^+$, la qual cosa li permet confirmar la presència d'aquest compost. Allegrone i cols,. 2008 en canvi proposen utilitzar la cromatografia de gasos acoblada a l'espectrometria de masses en tàndem (GC-MS/MS) emprant com a analitzador una trampa d'ions i monitoritzant les transicions *m/z* 254 > 239 i *m/z* 254 > 196 per a l'anàlisi de ITX en llet, mentre que Van Hoek i cols., 2010 analitzen la presència de benzofenona en mostres de cereals seguint les transicions *m/z* 182 > 153 i *m/z* 182 > 181.

Pel que fa a l'anàlisi quantitativa d'aquests compostos la dilució isotòpica és el mètode seleccionat en el cas que es disposi d'estàndards comercials marcats isotòpicament com per exemple pe l'ITX per al que es pot emprar el 2-ITX-d_7 i o 2-

ITX-d_3 i per la benzofenona la BP-d_{10}. Per als altres fotoiniciadors cal emprar calibració externa.

Taula 1.5. Anàlisis de fotoiniciadors mitjançant LC-MS i GC-MS.

Compost	**Tècnica** **Font d'ionització** **Analitzador**	**Mode d'adquisició**	**Ions o transicions monitoritzades**	**Ref.**
BP ITX	GC-MS/MS EI+ IT	SRM	BP: *m/z* 182 → 153 *m/z* 182 → 181 ITX: *m/z* 254 → 239 *m/z* 254 → 196	Van Hoek i cols., 2010; Allergone i cols., 2008
ITX EHDAB EDAB BP HCPK	GC-MS EI+ IT	SIM	ITX: *m/z* 239, 254, 184 EHDAB: *m/z* 148, 165, 177 EDAB: *m/z* 81, 99, 148, 164, 193 BP: *m/z* 105, 182 HCPK: *m/z* 81, 99, 148, 164, 193	Sagratini i cols., 2008; Gil-Vergara i cols., 2007
ITX EHDAB EDAB BP HCPK	LC-MS ESI+ Q	SIM	ITX: *m/z* 300, 255, 213 EHDAB: *m/z* 277 EDAB: *m/z* 216 BP: *m/z* 205 HCPK: *m/z* 227	Benetti i cols., 2008; Sagratini i cols., 2008.
ITX EHDAB EDAB BP HCPK	LC-MS/MS ESI+ QqQ/Q-Trap	SRM	ITX: *m/z* 255 → 184 *m/z* 255 → 213 EHDAB: *m/z* 278 → 151 *m/z* 278 → 166 EDAB: *m/z* 194 → 166 BP: *m/z* 183 → 105 *m/z* 183 → 77 HCPK: *m/z* 205 → 239 *m/z* 205 → 187	Shen i cols., 2009; Sagratini i cols., 2008; Benetti i cols., 2008; Bagnati i cols., 2008; Sun i cols., 2007

La majoria dels mètodes emprats per a l'anàlisi d'aquests compostos en mostres d'aliments utilitzen espectrometria de masses de baixa resolució, però Morlock and Schwach (2006 i 2007) han desenvolupat un mètode ràpid d'*screening* basat en cromatografia de capa fina acoblada a espectrometria de masses (HPTLC-MS), utilitzant *Direct Analysis in Real Time* (DART) i un analitzador de temps de

vol (TOF) que els ha permès analitzar ITX en mostres de llet, iogurt amb un elevat contingut de greix.

CAPÍTOL 2

ESPECTROMETRIA DE MASSES: ESTUDIS DE FRAGMENTACIÓ

2.1. INTRODUCCIÓ

Aquest capítol està dedicat a estudiar de forma exhaustiva la fragmentació en espectrometria de masses dels compostos estudiats en aquesta Tesis mitjançant la combinació de l'informació obtinguda amb un analitzador que ens permet realitzar fragmentacions en múltiples etapes (trampa d'ions) i l'obtenció de mesures de massa exacta amb un instrument de triple quadrupol de nova generació per tal de poder establir i confirmar les composicions elementals dels ions producte obtinguts.

Per establir rutes de fragmentació és necessari disposar d'un espectròmetre de masses que permeti fragmentar els anàlits en múltiples etapes de manera que es puguin aïllar els diferents ions producte. Molts dels analitzadors disponibles avui dia permeten realitzar estudis de fragmentació, ja sigui utilitzant-los de forma individual o bé en instruments híbrids amb dos o més analitzadors. Al mercat existeixen una gran diversitat d'analitzadors entre els que cal citar els quadrupolars (Q), les trampes d'ions (IT), els sectors (magnètic i electrostàtic), els de temps de vol (TOF), els de ressonància ciclotrònica d'ions amb transformada de Fourier (FT-ICR) i el més recentment desenvolupat l'Orbitrap (Makarov A. 2000). A la Taula 2.1 es resumeixen algunes de les característiques més rellevants d'aquests analitzadors.

La necessitat de disposar d'analitzadors cada vegada més sensibles i amb capacitat augmentar la resolució i l'exactitud en la mesura de la massa ha anat augmentant amb els anys. Per exemple, en els darrers anys s'ha millorat considerablement la transmissió dels ions en els sistemes quadrupolars desenvolupant analitzadors molt sensibles i ràpids, a la vegada que s'ha estès l'interval de *m/z* de treball. Aquests analitzadors són molt eficaços a baixa resolució (0,7 *m/z Full Width Half Maximum,* FWHM), però no poden precisar més enllà de la dècima de *m/z* en la mesura de la massa (Taula 2.1). Actualment es disposa d'una nova generació de quadrupols que presenten una geometria hiperbòlica que permeten generar un camp quadrupolar pur i que, tal i com es discutirà posteriorment en aquest capítol, permeten augmentar considerablement la resolució (fins a 0,04 *m/z* FWHM) sense una pèrdua significativa del senyal. A més, l'estabilitat de l'eix *m/z* combinada amb una electrònica adequada fa possible una mesura prou acurada de la massa (fins a 5 mDa d'error) (Taula 2.1). Aquestes capacitats són adequades i suficients per a resoldre alguns problemes com es

demostrarà al llarg d'aquesta Tesis. Si és necessita una alta resolució (poder de resolució > 10.000, *m/z* 500 FWHM) s'ha de treballar amb analitzadors com els TOF o els Orbitraps. Amb els TOFs es possible aconseguir poders de resolució de >10000 i errors en les mesures de massa exacta per sota de <10 ppm. En relació a l'Orbitrap la resolució d'aquest nou analitzador és molt bona arribant a un poder de resolució de 100.000 i una exactitud en la mesura de la massa per sota de les dècimes de mDa obtenint errors <5 ppm.

Taula 2.1. Característiques dels analitzadors de masses més freqüents en LC-MS.

Criteri	**Quadrupol**	**Quadrupol hiperbòlic**	**Trampa d'ions**	**Temps de vol**	**Orbitrap**
Interval *m/z*	30-3000	30-1500	50-2000	20-40000	50-5000
Exactitud en la massa	1000 ppm	5 ppm (per a *m/z* 1000)	500 ppm	5-10 ppm	<5 ppm
Velocitat	Baixa	Mitjana	Baixa-mitjana	Molt alta	Baixa
Sensibilitat	Mitjana	Alta	Mitjana (elevada a la trampa lineal)	Mitjana	Mitjana
Resolució (FWHM)*	0.7	0.7 fins a 0.04	0.5	$\leq 2.10^4$	$\leq 2.10^6$
MS/MS	No (excepte en triple quadrupol)	No (excepte en triple quadrupol)	MS^n	No (excepte en Q-TOF)	No (excepte en IT-Orbitrap)

*FWHM: *full width at half maximum*

Per altra banda, en cromatografia de líquids les fonts de ionització més comunament emprades (electroesprai i ionització química a pressió atmosfèrica) proporcionen els ions generats per protonació o desprotonació de les molècules, la qual cosa fa que sigui indispensable recórrer a l'espectrometria de masses en tàndem per obtenir informació estructural. Aquest fet, ha estat possiblement una de les causes del desenvolupament d'analitzadors amb capacitat de dur a terme experiments de fragmentació en tàndem entre els que cal citar les trampes d'ions. Aquests analitzadors han evolucionat d'una manera espectacular des de la clàssica trampa d'ions 3D fins al nou analitzador Orbitrap. Les trampes d'ions més habituals avui dia són les trampes lineals ja que aquest disseny proporciona més sensibilitat que la clàssica 3D, atesa la seva elevada eficàcia d'emmagatzemament dels ions, i a la rapidesa en l'escombratge de l'eix *m/z* (Hager 2002). Tant les

trampes d'ions 3D com les lineals, així com els analitzadors de ressonància ciclotrònica d'ions amb transformada de Fourier (FT-ICR) presenten una característica comuna, la capacitat de fragmentar els ions en el temps, que permet dur a terme la fragmentació un nombre determinat de vegades seleccionant cada vegada el nou ió producte com a ió precursor (experiments de fragmentació en múltiples etapes, MS^n) i que fa possible, d'una manera relativament fàcil, establir l'ordre genealògic dels ions. Respecte a la resolució, només el FT-ICR pot treballar a alta resolució arribant fins a 1.000.000 de poder de resolució i mesurant la massa amb un error inferior a poques dècimes de mDa. Un aspecte que també cal tenir en compte es la velocitat d'escombratge d'aquests analitzadors que disminueix en augmentar tant el nombre d'etapes de fragmentació com la resolució. També cal dir que en les trampes d'ions es possible fer escombratges més lents per tal d'augmentar la resolució fins a 0,05 *m/z* FWHM la qual cosa s'aconsegueix treballant en els modes *zoom scan i ultra-zoom scan*.

Pel que fa a l'Orbitrap, encara que és un analitzador de trampa d'ions, avui dia encara no és possible dur a terme experiments de fragmentació en múltiples etapes. Ara bé, la capacitat d'aquest analitzador per treballar a alta resolució (fins a 100.000 de poder de resolució) i proporcionar mesures de massa exacta amb errors inferiors a les dècimes de mDa, es combina amb una relativa facilitat d'operació i una gran estabilitat del eix de masses, característiques que fan d'aquest analitzador una molt bona alternativa als TOFs i els FT-ICR.

Per obtenir informació estructural amb analitzadors quadrupolars, TOFs o Orbitraps és necessari recórrer a configuracions híbrides on es treballa amb un parell d'analitzadors en sèrie i duent a terme experiments d'espectrometria de masses en l'espai mitjançant una cel·la de col·lisió inserida entre els dos analitzadors o emprant una trampa d'ions. Els analitzadors quadrupolars, possiblement degut a la seva senzillesa i robustesa, són emprats en molts sistemes híbrids com el triple quadrupol, el QTOF o el Q-Trap. En tots aquests instruments la selecció d'ions precursors es duu a terme a baixa resolució, amb l'excepció dels quadrupols hiperbòlics de l'instrument que s'ha emprat en aquesta Tesi que permet seleccionar els ions precursors amb una resolució de fins a 0,04 *m/z* FWHM. Les trampes lineals també estant sent emprades en instruments híbrids. Per exemple en el Q-Trap el tercer quadrupol s'ha substituït per una trampa lineal (Q-Trap) la qual cosa permet dur a terme escombratges d'ions precursors i de pèrdua de neutres que no són possible en una trampa d'ions. En el cas de l'Orbitrap i del FT-

ICR normalment s'utilitza una trampa lineal per tal d'acumular els ions i dur a terme les fragmentacions abans d'enviar els ions a l'analitzador d'alta resolució per a la mesura de massa exacta.

S'ha de tenir en compte que en els sistemes híbrids que proporcionen fragmentacions via tàndem en l'espai (QqQ o QTOF) es possible observar tant els productes de fragmentació directa com els generats per col·lisions múltiples, ja que els ions poden adquirir energia suficient per fragmentar-se durant el seu pas per la cel·la de col·lisió. Com a exemple d'aquest fenomen a la Figura 2.1 es mostren les corbes de fragmentació corresponents al tetraclorobisfenol A (TeCBPA) i a la 4,4'-bis(dietilamino)-benzofenona (DEAB) en un analitzador de triple quadrupol. Per al TeCBPA el desplaçament dels màxims en les corbes corresponents als ions *m/z* 350, 314, 286 i 250 suggereixen que els tres últims ions (*m/z* 314, 286 i 250) podrien provenir de la fragmentació per múltiples col·lisions de l'ió radical a *m/z* 350 que s'ha originat per la pèrdua radicalària d'un dels metils de la molècula desprotonada, $[M\text{-}H\text{-}CH_3]^{-\cdot}$. Això permet una possible primera assignació d'aquests ions, $[M\text{-}CH_3\text{-}HCl]^{-\cdot}$ (*m/z* 314), $[M\text{-}CH_3\text{-}HCl\text{-}C_2H_3O]^{-\cdot}$ (*m/z* 286) i $[M\text{-}CH_3\text{-}HCl\text{-}C_2H_3O\text{-}HCl]^{-\cdot}$ (*m/z* 250). De la mateixa manera, per al DEAB els ions de *m/z* 281 i 176 poden provenir de la fragmentació consecutiva de l'ió *m/z* 286 corresponent a la pèrdua de C_2H_5, ja que els màxims de les corbes d'aquests ions producte es troben lleugerament desplaçats respecte a l'ió a *m/z* 296. Per aquest compost els ions a *m/z* 133 i 148 poden provenir de la fragmentació dels ions 176 i 281 respectivament. De tota manera, les assignacions seguint aquest criteri no són fàcils de fer i és necessari recórrer als analitzadors de trampa d'ions per garantir el correcte ordre genealògic dels ions producte. En el cas concret esmentat abans les primeres assignacions basades en les corbes de col·lisió en el triple quadrupol s'han confirmat mitjançant la fragmentació en un analitzador de trampa de ions (LCQ Classic de ThermoFinnigan). Cal posar de manifest que aquesta trampa d'ions 3D permet obtenir espectres de masses MS^n potencialment fins a n=10, però a la pràctica normalment només es realitzen espectres de masses fins a $MS^{3\text{-}5}$ degut a una disminució significativa de l'abundància dels ions en augmentar les etapes de fragmentació, "n".

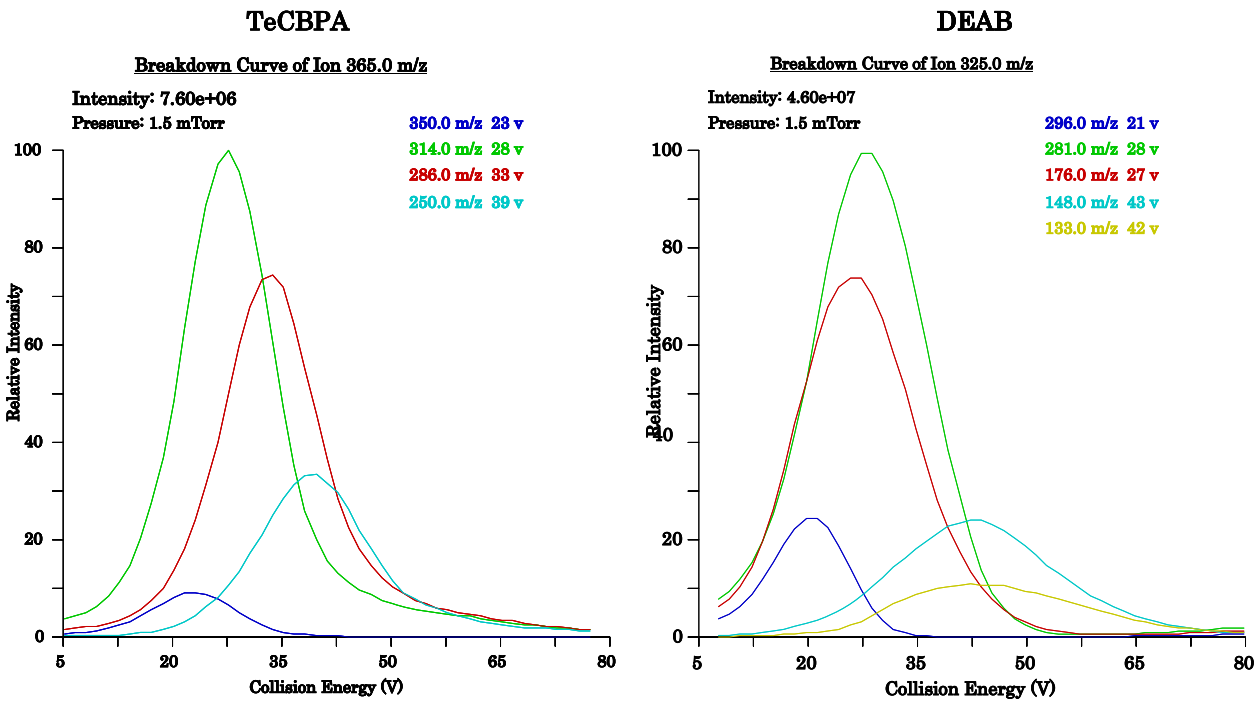


Figura 2.1. Corbes de fragmentació dels ions *m/z* 365 (TeCBPA) i *m/z* 325 (DEAB) emprant un analitzador de triple quadrupol.

Com a pas previ a la formulació d'una hipòtesi sobre l'estructura química dels ions que es generen tant en la font d'ionització com en els experiments d'espectrometria de masses en tàndem, és necessari assignar correctament la composició elemental de l'io en qüestió. Si les pèrdues son característiques de grups funcionals freqüents és relativament fàcil dur a terme aquesta assignació en baixa resolució. Ara bé, a vegades treballar d'aquesta manera no es suficient i és indispensable realitzar mesures de massa exacta tant d'ions precursors com d'ions producte. En l'acoblament LC-MS els analitzadors més emprats per aquests propòsit són els de temps de vol (TOF) donat que poden treballar a poders de resolució de fins a 20.000 i proporcionen una molt bona precisió i exactitud en la mesura de la massa (<10 ppm) treballant d'una forma relativament senzilla. Per obtenir mesures de massa exacta d'ions producte és necessari dur a terme la fragmentació a la font o emprar un sistema híbrid quadrupol temps de vol (QTOF). Malauradament, no és possible l'aïllament d'ions precursors en alta resolució per als experiments de tàndem. Avui dia tant sols el triple quadrupol que s'ha emprat en aquesta Tesi (quadrupols hiperbòlics) permet aïllar els ions precursors, així com mesurar els ions producte, amb una relativa bona resolució, de fins a 0,04 unitats

de *m/z* a FWHM en tot l'eix de masses, o el que és al mateix amb un poder de resolució de 25.000 per a una *m/z* de 1.000. Per altra banda permet realitzar mesures de massa exacta amb una molt bona precisió i exactitud (± 5 mDa en tot l'eix de masses, 5 ppm a *m/z* 1000). A la literatura existeixen alguns exemples d'utilització d'aquest triple quadrupol treballant a resolució elevada i realitzant mesures de massa exacta tant d'ions precursors com d'ions producte per a la identificació i caracterització d'impureses en fàrmacs (Paul i col., 2003), per a la identificació de metabòlits (Jemal i col., 2003), així com per a la elucidació estructural de nous fàrmacs (Pucci i col., 2007) i per a la identificació de compostos desconeguts (Grange i cols., 2005 RCM). Un dels avantatges que es destaca d'aquest instrument (Jemal i col., 2003) és la possibilitat de combinar les excel·lents capacitats dels instruments de triple quadrupol per a l'anàlisi quantitativa en els diferents modes d'escombratge amb les capacitats per a l'anàlisi qualitativa i la confirmació dels instruments de més elevada resolució. Així per exemple es pot combinar una primera etapa de cribratge *(screening)* mitjançant escombratges de pèrdua de neutres i de precursors amb la posterior confirmació de les composicions elementals mitjançant les mesures de massa exacta tant d'ions precursors com d'ions producte.

Per altra banda, encara que els analitzadors TOF i QqQ són els que més s'utilitzen en LC-MS, quan el problema analític requereix una molt elevada resolució és necessari recórrer a analitzadors com els de ressonància ciclotrònica d'ions amb transformada de Fourier (FT-ICR) i a l'Orbitrap.

2.2. TREBALL EXPERIMENTAL

L'estudi de les rutes de fragmentació dels compostos inclosos en aquesta tesis s'ha dut a terme a partir dels espectres de fragmentació en etapes successives (MS^n) obtinguts en un analitzador de trampa d'ions (LCQ Classic de Thermo-Finnigan). Aquest estudi s'ha realitzat en primer lloc per al BPA i alguns derivats halogenats com el TeCBPA i el TBBPA per als quals es disposen de patrons comercials. Per tal de completar l'estudi i caracteritzar correctament els ions producte obtinguts també s'han estudiat les fragmentacions del BPA i el TBBPA marcats isotòpicament, BPA-d_{16} i $^{13}C_{12}$-TBBPA (en els carbonis dels anells aromàtics). A més s'han obtingut espectres de fragmentació dels derivats clorats

del BPA (MCBPA, DCBPA i TCBPA), en aquest cas i atès que no són comercials aquests derivats clorats s'han sintetitzat mitjançant la cloració del BPA i s'han purificat per cromatografia de líquids semipreparativa. A l'article científic II inclòs en aquest capítol es detalla aquesta obtenció. També s'han estudiat les fragmentacions dels altres compostos inclosos en aquesta memòria, els diglicidil èters del BPA i el BPF i els seus derivats hidrolitzats i cloro-hidrolitzats (BADGEs i BFDGEs) (article científic III inclòs en aquest capítol) i els fotoiniciadors (ITX, DETX, HCPK, DMPA, DEAB, EHDAB, HMPP, PBZ i BP), apartat 2.2.3.3. Ara bé, treballar a baixa resolució comporta que alguns dels ions obtinguts en els espectres de MS^n amb la trampa d'ions es puguin assignar a més d'una composició elemental. Aquest fet obliga a realitzar mesures de massa exacta per tal d'obtenir una correcta assignació de la composició elemental. En aquesta tesi les mesures de massa exacta s'han realitzat amb un instrument triple quadrupol TSQ Quantum Ultra AM.

Com s'ha comentat anteriorment a la introducció d'aquest capítol l'any 2000 es van desenvolupar uns nous quadrupols de geometria hiperbòlica. L'analitzador que s'utilitza en aquesta tesis és d'aquest tipus i està equipat amb dos quadrupols de 250 mm de longitud i un radi intern de 6 mm (Figura 2.2A). La capacitat per generar camps quadrupolars purs (Figura 2.2B) te com a conseqüència una millora considerable tant en la transmissió dels ions com en la resolució. En aquests quadrupols es generen pics tubulars amb màxims relativament plans que permeten treballar a baixa resolució (0,7 *m/z* FWHM) amb una molt bona estabilitat de la massa i a més estrènyer la finestra d'aïllament en el quadrupol sense que es produeixi una pèrdua significativa de la intensitat del feix d'ions. Aquest fet permet desplaçar la línia d'escombratge cap al vèrtex del triangle d'estabilitat en el diagrama de Mathieu. Prop del vèrtex és on s'aconsegueix una major resolució i en aquest cas sense que es produeixi una pèrdua significativa del senyal degut a la bona transmissió dels ions, al contrari del que passa amb els quadrupols cilíndrics on la pèrdua de senyal amb l'augment de resolució és molt important (Blaum et al., 2000).

A) B)

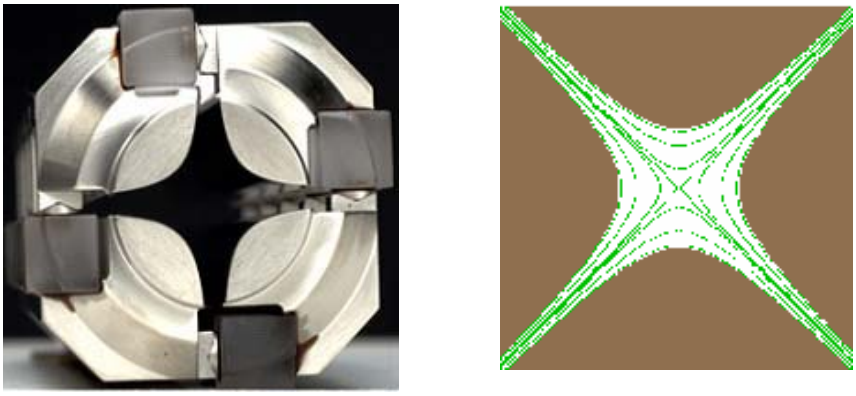


Figura 2.2. A) Quadrupols de geometria hiperbòlica del TSQ Quantum Ultra AM. B) línies de camp dels quadrupols hiperbòlics.

En començar a treballar amb aquest instrument no es disposava de dades sobre l'estabilitat de l'eix *m/z* ni d'exactituds en la mesura de la massa a valors baixos (<300 Da). Per aquesta raó es van planificar una sèrie d'experiments amb l'objectiu d'obtenir aquesta informació. Els patrons utilitzats van ser el clormequat (CQ) en mode positiu i el bisfenol A (BPA) en mode negatiu. A la Figura 2.3 A) es mostren els espectres de masses *(full-scan)* en la zona del clúster de l'ió molecular pel CQ (*m/z* 122, $[M]^+$) i de la molècula desprotonada pel BPA (*m/z* 227, $[M\text{-}H]^-$) a diferents resolucions. Com es pot observar la resposta és gairebé la mateixa pel CQ quan es treballa a una resolució de 0,4 *m/z* FWHM que quan s'augmenta la resolució fins a 0,1 *m/z* FWHM. En treballar a una resolució de 0,04 *m/z* FWHM s'observa una pèrdua de la intensitat dels ions (disminució del senyal en 2 ordres de magnitud). Ara bé fins i tot així, aquesta resposta és suficient per poder dur a terme mesures de massa exacta a nivells baixos de concentració com es posarà de manifest en les aplicacions incloses en aquesta Tesis. Pel BPA (Figura 2.3B) inclús treballant a la màxima resolució (0,04 *m/z* FWHM) no es produeix una pèrdua significativa de senyal, passant d'un fons d'escala de 1,6 10^7 a baixa resolució (0,7 *m/z* FWHM) a un fons d'escala de 7,27 10^6 a resolució de 0,04 *m/z* FWHM (Figura 2.3 B). La tendència en el comportament dels dos compostos a 0,04 *m/z* FWHM es pot deure a que pel BPA estem treballant a 100 unitats de *m/z* superiors que pel CQ.

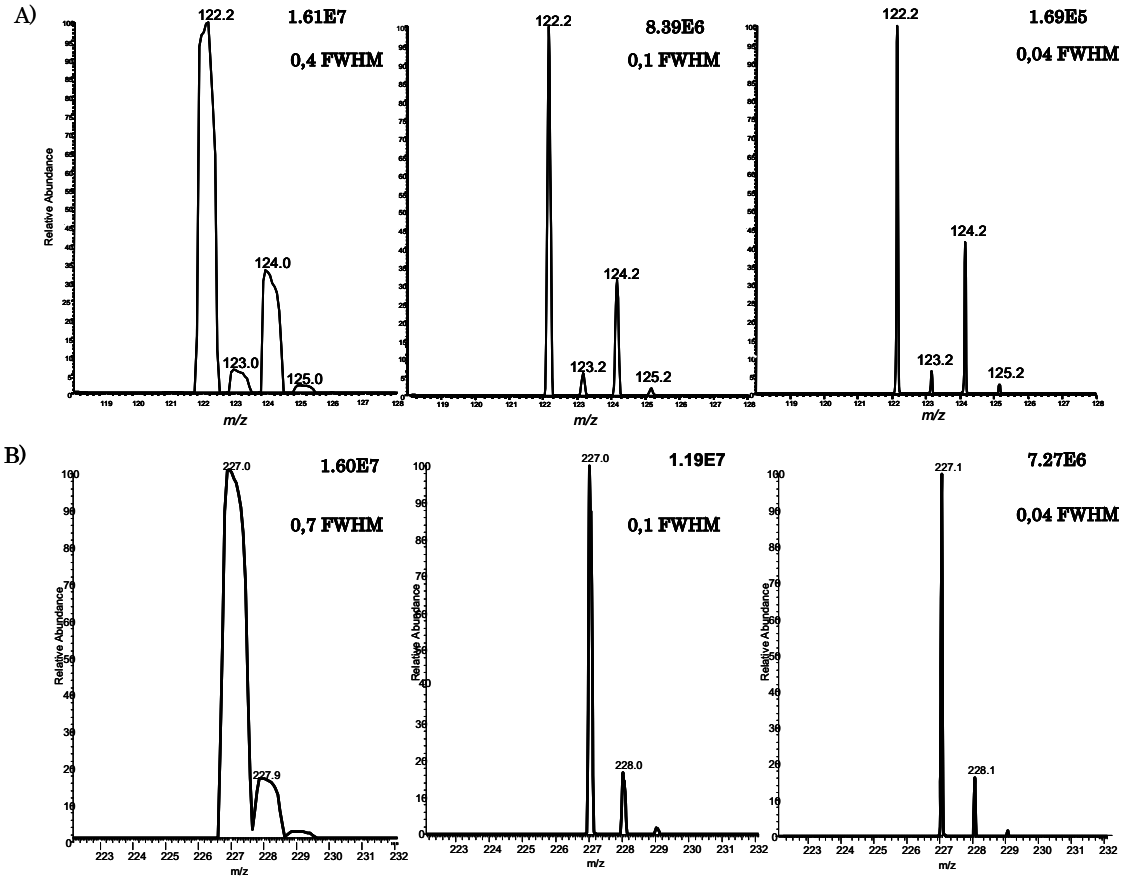


Figura 2.3. Espectres de MS adquirits a diferents resolucions (0,4, 0,1 i 0,04 *m/z* FWHM) amb l'instrument de triple quadrupol TSQ Quantum Ultra AM emprant electroesprai com a font d'ionització. A) Clormequat (CQ) en mode positiu i B) Bisphenol A (BPA) en mode negatiu.

L'escombratge que s'utilitza habitualment per obtenir la màxima sensibilitat en sistemes quadrupolars de baixa resolució és el SIM (*selected ion monitoring)* o el SRM (*selected reaction monitoring)* en treballar en tàndem. Amb el TSQ Quantum Ultra AM atès que és possible operar amb un o amb els dos quadrupols a una major resolució (0,04-0,1 *m/z* FWHM) es poden definir dos nous modes d'escombratge H-SIM (*highly-selective ion monitoring*) i H-SRM (*highly-selective selected reaction monitoring*). Aquests modes de treball permeten millorar la selectivitat i la sensibilitat dels mètodes filtrant selectivament el soroll químic de fons i les interferències, sense una pèrdua important del senyal i augmentant conseqüentment la relació S/N. Per altra banda, la capacitat de poder augmentar la resolució fins a 0,04 unitats de *m/z* a FWHM, combinada amb un acurat calibratge

de l'instrument fa possible realitzar mesures de massa exacta amb una bona precisió i exactitud obtenint errors absoluts en la mesura inferiors als ±5 mDa. Cal remarcar que en la mesura de la massa de molècules petites (*m/z* 50-400) un error de 5mDa és en molts casos suficient per poder assignar correctament una composició elemental ja que hi ha molt pocs candidats per a una determinada massa mesurada amb un error tant petit (Guide for authors JASMS).

Calibratge

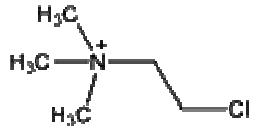
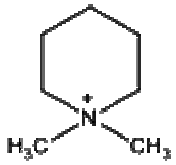
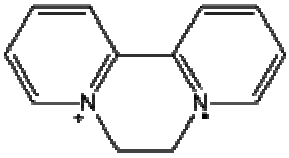
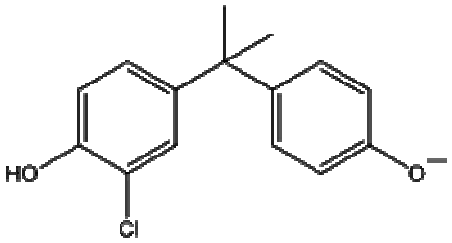
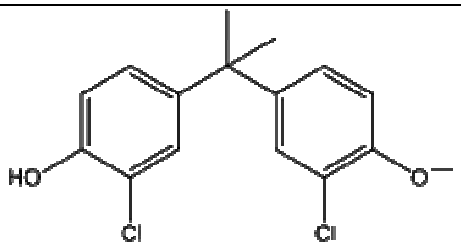
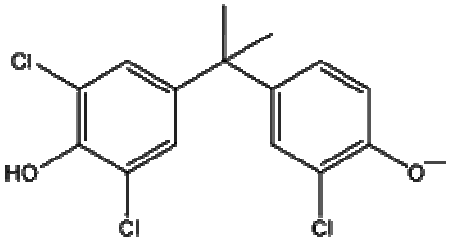
Per poder treballar a una resolució superior a 0,7 *m/z* FWHM i realitzar una mesura de la massa precisa i amb una bona exactitud s'ha de dur a terme un acurat calibratge de l'eix de *m/z*. Aquest calibratge es sol realitzar utilitzant un o més compostos de referència amb valors de *m/z* perfectament coneguts (massa exacta) que permeten que durant el procés de calibratge s'ajustin els paràmetres instrumentals obtenint els coeficients de calibració d'una equació polinòmica. En general amb els instruments d'alta resolució (TOF, FT-ICR, Orbitrap) es sol operar en dues etapes, primer es fa un calibratge extern mitjançant la infusió d'una solució d'estàndards de referència que permet ajustar l'eix *m/z* i posteriorment es dur a terme un calibratge intern en temps real mitjançant compostos de referència anomenats *"Lock Masses"* que es mesuren alhora que els anàlits i que permeten corregir petits desajusts que es produeixen en el transcurs de la mesura. Ara bé, en el cas de l'instrument TSQ Quantum Ultra AM utilitzat en aquesta Tesi pot ser necessari realitzar fins a quatre etapes de calibratge depenen del mode de treball.

Quan s'opera a baixa resolució (0,7 *m/z* FWHM) es duu a terme un calibratge extern de l'eix de *m/z* amb una mescla de compostos de referència, en aquest cas generalment es fa servir una mescla de la 1, la 3 i la 6-politirosina, en aquesta etapa també s'ajusta la forma dels pics de l'espectre (tubulars) i la intensitat del senyal. Aquest calibratge, com a la resta d'instruments quadrupolars i de baixa resolució es sol realitzar periòdicament en general cada 6 mesos. Posteriorment, quan es vol treballar a una resolució més elevada s'ha d'efectuar un calibratge de l'eix *m/z* a elevada resolució el qual mitjançant un algoritme que optimitza els potencials del corrent continu (DC *offset*) permet millorar la forma del pic en treballar a resolucions elevades amb una amplada de pic inferior a 0,5 *m/z* FWHM a la vegada que s'ajusta l'eix *m/z* en l'interval de valors de *m/z* de treball.

Encara que en aquest instrument l'eix *m/z* és molt estable en aquestes condicions, és recomanable realitzar aquesta etapa de calibratge setmanalment si es treballa per sota de 0,5 *m/z* FWHM). En els treballs inclosos en aquesta tesis aquesta etapa de calibratge s'ha realitzat seleccionant un set de compostos de *m/z* coneguda dependents de l'interval de treball i del mode d'ionització (positiu o negatiu) (Taula 2.2).

En aquesta memòria i una vegada calibrat l'instrument a 0,1 i 0,04 *m/z* FWHM amb una mescla de tres sals d'amoni quaternari (clormequat, mepiquat i diquat) (Taula 2.2) es van dur a terme una sèrie d'experiments per tal d'avaluar l'estabilitat de l'eix *m/z* i la reproductibilitat de la mesura de la massa amb el temps. Amb aquest objectiu es va injectar 158 vegades una dissolució estàndard de 50 μg L^{-1} de CQ (*m/z* 122,0731) en una columna HILIC (150 mm x 2,1 mm i.d., 3,5 μm) (Waters, Milford, MS, USA) utilitzant ACN:àcid fòrmic-formiat d'amoni 50 mM (pH 3,75) com a fase mòbil a 400 μL min^{-1}. Aquest sistema cromatogràfic es va acoblar a l'espectròmetre de masses de triple quadrupol emprant com a font d'ionització l'electroesprai en mode positiu. Es van enregistrar els espectres de masses en mode d'escombratge total d'ions (*full-scan*) entre *m/z* 30 i *m/z* 150 i treballant a una resolució de 0,1 *m/z* FWHM. A la Figura 2.4 es mostren els resultats dels valors de *m/z* mesurats després del calibratge a aquesta resolució. En aquesta figura l'eix d'ordenades s'ha limitat al valor corresponent a l'amplada de la finestra de *m/z* utilitzada (Δ*m/z* 0,1) i com es pot observar es va obtenir una molt bona estabilitat de les mesures de *m/z* amb una desviació estàndard inferior al 10 %. Aquest mateix estudi es va repetir a la màxima resolució que permet treballar l'instrument (0,04 *m/z* FWHM). En aquest cas es van realitzar 100 injeccions de l'estàndard de CQ i a la Figura 2.5 es mostra la representació gràfica dels valors de *m/z* mesurats per a cada injecció així com el valor *m/z* corresponent al valor teòric pel CQ i l'interval per una finestra de 0,04 *m/z*. En aquest cas també es va observar una bona estabilitat de la mesura de *m/z* amb una dispersió de valors similar (RSD< 10%) a la del cas anterior i dins de la finestra de 0,04 *m/z*. Pel que fa referència al valor teòric de la massa del CQ, per a les 100 injeccions es va obtenir un valor de *m/z* promig de 122.0536 que correspon a un error absolut de 19.5 mDa respecte a la massa exacta del CQ (122,0731 Da).

Taula 2.2. Estructures, composició elemental i massa teòrica dels internal lock mass utilitzats

Compost	**Estructura**	**Composició elemental de l'ió**	**m/z (assignació)**	**Massa exacta calculada**
Clormequat (CQ)		$C_5H_{13}NCl^+$	*m/z* 122, $[M]^+$	122,0731
Mepiquat (MQ)		$C_7H_{16}N^+$	*m/z* 114, $[M]^+$	114,1277
Diquat (DQ)		$C_{12}H_{12}N_2^{+\cdot}$	*m/z* 184, $[M]^{+\cdot}$	184,0995
MCBPA		$C_{15}H_{14}O_2Cl^-$	*m/z* 261, $[M-H]^-$	261,0688
DCBPA		$C_{15}H_{13}O_2Cl_2^-$	*m/z* 295, $[M-H]^-$	295,0298
TCBPA		$C_{15}H_{12}O_2Cl_3^-$	*m/z* 329, $[M-H]^-$	328,9908

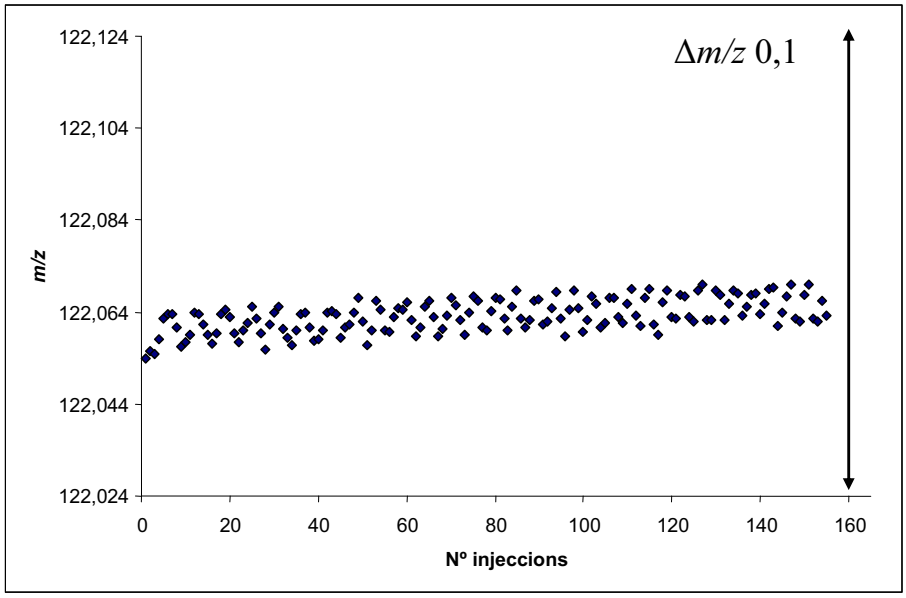


Figura 2.4. Mesures de *m/z* corresponents al [M+] del CQ (LC-MS, n:158) Q1 a 0,1 *m/z* FWHM.

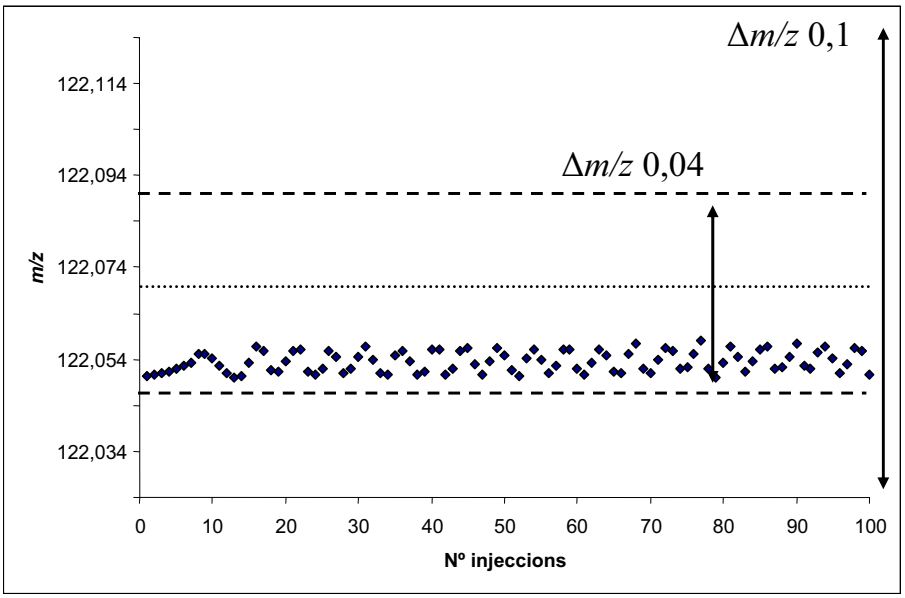


Figura 2.5. Mesures de *m/z* corresponent al [M+] del CQ (LC-MS, n:100) Q1 a 0,04 *m/z* FWHM.

Aquest experiment es va tornar a repetir després d'efectuar un *calibratge* extern *de l'instrument en massa exacta*. En aquesta etapa es va calibrar l'eix de *m/z* ajustant els paràmetres instrumentals de l'equip mitjançant la mesura de *m/z* d'ions de referència i comparant els valors experimentals amb els valors teòrics. En

aquets cas els compostos utilitzats van ser el mepiquat (MQ), el clormequat (CQ) i el diquat (DQ) (Taula 2.2). El valors observats presenten també petites dispersions (<10%) però amb errors importants (>15 mDa) respecte al valor teòric. S'ha de tenir en compte que encara que aquest calibratge es realitza sempre abans de dur a terme mesures de massa exacta, factors externs com per exemple les variacions en la temperatura ambient poden provocar una deriva (*drift*) de l'eix *m/z* que porti a errors en la mesura de la massa exacta. Per tal d'obtenir mesures més precises i exactes es van repetir els experiments corregint les mesures de *m/z* dels compostos amb *m/z* de referència perfectament coneguts, MQ (*m/z* 114,1277) i DQ (m/z 184,0995) (Taula 2.2). Un d'aquests tipus d'ajustos és el mode extern on primer s'enregistra l'espectre de masses dels compostos de referència *(external lock masses)* per tal d'avaluar la diferència entre el valor teòric i el valor mesurat i calcular uns coeficients de calibratge que posteriorment s'apliquen a las adquisicions posteriors per tal d'obtenir els espectres de masses problema corregits. Encara que l'exactitud en la mesura de *m/z* millora (errors entre 10 i 15 mDa) un inconvenient d'aquest mètode és que no permet corregir petites diferencies que puguin produir-se entre la mesura dels compostos de referència i la dels anàlits. Aquest problema es soluciona emprant el calibratge intern on els compostos de referència *(internal lock masses)* s'introdueixen en l'espectròmetre de masses al mateix temps que els anàlits, d'aquesta manera la deriva en l'eix *m/z* queda corregida en temps real. Amb aquest mètode s'aconsegueix una millor exactitud en la mesura de la massa que és més bona quan més pròxim sigui el *lock mass* al *m/z* de l'ió que es vol mesurar. En el cas concret del TSQ Quantum Ultra AM el calibratge tant intern com extern s'ha de realitzar emprant dos *lock masses*, un per sota i l'altre per sobre del *m/z* de l'ió que es vol mesurar. Altres instruments com els TOF i instruments d'alta resolució permeten corregir l'eix de masses amb un sol *internal lock mass* i a més presenten també l'opció de poder corregir les masses dels anàlits després de l'adquisició amb el mode anomenat *"post processing"*.

Aquest mode de treball (calibratge intern) es va avaluar inicialment mesurant la massa exacta del CQ (*m/z* 122,0731) utilitzant com a internal *lock masses* el mepiquat (MQ, *m/z* 114,1277 $[M]^+$) i el diquat (DQ, *m/z* 184,0995 $[M]^{+\bullet}$), les estructures d'aquests compostos es troben a la Taula 2.2. L'addició d'aquests compostos de referència es va realitzar mitjançant una unió de volum mort col·locada a l'entrada a la font d'ionització a un cabal de 5 μL/min emprant la bomba de xeringa integrada en el mateix espectròmetre de masses que contenia la

mescla dels diferents compostos a una concentració de 1 µg/L. Això permet obtenir un senyal continu de les masses de referència durant el procés de mesura. Aquest mètode té l'inconvenient de que en alguns casos es poden produir efectes de supressió de la ionització i per tant les substàncies que han d'actuar com a *internal lock masses* s'han d'escollir amb cura. És per aquesta darrera raó que en algunes ocasions s'ha de emprar el calibratge extern que evita els fenòmens de supressió iònica. De fet alguns instruments com el Q-TOF de Micromass incorporen un sistema dual d'esprai, un per la mostra i un altre per el calibratge intern, denominat *lock spray.* En aquesta tesis es va dur a terme el calibratge intern escollint en cada cas la mescla de *lock mass* més adient per tal d'evitar la supressió iònica. A la Figura 2.6 es mostren els resultats obtinguts en la mesura de massa exacta del CQ realitzant 10 injeccions successives d'un estàndard de 50 µg L^{-1}. La mesura de la massa es va obtenir amb una bona exactitud i a més es pot observar a la Figura 2.6 una molt bona estabilitat en la mesura tenint en tots els casos errors inferiors als 5 mDa i una bona repetibilitat amb una desviació estàndard (SD) de 0,00095 en el valor de *m/z*.

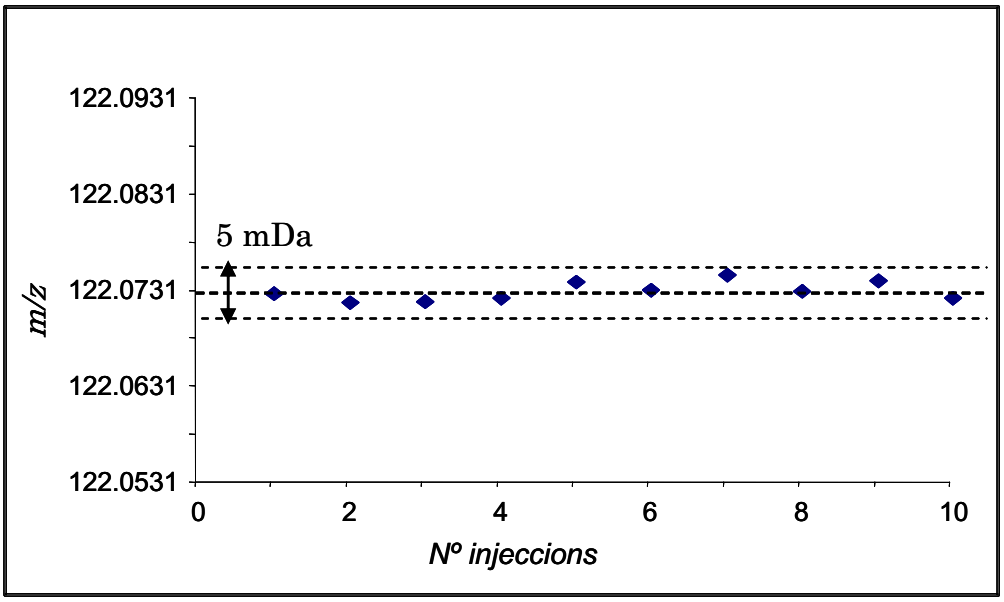


Figura 2.6. Estabilitat en la mesura de la massa exacta (10 injeccions successives CQ d'un patró de 50 µg L^{-1}). Resolució 0,04 *m/z* FWHM.

Aquesta avaluació de l'estabilitat en la mesura de la massa també es va realitzar en mode negatiu utilitzant com a patró un dels derivats clorats del BPA,

el DCBPA obtenint també en aquest cas una molt bona exactitud en la mesura de la massa així com una bona repetibilitat (Figura 2.7) amb una desviació estàndard de 0,00057 en el valor de *m/z* amb errors en la mesura de la massa en mode negatiu també inferiors als 5 mDa. En aquest cas es va utilitzar com a *internal lock masses el* monoclorobisfenol A (MCBPA, m/z 261,0688) i el triclorobisfenol A (TCBPA, 329,9908) (Taula 2.2).

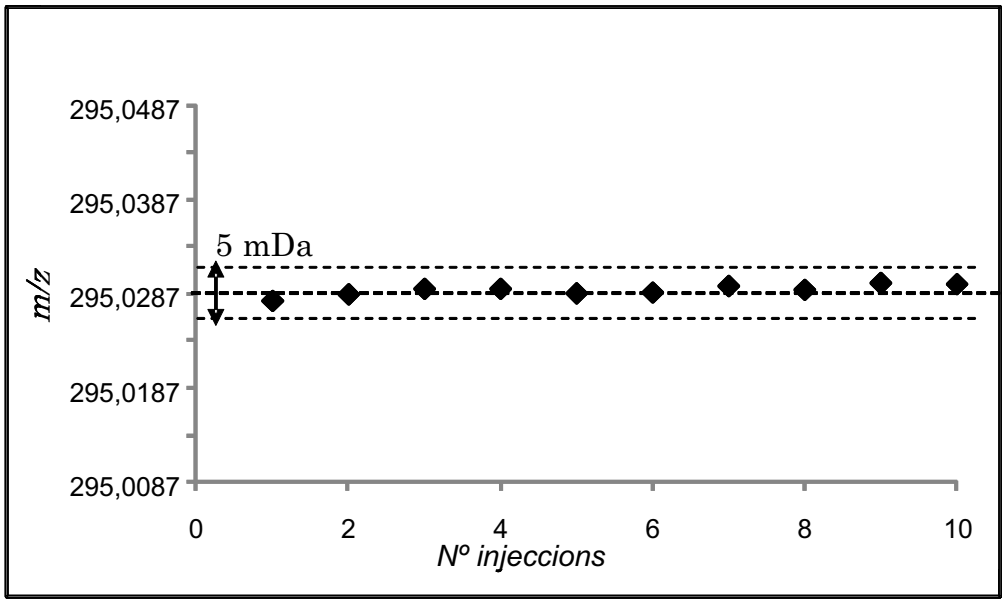


Figura 2.7. Estabilitat en la mesura de la massa exacta (10 injeccions successives d'un patró de DCBPA de 50 μg L^{-1}). Resolució 0,04 *m/z* FWHM.

Atès que en molts casos es realitzen mesures de massa exacta per tal d'assignar la composició elemental d'ions producte també es va avaluar l'exactitud en la mesura de la massa en el mode H-SRM treballant a baixa resolució en el primer quadrupol (0,7 *m/z* FWHM) i augmentant la resolució del tercer quadrupol fins a 0,04 *m/z* FWHM obtenint com en els casos anteriors errors inferiors als 5 mDa. Cal indicar que en la majoria dels casos es va emprar una energia de col·lisió de 5 eV per tal d'evitar la fragmentació dels ions precursor dels *internal lock masses* utilitzats i poder utilitzar aquests ions per corregir les mesures de *m/z* dels ions producte dels compostos estudiats.

2.2. TREBALL EXPERIMETAL

2.2.1. ESTUDIS DE FRAGMENTACIÓ

En aquest apartat s'inclou l'estudi de la fragmentació dels compostos estudiats en aquesta memòria on es mostra com la informació obtinguda amb diferents analitzador de masses s'utilitza de forma complementaria. En concret s'aprofiten els avantatges de les fragmentacions successives en el temps (MS^n) per establir l'ordre genealògic dels ions producte en treballar amb la trampa d'ions. Aquests estudis es complementen amb les mesures de massa exacta, tant dels ions precursors com dels ions producte dutes a terme amb l'analitzador de triple quadrupol treballant a una elevada resolució en el tercer quadrupol. El desenvolupament experimental dels estudis de fragmentació, així com les rutes de fragmentació proposades pel BPA i els seus derivats halogenats es troben descrits a l'article II (Apartat 2.2.1.1), intitulat *"Liquid Chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives"*. Pel que fa referència a les rutes de fragmentació proposades a partir dels estudis de fragmentació dels BADGEs i BFDGEs, aquestes s'inclouen a l'article III (Apartat 2.2.1.2), intitulat *"Multiple-stage mass spectrometry analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives",* d'aquest capítol. El treball experimental realitzat en relació a l'estudi de la fragmentació dels fotoiniciadors es descriu a l'apartat 2.2.1.3 on es descriuen els resultats obtinguts en els estudis de fragmentació d'aquests compostos emprant un analitzador de triple quadrupol i una trampa d'ions.

2.2.1.1 ARTICLE CIENTÍFIC II.

Liquid Chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives

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Liquid chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives

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We report a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for analyzing bisphenol A (BPA) and its halogenated derivatives. Since only tetrachlorobisphenol A and tetrabromobisphenol A (TBBPA) are commercially available, mono-, di- and trichlorobisphenol A were synthesized and purified in order to be used as analytical standards. This family of compounds was studied using electrospray ionization and an ion trap mass analyzer in order to characterize the new compounds and to propose fragmentation pathways. Multi-stage mass spectrometry was used to confirm the genealogical relationship between the ions. Some product ions were traced from MS/MS to MS^4 and the labelled compounds BPA-d_{16} and TBBPA-$^{13}C_{12}$ were used to assign some product ion structures. In general, the deprotonated molecule $[M–H]^-$ loses a methyl and/or a halogen group during both MS/MS and MS^3, while the neutral loss of CO was also observed in MS^3 spectra. We selected the most intense and characteristic MS/MS transitions for LC/MS/MS analysis. LC separation was performed in a reversed-phase column; methanol/water (no additives) was used as the mobile phase in gradient elution mode; and BPA-d_{16} was chosen as the internal standard. Solid-phase extraction (SPE) was used to pre-concentrate and to clean up water samples. The SPE LC/MS/MS method allows BPA and its halogenated derivatives to be detected at a few parts-per-billion (ppb) in surface water. Copyright © 2007 John Wiley & Sons, Ltd.

Bisphenol A (BPA) is widely used in the production of epoxy resins and polycarbonate plastics, which are employed as coatings especially for food-contact surfaces in cans, for electric and electronic equipment, and for digital supports such as CDs and DVDs. Hydrolysis of polycarbonate plastics and epoxy resins results in BPA monomer leaching into the environment. BPA has therefore been found in sediments (0.6–5.0 ng g^{-1}),[1,2] sewage sludge (25–325 μg g^{-1}),[3] and environmental water samples (20–1300 ng L^{-1}).[4–11] Some halogenated derivatives of BPA – such as tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TeCBPA) – are commonly used as flame retardants in polymers and due to their extensive use they are also found in the environment. For instance, TBBPA has been detected in sediments (2–300 ng/g),[8,12,13] sewage sludge (65–100 ng g^{-1}),[13] and air (14–150 ng m^{-3} in indoor air).[14,15] During water disinfection and blenched paper recycling, BPA can become chlorinated and its derivatives (monochloro-, dichloro-, trichloro- and tetrachlorobisphenol A) can be released into the environment. Final effluents from paper recycling plants have been found to contain 0.2–2.0 μg L^{-1} of these compounds.[16,17]

BPA is acutely toxic to aquatic organisms,[19–22] and, at low doses (below 5 mg/kg/day),[18–20] it has been reported to disrupt endocrine function. Toxicity studies of the chlorinated derivatives suggest that their estrogenic activity is stronger than that of BPA,[16,23–25] and TeCBPA also shows thyroid hormonal activity.[26] Furthermore, *in vitro* toxicity experiments indicate that TBBPA is an immunotoxic compound[27] that disrupts endocrine function. At present there are no restrictions on any of these compounds, although TBBPA is currently being evaluated in the EU, US and Asian-Pacific countries.[28] Nevertheless, as more than 1000 tons/year of TBBPA is produced, it is now considered a high volume substance under REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and it will have to be registered within 3 years of REACH coming into force in the EU.

BPA is usually detected by using liquid chromatography (LC) with different detection systems, such as spectrophotometry (UV),[11,29,30] mass spectrometry (MS),[2,4,31] or fluorescence,[30,32] although some methods based on gas chromatography coupled to mass spectrometry (GC/MS) have also been used.[2,3,5–7] For TBBPA, both LC and GC coupled to MS are used.[12,14,15,33] Chlorinated derivatives of BPA are generally analyzed by GC/MS after derivatization of the phenolic groups with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)[25,26] or *N*-(*tert*-butyldimethylsilyl)-*N*- methyltrifluoroacetamide (MTBSTFA).[34,35] Since there are no published LC/MS and LC/MS/MS methods for the simultaneous detection of BPA and its halogenated derivatives (MCBPA, DCBPA,

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TCBPA, TeCBPA and TBBPA), the aim of the present work is to develop a sensitive and selective LC/MS/MS method for their detection in water samples. Furthermore, we apply multi-stage ion trap mass spectrometry (MS^n) to study the fragmentation pathways of this family of compounds.

EXPERIMENTAL

Chemicals

Bisphenol A, 2,2-bis(4-hydroxyphenyl)propane (BPA) (Table 1), bisphenol A-d_{16} (BPA-d_{16}), tetrachlorobisphenol A, 2,2-bis(3,5-dichloro-4-hydroxyphenyl)propane (TeCBPA), tetrabromobisphenol A, 2,2-bis(3,5-dibromo-4-hydroxyphenyl)propane (TBBPA) were obtained from Sigma-Aldrich (Steinheim, Germany), and tetrabromobisphenol A (Ring-$^{13}C_{12}$) (TBBPA-$^{13}C_{12}$) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). HPLC-gradient grade methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), dichloromethane (DCM) and water as well as hydrochloric acid (25%) and sodium hydroxide for analysis were purchased from Merck (Darmstadt, Germany), while sodium hypochlorite solution (10% chlorine) and anhydrous sodium sulfate were obtained from Flucka (Buchs, Germany). $CDCl_3$ purchased from Sigma-Aldrich was used as the solvent for NMR. Stock standard solutions of individual compounds and BPA-d_{16} (10 mg L^{-1}) were prepared in MeOH and stored at 4°C. Mobile phases were filtered using a 0.45 μm nylon filter (Whatman, Clifton, NJ, USA). Bond Elute C_{18} (500 mg) cartridges purchased from Varian (Harbor City, CA, USA) were used for solid-phase extraction (SPE).

Nitrogen (99.8% pure) supplied by a Claind N_2 FLO nitrogen generator (Lenno, Italy) was used in the atmospheric pressure ionization (API) source. Helium of high purity purchased from Air Liquide (Madrid, Spain) was used as a damper gas for the ion trap.

Synthesis of chlorinated derivatives of bisphenol A

Chlorinated derivatives of BPA (MCBPA, DCBPA and TCBPA) (Table 1) were synthesized and purified to be used as standards. The synthesis was based on the chlorination of BPA by aromatic electrophilic substitution since the hydroxyl group activates the *ortho* position.

Sodium hypochlorite solution (75 mL, >10% chlorine available) and hydrochloric acid (25%) were mixed to generate a chlorine gas current that was bubbled, at room temperature, into a stirred solution of BPA (0.5 g) previously prepared in EtOH/H_2O (20:80 v/v) and adjusted to pH 8 with sodium hydroxide (0.1 M). After the chlorination step, the solution was heated to 60°C to eliminate the residual chlorine and centrifuged (45 min, 2000 rpm). The aqueous solution was decanted and extracted with DCM (5 × 20 mL). The solid residue was dissolved in the DCM extract and this organic solution was dried using anhydrous sodium sulfate and then evaporated to dryness. The residue was reconstituted in 30 mL of ACN/water (50:50). A reversed-phase C_{18} semi-preparative column (eluent: ACN/water, 50:50) was used to purify the extract. BPA and its chlorinated derivatives were separated at base line resolution and multiple injections (500 μL) were performed to collect combined individual fractions of each compound. Each combined fraction was evaporated using a Turbo Vap II concentration workstation (Zymark Corp., Hopkinton, MA, USA) (30 min at 8 psi and 25°C) to eliminate the ACN. The aqueous extract was re-extracted with DCM (5 × 20 mL). Finally, the solvent was evaporated to leave a crystalline product. The purity (>99%) of these compounds was established by ^{1}H NMR and confirmed by LC/MS.

Monochlorobisphenol A (MCBPA) ^{1}H NMR ($CDCl_3$); δ: 1.60 ppm (6H, s, 2 × CH_3), 4.98 ppm (1H, s, OH), 5.45 ppm (1H, s, OH), 6.74 ppm (2H, dt, $^3J_{ortho} = 8.8$ Hz, $^4J_{meta} = 2.6$ Hz, 2 × ArH), 6.89 ppm (1H, d, $^3J_{ortho} = 8.4$ Hz, ArH), 7.00 ppm (1H, dd, $^3J_{ortho} = 8.4$ Hz, $^4J_{meta} = 2.4$ Hz, ArH), 7.07 ppm (2H, dt, $^3J_{ortho} = 9.2$ Hz, $^4J_{meta} = 2.6$ Hz, 2 × ArH), 7.17 ppm (1H, d, $^4J_{meta} = 2.4$ Hz, ArH).

Dichlorobisphenol A (DCBPA) ^{1}H NMR ($CDCl_3$); δ: 1.60 ppm (6H, s, 2 × CH_3), 5.47 ppm (2H, s, 2 × OH), 6.91 ppm (2H, d, $^3J_{ortho} = 8.4$ Hz, 2 × ArH), 7.00 ppm (2H, dd, $^3J_{ortho} = 8.6$ Hz, $^4J_{meta} = 2.2$ Hz, 2 × ArH), 7.16 ppm (2H, d, $^4J_{meta} = 2.00$ Hz, 2 × ArH). Only 3,3′-dichlorobisphenol A was obtained.

Trichlorobisphenol A (TCBPA) ^{1}H NMR ($CDCl_3$); δ: 1.60 ppm (6H, s, 2 × CH_3), 5.51 ppm (1H, s, OH), 5.77 ppm (1H, s, OH), 6.93 ppm (1H, d, $^3J_{ortho} = 8.4$ Hz, ArH), 6.98 ppm (1H, dd, $^3J_{ortho} = 8.6$ Hz, $^4J_{meta} = 2.2$ Hz, ArH), 7.15 ppm (1H, d, $^4J_{meta} = 2.4$ Hz, ArH), 7.08 ppm (2H, s, 2 × ArH).

These spectra match the published[17] spectra for these compounds perfectly and no signal from impurities was found in the synthesized compounds.

Instrumentation

^{1}H NMR

^{1}H NMR spectra of the synthesized compounds dissolved in deuterated chloroform were obtained using a Varian Model Mercury 400 NMR spectrometer (400 MHz; Varian, Palo Alto, CA, USA).

Liquid chromatography

A liquid chromatograph (Alliance 2695 separations module; Waters, Milford, MA, USA) equipped with a low-pressure quaternary solvent pumping system and an autosampler was used. The chromatographic separation was carried out in a SunFire™ C_{18} column, 150 × 2.1 mm i.d., 3.5 μm particle size (Waters), using MeOH/water as the mobile phase, a gradient elution program, a flow rate of 200 μL min^{-1} and an injection volume of 10 μL. Gradient 1: started at 65% MeOH and rose to 85% MeOH in 5 min; this percentage was maintained for 9 min. Finally, the mobile phase was returned to the initial conditions over 5 min and equilibrated for a further 10 min. Gradient 2: started at 50% MeOH for 1.5 min followed by a linear gradient up to 65% MeOH in 0.5 min and a second linear gradient step up to 85% MeOH in 5 min; this percentage was maintained for 8 min. Finally, the mobile phase was returned to the initial conditions over 5 min and this was followed by a 10 min equilibration step.

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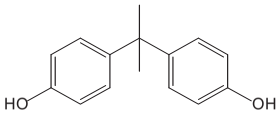
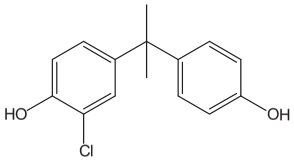
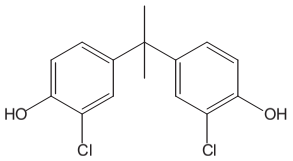
Table 1. Optimized CID conditions in MS^2 and MS^3 and main product ions obtained for BPA and its halogenated compounds and the corresponding labelled compounds

	MS		MS/MS				MS^3			
Compound	*m/z* (% Rel.Ab.)	Assignment	NCE (%)	AQ	*m/z* (% Rel.Ab.)	Assignment	NCE (%)	AQ	*m/z* (% Rel.Ab.)	Assignment
Bisphenol A (BPA) 	227 (100)	$[M–H]^-$	44	0.35	227 (23)	$[M–H]^-$				
					212 (100)	$[M–H–CH_3]^{-\bullet}$	43	0.35	212 (10)	$[M–H–CH_3]^{-\bullet}$
									211 (100)	$[M–H–CH_3–H]^-$
					211 (35)	$[M–H–CH_4]^-$			196 (21)	$[M–H–CH_3–CH_4]^{-\bullet}$
									117 (34)	$[M–H–CH_3–C_6H_7O]^-$
									93 (16)	$[M–H–CH_3–C_8H_7O]^-$
					133 (85)	$[M–H–C_6H_6O]^-$	44	0.35	133 (100)	$[M–H–C_6H_6O]^-$
									93 (22)	$[M–H–C_6H_6O–C_3H_4]^-$
					93 (20)	$[M–H–C_9H_{10}O]^-$				
Monochlorobisphenol A (MCBPA) 	261 (100) 263 (34)	$[M–H]^-$	44	0.45	261 (8)	$[M–H]^-$				
					246 (100)	$[M–H–CH_3]^{-\bullet}$	44	0.45	246 (11)	$[M–H–CH_3]^{-\bullet}$
									210 (100)	$[M–H–CH_3–HCl]^{-\bullet}$
									182 (4)	$[M–H–CH_3–CHOCl]^{-\bullet}$
					225 (4)	$[M–H–HCl]^-$				
					210 (68)	$[M–H–CH_4Cl]^{-\bullet}$	32	0.45	210 (20)	$[M–H–CH_4Cl]^{-\bullet}$
									209 (10)	$[M–H–CH_4Cl–H]^-$
									195 (6)	$[M–H–CH_4Cl–CH_3]^-$
									182 (100)	$[M–H–CH_4Cl–CO]^{-\bullet}$
					195 (<5)	$[M–H–C_2H_7Cl]^-$				
					182 (7)	$[M–H–C_2H_4OCl]^{-\bullet}$				
					167 (<5)	$[M–H–C_6H_6O]^-$				
					133 (<5)	$[M–H–C_6H_5OCl]^-$				
Dichlorobisphenol A (DCBPA) 	295 (100) 297 (66) 299 (11)	$[M–H]^-$	44	0.45	295 (5)	$[M–H]^-$				
					280 (26)	$[M–H–CH_3]^{-\bullet}$	28	0.45	280 (10)	$[M–H–CH_3]^{-\bullet}$
									244 (100)	$[M–H–CH_3–HCl]^{-\bullet}$
					259 (6)	$[M–H–HCl]^-$	39	0.45	259 (60)	$[M–H–HCl]^-$
									244 (100)	$[M–H–HCl–CH_3]^{-\bullet}$
									216 (64)	$[M–H–HCl–C_2H_3O]^{-\bullet}$
									208 (24)	$[M–H–HCl–CH_4Cl]^{-\bullet}$
					244 (100)	$[M–H–CH_4Cl]^{-\bullet}$	34	0.45	244 (15)	$[M–H–CH_4Cl]^{-\bullet}$
									229 (11)	$[M–H–CH_4Cl–CH_3]^-$
									216 (100)	$[M–H–CH_4Cl–CO]^{-\bullet}$
					216 (5)	$[M–H–C_2H_4OCl]^{-\bullet}$				
					167 (<5)	$[M–H–C_6H_5OCl]^-$				

(*Continues*)

DOI: 10.1002/rcm

RCM

Table 1. (Continued)

Compound	MS		MS/MS			MS^3			
	m/z (% Rel.Ab.)	Assignment	NCE (%)	AQ	*m/z* (% Rel.Ab.)	Assignment	NCE (%)	AQ	*m/z* (% Rel.Ab.)	Assignment
Trichlorobisphenol A (TCBPA)	329 (100)	$[M–H]^-$	44	0.45	329 (7)	$[M–H]^-$				
	331 (99)									
	333 (32)				314 (29)	$[M–H–CH_3]^{-\bullet}$	28	0.45	314 (11)	$[M–H–CH_3]^{-\bullet}$
	335 (3)								278 (100)	$[M–H–CH_3–HCl]^{-\bullet}$
					293 (5)	$[M–H–HCl]^-$	38	0.45	293 (100)	$[M–H–HCl]^-$
									278 (94)	$[M–H–HCl–CH_3]^{-\bullet}$
									250 (51)	$[M–H–HCl–C_2H_3O]^{-\bullet}$
					278 (100)	$[M–H–CH_4Cl]^{-\bullet}$	32	0.45	278 (19)	$[M–H–CH_4Cl]^{-\bullet}$
									263 (<5)	$[M–H–CH_4Cl–CH_3]^-$
									250 (100)	$[M–H–CH_4Cl–CO]^{-\bullet}$
					250 (6)	$[M–H–C_2H_4OCl]^{-\bullet}$	31	0.45	250 (100)	$[M–C_2H_4OCl]^{-\bullet}$
									249 (46)	$[M–C_2H_4OCl–H]^-$
									214 (16)	$[M–C_2H_4OCl–HCl]^{-\bullet}$
					201 (<5)	$[M–H–C_6H_5OCl]^-$				
Tetrachlorobisphenol A (TeCBPA)	363 (76)	$[M–H]^-$	46	0.45	363 (32)	$[M–H]^-$				
	365 (100)									
	367 (47)				348 (20)	$[M–H–CH_3]^{-\bullet}$	30	0.45	348 (19)	$[M–H–CH_3]^{-\bullet}$
	369 (10)								312 (100)	$[M–H–CH_3–HCl]^{-\bullet}$
					327 (<5)	$[M–H–HCl]^-$	46	0.45	327 (36)	$[M–H–HCl]^-$
									312 (49)	$[M–H–HCl–CH_3]^{-\bullet}$
									284 (100)	$[M–H–HCl–C_2H_3O]^{-\bullet}$
					312 (100)	$[M–H–CH_4Cl]^{-\bullet}$	35	0.45	312 (21)	$[M–H–CH_4Cl]^{-\bullet}$
									297 (<5)	$[M–H–CH_4Cl–CH_3]^-$
									284 (100)	$[M–H–CH_4Cl–CO]^{-\bullet}$
					284 (<5)	$[M–H–C_2H_4OCl]^{-\bullet}$	38	0.45	284 (30)	$[M–H–C_2H_4OCl]^{-\bullet}$
									248 (100)	$[M–H–C_2H_4OCl–HCl]^{-\bullet}$
					201 (<5)	$[M–H–C_6H_4OCl_2]^-$				
Tetrabromobisphenol A (TBBPA)	539 (18)	$[M–H]^-$	40	0.45	539 (20)	$[M–H]^-$				
	541 (70)									
	543 (100)				524 (65)	$[M–H–CH_3]^{-\bullet}$	27	0.45	524 (32)	$[M–H–CH_3]^{-\bullet}$
	545 (63)								444 (100)	$[M–H–CH_3–HBr]^{-\bullet}$
	547 (15)									
					459 (15)	$[M–H–HBr]^-$				
					444 (100)	$[M–H–CH_4Br]^{-\bullet}$	34	0.45	444 (90)	$[M–H–CH_4Br]^{-\bullet}$
									429 (30)	$[M–H–CH_4Br–CH_3]^-$
									416 (100)	$[M–H–CH_4Br–CO]^{-\bullet}$
					416 (<5)	$[M–H–C_2H_4OBr]^{-\bullet}$				
					289 (<5)	$[M–H–C_6H_4OBr_2]^-$				

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Semi-preparative liquid chromatography

A Waters 600E liquid chromatograph equipped with a Rheodyne injection valve (Cotati, CA, USA) with a 500 μL loop and a model 432 fixed wavelength detector (Kontro Instruments, Fantoly, Italy) was used to purify the chlorinated BPA derivatives. A SunFire™ Prep C_{18} column, 100 × 10 mm i.d., 5 μm particle size (Waters), was used for the chromatographic separation at a flow rate of 3 mL min^{-1}, injecting 500 μL onto the column. An ACN/water gradient elution was used for the separation of BPA and its halogenated derivatives. The elution program started with 40% ACN and proceeded with a linear gradient up to 60% ACN over 10 min. This was followed by a 10 min isocratic step. Finally, the mobile phase was returned to the initial conditions over 5 min, and the column was equilibrated for 5 min. A model II fraction collector from Waters was used to collect peak fractions. Data were processed using Chromatography Software from Borwin (Vienna, VA, USA).

Mass spectrometry

The liquid chromatographic system (Alliance 2695) was coupled to a Classic LCQ instrument (ThermoFinningan, San Jose, CA, USA) equipped with an ion trap mass analyzer and both a coaxial pneumatically assisted electrospray ionization (ESI) and an atmospheric pressure chemical ionization (APCI) source. Data acquisition was performed in the negative ion mode and the Xcalibur software version 1.4 (ThermoFinnigan) was used to control the LC/MS system and to process data.

The ESI working conditions were: sheath gas and auxiliary gas (N_2) flow rates 74 a.u. (arbitrary units) and 52 a.u., respectively; capillary heater temperature 280°C; electrospray needle voltage −4.0 kV; and tube lens offset voltage −13.0 V. The APCI working parameters were: sheath gas flow rate 35 a.u. and no auxiliary gas; vaporizer temperature 275°C; capillary heater temperature 200°C; corona discharge voltage −4.0 kV; spray current 4.5 μA; and tube lens offset voltage −10.0 V. Product ion spectra from the multi-stage mass spectrometry (MS^n) experiments were acquired (m/z 50–600) using profile mode. The $[M–H]^-$ ion was used as the precursor ion for tandem mass spectrometry experiments under the following working conditions: isolation width of 1.5 m/z units; 5 μscans; maximum injection time 200 ms; and activation time 30 ms. The trapping radio-frequency voltage (AQ) was set at a value between 0.35 and 0.45 and the normalized collision energy (NCE%, amplitude of the voltage applied to the end-cap electrode) was from 38 to 44%. Table 1 gives the AQ and NCE% values selected for each analyte.

A 10 mg L^{-1} stock standard solution of each compound prepared in MeOH was infused at a flow rate of 10 μL min^{-1} using the syringe pump integrated in the LCQ instrument. It was mixed with the mobile phase (200 μL min^{-1}, MeOH/H_2O (75:25 v/v)) by means a Valco zero dead volume tee piece (Supelco, Alcobendas, Spain) to optimize the source working conditions and to carry out the MS^n experiments. Quantitative analysis was carried out by the standard addition method and using BPA-d_{16} as the internal standard.

Sample treatment

Wastewater samples from a paper recycling plant were collected in 1-L glass bottles and 1 mL of ascorbic acid (0.1 M) was added in order to avoid chlorination during storage (4°C) due to the residual chlorine. Off-line SPE using reversed-phase C_{18} cartridges (Bond Elute, 500 mg; Varian) was used as a clean-up and pre-concentration step before LC/MS/MS analysis. First, the C_{18} cartridge was conditioned using 10 mL of MeOH and 6 mL of water, then 50 mL of a water sample was loaded and the cartridge was washed with 10 mL of water and 10 mL of MeOH/water (20:80 v/v). Finally it was dried and the analytes were eluted using 5 mL of MeOH. The collected fraction was evaporated to dryness, the extract was reconstituted with 500 μL of an internal standard MeOH solution (BPA-d_{16} at 145 μg L^{-1}) and 10 μL of this extract was injected into the LC/MS/MS system. A Supelco Visisprep and a Supelco Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for SPE and solvent evaporation.

RESULTS AND DISCUSSION

Chlorinated derivatives of BPA were synthesized and purified to provide analytical standards of mono-, di- and trichloro-BPA since these are not commercially available. ^{1}H NMR allowed us to identify the DCBPA isomer synthesized, since two isomers, 3,3′-DCBPA and 3,5-DCBPA, can be obtained. The analysis of the aromatic range of the ^{1}H NMR spectrum (Fig. 1) revealed signals associated with three different types of aromatic protons and the multiplicity of these signals agreed with those of the 3,3′-DCBPA isomer. The doublet (d) at 6.91 ppm (2H, d, $^3J_{ortho} = 8.4$ Hz, 2 × ArH) corresponded to ortho-protons, the doublet doublet (dd) at 7.00 ppm (2H, dd, $^3J_{ortho} = 8.6$ Hz, $^4J_{meta} = 2.2$ Hz, 2 × ArH) could be related to meta-protons and the doublet at 7.16 ppm (2H, d, $^4J_{meta} = 2.00$ Hz, 2 × ArH) was due to meta-protons in the *ortho* position with respect to the chlorine atoms. Moreover, no signals that could be associated with the presence of the other isomer were present.

Mass spectrometry fragmentation studies

Electrospray ionization (ESI) in negative ion mode was used for the fragmentation studies. This ionization technique provided a full scan single MS spectrum where the base peak was the isotopic cluster corresponding to the deprotonation of one hydroxyl group $[M–H]^-$ and no additional fragments or adducts were observed.

Multi-stage mass spectrometry (MS^n) was used to study the fragmentation of BPA and its halogenated derivatives (MCBPA, DCBPA, TCBPA, TeCBPA and TBBPA). Labelled compounds (BPA-d_{16} and TBBPA-$^{13}C_{12}$) were also fragmented to help interpret the MS^n spectra. The lightest ion in the isotopic cluster (lowest m/z value) was used as the precursor ion for tandem mass spectrometry experiments. For further MS^n experiments the most abundant product ions or the most characteristic ones were used as precursor ions. Table 1 summarizes the collision-induced dissociation (CID) working conditions, the main product ions and their

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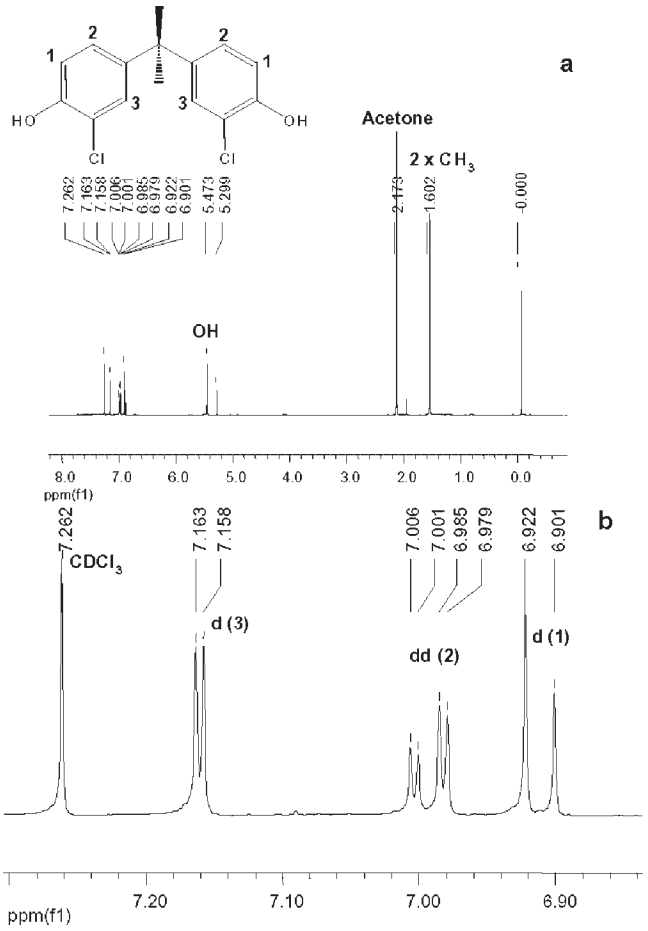

Figure 1. ^{1}H NMR spectra of DCBPA: (a) complete spectrum and (b) aromatic range enlargement.

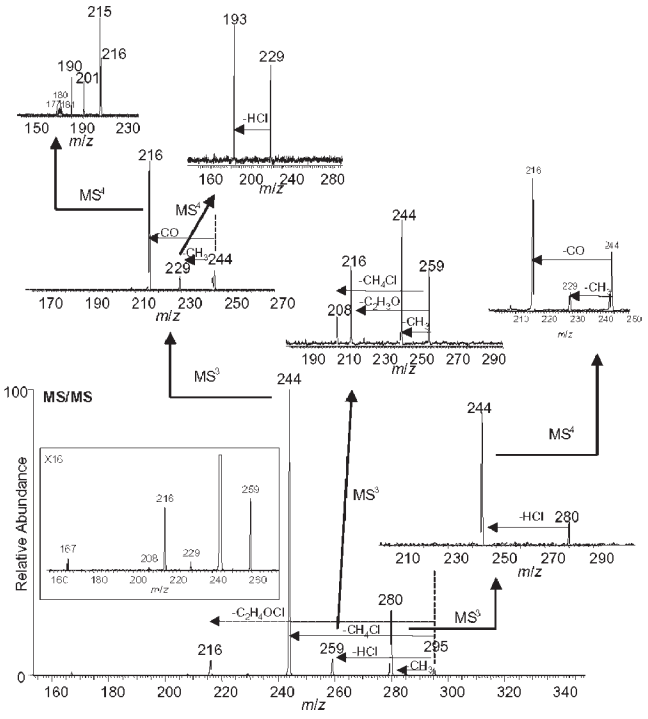

Figure 2. MS^2 spectra of DCBPA and higher-order mass spectra of some of the most important product ions. Working conditions as detailed in the Experimental section; CID conditions as given in Table 1.

assignment in MS/MS and MS^3 for BPA and its halogenated compounds. Figure 2 shows the MS/MS spectrum of the selected precursor ion of DCBPA and MS^n spectra of some of the most abundant product ions.

For BPA and MCBPA the MS/MS spectra show as base peaks the product ion resulting from the loss of a methyl group $[M–H–CH_3]^{-\bullet}$ (*m/z* 212 and 246, respectively). BPA also produced an abundant product ion resulting from the cleavage of the hydroxybenzyl group $[M–H–C_6H_5OH]^-$ (*m/z* 133, 85%) while for MCBPA this ion had a low relative abundance, <5%. Although the $[M–H–CH_3]^{-\bullet}$ ion is also present in the MS/MS spectra of the other halogenated derivatives of BPA, for these compounds the base peak is an ion resulting from the combined loss of a methyl group and HCl or HBr, $[M–H–CH_4X]^{-\bullet}$ (X = Cl, Br). Moreover, for chlorinated derivatives, the $[M–H–HCl]^-$ product ions had relative abundances of less than 6%, while for TBBPA the $[M–H–HBr]^-$ ion was slightly more abundant, 15%. In the MS/MS fragmentation of BPA, a product ion was observed at *m/z* 93, probably due to the cleavage of the hydroxyphenyl propyl bond $[M–H–C_9H_{10}O]^-$. This product ion also appeared in the MS^3 spectra, and it was generated along two different pathways: (i) the consecutive losses of $CH_3^{\bullet}$ (*m/z* 212) and the hydroxyphenylethyl (C_8H_7O) group, and (ii) the loss of the hydroxyphenyl (C_6H_6O) group (*m/z* 133) followed by the loss of the propyl group.

Halogenated derivatives of BPA showed a $[M–H–CH_4X–28]^{-\bullet}$ MS^3 product ion. To investigate this fragmentation the MS^n spectra of TBBPA-$^{13}C_{12}$ (^{13}C in the aromatic rings) were studied. Figure 3 shows the MS/MS spectrum of the most abundant ion in the isotopic cluster ($^{79}Br_2$ $^{81}Br_2$, *m/z* 555) and higher order fragmentation spectra of the main product ions. The MS^3 spectra obtained from precursor ions

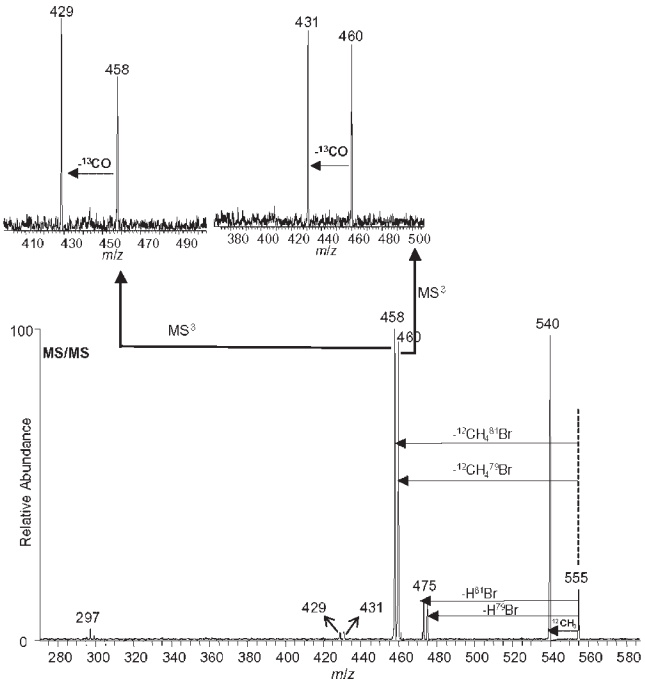

Figure 3. MS^2 spectra of TBBPA-$^{13}C_{12}$ (ring) and higher-order mass spectra of some of the most important product ions.

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at *m/z* 460 and 458 show a product ion formed by loss of 29 Da, indicating that a ^{13}C atom is involved in this fragmentation step; this is due to the neutral loss of a CO group, a characteristic fragmentation of phenolic compounds.

These fragmentation study results lead us to propose a common fragmentation pathway for this family of compounds (Fig. 4). First a $CH_3^{\bullet}$ and/or HCl or HBr are lost, followed by the loss of HCl, HBr or $CH_3^{\bullet}$ depending on the first fragmentation step. Later a neutral loss of CO takes place, and finally in the MS^4 or MS^5 spectra the cleavage of the other methyl and the other halogen group occurred. Moreover, the elimination of one aromatic ring in MS/MS is another important fragmentation that provides information about the number of halogen substitutions in each phenolic group.

We also observed in the MS/MS spectra of BPA and BPA-d_{16} (used as internal standard, IS) ions at *m/z* values – *m/z* 244 and 255, respectively – higher than the ions for the deprotonated molecules. These ions could be obtained from an ion-molecule reaction[36] between the ion originating from the loss of the methyl group and MeOH from the mobile phase. As an example, Fig. 5 shows the MS/MS spectra of BPA-d_{16} where an ion is observed at *m/z* 255. Moreover, the single MS spectrum of this compound showed an ion at *m/z* 241 $[(M-D_2+H_2)-H]^-$ instead of at *m/z* 242 $[M-D]^-$,[37] due to a deuterium/hydrogen (D/H) exchange in the hydroxyl groups. D/H exchange was also observed in the MS^3 spectrum of the adduct ion at *m/z* 255 that shows the loss of one $H^{\bullet}$ (from the hydroxyl group) to *m/z* 254 and the loss of MeOH (*m/z* 222) or MeOD (*m/z* 221).

For quantitative proposes, transitions between the most abundant ion of the molecular cluster and the most abundant product ion were used.

Liquid chromatography/mass spectrometry

When analyzing this family of compounds by LC/MS, it was observed that mobile phase composition had an important effect on sensitivity, especially in the response of BPA. Different elution gradients were tested in order to optimize the separation. The best conditions were obtained using an MeOH/water gradient from 65 to 85% MeOH (gradient 1). In order to promote deprotonation of BPA and its halogenated derivatives, a post-column addition using different bases (ammonia, triethylamine and dimethylamine, 100 mM at a flow rate of 100 μL min^{-1}) was tested. The addition of basic additives to the mobile phase produced important signal suppression – especially for BPA – in agreement with Benijts *et al*.[38] Therefore, no mobile phase additives were used.

When coupling liquid chromatography to mass spectrometry we tested both atmospheric pressure ionization sources: electrospray (ESI) and atmospheric pressure chemical ionization (APCI), in negative ion mode. The ESI source

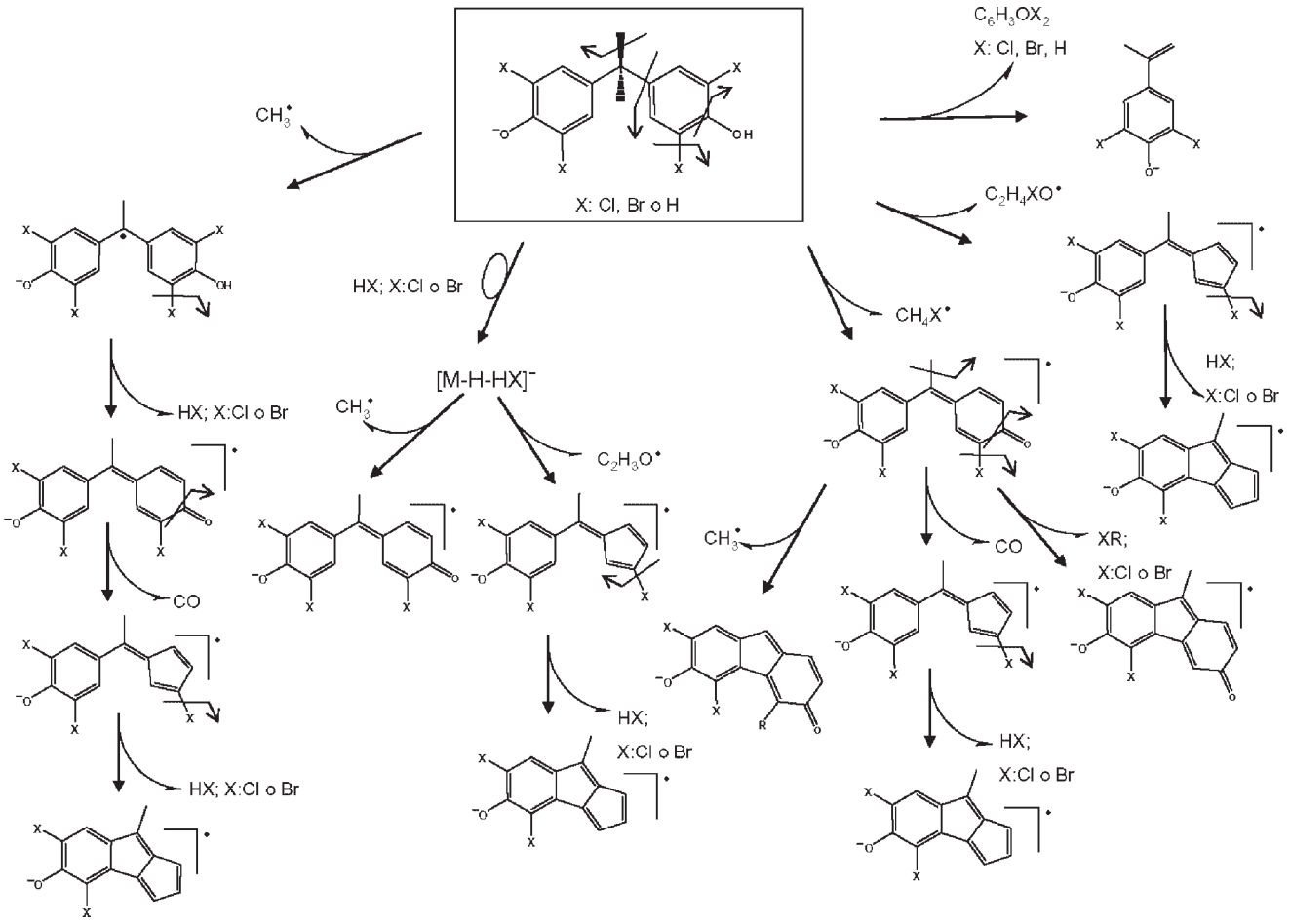

Figure 4. Fragmentation pathway of BPA and its halogenated derivatives.

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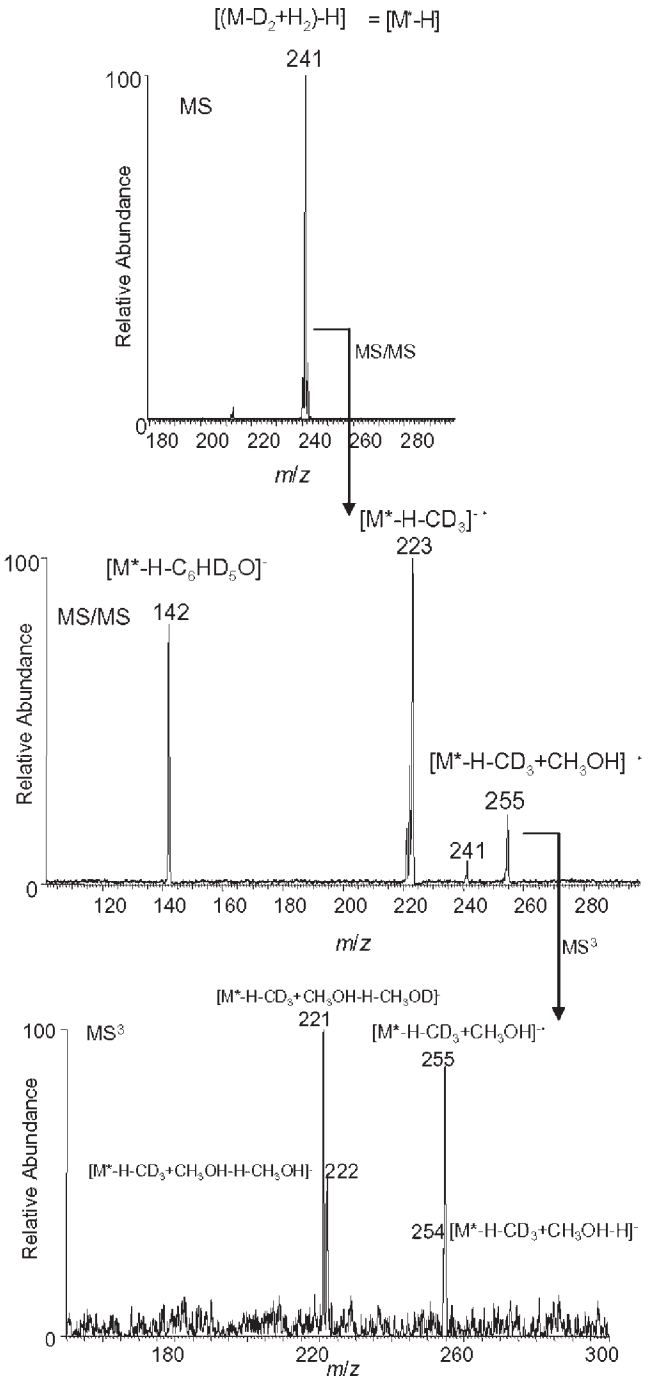

Figure 5. MS, MS/MS and MS^3 spectra of BPA-d_{16}.

provided better responses than the APCI source; at least six times higher (Table 2), since fragmentation of the deprotonated molecule by loss of $CH_3^{\bullet}$ and/or HX occurred in APCI. This fragmentation occurred even under mild temperature conditions and an important increase was observed at high vaporizer and heated capillary temperatures. Moreover, this fragmentation in the APCI source was more important for the highly halogenated compounds: TeCBPA and TBBPA. Therefore, we chose ESI in negative ion mode (which provides soft ionization without in-source fragmentation) for the determination of BPA and its halogenated derivatives in water samples by LC/MS/MS. Under these conditions, detection limits (signal-to-noise (S/N) ratio 3:1) for halogenated derivatives were 10–30 times lower than those for BPA, due to the lower fragmentation efficiency for this compound. In contrast, in single MS (Table 2) the limits of detection (LODs) for BPA were only slightly higher than for the halogenated derivatives compounds.

Water sample analysis

In a preliminary study, surface water samples free from BPA and its halogenated derivatives were spiked (200 $\mu g\,L^{-1}$), pre-concentrated by SPE, and analyzed by LC/MS/MS. The responses for BPA and MCBPA were much lower than those obtained when we analyzed water (HPLC-gradient) spiked at the same level. This can be explained by a matrix ion suppression effect, since both BPA and MCBPA eluted at the beginning of the chromatogram where matrix components also eluted. To avoid ion suppression and thereby enhance the signal for BPA and MCBPA, the gradient elution program was modified: we decreased the methanol percentage of the initial conditions to 50% and included an isocratic step (gradient 2). Under these conditions responses were enhanced. Figure 6 shows the LC/MS chromatograms obtained for mountain river water (Garona, Vall d'Aran, Spain) spiked with BPA and halogenated derivatives at 200 $\mu g\,L^{-1}$ analyzed using both gradient elution programs. At the lower methanol percentage in the mobile phase, the isocratic step increased retention times, reducing the matrix effect and producing similar responses to those obtained with the spiked water (HPLC-gradient). Under these new separation conditions, recovery values and LODs of the off-line SPE-LC/MS/MS method were estimated. We obtained recovery values of more than 85% for all the compounds. Method limits of detection (MLODs), based on a S/N ratio of 3:1, were determined using different aliquots of

Table 2. LC/MS instrumental LODs using ESI and APCI and MLODs for BPA and its halogenated compounds using a river water sample by LC/MS/MS

	Standard solution (LODs)			Water sample (MLODs)	
Compound	LC/APCI-MS (pg inject)	LC/ESI-MS (pg inject)	LC/ESI-MS/MS (pg inject)	SPE *off-line* LC/ESI-MS/MS ($\mu g\,L^{-1}$)	SPE *off-line* LC/ESI-MS/MS (pg inject)
BPA	260	44	182	0.38	324
MCBPA	218	28	20	0.23	197
DCBPA	166	26	10	0.062	53
TCBPA	128	20	6	0.067	57
TeCBPA	186	28	10	0.016	14
TBBPA	232	34	20	0.02	18

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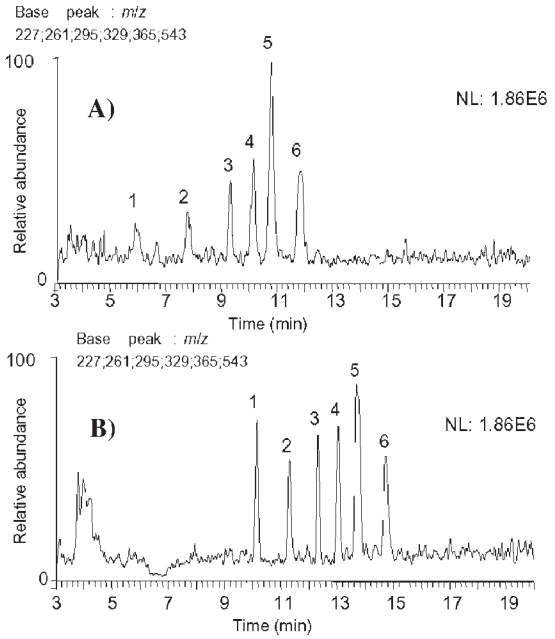

Figure 6. Full scan LC/MS chromatogram of BPA and its halogenated derivatives using two gradient elution programs: (A) gradient 1 and (B) gradient 2. Peak numbers: 1. BPA, 2. MCBPA, 3. DCBPA, 4. TCBPA, 5. TeCBPA, 6. TBBPA.

a mountain river water sample spiked at low concentrations (0.05–0.5 $\mu g\,L^{-1}$). LODs ranging from 16 to 230 $ng\,L^{-1}$ (Table 2) for the halogenated derivatives and of 380 $ng\,L^{-1}$ for BPA were obtained. However, MLODs 2 to 10 times higher than the instrumental LODs were obtained for the first four compounds eluted in the chromatogram (BPA, MCBPA, DCBPA and TCBPA). This could be explained by a residual matrix effect.

To assess the applicability of the optimized off-line SPE-LC/MS/MS method, a water sample from a paper recycling plant was analyzed. Figure 7 shows the LC/MS/MS chromatograms and the mass spectra of the chromatographic peaks obtained for this water sample. Some chlorinated derivatives of BPA (MCBPA, DCBPA, TCBPA and TeCBPA) were identified. A standard addition method (3 zero levels and 4 addition levels) was used for quantification and the concentrations for the identified chlorinated compounds ranged from 464 to 810 $ng\,L^{-1}$ with acceptable relative standard deviations (%RSD from 5 to 16). These concentrations agree with those reported by other authors when analyzing water samples from paper recycling plants in Japan.[22]

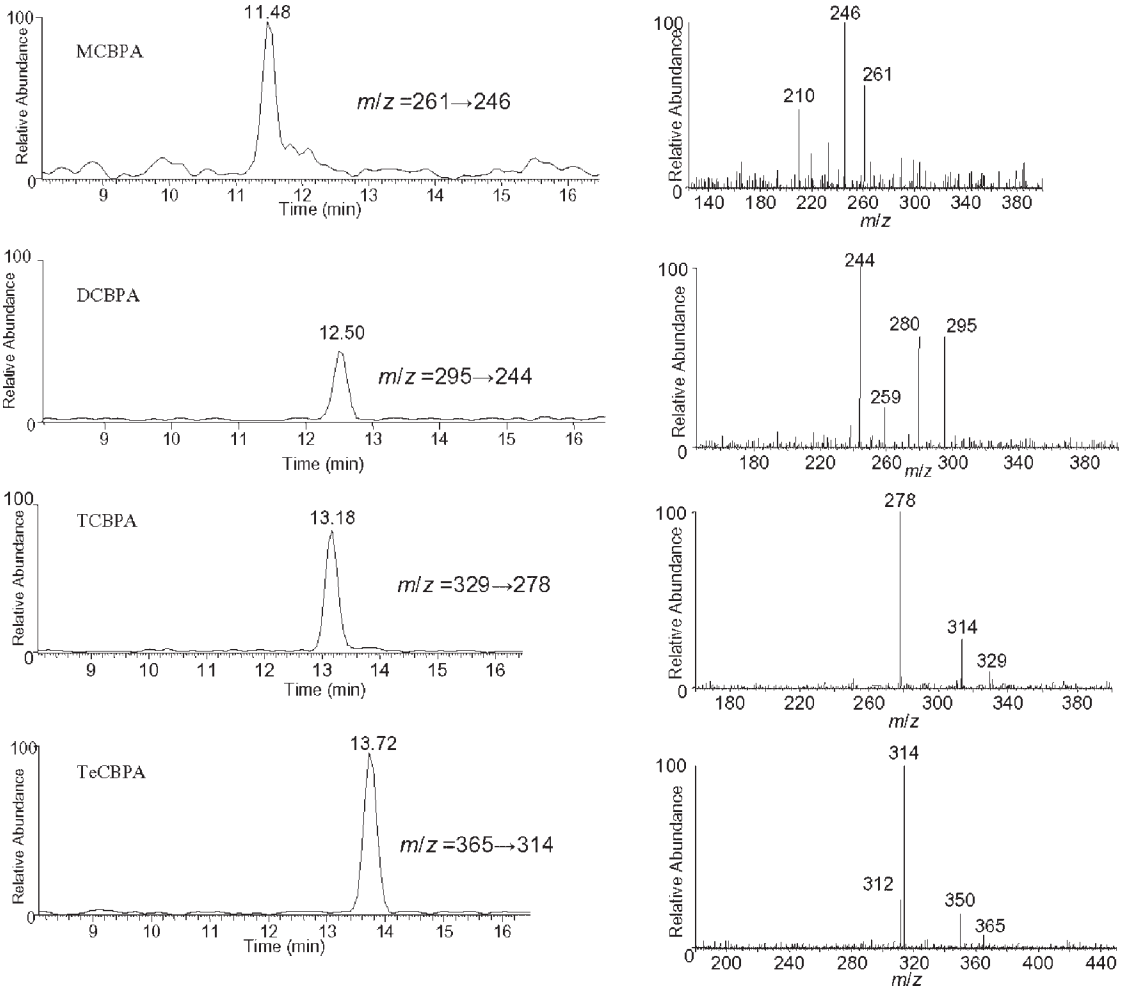

Figure 7. LC/MS/MS chromatograms and product ion scan spectra obtained for the effluent from a paper recycling plant contained: MCBPA, 0.63 $\mu g\,L^{-1}$ (%RSD, 16); DCBPA, 0.76 $\mu g\,L^{-1}$ (%RSD, 7); TCBPA, 0.81 $\mu g\,L^{-1}$ (%RSD, 5); and TeCBPA, 0.46 $\mu g\,L^{-1}$ (%RSD, 15).

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CONCLUSIONS

In the present study, several halogenated derivatives of BPA (MCBPA, DCBPA and TCBPA) were synthesized to be used as analytical standards for their analysis in water samples. We used multi-stage mass spectrometry in an ion trap mass analyzer to establish, for the first time, the fragmentation pathways of BPA and its halogenated derivatives. Generally, the fragmentation behavior of this family of compounds starts with the loss of $CH_3^{\bullet}$ and/or HCl or HBr and is followed by the neutral loss of CO. For BPA, ion-molecule reactions in the ion trap occurred between the radical anion $[M–H–CH_3]^{-\bullet}$ and MeOH. We have also developed a selective and sensitive off-line SPE-LC/MS/MS method for the simultaneous determination of BPA and its halogenated derivatives in water samples, with LODs in the low ppb range. This method has been applied to the analysis of water from a paper recycling plant.

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2.2.1.2 ARTICLE CIENTÍFIC III.

Multiple-stage mass spectrometry analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives

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MULTIPLE-STAGE MASS SPECTROMETRY ANALYSIS OF BISPHENOL A DIGLYCIDYL ETHER, BISPHENOL F DIGLYCIDYL ETHER AND THEIR DERIVATIVES

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Abstract

The fragmentation of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives was studied by electrospray tandem mass spectrometry. Multiple-stage mass spectrometry and accurate mass measurements were combined to establish the fragmentation pathways. BADGEs and BFDGEs tend to form ammonium adducts under electrospray conditions which fragmented easily. The fragmentation of $[M+NH_4]^+$ for BADGEs started with the cleavage of the phenyl-alkyl bond, which was followed by the α-cleavage of the ether group to generate the characteristic product ions at *m/z* 135, $[C_9H_{11}O]^+$ and *m/z* 107, $[C_7H_7O]^+$. The fragmentation of the BFDGE isomer mixtures was studied by on-line reversed phase liquid chromatography coupled to multiple-stage mass spectrometry ($LC\text{-}MS^n$). Information obtained from product ion spectra for each BFDGE isomer and its

comparison with the fragmentation pathway of BADGE allowed each isomer and the chromatographic elution order to be identified.

1. Introduction

Epoxy phenolic resins obtained by polymerization of bisphenol A diglycidyl ether (BADGE) or bisphenol F diglycidyl ether (BFDGE) are currently used as protective coatings for the interior of food cans. BFDGE (also known as epoxy novolac or NOGE) and BADGE have also been used as additives to eliminate the hydrochloric acid that forms during the heat treatment used in the coating of polyvinyl-based polymer. It is well documented that these monomers can migrate into the food product during autoclavation if the curing of the lacquer is unsuccessful, and that several reactions can take place in the food products. For instance, the epoxy groups may be hydrolyzed when they come into contact with aqueous or acidic food during storage, thus generating the corresponding hydrolyzed derivatives (BADGE·H_2O, BADGE·$2H_2O$, BADGE·HCl, BADGE·2HCl, BADGE·HCl·H_2O, BFDGE·$2H_2O$ and BFDGE·2HCl). Because of this, the European Union (EU) has set specific migration limits (SML) for the sum of BADGE and its hydrolyzed derivatives in food[1-2]. This regulation specifies an SML of 9 mg kg^{-1} or 9 mg/6 dm^2 for the sum of BADGE and its hydrolyzed derivatives and 1 mg kg^{-1} or 1 mg/6 dm^2 for the sum of BADGE·HCl, BADGE·2HCl and BADGE·HCl·H_2O, while the use of BFDGE and NOGE have been prohibited since 2005 because of a lack of toxicological interpretation.

BADGE, BFDGE and their hydrolyzed derivatives are usually analyzed by gas chromatography coupled to mass spectrometry (GC-MS) or by liquid chromatography with fluorescence detection (LC-FD)[1-3]. In contrast, liquid chromatography coupled to

mass spectrometry (LC-MS) has scarcely been used to analyze these compounds[7-11]. Because of their high tendency to form adducts in positive mode, acetonitrile adduct ions and ammonium adducts of BADGEs and BFDGEs have been proposed as diagnostic ions in LC-MS and as precursor ions in LC-MS/MS to analyze these compounds in cans and canned foods[7-10]. Although negative-ESI is not frequently used for the analysis of BADGEs, since only the hydrolyzed compounds are ionizable, this ionization mode has been proposed for the analysis of BADGE·$2H_2O$[7,10,11].

In this work, the elution order of the three isomers of BFDGE (*ortho,ortho-, ortho,para-* and *para,para*-BFDGEs) was studied using the mass spectral information provided by reversed phase liquid chromatography coupled to multiple-stage mass spectrometry (MS^n) on an ion trap analyzer. Moreover, the study of the fragmentation of BADGEs helped to identify the most common characteristic fragmentations that contribute to the identification of BFDGE isomers. Finally, high mass accuracy measurements were used to confirm the elemental composition of fragment ions.

2. Experimental

2.1 Chemicals and Consumables

Bisphenol A diglycidyl ether (BADGE, CAS No: 1675-54-3), bisphenol A (2,3-dihydroxypropyl) glycidyl ether (BADGE·H_2O, CAS No: 76002-91-0), bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE·$2H_2O$, CAS No: 5581-32-8), bisphenol A (3-chloro-2-hydroxypropyl) glycidyl ether (BADGE·HCl, CAS No: 13836-48-1), bisphenol A bis(3-chloro-2-hydroxypropyl) ether (BADGE·2HCl, CAS No: 4809-35-2), and bisphenol A (3-chloro-2-hydroxypropyl)(2,3-dihydroxypropyl ether (BADGE·HCl·H_2O, CAS No: 227947-06-0) standards of analytical grade were obtained

from Sigma-Aldrich (Steinheim, Germany). Bisphenol F diglycidyl ether (BFDGE mixture of *ortho,ortho, ortho,para* and *para,para* isomers), bisphenol F bis(2,3-dihydroxypropyl) ether (BFDGE·$2H_2O$ mixture of *ortho,ortho, ortho,para* and *para,para* isomers) and bisphenol F bis(3-chloro-2-hydroxypropyl) ether (BFDGE·2HCl, *ortho,ortho* CAS No: 338974-98-4, *ortho,para* CAS No 338974-97-3, *para,para* CAS No 235741-59-0) were also obtained from Sigma-Aldrich (Steinheim, Germany). The structures of the studied compounds are given in Figure 1.

Methanol (MeOH) and water (LC-MS grade) were purchased from Riedel-de Haën (Seelze, Germany). Ammonium formate (≥ 99.0%) was obtained from Fluka and formic acid (98-100%) from Merck (Darmstadt, Germany). Stock standard solutions (10 mg kg^{-1}) were individually prepared by weight in methanol:formic acid/ammonium formate (25 mM, pH 3.75) (50:50) and stored at 4ºC. A standard solution of a mixture of diethylamine purchased from Sigma-Aldrich, mepiquat chloride (1,1'-dimethylpyperidinium chloride, MQ) and difenzoquat methyl sulfate (1,2-dimethyl-3,5-diphenylpyrazolium methyl sulfate, DF) were purchased from Chem Service (West Chester, PA, USA) and polytyrosine standard (1-3-6) from CS Bio Company (Menlo Park, CA, USA), was used for accurate mass calibration and as internal lock masses.

Nitrogen (99.98% pure) supplied by a Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the API source. High-purity argon (Ar_1) purchased from Air Liquide (Madrid, Spain) was used as a collision-induced dissociation gas (CID gas) in the triple quadrupole and helium also purchased from Air Liquid was used as damper gas for the ion trap,

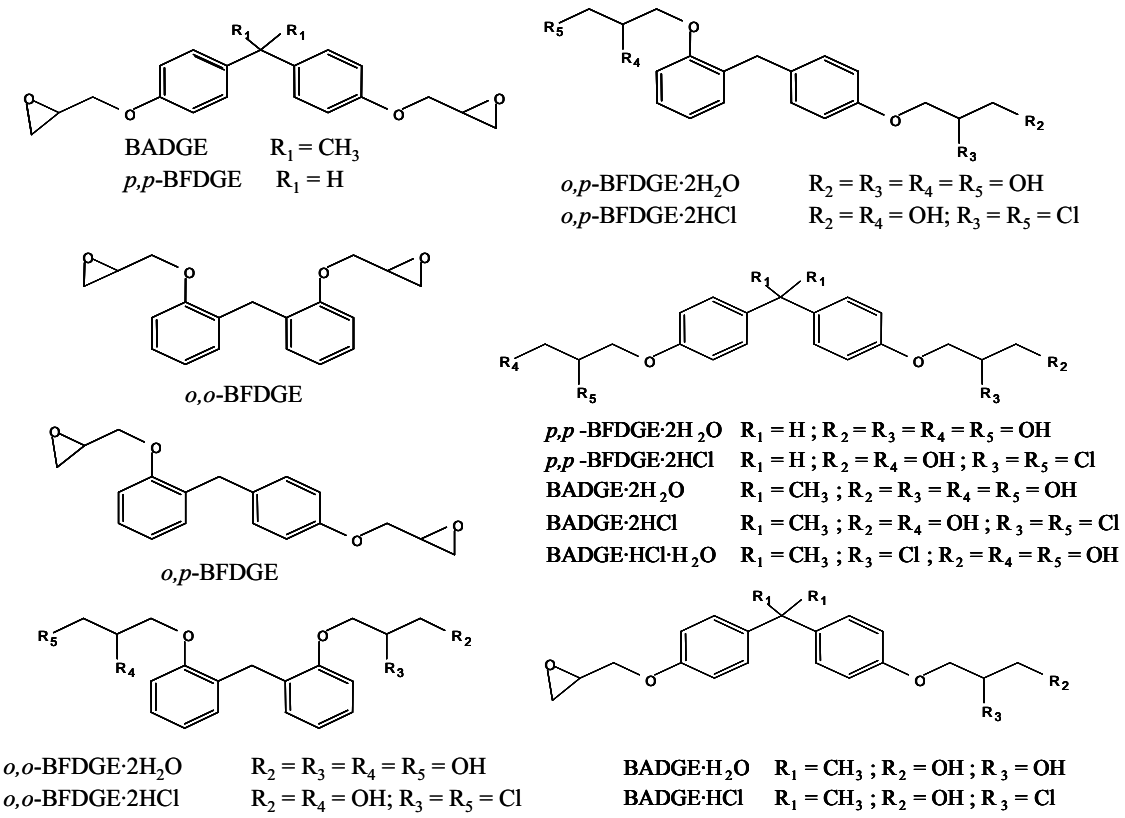

Figure 1. Chemical structures of BPA- and BPF-diglycidyl ethers.

2.2 Liquid Chromatography

A liquid chromatograph (Alliance 2695 Separations Module, Waters, Milford, MA, USA) equipped with a low-pressure quaternary solvent pumping system and an autosampler was used. The chromatographic separation of BFDGE isomers was carried out in an Ascentis Express C_{18} column (50 x 2.1 mm i.d., 2.7 µm particle size) (Supelco, Sigma-Aldrich) using methanol:formic acid/ammonium formate (25 mM, pH 3.75; 40 ºC) gradient elution at a flow rate of 600 µL min^{-1} and 20 µL as injection volume. The gradient elution program started with 50% methanol for 2 minutes followed by a linear gradient up to 75% methanol in 5-minute and 8-minute isocratic steps at 75% methanol.

In negative ESI MeOH:water was used as a mobile phase with the same gradient elution program.

2.3 Mass Spectrometry

Multiple-stage mass spectrometry experiments were performed in an LCQ Classic ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Both positive and negative ions were acquired and Xcalibur software version 1.4 was used to control the LC-MS system and process data.

ESI working conditions were as follows: sheath gas and auxiliary gas (N_2) flow-rates 92 a.u. (arbitrary units) and 45 a.u., respectively; heated capillary temperature 200ºC; electrospray needle voltage 4.0 kV and tube lens offset voltage 5 V (in both positive and negative modes). Data from multiple-stage mass spectrometry (MS^n) experiments were acquired in profile full scan mode (*m/z* 50-600). In positive mode $[M+NH_4]^+$ was selected as precursor ion, whereas in negative mode the precursor ion was $[M-H]^-$. The multiple-stage mass spectrometry working conditions were as follows: isolation width of *m/z* 1.5; 6 μscans; maximum injection time 50 ms and activation time 30 ms. The trapping radio frequency voltage (AQ) was set at 0.3 V and the normalized collision energy (NCE%) was optimized for each compound and ranged from 24 to 32%. The most abundant and characteristic ions in the MS/MS spectra were used as precursor ions for further MS^n experiments.

To optimize the source working conditions and to carry out the multiple-stage mass spectrometry experiments in positive ESI, a 10 mg L^{-1} stock standard solution prepared in methanol:formic acid/ammonium formate (25 mM, pH 3.75) (50:50) was infused at a flow-rate of 10 μL min^{-1} using the syringe pump integrated in the LCQ

instrument and mixed with the mobile phase (600 μL min^{-1}, MeOH:formic acid/ammonium formate 25 mM at pH 3.75, 75:25) using a Valco zero dead volume tee piece (Supelco, Alcobendas, Spain). In negative ESI, MeOH:water (50:50) was used as a mobile phase.

A TSQ Quantum Ultra AM (Thermo Fisher Scientific) triple quadrupole instrument equipped with an electrospray ionization source (ESI) and hyperbolic quadrupoles with accurate mass capabilities was used to provide accurate product ion mass measurements. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow rates of 60, 20 and 40 a.u. (arbitrary units), respectively. The ion transfer tube temperature was set at 375ºC and electrospray voltage at 4 kV. The first quadrupole (Q1) operated at low resolution, 0.7 *m/z* full-width half maximum (FWHM), while the third quadrupole (Q3) operated in enhanced mass-resolution mode, 0.04 *m/z* FWHM.

Argon was used as collision gas at 1.5 mTorr and the optimum collision energy (CE) was selected for each compound, ranging from 10 to 25 eV. Xcalibur software version 2.0 (Thermo Fisher Scientific) was used to control the LC-MS system and process data.

For the accurate mass (AM) measurements in highly-selective reaction monitoring (H-SRM) mode, the TSQ Quantum Ultra AM was first calibrated following the manufacturer's recommended calibration procedures. A full calibration of the triple quadrupole mass spectrometer was carried out before accurate mass measurements were taken. Moreover, internal lock masses were used to correct accurate mass measurements. The reference compounds used as lock masses were: protonated diethyl amine ($[M+H]^+$, *m/z* 74.0964), MQ ($[M]^+$, *m/z* 114.1280), DF ($[M]^+$, m/z 249.1390) and polytyrosine (1-3-6) (only *m/z* 508.2080 corresponding to the protonated trimer,

$[M+H]^+$ was selected). The lock masses standard solution was mixed with the studied analytes eluted from the LC system using a Valco zero dead volume tee piece. In order to prevent fragmentation of the ions used as internal lock masses in MS/MS experiments, a low collision energy (5V) was applied. Accurate mass values were the average of ten individual measurements. Mass errors were estimated taking into account the expanded uncertainty at 95% confidence level for ten degrees of freedom. Additionally, Mass Frontier software (HighChem^{TM}) was used to contribute to structural characterization.

3. Results and Discussion

In this work, mass spectral information was used in order to identify BFDGE isomers and propose an elution order in a reversed phase column. To achieve this goal, the multiple-stage mass spectra of both BFDGEs and BADGEs were obtained in an ion trap mass analyzer using both positive and negative electrospray. These compounds showed good ionization in positive mode, but in negative ESI only the hydrolyzed compounds (BADGE·H_2O, BADGE·$2H_2O$ and BFDGE·$2H_2O$) provided a good signal. MS^n spectra of BADGEs were obtained by infusing standard solutions (section 2.3); however, since commercially available standards for BFDGEs are a mixture of three isomers (*ortho-ortho, ortho-para* and *para-para*, Figure 1), reversed phase LC was required to study their fragmentation.

Both BADGEs and BFDGEs tend to form adducts in both positive and negative mode. In positive electrospray sodium, ammonium and potassium adduct ions were obtained. The sodium adduct was always observed instead of the protonated molecule, although no additives were used in the mobile phase. In tandem mass spectrometry experiments, this adduct was not fragmented even at high collision energy, while

efficient fragmentation occurred for ammonium adducts with a stable signal under tandem mass spectrometry, consistent with that observed by Søeborg *et al.*[11]. To enable the formation of ammonium adducts and ensure signal reproducibility, formic acid/ammonium formate buffer (25 mM, pH 3.75) at 50% was used as an additive in the mobile phase in positive ESI. At these conditions, the hydrolyzed compounds (BADGE·H_2O, BADGE·$2H_2O$ and BFDGE·$2H_2O$) showed adducts with alkaline metals (Na^+ < 20% relative abundance and K^+<10% relative abundance) in the MS spectra, even though the ammonium adduct was always the base peak (Table 1). Additionally, in the ion trap, MeOH adducts with a relative abundance between 10 and 25% were observed for BADGEs that contained a non-hydrolyzed epoxy group.

In negative ESI, MeOH:water[12] with no additives was used as a mobile phase to prevent ion suppression. Full-scan mass spectra were obtained for BADGE·H_2O, BADGE·$2H_2O$ and BFDGE·$2H_2O$, which were generally dominated by the deprotonated molecule $[M-H]^-$. However, in the MS spectrum of BADGE·$2H_2O$ and BFDGE·$2H_2O$ the formate adducts were also observed at a high relative abundance (Table 2). This may have been due to the presence of residual formic acid/formate in the LC-MS system, which could not be eliminated even after an accurate cleaning. This behaviour is consistent with literature data[11] and shows the high tendency of these compounds to form adducts in negative ESI. In addition, ion source fragmentation originating from the α-cleavage of the ether group $[M-H-C_3H_6O_2]^-$ was observed for BADGE·H_2O.

Table 1. Ion trap mass spectral data (MS^n, n:2-4) of BADGEs and BFDGEs using ESI in positive mode.

Compound	MS		MS/MS		MS^3		MS^4	
	m/z **(%Rel.Ab.)**	**Assignment**	***m/z*** **(%Rel.Ab.)**	**Assignment**	***m/z*** **(%Rel.Ab.)**	**Assignment**	***m/z*** **(%Rel.Ab.)**	**Assignment**
BADGE	358 (100)	$[M+NH_4]^+$	191 (100)	$[M+NH_4-C_9H_{13}O_2N]^+$	161 (<10)	$[M+NH_4-C_9H_{13}O_2N-CH_2O]^+$		
					147 (<10)	$[C_9H_7O_2]^+$		
					135 (100)	$[C_9H_{11}O]^+$	107* (100)	$[C_7H_7O]^+$
							95 (10)	$[C_6H_7O]^+$
					107* (<10)	$[C_7H_7O]^+$		
BADGE·2H_2O	394 (100)	$[M+NH_4]^+$	377 (100)	$[M+NH_4-NH_3]^+$	209 (100)	$[M+NH_4-NH_3-C_9H_{12}O_3]^+$		
			209 (10)	$[M+NH_4-C_9H_{15}O_3N]^+$	135 (100)	$[C_9H_{11}O]^+$	107 (100)	$[C_7H_7O]^+$
							95 (10)	$[C_6H_7O]^+$
BADGE·H_2O	376 (100)	$[M+NH_4]^+$	359 (<10)	$[M+NH_4-NH_3]^+$				
			209 (100)	$[M+NH_4-C_9H_{13}O_2N]^+$	135 (100)	$[C_9H_{11}O]^+$	107* (100)	$[C_7H_7O]^+$
							95 (15)	$[C_6H_7O]^+$
					107* (<10)	$[C_7H_7O]^+$		
			191 (20)	$[M+NH_4-C_9H_{15}O_3N]^+$	173 (70)	$[M+NH_4-C_9H_{15}O_3N-H_2O]^+$		
					147 (50)	$[C_9H_7O_2]^+$		
					135 (100)	$[C_9H_{11}O]^+$		
			173 (10)	$[M+NH_4-C_9H_{17}O_4N]^+$				
BADGE·2HCl[#]	430 (100)	$[M+NH_4]^+$	413 (50)	$[M+NH_4-NH_3]^+$	227 (100)	$[M+NH_4-NH_3-C_9H_{11}O_2Cl]^+$		
			227 (100)	$[M+NH_4-C_9H_{14}O_2ClN]^+$	185 (<5)	$[M+NH_4-C_9H_{14}O_2ClN-C_3H_6]^+$		
					167 (15)	$[M+NH_4-C_9H_{14}O_2ClN-C_3H_8O]^+$		
					135 (100)	$[C_9H_{11}O]^+$	107* (100)	$[C_7H_7O]^+$
							95 (15)	$[C_6H_7O]^+$
					107* (10)	$[C_7H_7O]^+$		
BADGE·HCl[#]	394 (100)	$[M+NH_4]^+$	377 (<5)	$[M+NH_4-NH_3]^+$				
			227 (100)	$[M+NH_4-C_9H_{13}O_2N]^+$	135 (100)	$[C_9H_{11}O]^+$	107* (100)	$[C_7H_7O]^+$
							95 (20)	$[C_6H_7O]^+$
					107* (10)	$[C_7H_7O]^+$		
			191 (10)	$[M+NH_4-C_9H_{14}O_2ClN]^+$				
BADGE·HCl·H_2O[#]	412 (100)	$[M+NH_4]^+$	395 (100)	$[M+NH_4-NH_3]^+$	227 (100)	$[M+NH_4-NH_3-C_9H_{12}O_3]^+$		
					209 (25)	$[M+NH_4-NH_3-C_9H_{11}O_2Cl]^+$		
			227 (55)	$[M+NH_4-C_9H_{15}O_3N]^+$	135 (100)	$[C_9H_{11}O]^+$	107* (100)	$[C_7H_7O]^+$

							95 (10)	$[C_6H_7O]^+$
					107* (15)	$[C_7H_7O]^+$		
			209 (15)	$[M+NH_4-C_9H_{14}O_2ClN]^+$				
BFDGE·2H_2O	366 (100)	$[M+NH_4]^+$	349 (100)	$[M+NH_4-NH_3]^+$	181 (100)	$[M+NH_4-NH_3-C_9H_{12}O_3]^+$		
o,o-			181 (15)	$[M+NH_4-C_9H_{15}O_3N]^+$	107* (100)	$[C_7H_7O]^+$	94 (40)	$[C_6H_6O]^{+\bullet}$
o,p-							95 (60)	$[C_6H_7O]^+$
p,p-							79 (100)	$[C_6H_7]^+$
							77 (85)	$[C_6H_5]^+$
			163 (<10)	$[M+NH_4-C_9H_{17}O_4N]^+$	145 (30)	$[M+NH_4-C_9H_{17}O_4N-H_2O]^+$		
					121 (100)	$[C_8H_9O]^+$		
	349 (60)	$[M+H]^+$						
BFDGE·2HCl[#]	402 (100)	$[M+NH_4]^+$	385 (100)	$[M+NH_4-NH_3]^+$	199 (100)	$[M+NH_4-NH_3-C_9H_{11}O_2Cl]^+$	181 (100)	$[M+NH_4-NH_3-C_9H_{11}O_2Cl-H_2O]^+$
o,o-					181 (20)	$[M+NH_4-NH_3-C_9H_{13}O_3Cl]^+$		
o,p-								
p,p-			199 (10)	$[M+NH_4-C_9H_{14}O_2ClN]^+$	181 (100)	$[M+NH_4-C_9H_{14}O_2ClN-H_2O]^+$	145 (100)	$[M+NH_4-C_9H_{14}O_2ClN-H_2O-HCl]^+$
					145 (<10)	$[M+NH_4-C_9H_{14}O_2ClN-H_3OCl]^+$		
					107* (<10)	$[C_7H_7O]^+$		
BFDGE								
Isomer 1 (*p,p-)*	330 (100)	$[M+NH_4]^+$	163 (100)	$[M+NH_4-C_9H_{13}O_2N]^+$	107* (100)	$[C_7H_7O]^+$		
Isomer 2 (*o,p-*)	330 (100)	$[M+NH_4]^+$	313 (<10)	$[M+NH_4-NH_3]^+$				
			295 (40)	$[M+NH_4-NH_3-H_2O]^+$				
			277 (25)	$[M+NH_4-NH_3-H_4O_2]^+$				
			265 (40)	$[M+NH_4-NH_3-CH_4O_2]^+$				
			237 (25)	$[M+NH_4-C_3H_{11}O_2N]^+$				
			189* (100)	$[M+NH_4-C_7H_{11}O_2N]^+$	171 (50)	$[C_{12}H_{11}O]^+$		
					133 (100)	$[C_9H_9O]^+$		
			163 (60)	$[M+NH_4-C_9H_{13}O_2N]^+$	145 (45)	$[M+NH_4-C_9H_{13}O_2N-H_2O]^+$		
					121 (100)	$[C_8H_9O]^+$		
					107* (40)	$[C_7H_7O]^+$		
Isomer 3 (*o,o-*)	330 (100)	$[M+NH_4]^+$	277 (40)	$[M+NH_4-NH_3-H_4O_2]^+$				
			239 (50)	$[M+NH_4-C_3H_9O_2N]^+$				
			211 (25)	$[M+NH_4-C_4H_9O_3N]^+$				

171 (30)	$[C_{12}H_{11}O]^+$		
163 (100)	$[M+NH_4-C_9H_{13}O_2N]^+$	145 (45)	$[M+NH_4-C_9H_{13}O_2N-H_2O]^+$
		121 (100)	$[C_8H_9O]^+$
		107* (40)	$[C_7H_7O]^+$
147 (60)	$[M+NH_4-C_{10}H_{17}O_2N]^+$	119 (100)	$[M+NH_4-C_{10}H_{17}O_2N-CO]^+$
145 (60)	$[M+NH_4-C_{10}H_{19}O_2N]^+$		
133 (75)	$[C_9H_9O]^+$		

*The elemental composition was established by accurate mass measurement.
#The isotope ions corresponding to ^{35}Cl have been selected.
AQ: 0.3V
NCE: 24-32%

Table 2. Ion trap mass spectral data (MS^n, n:2-4) of BADGEs and BFDGEs using ESI in negative mode.

Compound	MS	MS/MS	MS^3	MS^4				
	m/z **(%Rel.Ab.)**	**Assignment**	***m/z*** **(%Rel.Ab.)**	**Assignment**	***m/z*** **(%Rel.Ab.)**	**Assignment**	***m/z*** **(%Rel.Ab.)**	**Assignment**
BADGE·$2H_2O$	421 (50)	$[M+HCOO]^-$	375 (100)	$[M+HCOO-HCOOH]^-$				
	375 (100)	$[M-H]^-$	301 (100)	$[M-H-C_3H_6O_2]^-$	227 (100)	$[M-H-C_3H_6O_2-C_3H_6O_2]^-$	212 (100)	$[M-H-C_3H_6O_2-C_3H_6O_2-CH_3]^{-\bullet}$
							133 (65)	$[C_9H_9O]^-$
BADGE·H_2O	357 (100)	$[M-H]^-$	283 (20)	$[M-H-C_3H_6O_2]^-$				
			227 (100)	$[M-H-C_6H_{12}O_4]^-$	133 (100)	$[C_9H_9O]^-$		
					93 (50)	$[C_6H_5O]^-$		
			133 (15)	$[C_9H_9O]^-$				
	283 (90)	$[M-H-C_3H_6O_2]^-$	226 (100)	$[M-H-C_3H_6O_2-C_3H_5O]^{-\bullet}$	211 (100)	$[M-H-C_3H_6O_2-C_3H_5O-CH_3]^-$	117 (75)	$[M-H-C_3H_6O_2-C_3H_5O-CH_3-C_6H_6O]^-$
							93 (40)	$[C_6H_5O]^-$
BFDGE·$2H_2O$	393 (100)	$[M+HCOO]^-$	347 (100)	$[M+HCOO-HCOOH]^-$				
	347 (100)	$[M-H]^-$	273 (100)	$[M-H-C_3H_6O_2]^-$	199 (100)	$[M-H-C_3H_6O_2-C_3H_6O_2]^-$	93 (100)	$[C_6H_5O]^-$

AQ: 0.3V
NCE: 24-32%

Multiple-Stage Mass Spectrometry

MS^n studies of positive ions

Multiple-stage mass spectra (MS^n) of ammonium adduct and the most abundant or most characteristic product ions were recorded. Table 1 summarizes the main product ions obtained in MS/MS, MS^3 and MS^4 and their assignment for all the compounds. BADGEs showed as characteristic fragmentation the cleavage of the phenyl-alkyl bond with the simultaneous loss of NH_3. Only one fragment ion was observed for those compounds with a symmetrical chemical structure, while for those with two different ether chains, two product ions were obtained, the base peak being the ion keeping the hydrolyzed epoxy group. For instance, the tandem mass spectrum of BADGE·H_2O (Table 1) showed an ion at *m/z* 209 (relative abundance 100%) corresponding to this fragmentation, and an ion at *m/z* 191 (relative abundance 20%) which contained the epoxy group. In addition to this fragmentation a product ion due to the neutral loss of NH_3 (17 Da) was also observed (<10% relative abundance). This fragmentation was obtained with a higher relative abundance (50-100%) for compounds which have two hydrolyzed and/or chloro-hydrolyzed epoxy groups (BADGE·$2H_2O$, BADGE·2HCland BADGE·HCl·H_2O). The characteristic cleavage of the phenyl-alkyl bond was also observed inthe MS^3 spectra of this ion.

A common product ion at *m/z* 135, $[C_9H_{11}O]^+$ due to the α-cleavage of the ether group was observed in the MS^3 spectra of BADGEs (Table 1). In the MS^4 spectra (*m/z* 135) another common product ion of this family of compounds at *m/z* 107 due to the loss of 28 Da was obtained. This product ion can be generated by the loss of C_2H_4 or CO. In order to correctly assign this loss, accurate mass measurement of the generated product ion was performed. High collision energy was used in order to favour multiple

collisions in the triple quadrupole analyzer to obtain fourth generation product ions. An accurate *m/z* value of 107.049±0.002 was obtained, providing errors of 3±2 mDa for the loss of C_2H_4 and 23±3 mDa for the loss of CO. Consequently, the ion at *m/z* 107 was assigned to $[C_7H_7O]^+$ (Table 1). Finally, the characteristic product ion of phenolic compounds *m/z* 95 was observed in the MS^4 or MS^5 spectra of BADGEs. In the MS^3 spectrum of BADGE·2HCl (Table 1), in addition to the characteristic fragmentation observed for this family of compounds, two product ions at *m/z* 185 and *m/z* 167 (Table 1) with low intensity (<20%) corresponding to the loss of C_3H_6 and to the consecutive losses of H_2O and C_3H_6, respectively, were observed. These product ions were probably favoured by the presence of chlorine in the molecule that stabilizes the positive charge in the aromatic ring.

For BFDGE fragmentation, studies were performed after liquid chromatography in order to separate the commercial mixture (*ortho,ortho-, ortho,para- and para,para*). The three isomers of BFDGE·2H_2O (Figure 2A) and BFDGE (Figure 3) were separated using a reversed phase C_{18} column, while partial overlapping was observed for BFDGE·2HCl (Figure 2B). These separations were enough to study each isomer individually. When performing multiple-stage mass spectrometry experiments (up to MS^3), the three isomers of BFDGE·2H_2O and BFDGE·2HCl showed the same product ion spectra, while for BFDGE the three isomers provided different spectra. The MS/MS spectra for BFDGE·2H_2O and BFDGE·2HCl (Table 1, Figure 2) showed the ion corresponding to the neutral loss of NH_3 as base peak and the characteristic cleavage of the phenyl-alkyl bond was observed with relatively low abundance (<40%). This fragmentation pattern was similar to that observed previously for hydrolyzed BADGEs. Moreover, in the MS^3 spectrum of the BFDGE·2H_2O, the characteristic product ion of

this family of compounds at *m/z* 107 assigned to $[C_7H_7O]^+$ was also observed (Table 1, Figure 2). MS^3 and MS^4 spectra of BFDGE·2HCl also showed the product ion at *m/z* 145 corresponding to the consecutive losses of H_2O and HCl, and in this case, the characteristic ion of this family of compounds generally found at high fragmentation stages (*m/z* 95) was not obtained (Table 1).

As mentioned above, the three BFDGE isomers showed a different MS/MS spectrum, as can be seen in Figure 3. Table 1 summarizes the most characteristic product ions in MS/MS and MS^3 spectra of each isomer. The first eluted isomer (isomer 1) only showed an intense product ion in the MS/MS spectrum at *m/z* 163 due to cleavage of the phenyl-alkyl bond, as occurred previously for BADGE. The MS^3 spectrum of this isomer showed a product ion at *m/z* 107 corresponding to the loss of 56 Da due to the α-cleavage of the ether bond, as occurred for BADGE. To confirm the elemental composition of this product ion (*m/z* 107), the accurate mass measurement was performed providing an *m/z* value of 107.049±0.003 that can be related to an elemental composition of $[C_7H_7O]^+$ with a mass error of 1.6±5.0 mDa. This fragmentation is quite similar to that observed for BADGE, which presents two *para-para* glycidyl ether groups, so this chromatographic peak might be assigned to the *para,para*-BFDGE isomer.

The other two isomers (isomer 2 and isomer 3) (Figure 3) showed a noisy spectra with higher fragmentation than isomer 1 under MS/MS conditions. In the MS/MS spectrum of isomer 2, a characteristic product ion at *m/z* 295 was observed. This may have been generated by the simultaneous neutral loss of NH_3 and H_2O. The loss of water may be explained by the opening of the epoxy group involving a hydrogen of the central carbon atom and the subsequent condensation of the ether chain (Figure 5). Therefore, this fragmentation behaviour indicates that isomer 2 contains a glycidyl

ether in *ortho* position. Additionally, if the two groups are in *ortho* position, the steric hindrance would prevent this loss. Consequently, isomer 2 might be assigned to the *ortho,para*-BFDGE isomer, which is supported by the fact that the ion at *m/z* 295 is not present in the spectrum of isomer 3 (Figure 3). Furthermore, the base peak in the MS/MS spectrum of isomer 2 is the ion at *m/z* 189, which was not observed for the other two isomers. The accurate mass measurement using the triple quadrupole instrument for this ion was *m/z* 189.093±0.002. Within a mass error of ±5mDa, only one elemental composition ($C_{12}H_{13}O_2$) with an RDB (rings and double bonds) value of 6.5 was possible, indicating that one of the aromatic rings had been opened. Taking into account this elemental composition, the unknown product ion may originate from the simultaneous losses of NH_3 and $C_7H_8O_2$. Two structures can be proposed for this elemental composition; one containing the glycidyl ether group in *ortho* position and the other with the glycidyl ether group in *para* position. In order to characterize this ion (*m/z* 189), an MS^3 spectrum was obtained (Figure 3). The ion at *m/z* 171 corresponded to the loss of 18 Da due to the neutral loss of a water molecule which, as previously mentioned, is favoured by the *ortho* position of the glycidyl ether group. In order to characterize the product ion at *m/z* 133, accurate mass measurement was also performed, providing an elemental composition of $[C_9H_9O]^+$ (7.5±3 mDa of mass error).

After the identification of isomers 1 and 2, isomer 3 might be assigned to the *ortho,ortho*-BFDGE. This compound showed in the MS/MS spectrum characteristic and specific ions at *m/z* 239 and *m/z* 147 (Figure 3). The product ion at *m/z* 239 may originate from the loss of a glycidyl ether group favoured by the steric hindrance of the *ortho,ortho* position. The other characteristic product ion at *m/z* 147 can be assigned to the simultaneous neutral loss of NH_3 and the cleavage of the phenyl-alkyl bond. The

elemental composition of this last product ion was also confirmed by accurate mass measurement with a mass error of 10±3 mDa. Even though the low quality of the MS^3 spectrum of the ion at *m/z* 147 the product ion at *m/z* 119 in MS^3 assigned to the neutral loss of CO showed a signal-to-noise ration >3. Elemental composition (C_8H_7O) of this ion was also confirmed by accurate mass measurement (*m/z* 119.049±0.001, mass error 4.2±2 mDa).

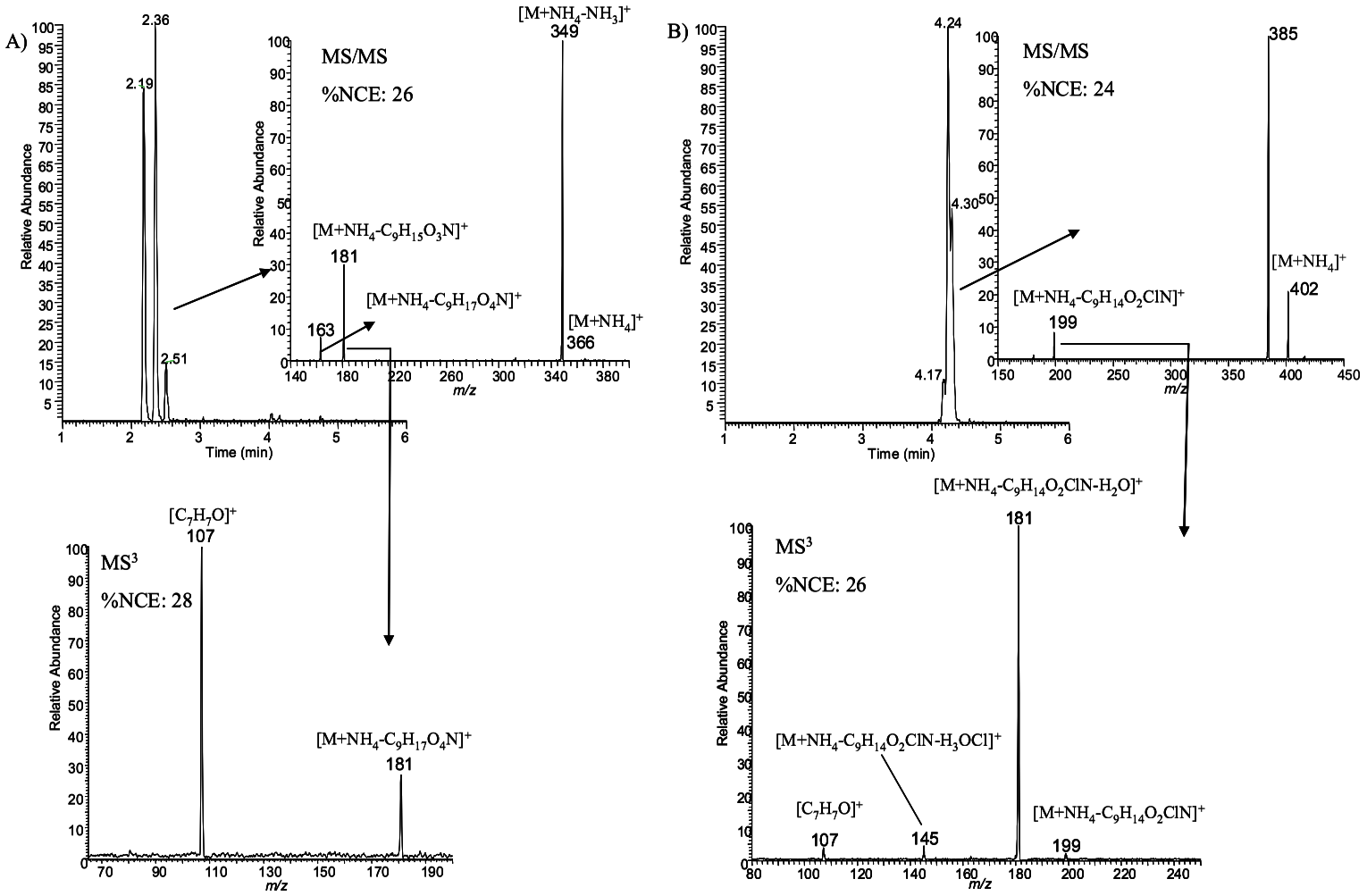

Figure 2. LC-MS/MS chromatograms in ESI positive mode and an ion trap analyzer, MS/MS and MS^3 spectra of: A) BFDGE·$2H_2O$ isomers and B) BFDGE·2HCl isomers. Conditions as indicated in the experimental section.

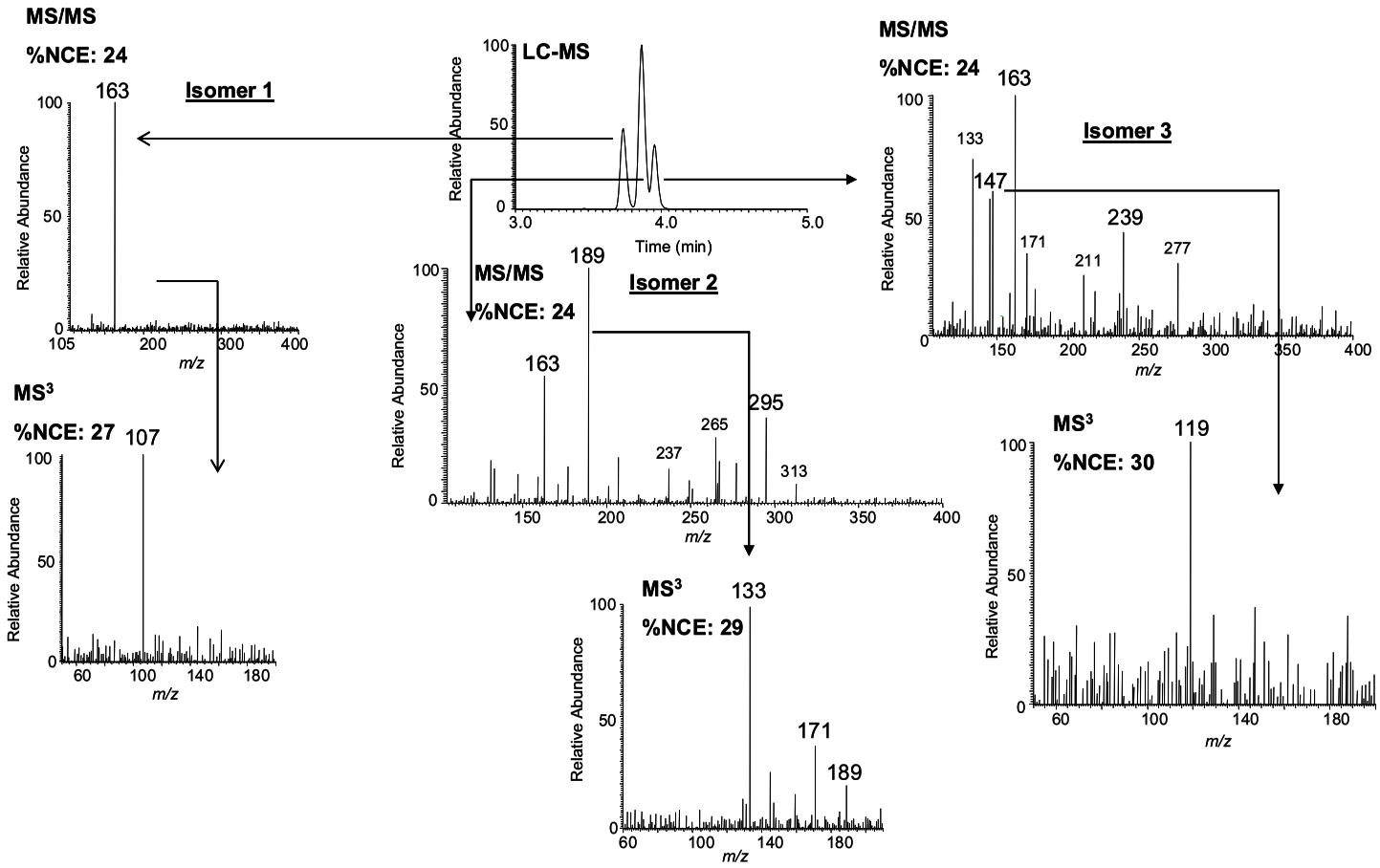

Figure 3. LC-MS chromatogram in ESI positive mode and an ion trap analyzer. MS/MS and MS^3 spectra of each BFDGE isomer. Conditions as indicated in the experimental section.

A common product ion at *m/z* 163 due to cleavage of the phenyl-alkyl bond was observed in the MS/MS spectra of the three isomers, which give the characteristic product ion at *m/z* 107 well described above for BADGEs (Table 1). Figure 4 shows the tentative chemical structures for the most characteristic product ions of the three isomers.

MS^n studies of negative ions

In negative ESI mode, only the hydrolyzed compounds (BADGE·H_2O, BADGE·$2H_2O$ and BFDGE·$2H_2O$) were ionized (Table 2). In addition to the deprotonated molecule $[M-H]^-$, the hydroxyl compounds of both BADGE and BFDGE tend to form adduct ions with the LC mobile phases residual components in the system such as formate $[M+HCOO]^-$ which could not be eliminated even after an accurate cleaning, as can be observed in Table 2. For BADGE·H_2O, an intense ion was observed (*m/z* 283) coming from the in-source α-cleavage of the ether bond, which generated a radical product ion at *m/z* 226. The MS/MS spectra of $[M-H]^-$ is dominated by the product ion originated from the α-cleavage of the ether bond as a base peak. In MS^3 for BADGE·$2H_2O$ and BFDGE·$2H_2O$, the loss of the remaining glycidyl ether group generated the ions corresponding to the deprotonated molecules of BPA and BPF (*m/z* 227 for BADGEs and *m/z* 199 for BFDGE). In MS^4, the fragmentation pathway of these ions (Table 2) is consistent with that described previously for bisphenols[13].

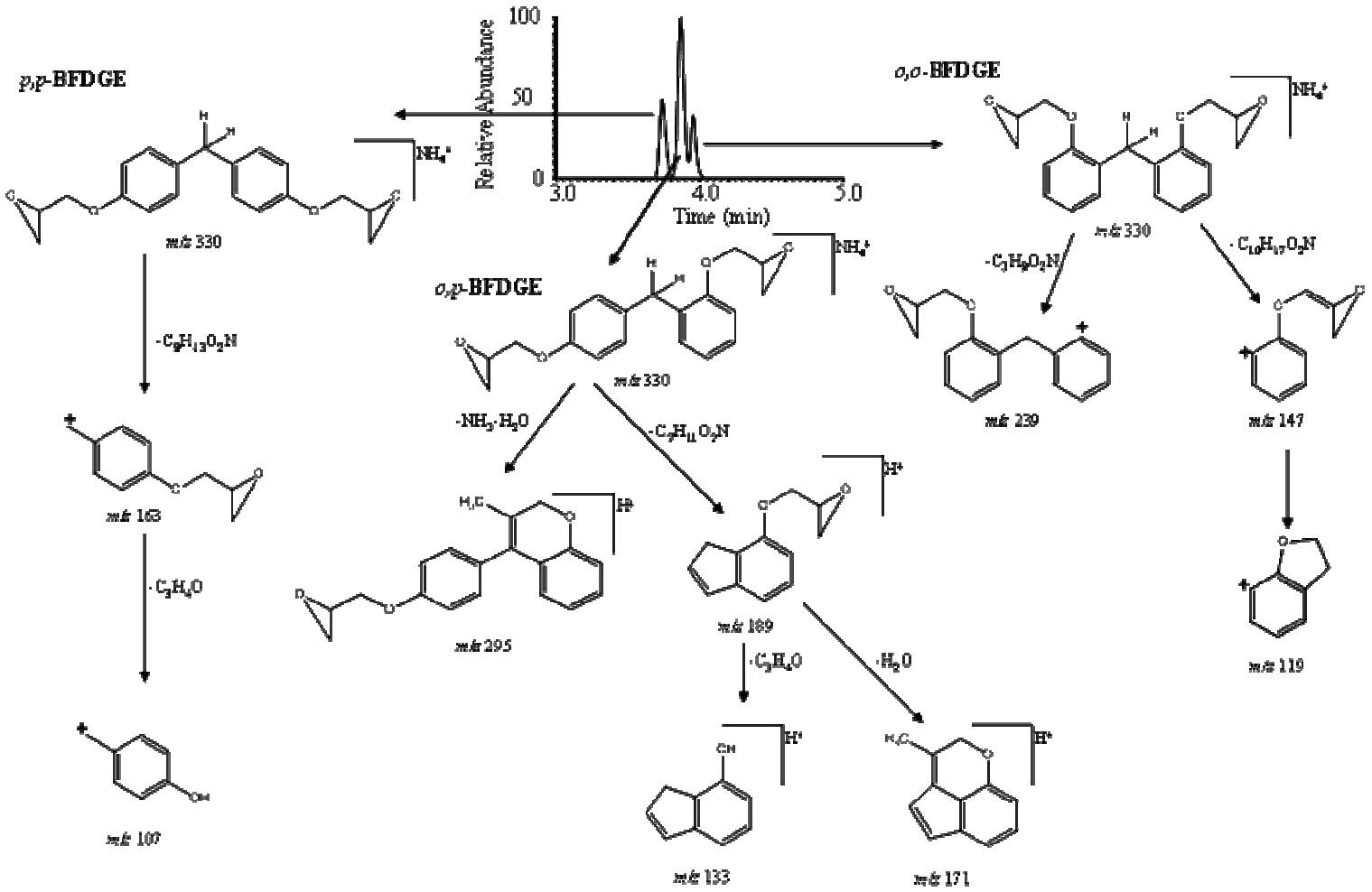

Figure 4. Tentative chemical structures of BFDGE isomers product ions

CONCLUDING REMARKS

In the present study, multiple-stage mass spectrometry and accurate mass measurements using electrospray ionization in both positive and negative modes, were used to establish the fragmentation pathway of BADGEs and BFDGEs. Only the hydrolyzed compounds (BADGE·$2H_2O$, BADGE·H_2O and BFDGE·$2H_2O$) could be ionized using ESI in negative mode. In contrast, important structural information was obtained for this family of compounds in positive mode. Generally, the fragmentation behaviour starts with the loss of one phenyl-glycidyl ether group followed by the α-cleavage of the remaining ether group. These fragmentations generate a common product ion at *m/z* 135 for BADGEs and at *m/z* 107 for BFDGEs. This latter product ion was also observed in the fragmentation pathway of BADGEs due to the further loss of the two methyl groups. These fragmentation studies can be useful for the identification and confirmation of BADGEs and BFDGEs in complex matrices and for the identification of new related compounds, such as new transformation products and metabolites. The advantage of using fragmentation pathways for solving analytical problems has been demonstrated with the establishment of the chromatographic elution order of BFDGE isomer mixture in a C_{18} column.

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2.2.1.3. FOTOINICIADORS.

Els estudis de fragmentació d'aquesta família de compostos (Taula 1.3) s'han realitzat per tal de caracteritzar les transicions proposades per a l'anàlisi, quantificació i confirmació, per LC-MS/MS (aquesta aplicació s'inclou a l'apartat 3.2.3 d'aquesta memòria). En primer lloc es van obtenir els espectres de MS de cadascun dels compostos utilitzant electroesprai en mode positiu com a font d'ionització. Generalment la molècula protonada, $[M+H]^+$, és l'ió més abundant en els espectres de MS d'aquests compostos encara que en alguns casos com pe exemple per la 1-hidroxifenilciclohexil fenil cetona (HCPK), la 2,2-dimetoxi-2-fenilacetofenona (DMPA) i la 2-hidroxi-2-metilpropiofenona (HMPP) apareixen altres ions degut a una important fragmentació a la font, tal i com es pot observar a la Taula 2.3 on s'indiquen els ions observats en electroesprai. Aquestes fragmentacions a la font corresponen a la pèrdua d'una molècula d'aigua en el cas del HCPK i el HMPP i a la pèrdua d'un dels grups metoxi (CH_3O) en el cas del DMPA. Per a tots aquests ions es va estudiar la seva fragmentació en una trampa d'ions emprant múltiples etapes (fins a MS^4). Els compostos d'aquesta família es poden subdividir en quatre grups en funció de la seva estructura química i dels grups funcionals que presenten. En un primer grup es poden incloure els isòmers de l'ITX (2- i 4-ITX) i el DETX que presenten un grup tioxanté a la seva estructura. La BP i la PBZ que tenen anells aromàtics units mitjançant un grup cetona formen el segon grup, mentre que el EDMAB, el EHDAB i el DEAB que presenten grups aminofenils es poden englobar en un tercer grup. Finalment la HCPK, la HMPP i la DMPA amb estructures molt diferents a les dels altres compostos estudiats formen el quart grup.

El pic base en l'espectre de MS/MS dels isòmers de l'ITX i el DETX és l'ió producte corresponent a la pèrdua de la cadena alquílica (Taula 2.3). En el cas del DETX, que presenta dos grups etil a la seva estructura, la pèrdua de la segona cadena alquílica té lloc en el MS^3 originant el mateix ió (*m/z* 213) que s'obté pel ITX en l'espectre de MS/MS. En ambdós compostos aquest ió a *m/z* 213 perd un grup CHO en la etapa posterior de fragmentació (MS^3 pel ITX i MS^4 pel DETX). Aquestes fragmentacions també s'observen en l'espectre MS/MS obtingut amb un analitzador de triple quadrupol (Figura 2.8A). En aquest cas l'estudi de les corbes de fragmentació, a les quals es representa la intensitat dels ions a diferents energies de col·lisió (5-80V) mostrant com els màxims dels ions estudiats es troben

desplaçats en l'eix d'energia la qual cosa ens indica que aquestes tenen lloc de forma consecutiva (Figura 2.8B). En aquesta figura a més es mostra la ruta de fragmentació proposada pel DETX (Figura 2.8C).

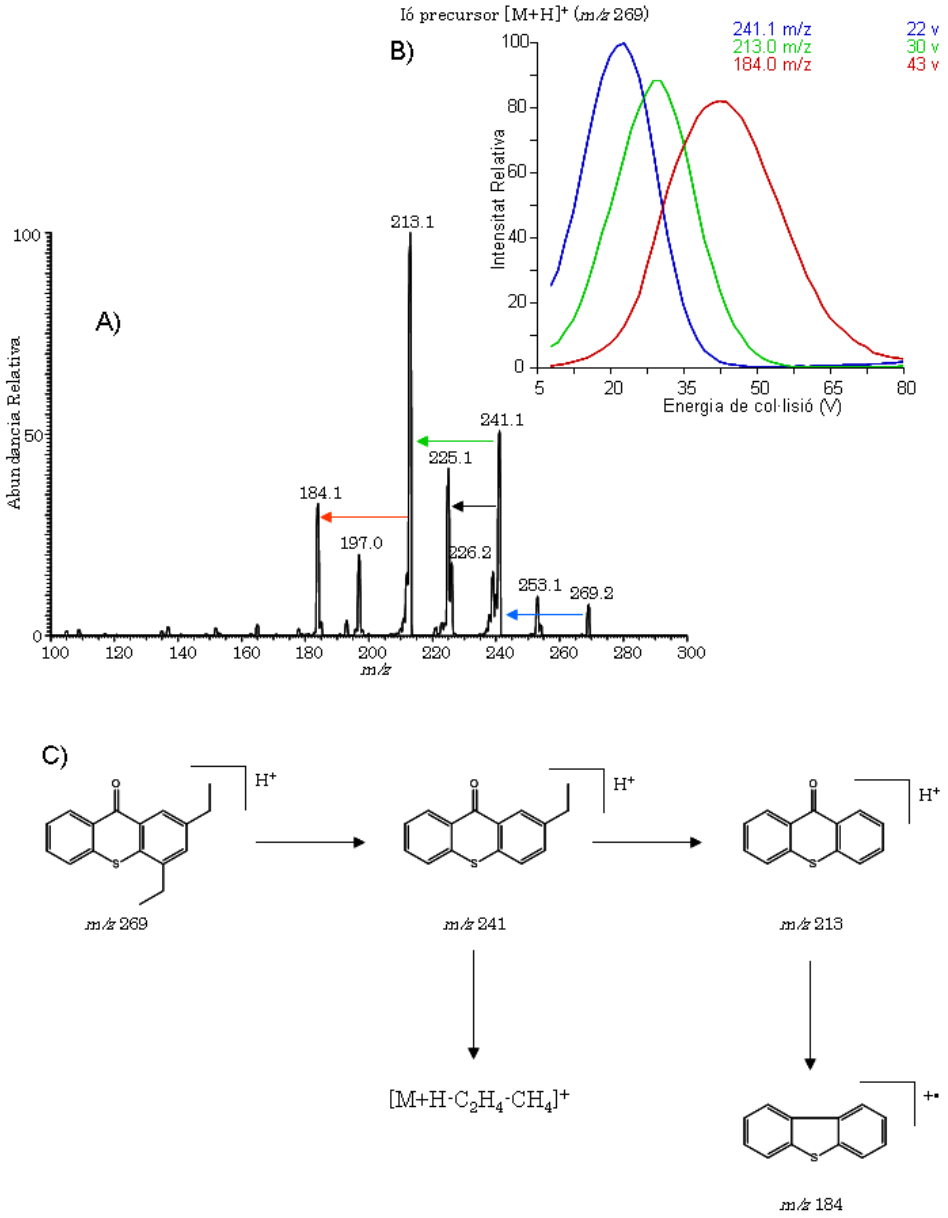

Figura 2.8. A) Espectre MS/MS obtingut amb l'instrument triple quadrupol per al DETX, B) corbes de fragmentació i C) ruta de fragmentació.

Per altra banda a l'espectre d'ions producte de la BP i la PBZ, les quals presenten una estructura amb anells aromàtics units mitjançant un grup carbonil, en l'espectre d'ions producte el pic base correspon a l'ió de *m/z* 105 que es pot assignar a $[C_7H_5O]^+$ (Taula 2.3) corresponent a la ruptura de l'enllaç entre el grup carbonil i el fenil en el cas de la BP i el bifenil en la PBZ. En el cas de la PBZ també

s'observa l'ió a *m/z* 181 que s'obté per la pèrdua del grup fenil, $[M+H-C_6H_6]^+$. L'ió *m/z* 105 en el MS^3 dóna lloc a l'ió *m/z* 77 $[C_6H_5]^+$ característica de compostos aromàtics que prové de la pèrdua del grup CO. Aquestes fragmentacions s'observen també en l'espectre de MS/MS obtingut en el triple quadrupol tal i com es mostra a mode d'exemple pel PBZ (Figura 2.9A). En aquest cas per tal d'assignar correctament les fragmentacions observades es van realitzar els estudis de les corbes de fragmentació, les quals en aquest cas ens indiquen que els ions a m/z 181 i 105 s'obtenen per via directa mentre que els ions a m/z 153 i 77 s'obtenen per via consecutiva d'aquests tal i com es pot observar a la Figura 2.9B). Aquests estudis ens han permès establir la ruta de fragmentació d'aquests compostos i caracteritzar els ions producte obtinguts (Figura 2.9C).

En el cas del EHDAB i el EDMAB, que presenten un grup èster a la seva estructura, el pic base de l'espectre de tàndem correspon a l'ió de *m/z* 166 originat per la pèrdua de la cadena alquílica del grup èster, $[M+H-C_2H_4]^+$ i $[M+H-C_9H_{19}]^+$, respectivament (Taula 2.3). Posteriorment aquest ió en el MS^3 es fragmenta originant l'ió radical de *m/z* 151 corresponent a la pèrdua radicalària d'un dels metils del grup amino. Aquest ió que s'observa en MS^3 és el pic base de l'espectre de MS/MS en el triple quadrupol. Finalment en el MS^4 aquests compostos presenten la pèrdua de 17 uma corresponents al •OH del grup carboxílic originant l'ió a *m/z* 134. Aquests estudis de fragmentació realitzats amb la trampa d'ions juntament amb els estudis de les corbes d'energia de col·lisió obtingudes en el triple quadrupol permeten caracteritzar els ions producte obtinguts i establir si les pèrdues tenen lloc per via directa o consecutiva. A la Figura 2.10 es mostra a mode d'exemple la ruta de fragmentació obtinguda pel EDMAB. En el cas del DEAB el qual presenta dos grups amino quaternaris a la seva estructura el pic base de l'espectre de MS/MS correspon a l'ió originat per la ruptura de l'enllaç en α al grup carbonil que conté el grup amino protonat donant lloc a l'ió de *m/z* 176, $[M+H-C_{10}H_{15}N]^+$, aquest mateix ió s'observa també a l'espectre de MS degut a fragmentació a la font (Taula 2.3). Aquest ió es fragmenta en MS^3 perdent les cadenes alquíliques del grup amino originant els ions a *m/z* 148 (abundancia relativa 100%), $[M+H—C_{10}H_{15}N—C_2H_4]^+$ i l'ió a *m/z* 133 (abundància relativa 50%), $[M+H—C_{10}H_{15}N—C_3H_7]^+$. A més, s'observa l'ió de *m/z* 105, $[C_7H_5O]^+$ degut a la pèrdua de tot el grup amino donant el mateix ió que la PBZ i la BP comentat anteriorment.

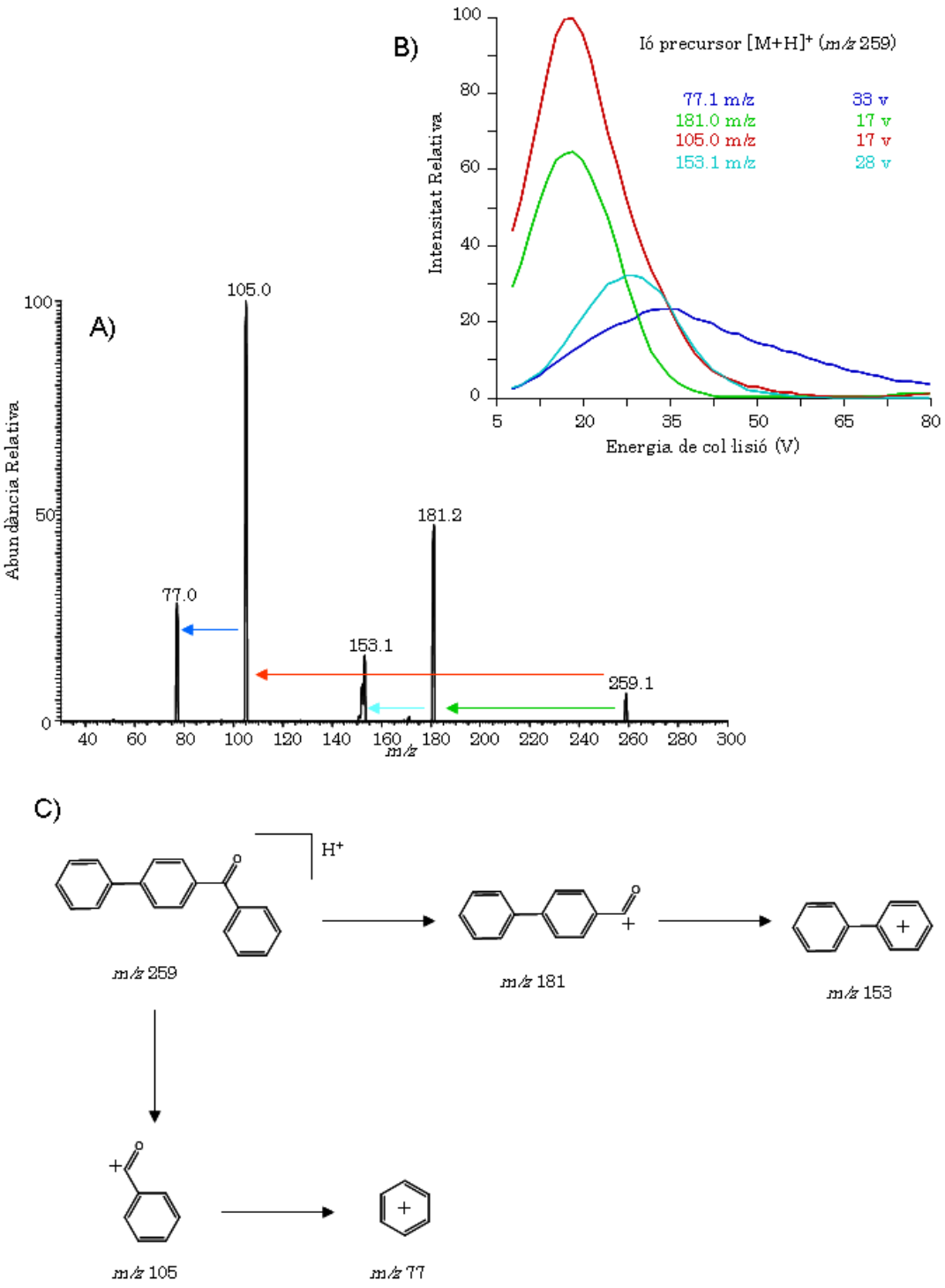

Figura 2.9. A) Espectre MS/MS obtingut amb l'instrument triple quadrupol pel PBZ, B) corbes de fragmentació i C) ruta de fragmentació.

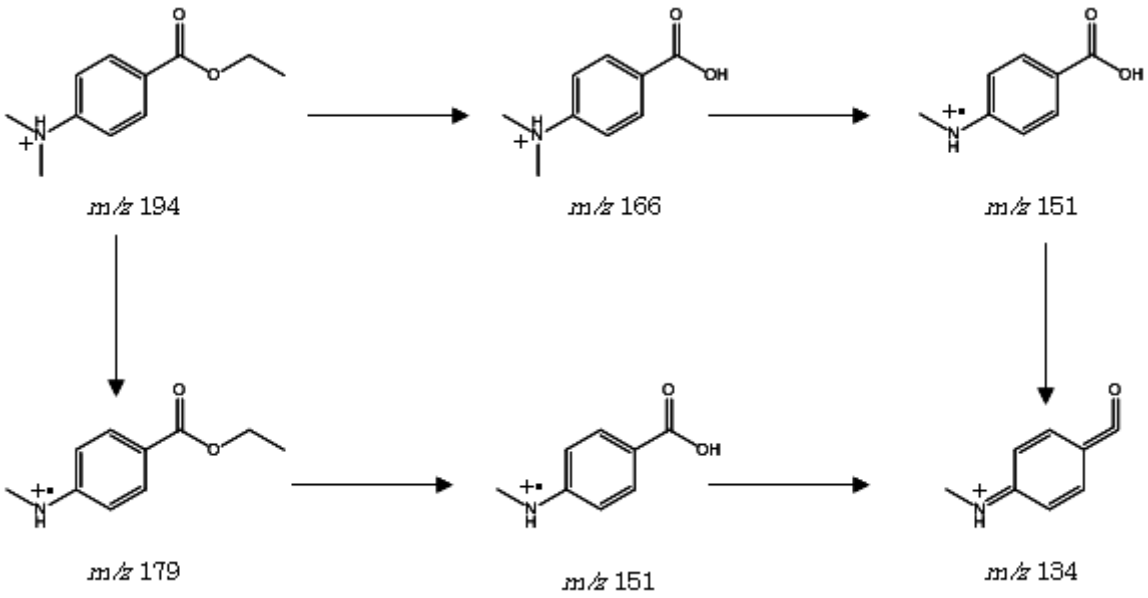

Figura 2.10. Ruta de fragmentació del EDMAB.

Els altres fotoiniciadors estudiats en aquesta memòria, HCPK, DMPA i HMPP presenten una fragmentació una mica diferent a la observada en els casos comentats anteriorment degut als diferents grups funcionals que presenten a la seva estructura. A més, tal i com podem observar a la Taula 2.2, presenten una important fragmentació a la font. En el cas del HMPP i el HCPK en fragmentar la molècula protonada $[M+H]^+$ en MS/MS s'observa la pèrdua d'una molècula d'aigua. Aquesta fragmentació és la mateixa que s'observa en l'espectre de MS degut a fragmentacions a la font (Taula 2.3). L'espectre MS/MS d'aquest ió $[M+H\text{-}H_2O]^+$ generat a la font dóna com a ions producte una segona pèrdua d'aigua i la pèrdua de la cadena alquílica (C_2H_4) en el cas del HMPP i del ciclohexà en el cas del HCPK. A més, pel HCPK s'obté l'ió a *m/z* 105 observat també per altres fotoiniciadors, mentre que per al HMPP s'observa l'ió tropili a *m/z* 91, $[C_7H_7]^+$ característic de compostos aromàtics. Finalment, el DMPA es fragmenta a la font perdent el grup metoxi (*m/z* 225). La fragmentació en MS/MS d'aquest ió *m/z* 225 origina la pèrdua de 28 unitats de massa corresponents al CO (*m/z* 197) ió que posteriorment en MS^3 dóna lloc a l'ió a *m/z* 105. Aquests ions junt amb el de *m/z* 165 s'observen en l'espectre de MS/MS obtingut amb l'instrument triple quadrupol. A la Figura 2.11 es mostra la ruta de fragmentació obtinguda pel DMPA.

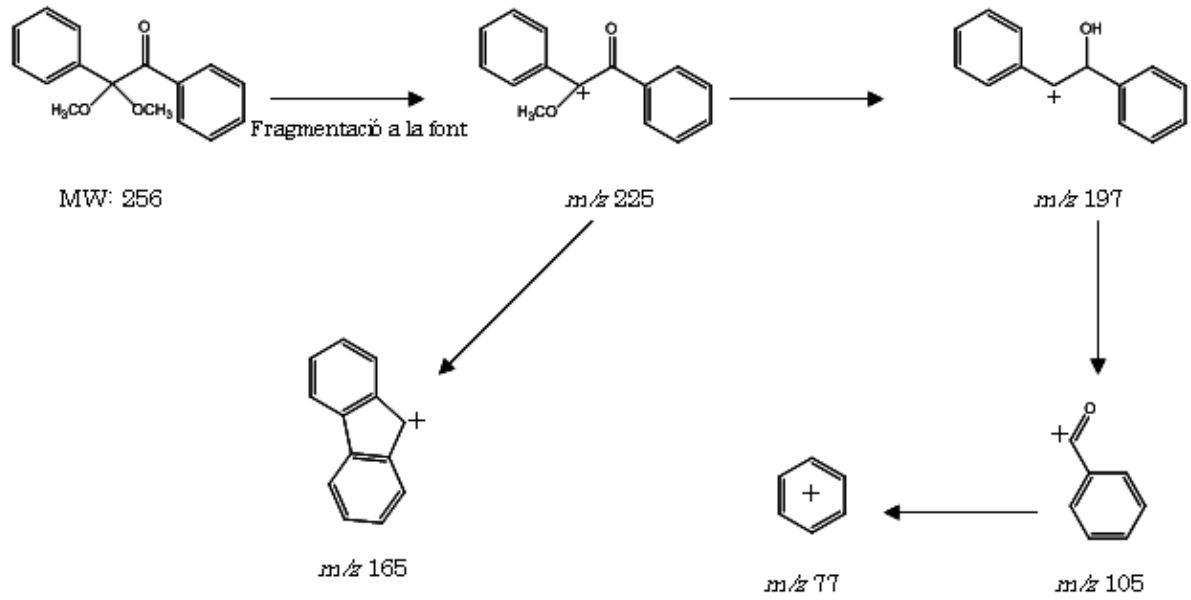

Figura 2.11. Ruta de fragmentació del DMPA.

Taula 2.3. Fragmentacions dels fotoiniciadors obtingudes en una trampa d'ions.

Compost	MS		MS/MS		MS^3		MS^4	
	m/z (%Ab.Rel.)	Assignació	*m/z* (%Ab.Rel.)	Assignació	*m/z* (%Ab.Rel.)	Assignació	*m/z* (%Ab.Rel.)	Assignació
2- i 4-ITX	255 (100)	$[M+H]^+$	213 (100)	$[M+H-C_3H_6]^+$	185 (50)	$[M+H-C_3H_6-CO]^+$		
					184 (100)	$[M+H-C_3H_6-CHO]^{+\bullet}$		
DETX	269 (100)	$[M+H]^+$	241 (100)	$[M+H-C_2H_4]^+$	225 (15)	$[M+H-C_2H_4-CH_4]^+$		
					213 (100)	$[M+H-C_2H_4-C_2H_4]^+$	184 (100)	$[M+H-C_2H_4-C_2H_4-CHO]^{+\bullet}$
					185 (<10)	$[M+H-C_2H_4-C_3H_4O]^+$		
BP	183 (100)	$[M+H]^+$	105 (100)	$[C_7H_5O]^+$	77 (100)	$[C_6H_5]^+$		
PBZ	259 (100)	$[M+H]^+$	181 (80)	$[M+H-C_6H_6]^+$	153 (100)	$[M+H-C_6H_6-CO]^+$		
			105 (100)	$[C_7H_5O]^+$	77 (100)	$[C_6H_5]^+$		
EDMAB	194 (100)	$[M+H]^+$	179 (25)	$[M+H-CH_3]^{+\bullet}$	164 (<10)	$[M+H-CH_3-CH_3]^+$		
					151 (100)	$[M+H-CH_3-C_2H_4]^{+\bullet}$		
					134 (15)	$[M+H-CH_3-C_2H_5O]^+$		
			166 (100)	$[M+H-C_2H_4]^+$	151 (100)	$[M+H-C_2H_4-CH_3]^{+\bullet}$	134 (30)	$[M+H-C_2H_4-CH_3-OH]^+$
					122 (<10)	$[M+H-C_2H_4-C_2H_6N]^+$		
EHDAB	278	$[M+H]^+$	166 (100)	$[M+H-C_8H_{16}]^+$	151 (100)	$[M+H-C_8H_{16}-CH_3]^{+\bullet}$	134 (100)	$[M+H-C_8H_{16}-CH_3-OH]^+$
							105 (25)	$[M+H-C_8H_{16}-CH_3-CH_4ON]^{+\bullet}$
					122 (<10)	$[M+H-C_8H_{16}-C_2H_6N]^+$		
			151 (15)	$[M+H-C_9H_{19}]^+$				
HCPK	205 (<10)	$[M+H]^+$						
	187 (100)	$[M+H-H_2O]^+$	169 (65)	$[M+H-H_2O-H_2O]^+$	141 (100)	$[M+H-H_2O-H_2O-C_2H_4]^+$		
			105 (100)	$[C_7H_5O]^+$				
HMPP	165 (100)	$[M+H]^+$	147 (100)	$[M+H-H_2O]^+$	119 (100)	$[M+H-H_2O-C_2H_4]^+$		
			119 (45)	$[M+H-C_2H_6O]^+$	91 (100)	$[C_7H_7]^+$		
	147 (85)	$[M+H-H_2O]^+$						
	119 (80)	$[M+H-H_2O-C_2H_4]^+$						
DEAB	325 (100)	$[M+H]^+$	296 (35)	$[M+H-C_2H_5]^{+\bullet}$	281 (100)	$[M+H-C_2H_5-CH_3]^+$	253 (100)	$[M+H-C_2H_5-CH_3-C_2H_4]^+$
							148 (30)	$[M+H-C_2H_5-CH_3-C_9H_{11}N]^+$
			176 (100)	$[M+H-C_{10}H_{15}N]^+$	148 (100)	$[M+H-C_{10}H_{15}N-C_2H_4]^+$		
					133 (50)	$[M+H-C_{10}H_{15}N-C_3H_7]^{+\bullet}$		
					120 (50)	$[M+H-C_{10}H_{15}N-C_4H_8]^+$		
					105 (35)	$[C_7H_5O]^+$		
					91 (20)	$[C_7H_7]^+$		
	176 (20)	$[M+H-C_{10}H_{15}N]^{+\bullet}$						
DMPA	225 (100)	$[M-CH_3O]^+$	197 (100)	$[M-CH_3O-CO]^+$	105 (100)	$[C_7H_5O]^+$		
					77 (<10)	$[C_6H_5]^+$		
			165 (40)	$[M-CH_3O-C_2H_4O]^+$	139 (100)	$[M-CH_3O-C_2H_4O-C_2H_2]^+$		

2.3. DISCUSSIÓ DE RESULTATS

Com ja hem comentat anteriorment, l'objectiu d'aquest capítol és l'estudi de la fragmentació dels compostos inclosos en aquesta Tesi per tal d'establir rutes de fragmentació comunes per a cadascuna de les famílies de compostos estudiats que permetin identificar i caracteritzar les fragmentacions més característiques i útils per la seva identificació, confirmació i quantificació. Aquests estudis de fragmentació s'han realitzant mitjançant la combinació de la informació proporcionada pels espectròmetres de massa trampa d'ions i triple quadrupol equipat amb quadrupols hiperbòlics que ens ha permès augmentar la resolució i realitzar mesures de massa exacta amb una bona precisió i exactitud. L'estudi de la fragmentació del BPA i dels seus derivats halogenats, així com dels diglicidil èters es troben inclosos en els articles dels apartats 2.2.1.1 i 2.2.1.2, respectivament, mentre que la fragmentació dels fotoiniciadors es detalla a l'apartat 2.2.1.3.

La primera etapa en els estudis de fragmentació duts a terme en aquesta Tesi ha estat l'obtenció dels espectres MS per a cada compost emprant polaritat positiva o negativa en funció de la resposta i de les característiques àcid-base de cadascun d'ells. En aquests espectres cal identificar l'io molecular, els adductes i les fragmentacions a la font que es puguin produir, ja que tots aquests ions són susceptibles de ser emprats com a ions precursors en els experiments de tàndem. En el nostre cas, en general els espectres de MS han estat dominats per la molècula protonada, $[M+H]^+$ o desprotonada, $[M-H]^-$, segons la polaritat de treball tal i com es mostra a la Figura 2.12 on a mode d'exemple es mostren els espectres de MS obtinguts per alguns dels compostos estudiats en aquesta tesi. Per al BPA i el BADGE·$2H_2O$ en mode negatiu s'obté com a pic base de l'espectre la molècula desprotonada $[M-H]^-$ (Figura 2.12a i b) mentre que per fer el EDMAB el pic base de l'espectre correspon a la molècula protonada $[M+H]^+$ (Figura 2.12c). Els derivats diglicidil èters del BPA i del BPF (BADGEs i BFDGEs) que tal i com es pot observar a la Taula 2 de l'article científic III (Apartat 2.2.1.2) presenten una gran tendència a formar adductes amb l'amoni (NH_4^+) i altres ions presents a la fase mòbil i en el sistema com poden ser el Na^+ i el K^+. A mode d'exemple a la Figura 2.12d es mostra l'espectre de MS del BADGE·$2H_2O$ on s'observa la presencia d'aquests adductes, essent l'adducte amb amoni el pic base de l'espectre. Els derivats hidrolitzats d'aquests compostos són també ionitzables en mode negatiu i

de la mateixa manera també presenten una elevada tendència a formar adductes, en aquest cas amb l'ió formiat present en la fase mòbil (Figura 2.12b) encara que el pic base de l'espectre correspon a la molècula desprotonada, $[M-H]^-$. Per aquesta raó, per als BADGEs i els BFDGEs en mode positiu es van seleccionar com a ions precursors per dur a terme els estudis de fragmentació els adductes amb amoni, $[M+NH_4]^+$. Per altra banda alguns dels fotoiniciadors estudiats en aquesta memòria en concret la DMPA, el HCPK i el HMPP (Taula 2.3, Apartat 2.2.1.3) presenten una important fragmentació a la font (abundància relativa >85%). A la Figura 2.12e) es mostra l'espectre de MS de la DMPA on s'observa com en aquest cas el pic base de l'espectre correspon a l'io $[M-CH_3O]^+$. En aquests casos per dur a terme els estudis de fragmentació es va seleccionar com a ió precursor l'origina't per aquesta fragmentació.

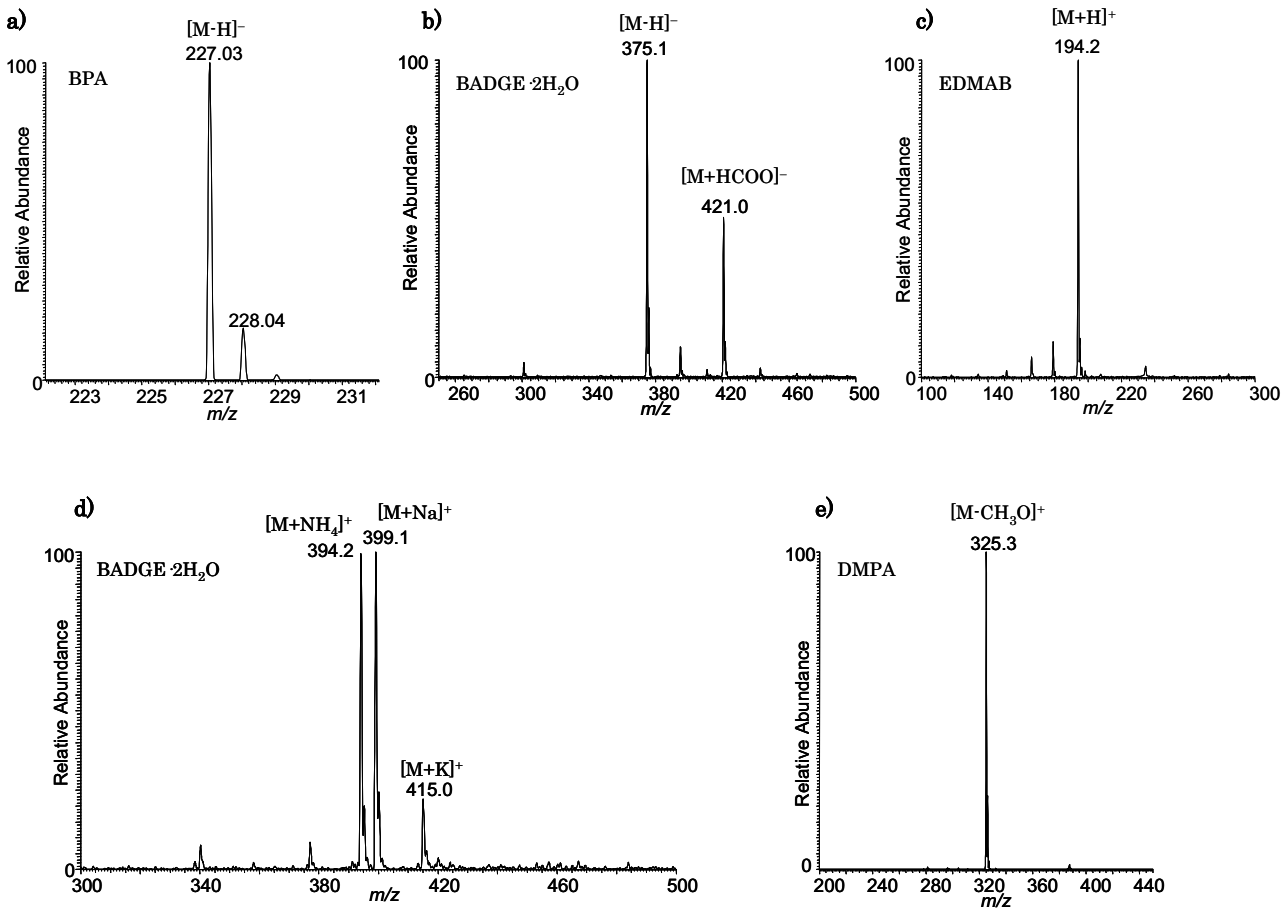

Figura 2.12. Espectres de MS obtinguts en ESI en polaritat negativa i positiva: A) Bisfenol A, b) BADGE·2H₂O, c) EDMAB, d) BADGE·2H₂O i e) DMPA.

Com ja s'ha comentat abans, els analitzadors que treballen en tàndem en el temps són els més adequats per dur a terme estudis de fragmentació, ja que permeten establir una relació genealògica entre els ions obtinguts. En el nostre cas aquests estudis de fragmentació en el temps es van dur a terme en una trampa d'ions realitzant experiments de tàndem en successives etapes fins a MS^4 i establint la relació genealògica entre els ions més importants i més característics per cada compost i per cada una de les famílies de compostos. Aquesta primera assignació dels ions es duu a terme a baixa resolució i en molts casos és suficient considerant els mecanismes de fragmentació més habituals.

Els analitzadors de triple quadrupol (QqQ) són el més emprats en l'anàlisi quantitativa per LC-MS/MS ja que proporcionen una molt bona sensibilitat, selectivitat i robustesa treballant en el mode SRM (*selected reaction monitoring*). Encara que és important seleccionar les transicions més sensibles i selectives per realitzar les anàlisis en LC-MS/MS, convé dur a terme l'assignació acurada dels ions dels espectres de MS/MS en l'espai. Degut a les múltiples col·lisions que es poden produir en la cambra de col·lisió del triple quadrupol no és fàcil a vegades esbrinar l'ordre genealògic dels ions en aquest tipus d'espectres. Així, en aquesta Tesis s'han emprat de manera combinada les corbes d'energia de col·lisió en el triple quadrupol i l'informació obtinguda dels estudis de fragmentació amb la trampa d'ions per assignar les fragmentacions observades, sent possible distingir entre fragmentacions directes o consecutives en la cambra de col·lisió, tal i com ocorre en el cas dels estudis de fragmentació dels fotoiniciadors (Apartat 2.2.1.3).

Una vegada obtinguts els espectres de masses en el temps i en l'espai per als compostos estudiats la tercera etapa a realitzar és assegurar la correcta assignació dels ions producte obtinguts. En alguns casos la primera assignació a baixa resolució pot resultar ambigua sent possible l'assignació de més d'una composició elemental. Per resoldre aquest problema es poden emprar compostos marcats isotòpicament (D o ^{13}C) en determinades posicions per tal de revelar la posició del trencament dels enllaços i confirmar de manera inequívoca algunes de les pèrdues observades. Per exemple, en el cas de la pèrdua de 28 unitats de massa observada en els estudis de fragmentació dels derivats halogenats del BPA aquesta pèrdua pot ser deguda tant a la pèrdua d'un grup CO com a la pèrdua de C_2H_4. En aquest cas es va estudiar la fragmentació del $^{13}C_{12}$–TBBPA on tots els carbonis dels anells

aromàtics són ^{13}C. En fragmentar aquest compost s'observa la pèrdua de 29 uma tal i com es mostra a la Figura 2.13 on es pot observar que la pèrdua de 28 unitats observada pel TBBPA (Figura 2.13A) es correspon a 29 en el cas del $^{13}C_{12}$–TBBPA (Figura 2.13B) la qual cosa indica que un carboni de l'anell aromàtic (^{13}C) es troba involucrat en la fragmentació i per tant ens permet confirmar, sense la necessitat de dur a terme mesures de massa exacta, que aquesta pèrdua és deguda al CO.

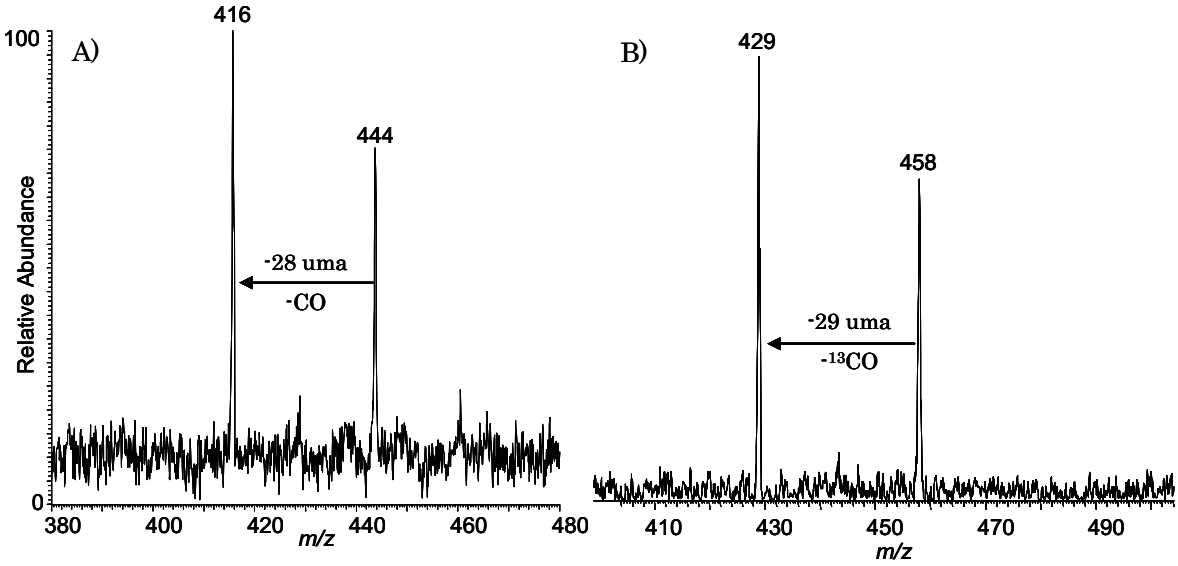

Figura 2.13. Espectre de MS^3 del TBBPA i el $^{13}C_{ring}$-TBBPA.

En estudis de fragmentació de compostos marcats isotòpicament cal tenir present que a vegades, la labilitat d'alguns hidrògens produeix intercanvis hidrògen-deuteri en els ions precursors la qual cosa no permet observar directament la molècula protonada o desprotonada. Per exemple, en el cas del BPA-*d*$_{16}$ s'observa aquest intercanvi en els grups hidroxils que comporta que en l'espectre de MS aparegui l'ió de *m/z* 241 corresponent a $[(M-2D+2H)-H]^-$, (Figura 5 de l'article II). Ara bé, no sempre es disposa comercialment dels compostos marcats isotòpicament tal i com passa amb la majoria dels compostos estudiats en aquesta tesis. En aquests casos hom pot recórrer a obtenir mesures de massa exacta tant dels ions precursors com dels ions producte per poder confirmar la composició elemental de les estructures proposades a partir dels estudis de fragmentació. En aquesta Tesi les mesures de massa exacta s'han realitzat amb el instrument triple quadrupol de quadrupols hiperbòlics. Aquestes mesures de massa exacta tal i com es posa de manifest en l'article científic III inclòs en aquest

capítol (Apartat 2.2.1.2) ens han permès assignar correctament alguns dels ions producte obtinguts en els estudis de fragmentació dels BADGEs i dels BFDGEs els quals no havien pogut ser assignats mitjançant els estudis de fragmentació realitzats amb la trampa d'ions. Aquesta correcta assignació de la composició elemental juntament amb l'estudi estructural obtingut a partir de les fragmentacions en el temps ha permès a més caracteritzar i identificar cadascun dels isòmers del BFDGE (*orto,orto-, orto,para- i para,para-*). Els estudis de fragmentació d'aquests isòmers es van realitzar en LC-MS^n utilitzant una columna de fase invertida per tal de poder separar els tres isòmers i estudiar la seva fragmentació per separat ja que comercialment únicament es disposa de la mescla dels tres isòmers. Cadascun dels isòmers va presentar un espectre de tàndem característic i a partir del seu estudi es va poder identificar els isòmers i proposar l'ordre d'elució en una columna de fase invertida.

Per tal de validar les composicions elementals obtingudes a partir de les mesures de massa exacta i avaluar la bondat de les mesures fetes amb un l'instrument de triple quadrupol es van realitzar les mesures de massa exacta amb un analitzador híbrid Q-TOF obtenint en tots els casos errors inferiors a ±1 mDa per a les composicions elementals establertes a partir de les mesures de massa exacta amb el triple quadrupol .

Finalment, els estudis de fragmentació realitzats per a les diferents famílies de compostos estudiades ens ha permès establir les rutes de fragmentació dels compostos estudiats. L'estudi d'aquestes rutes de fragmentació ens ha permès caracteritzar compostos relacionats com en el cas del derivats clorats del BPA. Alguns d'aquests compostos, monoclorobisfenol A (MCBPA), diclorobisfenol A (DCBPA) i triclorobisfenol A (TCBPA) van ser sintetitzats mitjançant la cloració del BPA tal i com es detalla a l'article científic II. En el procés de síntesi del DCBPA es poden obtenir dos isòmers el 3,3'-DCBPA o el 3,5-DCBPA segons tinguem els dos àtoms de clor en un anell aromàtic o un clor a cadascun dels anells. En aquest cas els estudis de fragmentació amb la trampa d'ions (MS^n) ens van permetre identificar que durant el procés de síntesi únicament s'ha obtingut l'isòmer 3,3'-DCBPA ja que en l'espectre de MS/MS s'observa l'ió producte de *m/z* 167 que conté un àtom de clor a la seva estructura, aquesta fragmentació té lloc degut a la pèrdua d'un dels grups hidoxibenzil. A més aquests compostos van ser caracteritzats mitjançant la ressonància magnètica nuclear de protó (^{1}H-NMR)

observant en el cas del DCBPA que efectivament durant el procés de síntesi únicament s'obté l'isòmer 3,3'-DCBPA tal i com es mostra a la Figura 1 de l'article científic II (Apartat 2.2.1.1) on es mostra l'espectre de ^{1}H-NMR obtingut pel DCBPA sintetitzat.

L'obtenció de les rutes de fragmentació d'aquestes famílies de compostos a la vegada ens han permès identificar els ions producte comuns així com les pèrdues característiques i selectives que posteriorment poden ser utilitzades per quantificar i confirmar la presència d'aquests compostos en mostres complexes, així com per a la identificació de compostos relacionats com poden ser els metabòlits o els productes de transformació. Aquestes tasques es poden dur a terme mitjançant els diferents modes d'adquisició en un analitzador triple quadrupol com són la pèrdua de neutres i l'escombra't d'ions precursors que podrien permetre cercar compostos d'una mateixa família en mostres complexes.

2.4. CONCLUSIONS

El treball experimental inclòs en aquest Capítol de la memòria on s'han dut a terme estudis de fragmentació en dos analitzadors que operen en dos modes diferents d'espectrometria de masses en tàndem (en el temps i l'espai) ha permès arribar a les següents conclusions:

- Les fragmentacions més característiques del bisfenol A i dels seus derivats halogenats (MCBPA, DCBPA. TCBPA, TeCBPA i TBBPA), alguns dels quals han estat sintetitzats en aquesta Tesi, corresponen en primer lloc a la pèrdua d'un dels metils del carboni central $[M\text{-}H\text{-}CH_3]^{-\bullet}$ seguida de la posterior pèrdua del grup hidroxibenzil. Els compostos que contenen halògens en l'estructura donen lloc a fragmentacions on es perd HCl o HBr. La pèrdua de CO, característica dels compostos fenòlics, no es produeix fins a les darreres etapes de fragmentació. Aquesta pèrdua s'ha confirmat estudiant la fragmentació del $^{13}C_{12}$-TBBPA compostos amb els carbonis aromàtics marcats isotòpicament.

- Els bisfenol A diglicidil èters (BADGEs) i els bisfenols F diglicidil èters (BFDGEs) presenten una elevada tendència a formar adductes amb els components de la fase mòbil, $[M+NH_4]^+$, $[M+Na]^+$ i $[M+K]^+$. D'aquests el que presenta una millor eficiència en la fragmentació per espectrometria de masses en tàndem és l'adducte amb amoni. Per tant, es recomana forçar les condicions de treball per tal d'obtenir els adductes d'amoni a la font d'ionització. La fragmentació més representativa dels adductes d'amoni s'inicia amb la pèrdua d'un dels grups fenil-glicidil èter i continua posteriorment amb el trencament en α del grup èter que resta a la molècula generant els ions a *m/z* 135 pels BADGEs i a *m/z* 107 pels BFDGEs característics d'aquesta família de compostos.

- Atès que els BFDGEs només estan disponibles com a mescles dels tres isòmers (*orto,orto-, ort,para- i para,para-*), els estudis de fragmentació s'han dut a terme acoblant la cromatografia de líquids en fase invertida a l'espectrometria de masses. La interpretació de la informació obtinguda en els estudis de fragmentació de cadascun dels tres isòmers dels BFDGEs ha

permès identificar cadascun dels isòmers i proposar un ordre d'elució en cromatografia de líquids en fase invertida.

- Els fotoiniciadors estudiats en aquesta memòria presenten tant grups funcionals com eficiències en la ionització en electrosprai molt diferents. En general, la molècula protonada $[M+H]^+$ domina l'espectre de masses en electrosprai positiu i la millor ionització s'obté per als compostos que contenen un grup tioxanté (2-ITX, 4-ITX i DETX). Els compostos amb un grup amino a la seva estructura (DEAB, EHDAB i EDMA) i els que tenen una fenona com la BP, la PBZ i la DMPA no es ionitzen tant bé, mentre que el HMPP i el HCPK donen les respostes pitjors.

- Per l'ITX i el DETX la fragmentació en tàndem més important es deu a la pèrdua de les cadenes alquíliques i la posterior pèrdua de CHO per donar lloc a l'ió a *m/z* 184. Per altra banda el pic base per a la BP, la PBZ i la HCPK correspon a l'ió a *m/z* 105 generat per la característica ruptura de l'enllaç en α del grup carbonil. Els compostos que contenen un grup amino i un grup èster, EHDAB, DEAB i EDMA, es caracteritzen per la pèrdua de la cadena alquílica del grup èster i la posterior pèrdua dels grups alquil dels grups amino. Els altres fotoiniciadors, HMPP i DMPA, presenten la pèrdua d'aigua seguida d'altres pèrdues més específiques com la de la cadena alquílica pel HMPP o del CO en el cas del DMPA.

CAPÍTOL 3

CROMATOGRAFIA DE LÍQUIDS-ESPECTROMETRIA DE MASSES

3.1. INTRODUCCIÓ

En els últims anys la tendència en l'evolució de la cromatografia de líquids ha estat millorar l'eficàcia de les columnes i reduir el temps d'anàlisi per tal d'obtenir una major productivitat. Amb aquest objectiu s'han desenvolupat nous rebliments porosos amb una mida de partícula inferior a 2 μm la utilització dels quals, ha permès una disminució substancial de l'eixamplament de les bandes cromatogràfiques i l'aparició d'una variant de la cromatografia de líquids anomenada "cromatografia de líquids d'ultra elevada eficàcia" (UHPLC). Un altre aspecte a destacar de la cromatografia de líquids d'avui dia és el renovat interès en la utilització de fases estacionàries de selectivitat diferent a la de les columnes de fase invertida, C8 i C18. En aquesta línia s'han desenvolupat noves fase estacionàries que en algun cas fins i tot han donat lloc a nous tipus de cromatografia i que comencen a ser emprades amb freqüència. Per exemple, es poden citar les fases estacionàries fluorades, les que contenen grups amida o fenil, les de diversos tipus de grafit o les HILIC (*Hydrophilic Interaction Liquid Chromatography*).

Per tal de reduir el temps d'anàlisi mantenint i fins i tot millorant l'eficàcia en general s'augmenta el cabal, es disminueix la longitud de la columna i es disminueix la mida de partícula. L'augment del cabal de la fase mòbil en columnes convencionals de cromatografia de líquids de mida de partícula de 3 μm i 5 μm produeix una disminució significativa de l'eficàcia (Figura 3.1). Per altra banda si es disminueix la llargada de la columna per tal de reduir el temps d'anàlisi es disminueix a la vegada el nombre de plats teòrics. Ara bé, si es redueix significativament la mida de la partícula, fins a valors de 1.8 μm, es poden assolir separacions amb una considerable eficàcia (Figura 3.1) aconseguint pics cromatogràfics estrets i bones resolucions amb l'avantatge addicional que aquestes partícules presenten poca pèrdua d'eficàcia a velocitats elevades de fase mòbil la qual cosa permet reduir el temps d'anàlisi. Ara bé, un paràmetre que s'ha de tenir en compte és la pressió necessària per mantenir el cabal en disminuir la mida de partícula ja que augmenta considerablement especialment quan s'empren cabals elevats. Per aquesta raó, per poder treballar amb columnes amb rebliments totalment porosos de mida de partícula inferior als 2 μm a elevats cabals de fase mòbil on es poden arribar a superar fàcilment pressions del sistema per sobre dels 6000 psi, es necessari utilitzar equips de cromatografia de líquids dissenyats per

suportar pressions elevades. Aquestes elevades pressions es poden reduir si s'augmenta la temperatura fins a 50-80ºC ja que la disminució de la viscositat de la fase mòbil produeix una disminució significativa de la pressió del sistema. La utilització de columnes amb rebliments de partícules de diàmetre inferior a les 2 μm ha estat important i continuada des de la seva aparició. Per exemple, a la literatura, des de l 'any 2005 han estat publicats més de 1000 treballs científics on s'utilitzen aquestes columnes en diferents camps com el medi ambient, l'anàlisi alimentària i farmacèutica i la bioanàlisi la qual cosa posa de manifest la força amb la que ha estat introduïda aquesta cromatografia i la seva aplicabilitat.

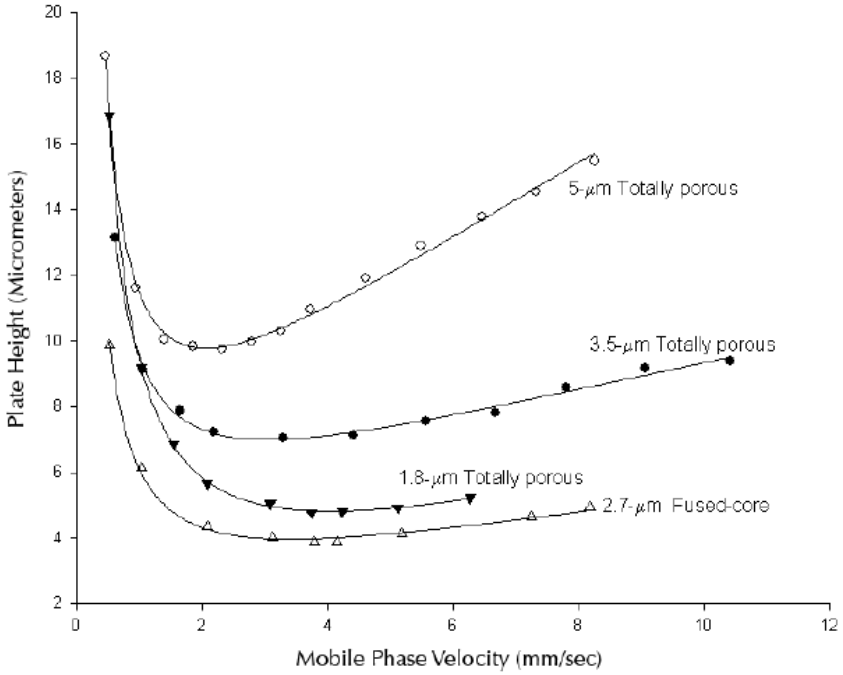

Figura 3.1. Corbes de van Deemter en funció de la mida de partícula i tipus.

Recentment i com alternativa a aquests rebliments sub-2 μm s'han desenvolupat uns nous rebliments amb partícules de 2.7 μm però que tant sols són poroses en la superfície (Figura 3.2) i que permeten assolir eficàcies similars a les dels rebliments porosos sub-2 μm amb l'avantatge de que les caigudes de pressió són molt menors i similars a les que s'obtenen amb columnes de partícules poroses de 3 μm. Aquest fet permet treballar amb equips convencionals de cromatografia de líquids que operen a pressions inferiors a 6000 psi. La utilització de partícules

superficialment poroses no és totalment nova en cromatografia de líquids i de fet han estat emprades des dels inicis d'aquesta tècnica. Per exemple, l'any 1967 Hovarth i cols., les van començar a utilitzar en cromatografia de bescanvi iònic tècnica en la qual han estat emprades fins avui dia. Aquestes primeres columnes contenien una fina capa semiporosa d'aproximadament el 5% del diàmetre total de la partícula presentant una transferència de massa favorable i una elevada eficàcia però amb algunes limitacions pel que fa a la retenció dels anàlits i a la càrrega de mostra. L'any 1969 Kirkland i cols., van desenvolupar columnes de fase invertida emprant aquest tipus de partícules i de diàmetres de 30—40 μm i l'any 1992 Kirkland i cols., (1992) van aconseguir preparar rebliments amb partícules de molt menor diàmetre, fins a 5 μm i amb una capa semiporosa que representa un 14%, del total del diàmetre, que proporcionaven millors paràmetres hidrodinàmics i millor transferència de massa que les partícules totalment poroses amb la mateixa mida de partícula. Són partícules semiporoses d'aquest tipus les comercialitzades per Agilent, les columnes anomenades *Poroshell*, amb un diàmetre de porus de 300Å especialment recomanades per separar macromolècules (Kirkland i cols., 2000). Actualment els rebliments d'aquest tipus que hi ha al mercat utilitzen partícules de 2.7 μm, amb un nucli sòlid de ~1.7 μm recobert per una capa porosa de ~0.5 μm (Figura 3.2) i reben el nom de *Porous Shell* (Phenomenex) o *Fused Core*™ (Supelco). En aquest cas la capa pel·licular representa un 20% del diàmetre total de la partícula la qual cosa permet assolir molt bones eficàcies en la separació cromatogràfica, poder carregar una quantitat de mostra similar a la que admeten les columnes amb partícules poroses i produir una baixa caiguda de pressió ja que el diàmetre és gran. A més, aquestes columnes també presenten poca pèrdua d'eficàcia en treballar a cabals elevats de fase mòbil amb un comportament semblant a l'observat per a les columnes sub-2μm (Figura 3.1). L'elevada eficàcia d'aquestes columnes és deguda primordialment a que facilita la transferència de massa i a que es redueix considerablement la difusió d'Eddy (efecte multi camí) probablement degut a la baixa dispersió en la mida de les partícules, del 5 al 6% enlloc del 19% de les partícules totalment poroses de 3 μm (Guiochon 2006; DeStefano i cols., 2008). Recentment aquestes columnes han començat a ser emprades en diverses aplicacions i a la literatura existeixen una sèrie de treballs que posen de manifest que són una bona alternativa a les columnes sub-2μm i que proporcionen separacions cromatogràfiques similars amb temps d'anàlisi molt curts

(Cundlife i col., 2007, Salisburg 2008, Mallet i col., 2009 i Fekete i col., 2009; Zheng i cols., 2009; Oláh i cols., 2010; Gritti i cols., 2010a i 2010b).

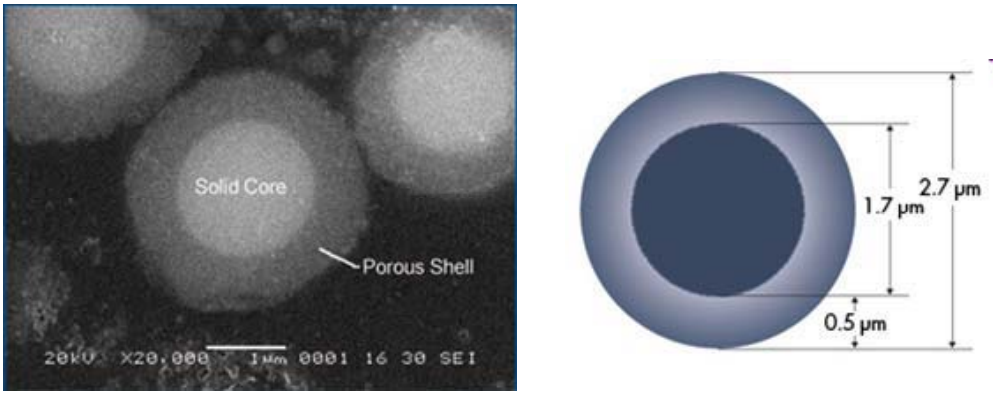

Figura 3.2. Imatge de microscopi electrònic corresponent a partícules semiporoses.

En aquesta Tesi es pretén desenvolupar mètodes de cromatografia de líquids d'ultra elevada eficàcia (UHPLC) emprant columnes de partícules semiporoses. En concret en aquest capítol s'han utilitzat per establir un mètode de cromatografia de líquids ràpida per a l'anàlisi de bisfenol A diglicidil èter i bisfenol F diglicidil èter i dels seus derivats hidrolitzats ja que fins a la data a la literatura la majoria de mètodes de LC publicats per a analitzar aquests compostos fan servir columnes de mida de partícula de 3 – 5 μm obtenint temps d'anàlisis superiors als 25 minuts (Leepipatpiboon i cols., 2005). Únicament Yonekubo i cols., 2008 proposen analitzar el BADGE i els seus derivats hidrolitzats en mostres d'aliments enllaunats emprant una columna de 1.8 μm de mida de partícula però la separació cromatogràfica obtinguda en aquest treball no pot ser avaluada ja que no s'indiquen els paràmetres cromatogràfics ni s'inclou un cromatograma de la separació obtinguda.

Pel que fa referència a la selectivitat en cromatografia de líquids, aquesta es pot modificar canviant la fase estacionaria i/o modificant la composició de la fase mòbil, encara que altres paràmetres físics com la temperatura també poden tenir efectes en la selectivitat. Generalment la composició de la fase mòbil és el paràmetre més estudiat en el desenvolupament de mètodes cromatogràfics per tal d'aconseguir millorar la resolució entre pics cromatogràfics, probablement perquè és fàcil de variar. Ara bé, cada dia és més habitual la utilització de fases

estacionaries diferents de les comunament utilitzades, fase invertida (C18), que permeten aconseguir selectivitats alternatives i ortogonals.

En aquest capítol es descriu la utilització d'una fase estacionària fluorada, més concretament una columna amb pentaflurofenil propil (PFPP) enllaçat químicament a un suport de base sílice. Aquestes fases estacionaries esdevenen una bona alternativa a les fases estacionaries alquíliques generalment emprades en LC fet que fa que les diferents cases comercials (Phenomenex, Supelco, Hypersil, Neos, etc) disposin de columnes amb aquest tipus de fase estacionaria. Tal i com es pot observar a la Figura 3.3 l'anell aromàtic pentafluorofenil s'enllaça a la sílice mitjançant un grup propil proporcionant una fase estacionaria que generalment dóna lloc a separacions ortogonals a les obtingudes amb columnes de fase invertida C8 o C18 i a més presenta una major retenció dels anàlits (Shao i cols., 1997; Emenhiser i cols., 1996; Richheimer i cols., 1994). Aquest increment en la retenció fa que aquesta fase estacionària sigui atractiva per a l'anàlisi de compostos polars que normalment no queden o queden molt poc retinguts en les columnes de fase invertida C18 o C8 (Needham i cols., 2000 i Teixidó i cols., 2008) sent possible augmentar el contingut de solvent orgànic a la fase mòbil la qual cosa comporta una millora en la resposta en dur a terme l'acoblament a l'espectrometria de masses (LC-MS). A més, les columnes amb aquesta fase estacionària presenten en ocasions mecanismes de retenció mixtes entre els de fase invertida i de fase normal en variar la composició de la fase mòbil aportant així una selectivitat característica (Havlíková i cols., 2008). La combinació de les interaccions addicionals de l'anàlit amb els dipols C-F de la fase estacionaria, de les interaccions π–π i l'augment de selectivitat degut a la rigidesa extra d'aquestes fases, aporta selectivitats úniques que permeten per exemple la separació d'isòmers posicionals i/o geomètrics (Monde i cols., 1996; Jinno i cols., 1994 i Csató i cols., 1990; Yamamoto i cols., 2000).

La fase estacionaria de fluorofenil ha estat utilitzada en diferents camps d'aplicació com el farmacèutic (Needham i cols., 1999 i 2000), toxicològic i anàlisis clíniques així com en l'anàlisi de productes naturals com esteroides (De Miguel i cols., 1999 i Büchele i cols., 2005), carotenoides i flavonoides (Monde i cols., 1996), polifenols (Monde i cols., 1996), taxans i catecolamines (Shao i cols., 1997), fosfolípids (De Miguel i cols., 1999), alcaloides (Pellati i cols., 2007 i 2008) i en

l'anàlisi d'isòmers posicionals de materials de partida en la síntesi de fàrmacs, herbicides i surfactants no iònics (Kamiusuki i cols., 1999).

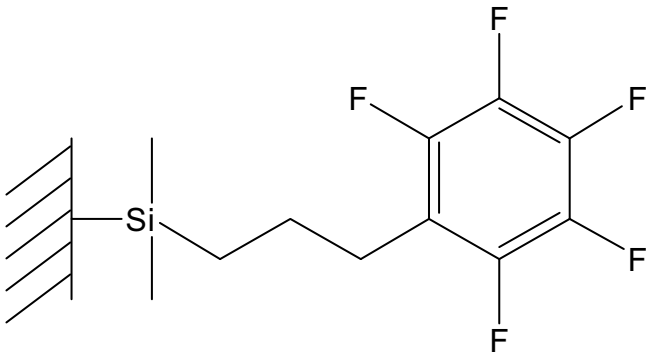

Figure 3.3. Estructura química de la fase estacionaria pentafenilfluorada (PFPP).

Un altre paràmetre que permet modificar la selectivitat de les separacions cromatogràfiques és la temperatura ja que per una banda tal i com hem comentat anteriorment, afecta a la viscositat dels solvents reduint la pressió del sistema cromatogràfic i per l'altra, afecta a la retenció cromatogràfica ja que es veuen modificats els equilibris d'interacció de l'anàlit amb la fase estacionària. En el cas de columnes de base sílice les variacions amb la temperatura són atribuïdes a les diferències d'entalpia involucrades en les interaccions solvofòbiques i silanofíliques i a fenòmens anomenats de "transició de fase". Aquesta transició és una conseqüència del canvi conformacional de la fase estacionaria, de l'estat sòlid (a temperatures baixes) a un estat líquid (a temperatures més elevades) (Morel i cols., 1981; Cole i cols., 1992; Zhu i cols., 1996; Dolan 1998a, 1998b, 1999a, 1999b, 1999c i 2002 i Liu i cols., 2005). Encara que generalment en cromatografia es tendeix a treballar a temperatura ambient o a temperatures per sobre de la temperatura ambient en alguns casos el fet de treballar a temperatures baixes, per sota de la temperatura ambient, aporta una selectivitat diferent que permet obtenir millors separacions. Per exemple per a l'anàlisi de proteïnes Wales i cols., 2008 proposen treballar a 0ºC i per a l'anàlisi de 17α-estradiol, 17β-estradiol i equilin en cromatografia de fase invertida alguns autors obtenen una millor resolució treballant a 5ºC (Lamparczyk i cols., 1995). En aquest capítol s'ha emprat aquesta

estratègia per optimitzar la separació d'alguns fotoiniciadors utilitzats en les tintes d'impressió.

3.2. TREBALL EXPERIMENTAL

Per tal de disminuir el temps d'anàlisi i obtenir separacions cromatogràfiques d'elevada eficàcia treballant a pressions moderades s'ha desenvolupat un mètode per a l'anàlisi de bisfenol A diglicidil èter i bisfenol F diglicidil èter de forma simultània amb els seus derivats hidrolitzats en mostres d'aliments enllaunats utilitzant una columna de partícules semiporoses. El treball experimental d'aquest estudi es troba recollit a l'article IV intitulat *"Fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A- and bisphenol F-diglycidyl ether derivatives in canned food and soft-drinks"* (Apartat 3.2.1).

Per altra banda, amb la finalitat d'augmentar la selectivitat en les separacions cromatogràfiques s'ha avaluat l'ús d'una columna de fase estacionaria pentafluorofenil propil (PFPP), per tal de separar els isòmers del ITX i que ha permès analitzar cadascun dels isòmers per separat. El treball experimental d'aquest estudi es troba recollit a l'article V inclòs en aquest capítol intitulat *"Liquid chromatography/tandem mass spectrometry (highly selective selected reaction monitoring) for the analysis of isopropylthioxanthone in packaged food"* (apartat 3.2.2). Posteriorment aquestes condicions cromatogràfiques es van prendre com a punt de partida per al desenvolupament d'un mètode per a l'anàlisi simultània de l'ITX i altres fotoiniciadors en mostres d'aliments envasats amb cartró. En aquest cas per tal de millorar l'eficàcia de separació i obtenir una bona resolució cromatogràfica es va avaluar l'efecte de treballar a temperatura per sota de la temperatura ambient tal i com s'indica a l'article científic VI *"Analysis of UV ink photoinitiators in packaged food by fast liquid chromatography at sub-ambient temperature coupled to tandem mass spectrometry"* (apartat 3.2.3).

A més, atesa la importància que té avui dia garantir les identificacions dels compostos analitzats i evitar falsos positius i falsos negatius en l'anàlisi per LC-MS/MS en aquesta Tesi s'ha avaluat la possibilitat d'obtenir una confirmació extra

de la presència dels anàlits mesurant la seva massa exacta emprant l'analitzador de triple quadrupol. Aquesta aplicació es descriu a l'apartat 3.2.4. d'aquest capítol.

3.2.1. ARTICLE CIENTÍFIC IV.

Fast liquid chromatography/tandem mass spectrometry for the analysis of bisphenol A- and bisphenol F-diglycidyl ether derivatives in canned food and soft-drinks.

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Journal of Chromatography A, Enviat

Fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives in canned food and beverages.

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Keywords: Bisphenol A diglycidyl ethers, Bisphenol F diglycidyl ethers, Fused Core^{TM}, tandem mass spectrometry, soft drinks, canned food.

Abstract

In this work a fast liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method using a C18 Fused Core^{TM} column, was developed for the simultaneous analysis of BADGEs (BADGE, BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·HCl and BADGE·2HCl) and BFDGEs (BFDGE, BFDGE·2H_2O and BFDGE·2HCl). The LC method was coupled with a triple quadrupole mass spectrometer, using an ESI source in positive mode and using the $[M+NH_4]^+$ adduct as precursor ion for tandem mass spectrometry experiments. The method developed was applied to the determination of these compounds in canned soft drinks and canned food. OASIS HLB solid phase extraction (SPE) cartridges were used for the analysis of soft drinks, while solid canned food was extracted with ethyl acetate. Method limits of quantitation ranged from 0.13 μg L^{-1} to 1.6 μg L^{-1} in soft drinks and 1.0 μg kg^{-1} to 4.0 μg kg^{-1} in food samples. BADGE·2H_2O was detected in all the analyzed samples, while other BADGEs such as BADGE·H_2O, BADGE·HCl·H_2O, BADGE·HCl and BADGE·2HCl were also detected in canned foods.

1. Introduction

Epoxy-based lacquers or vinylic organosol (PVC) materials are commonly used for coating the inside of food cans, big storage vessels and food containers to reduce food spoilage and to prevent degradation of the food can. These lacquers are epoxy phenolic resins based on polymerization products of bisphenol A-diglycidyl ether (BADGE) or bisphenol F-diglycidyl ether (BFDGE), so this coatings can release these compounds as well as oligomers and derivatives which can migrate into the packed foods. Chlorinated derivatives may be generated during the thermal coating treatment, since BADGE and BFDGE are also used as additives to remove the hydrochloric acid formed in this process. Moreover, hydrolyzed derivatives such as BADGE·$2H_2O$, BADGE·H_2O and BFDGE·$2H_2O$ can be produced during storage when the coating comes into contact with aqueous and acid foodstuffs. The presence of this family of compounds has received attention lately due to the suspected mutagenic, genotoxic and anti-androgenic effects of the compounds [1-4]. The toxicity of these epoxy compounds depends mainly on the fractional concentration of un-reacted epoxy groups [5], since they are alkylating agents and have rather specific cytotoxic actions in tissue [6]. However, the toxicity of BADGE·2HCl is probably due to the presence of halogen atoms in its chemical structure [5]. Regarding legislation, the European Union (EU) has set specific migration limits (SML), 9 mg kg^{-1} for the sum of BADGE and its hydrolyzed derivatives and 1 mg kg^{-1} for the sum of BADGE·HCl, BADGE·2HCl and BADGE·HCl·H_2O [7,8].

Bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their hydrolyzed derivatives are traditionally analyzed by gas chromatography coupled with mass spectrometry (GC-MS) or by liquid chromatography with fluorescence detection (LC-FLD) [9-12]. Liquid chromatography coupled with tandem mass spectrometry (LC-

MS/MS) is also used in the analysis of BADGEs and BFDGEs [13-15]. These methods use conventional reversed-phase C18 columns (3.5-5 μm particle size), providing long analysis times when both BADGEs and BFDGEs have to be analyzed in the same run. Recently, ultra-high performance liquid chromatography (UHPLC) has been used to analyze BADGEs in canned food, with its greater chromatographic efficiency shortening the analysis time [16]. Since the complexity of food matrixes usually requires extensive sample treatment, liquid-liquid extraction (LLE) [9,12,17-19] and solid phase extraction (SPE) [16,20] are the procedures most commonly used for the analysis of this family of compounds in canned foods, while other techniques such as pressurized liquid extraction (PLE) [21] have scarcely been employed.

The aim of this study was to develop a fast LC-MS/MS method for the analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives in canned food samples and soft-drink beverages after a simple sample treatment procedure. The applicability of a porous shell particle column was evaluated in order to provide short analysis times and high chromatographic efficiencies for the analysis of these compounds.

2. Experimental

2.1. Chemicals and reagents

Bisphenol A diglycidyl ether (BADGE), bisphenol A (2,3-dihydroxypropyl) glycidyl ether (BADGE·H_2O), bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE·$2H_2O$), bisphenol A (3-chloro-2-hydroxypropyl) glycidyl ether (BADGE·HCl), bisphenol A bis(3-chloro-2-hydroxypropyl) ether (BADGE·2HCl) and

bisphenol A (3-chloro-2-hydroxypropyl)(2,3-dihydroxypropyl ether (BADGE·HCl·H_2O) standards of analytical grade were obtained from Sigma-Aldrich (Steinheim, Germany). Bisphenol F diglycidyl ether (BFDGE), bisphenol F bis(2,3-dihydroxypropyl) ether (BFDGE·$2H_2O$), bisphenol F bis(3-chloro-2-hydroxypropyl) ether (BFDGE·2HCl) (all of them, mixtures of *ortho-ortho, ortho-para* and *para-para* isomers) were also obtained from Sigma-Aldrich (Steinheim, Germany). The chemical structures of the compounds studied are given in Figure 1.

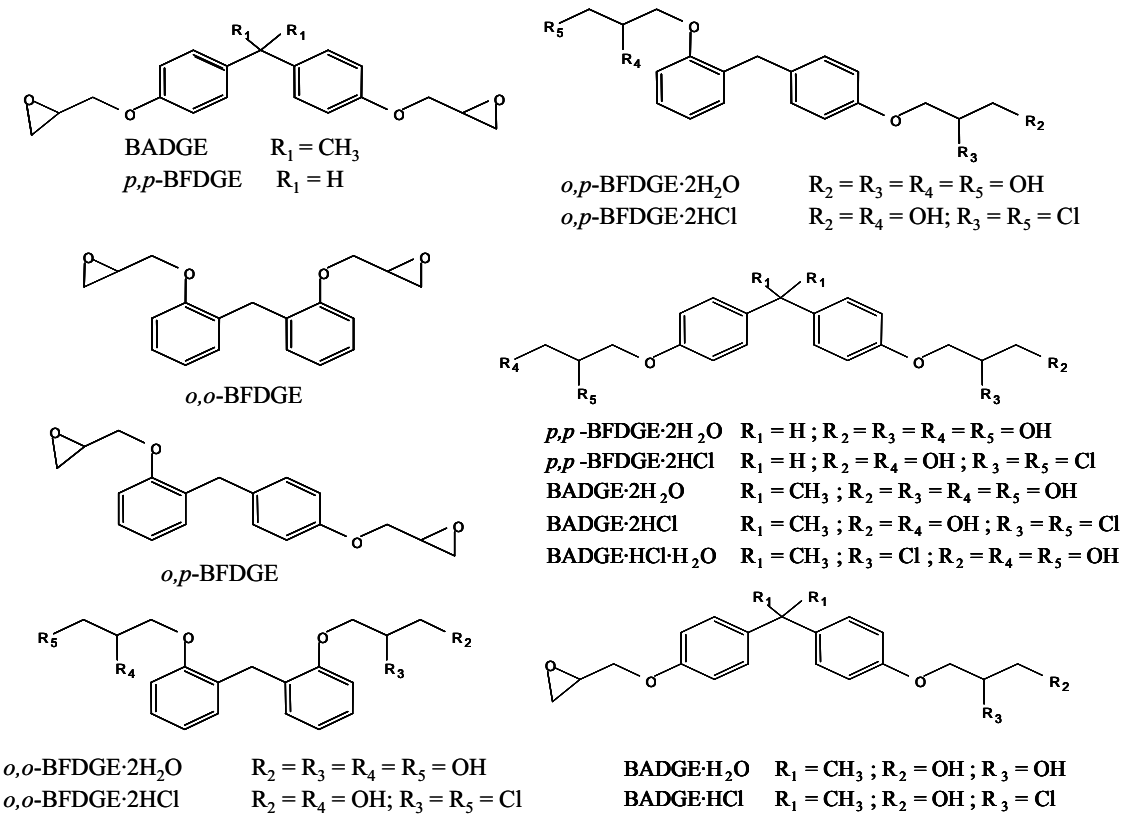

Figure 1. Chemical structures of Bisphenol A diglycidyl ethers and Bisphenol F diglycidyl ethers.

LC-MS grade methanol (MeOH), acetonitrile (ACN) and water were purchased from Riedel-de Haën (Seelze, Germany). Ammonium formate (≥ 99.0%) and ethyl acetate were obtained from Fluka (Steinheim, Sweden) and formic acid (98-100%) from Merck (Darmstadt, Germany). Stock standard solutions (200 mg kg^{-1}) were individually prepared by weight in methanol and stored at 4ºC. Intermediate solutions were prepared weekly from the stock standard solution by appropriate dilution in MeOH:water (1:1). Calibration standard solutions ranging from 0.5 μg kg^{-1} to 5,000 μg kg^{-1} were prepared daily. Mobile phases were filtered through 0.22 μm Nylon membrane filter (Whatman, Clifton, NJ, US) and sample extracts were filtered through 0.22 μm pore size Ultrafree-MC Centrifugal Filters (Millipore, Bedford, US). OASIS HLB cartridges (60 mg) purchased from Waters (Mildford, MA, US) were used for solid phase extraction (SPE).

Nitrogen (99.98% pure) supplied by Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the API source. High-purity Argon (Ar_1) purchased from Air Liquide (Madrid, Spain) was used as a collision-induced gas (CID gas).

2.2. Instruments, LC and MS conditions

A liquid chromatograph (Accela system; Thermo Fisher Scientific, San José, CA, US) equipped with a low-pressure quaternary pump, an autosampler and a column oven was coupled with a triple quadrupole mass spectrometer. The chromatographic separation was performed on a Fused Core™ Ascentis Express C18 column (150 x 2.1 mm i.d., 2.7 μm particle size) from Supelco (Bellefonte, PA, US), using as mobile phase methanol (solvent A) and 25 mM formic acid-ammonium formate buffer at pH 3.75 (solvent B) at 600 μL min^{-1}, at a column temperature of 50ºC. The gradient elution program started at 30% of solvent A (0.25 min), followed by a linear gradient up to

50% of solvent A in 0.75 min, then a second linear gradient up to 60% of solvent A in 0.5 min and finally a third linear gradient up to 80% of solvent A in 4 minutes. This composition was then maintained for 0.5 min.

The liquid chromatographic system was coupled with a triple quadrupole mass spectrometer TSQ Quantum Ultra AM (Thermo Fisher Scientific, San José, CA, US) equipped with a heated-electrospray ionization source (H-ESI I) working in positive mode. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow rates of 60, 20 and 40 a.u. (arbitrary units), respectively. Ion transfer tube temperature was set at 375ºC, vaporizer temperature at 25ºC and electrospray voltage at 4kV. When data were acquired in low-resolution selected reaction monitoring (SRM) mode, a resolution of 0.7 *m/z* full width half maximum (FWHM) on Q1 and Q3 and a scan width of 0.01 *m/z* were used. In highly-selective selected reaction monitoring (H-SRM), mode quadrupole Q1 operated at a mass resolution of 0.1 *m/z* FWHM with a scan width of 0.01 *m/z*, whereas Q3 operated at low resolution (0.7 *m/z* FWHM). Argon, used as collision gas at 1.5 mtorr, and the optimum collision energy (CE) selected for each transition are indicated in Table 1. Ammonium adducts $[M+NH_4]^+$ were used as precursor ions in tandem mass spectrometry and two transitions for each compound and a dwell time of 10 ms were chosen for quantitative analysis and confirmation purposes (Table 1).

To optimize source working conditions and to carry out multiple-stage mass spectrometry experiments, a standard solution (1 mg L^{-1}) prepared in methanol was infused at a flow-rate of 3µL min^{-1} using the syringe pump integrated in the TSQ instrument and was mixed with the mobile phase (600 µL min^{-1}, 60:40 v/v MeOH:

formic acid-ammonium formate) by a Valco zero dead volume tee piece (Supelco, Alcobendas, Spain).

2.3. *Sample treatment*

A total of six canned food and seven canned beverage samples were purchased at local supermarkets (Barcelona, Spain) and processed using two sample treatments: (i) canned food and (ii) canned beverages.

(i) For canned food samples of vegetables and fruits, the whole can content was homogenized using Ultra-Turrax TR-50 (Staufen, Germany). A subsample of 3 g was weighed into a 15-mL centrifuge tube, and 6 mL of ethyl acetate was added as extraction solvent. The resulting mixture was shaken for 20 min in a rotatory shaker and for 30 min in an ultrasonic bath. Then the mixture was centrifuged at 4,000 r.p.m. for 15 min using a Selecta Centronic centrifuge (Selecta, Barcelona, Spain). Five milliliters of the supernatant was transferred to an 8 mL vial and evaporated to dryness under nitrogen stream. Then, the extract was reconstituted in 1 mL of MeOH:water (1:1) and filtered before injection of 10 µL of it into the LC-MS/MS system.

(ii) The seven canned soft-drink beverages included soda, beer, cola, tea and a tonic drink, all of them carbonated except tea. They were stored unopened until analysis at 4ºC. Twenty milliliters of beverage samples were degassed by sonication for 20 minutes. After this step 3 mL were loaded into the OASIS HLB SPE cartridge, which was previously conditioned with 3 mL of MeOH and 3 mL of water. Finally, the analytes were eluted with 4 mL of MeOH. The collected fraction was evaporated to

dryness and the extract was reconstituted with 1 mL of MeOH:water (1:1) and filtered before injection of 10 µL of it into the LC-MS/MS system.

Supelco Visiprep and Supelco Visidry SPE (Supelco) vacuum manifold were used for SPE and solvent evaporation.

A soft-tonic beverage and a red pepper sample both packed in glass were submitted to the sample treatment detailed above and analyzed by the LC-MS/MS method. As they were shown to be free of BADGEs, BFDGEs and their derivatives, they were used to study the matrix effects and to evaluate the quantitative method. Analytes were determined in canned food and beverage samples by external calibration.

3. Results and discussion

3.1. Liquid Chromatography-Mass Spectrometry

In this study, a fast LC-MS/MS method was developed for the analysis of BADGE, BFDGE and their hydrolyzed derivatives in canned food and beverages. In a preliminary study, two short columns, a Fused Core™ (Ascentis Express C18 50 x 2.1 mm i.d., 2.7 µm) column and a total porous sub-2 µm particle size column (Acquite BEH C18 50 x 2.1 mm i.d., 1.7 µm), were evaluated for the separation of BFDGEs isomers. MeOH:ammonium formate/formic acid (25 mM, pH 3.75, 50ºC) was used as mobile phase at 600 µL min^{-1} in both cases. As can be seen in Figure 2, both columns provided similar resolutions and efficiencies for the separation of these isomers, although the Fused Core™ column showed a lower backpressure of 200 bar against 513 bar for the sub-2µm column. The Fused Core™ column was selected for further experiments, as its low backpressure permitted the increase of the column length to 150

mm, which allowed the separation of BADGEs, BFDGEs and their hydrolyzed derivatives.

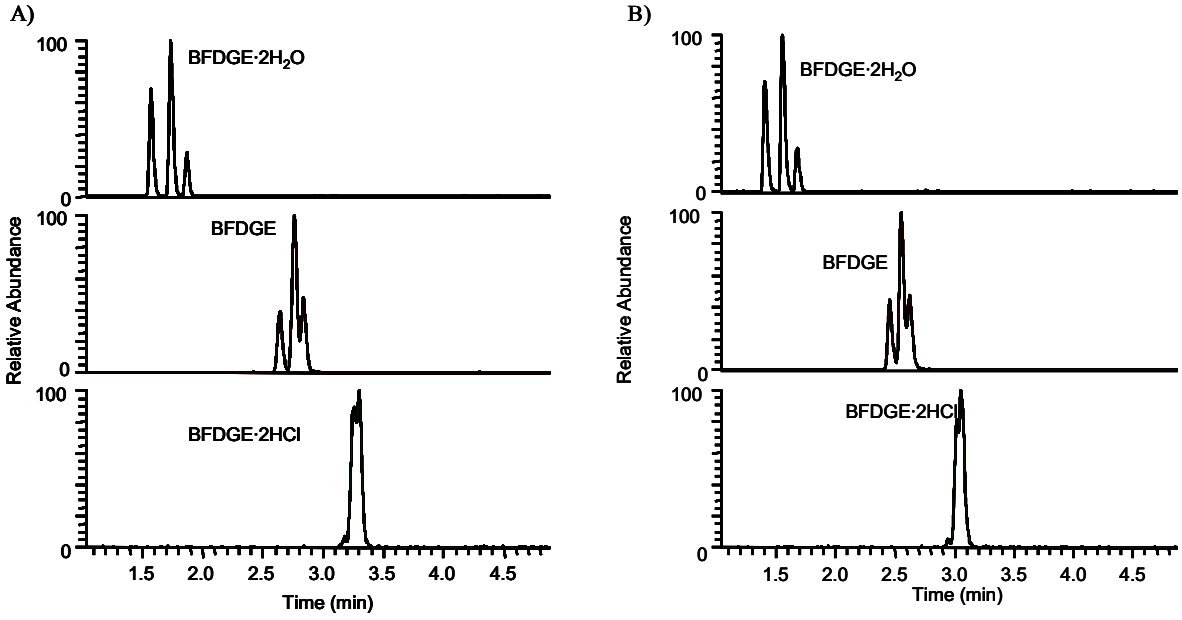

Figure 2. LC-MS/MS chromatogram of BFDGE·$2H_2O$ isomers, using A) Acquity BEH C18 column (50 mm x 2.1 mm i.d. 1.7 μm particle size) and B) Ascentis Express C18 column (50 mm x 2.1 mm i.d. 2.7 μm particle size).

Several mobile phase compositions and gradient elution programs were tested using the 150 mm Fused Core^{TM} C18 column and the whole set of compounds. The best separation was obtained in less than 5 minutes with MeOH:formic acid-ammonium formate buffer (25 mM, pH 3.75, 50ºC) and the gradient elution indicated in the experimental section at a flow rate of 600 μL min^{-1}. Figure 3A shows the chromatogram obtained for a standard solution under these conditions. It should be mentioned that acetonitrile as organic modifier in the mobile phase was also evaluated, but MeOH-based mobile phases produced, for most of the compounds, higher responses when in electrospray. This can be seen in Figure 3, where the chromatograms using both MeOH and ACN are shown. Moreover, in agreement with the results published by Lintschinder

et al., [17] the elution order for BADGE·HCl/BADGE·2HCl and BFDGE/BFDGE·2HCl changed when ACN was used instead of MeOH, probably due to the different proton donor/acceptor characteristics of the two solvents. However, ACN provided better chromatographic resolutions between BFDGE isomers (Figure 3B). Thus, MeOH can be proposed as an organic modifier to improve the sensitivity of the method, although ACN can be used in a second analysis if positive samples are detected and if it is important to know the distribution of BFDGE isomers.

The fast liquid chromatography separation was coupled to the triple quadrupole mass spectrometer, using an ESI source in positive mode. This family of compounds tends to form adducts and clusters in positive ionization mode, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$, as described in a previous work [22]. The mobile phase used favored the formation of ammonium adduct ions $[M+NH_4]^+$, which were the base peak of the full-scan spectra. The two most intense and characteristic fragmentations in tandem mass spectrometry provided by the $[M+NH_4]^+$ for BADGEs and BFDGEs were the cleavage of the phenyl-alkyl bond and the consecutive cleavages of the phenyl-alkyl bond and the α-cleavage of the ether bond [22], which were selected for quantification and confirmation (Table 1).

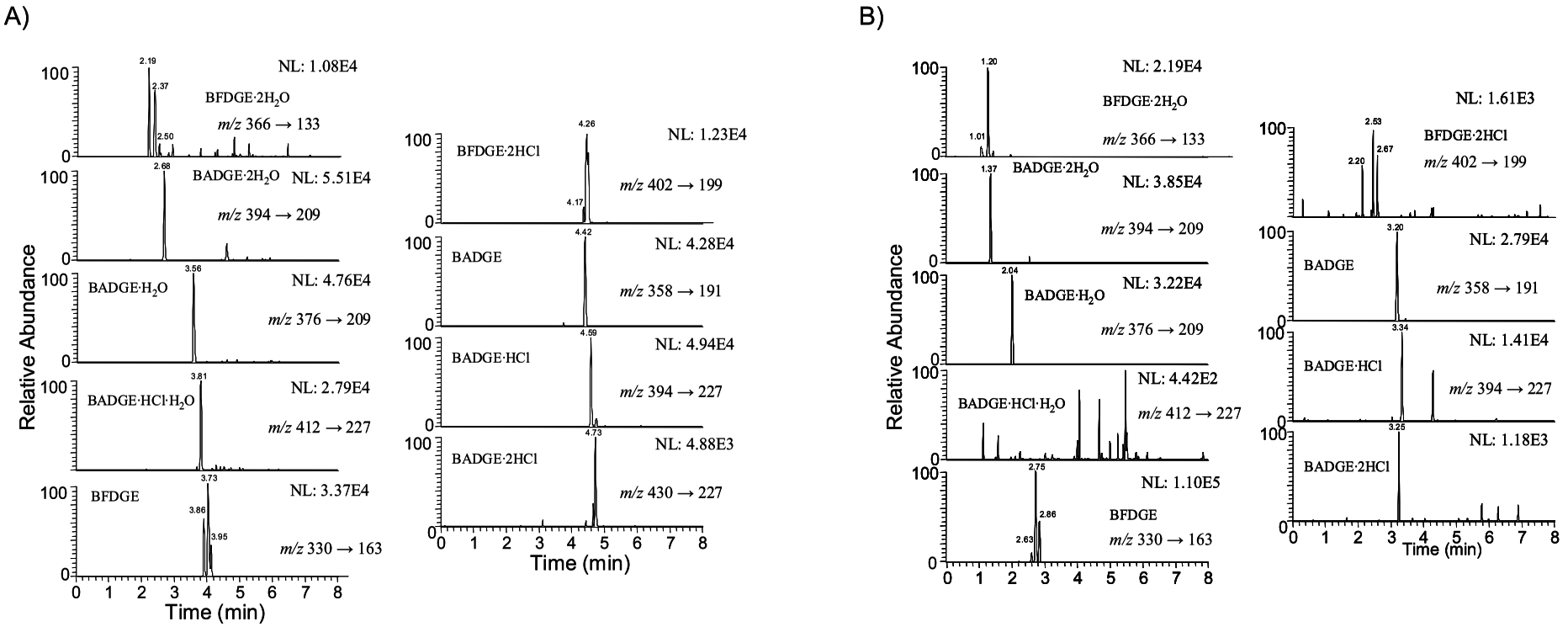

Figure 3. LC-MS/MS chromatograms of BADGEs and BFDGEs, using: A) Methanol: 25 mM formic acid-ammonium formate buffer at pH 3.75 gradient elution and B) ACN: 25 mM formic acid-ammonium formate buffer at pH 3.75 gradient elution.

Table 1. Tandem mass spectrometry transitions for SRM.

Compound	Precursor ion (*m/z*), $[M+NH_4]^+$	Quantitation		Confirmation		Ion Ratio ± SD[b]
		Product Ion (*m/z*)	CE[a] (V)	Product Ion (*m/z*)	CE[a] (V)	
BADGE·2H_2O	394.2	209.1	31	135.1	31	1.7 ± 0.1
BADGE·H_2O	376.2	209.1	29	135.1	29	1.9 ± 0.1
BADGE·HCl·H_2O	412.2	227.0	33	135.1	33	1.4 ± 0.1
BADGE	358.2	191.0	30	135.1	30	4.3 ± 02
BADGE·HCl	394.2	227.0	13	135.1	13	2.6 ± 0.3
BADGE·2HCl	430.2	227.0	30	135.1	30	2.0 ± 0.1
BFDGE·2H_2O	366.2	133.1	22	181.1	22	1.5 ± 0.1
BFDGE	330.2	163.1	12	189.1	12	1.3 ± 0.1
BFDGE·2HCl	402.1	199.1	20	181.1	20	1.7 ± 0.2

[a]CE: collision energy

[b]SD: Standard deviation (n :5)

Instrumental quality parameters such as limit of detection (LOD), limit of quantitation (LOQ), run-to-run precision, ion-ratio precision and linearity were studied in selected reaction monitoring (SRM). Limits of detection (LODs), based on a signal-to-noise ratio of 3 and limits of quantitation (LOQs), based on signal-to-noise 10 were estimated by the injection of 10 μL of standard solutions at low concentration levels (down to 200 ng kg^{-1}). LODs from 0.15 μg kg^{-1} for BADGE, BADGE·$2H_2O$, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·HCl and BFDGE to 8 μg kg^{-1} for BADGE·2HCl, BFDGE·$2H_2O$ and BFDGE·2HCl and LOQs from 0.5 μg kg^{-1} for BADGE, BADGE·$2H_2O$, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·HCl and BFDGE to 2.5 μg kg^{-1} for BADGE·2HCl, BFDGE·$2H_2O$ and BFDGE·2HCl were obtained. Good linearity ($r^2 > 0.999$) was observed for calibration curves for standard solutions ranging from 0.5 μg kg^{-1} to 5,000 μg kg^{-1}. Run-to-run precision (n=5) was evaluated at two concentration levels (0.5 μg kg^{-1} and 5,000 μg kg^{-1}) and the relative standard deviations (RSDs) based on concentration were lower than 10%. From these results it can be concluded that SRM mode provides good selectivity and is robust enough to be used as acquisition mode for the analysis of BADGEs and BFDGEs by LC-MS/MS.

3.2. Feasibility of the method

It is well documented that there are several critical factors that may contribute to poor results in the analysis of BPA. Special care must be taken during sample manipulation due to background contamination at ng L^{-1} mainly coming from solvents, SPE cartridges and plastic ware [23,24]. Since BPA and BPF are the monomers of BADGE and BFDGE, in this study, to ensure good quantitation results, blank samples were analyzed to evaluate possible contamination sources. Some BADGEs, BADGE·$2H_2O$, BADGE·H_2O, BADGE·HCl·H_2O, BADGE and BADGE·HCl, were

detected in the analysis of blank samples. To identify the source of contamination, all sample treatment steps were studied and some of the compounds were detected after transferring the extracts into the injection vials by syringes with metal needles. To evaluate the contamination, 1 mL of MeOH:water (1:1) was transferred to 2 mL injection vials from different suppliers by several syringe metal needles and then analyzed by LC-MS/MS. BADGE, BADGE·$2H_2O$ and BADGE·H_2O were detected at μg L^{-1} level in most of the needles (Table 2), while in two of them only BADGE·$2H_2O$ was detected at a concentration below LOQ. In addition, the needles were divided into three parts, each exposed to 1 mL of MeOH:water (1:1), and then the solution was analyzed. The results indicated that the contamination came from the adhesive used to cement the needles, probably an epoxy-resin based on BADGE. These results are consistent with those of Watabe et al. [25], who detected BPA in cemented syringe needles at similar concentration levels to these BADGEs. To prevent contamination in further studies, only Pasteur pipettes were used to transfer the extracts.

Table 2. Levels of BADGEs detected in cemented needles (μg L^{-1}).

Compound	Needle 1	Needle 2	Needle 3	Needle 4	Needle 5	Needle 6
BADGE·$2H_2O$	20.2	17.9	45.9	21.0	<LOQ	<LOQ
BADGE	n.d.	37.6	15.4	36.0	n.d.	n.d.
BADGE·HCl·H_2O	<LOQ	<LOQ	<LOQ	<LOQ	n.d.	n.d.
BADGE·H_2O	n.d.	63.3	35.2	74.1	n.d.	n.d.
BADGE·HCl	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.

n.d. not detected
<LOQ: below the instrumental limit of quantitation

Shorter chromatographic run times and simplified sample clean-up often lead to matrix suppression effects in electrospray. In this study, matrix effects were evaluated by means of two matrix samples (free of BADGEs and BFDGEs) selected as representative of the analyzed samples: a cola soft-drink beverage and red pepper (one

of the most complex vegetable samples), both in glass. These samples were analyzed by external and matrix-matched calibration. The results showed similar responses for both methods and matched calibration curves, indicating that no matrix effect occurred in the analysis of BADGEs and BFDGEs using the developed LC-MS/MS method.

To evaluate limits of quantitation, cola and red pepper samples were spiked with the studied compounds at low concentration levels (below 2.5 μg kg^{-1}) and submitted to the sample treatments detailed above in Section 2.3. The developed LC-MS/MS method using SRM acquisition mode provided a good method limit of quantitation (MLQ) (Table 3): between 0.13 μg L^{-1} and 1.6 μg L^{-1} in cola samples and between 1.0 μg kg^{-1} to 4.0 μg kg^{-1} in red pepper. This allowed the analysis of this family of compounds in canned food and beverages, since these values are 3 to 4 orders of magnitude lower than the specific migration limits (SML) established by the European Union (EU) [7,8].

Run-to-run precision was evaluated by analyzing six replicates of a red pepper sample and a cola sample spiked at two concentration levels. The low concentration level ranged from 0.15 μg L^{-1} to 2.0 μg L^{-1} (depending on the compound) for the cola sample and from 2.0 μg kg^{-1} to 15.0 μg kg^{-1} for the red pepper, while the medium level was ten times higher for both samples. The relative standard deviations (RSDs) based on concentration provided similar results for both sample matrices (cola and red pepper), ranging from 3 to 20% (Table 3). In addition, the ion ratios (quantitative *versus* confirmatory transitions) were calculated and errors (compared with standards) were always below 10%.

Finally, recoveries were calculated by addition of different amounts of the studied compounds (between LOQ and 250 μg kg^{-1}) to blank samples (cola and red pepper), which were analyzed by external calibration. The slope of the calculated

amount *versus* the added concentration, provided average recoveries ranging from 70% to 95% (Table 3).

In addition, to avoid false positive and false negative results, the use of enhanced mass resolution acquisition mode (H-SRM) was evaluated, since selectivity can be increased by filtering chemical background noise. Cola and red pepper blank samples spiked at low concentration levels (< 2.5 μg kg^{-1}) were analyzed by H-SRM on Q1 (Q1: 0.1 *m/z* FWHM, Q3: 0.7 *m/z* FWHM) mode, with cleaner chromatograms and limits of detection 2 to 10 times better than those obtained using SRM mode. Therefore, H-SRM on Q1 can be used as a complementary tool to confirm the results obtained by SRM.

Table 3. MLOQs, Run-to-Run precision, recoveries and ion ratio of the LC-MS/MS method.

Compound		Cola					Red pepper			
	MLOQ ($\mu g\ L^{-1}$)	Run to Run (% RSD)		Recovery (%)	Ion ratio*	MLOQ ($\mu g\ kg^{-1}$)	Run to Run (% RSD)		Recovery (%)	Ion ratio*
		Low concentration[a]	Medium concentration[b]				Low concentration[a]	Medium concentration[b]		
BADGE·2H_2O	0.13	7	3	95	1.8	1.0	13	4	70	1.7
BADGE·H_2O	0.14	12	3	83	1.8	1.1	20	5	60	1.8
BADGE·HCl·H_2O	0.14	20	9	95	1.5	1.1	7	7	69	1.3
BADGE	0.16	12	10	80	4.3	1.2	4	5	86	4.4
BADGE·HCl	0.16	3	11	70	2.4	1.3	9	5	60	2.5
BADGE·2HCl	1.6	14	10	82	2.1	3.4	8	6	80	1.9
BFDGE·2H_2O	1.5	16	8	85	1.3	1.4	16	8	90	1.5
BFDGE	0.7	20	10	70	1.6	4.0	17	6	89	1.6
BFDGE·2HCl	1.6	13	4	95	1.9	1.5	7	8	74	2.0

[a]Low concentration level: Cola sample (0.15 $\mu g\ L^{-1}$ to 2.0 $\mu g\ L^{-1}$) and red pepper (2.0 $\mu g\ kg^{-1}$ to 15.0 $\mu g\ kg^{-1}$).

[b]Medium concentration level: Cola sample (1.5 $\mu g\ L^{-1}$ to 20 $\mu g\ L^{-1}$) and red pepper (20 $\mu g\ kg^{-1}$ to 150 $\mu g\ kg^{-1}$).

*Ion ratio calculated at medium concentration level.

3.3. Sample Analysis

The LC-MS/MS method developed for the analysis of BADGEs and BFDGEs in canned food and soft-drinks has been employed to analyze six aqueous based canned foods and seven soft-drink samples (Table 4). Samples were prepared as described in Section 2.3 and analyzed by triplicate. In canned soft-drink beverages only BADGE·2H_2O was detected, at concentrations ranging from 2.3 μg L^{-1} to 5.1 μg L^{-1}, while other BADGEs and BFDGEs were not detected. In contrast, several BADGEs were found in canned food samples. BADGE·2H_2O was found in all food samples at concentrations between 2.7 μg kg^{-1} and 675 μg kg^{-1}, with the highest concentration level being in the asparagus sample. Other BADGEs detected in these samples were BADGE·H_2O at concentrations ranging from 35 μg kg^{-1} to 53 μg kg^{-1}, BADGE·HCl·H_2O (3.4 – 274 μg kg^{-1}) and BADGE·2HCl at concentrations between 0.9 μg kg^{-1} and 2.8 μg kg^{-1}. In contrast, the original monomer (BADGE) was not found in the samples, probably because it was easily hydrolyzed in these water-based samples. In addition, none of the BFDGEs were found, confirming the lesser use of BFDGE-based coatings. As an example, Figure 4 shows the LC-MS/MS chromatograms obtained for the asparagus samples in which BADGE.2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·HCl and BADGE·2HCl were detected. These samples were also analyzed by LC-MS/MS, using H-SRM on Q1. No false positives/negatives were detected in the samples analyzed.

Table 4. Canned soft-drinks and food samples analyzed using the developed LC-MS/MS method.

Samples	**BADGE·2H$_2$O** (μg kg^{-1}) ±SD	**BADGE·H$_2$O** (μg kg^{-1}) ±SD	**BADGE·HCl·H$_2$O** (μg kg^{-1}) ±SD	**BADGE·HCl** (μg kg^{-1}) ±SD	**BADGE·2HCl** (μg kg^{-1}) ±SD
Soft-drinks					
Cola	3.6±0.4	n.d.	n.d.	n.d.	n.d.
Tea	2.6±0.2	n.d.	n.d.	n.d.	n.d.
Beer 1	5.1±0.6	n.d.	n.d.	n.d.	n.d.
Beer 2	4.3±0.5	n.d.	n.d.	n.d.	n.d.
Lemon soda	2.1±0.1	n.d.	n.d.	n.d.	n.d.
Orange soda	2.8±0.1	n.d.	n.d.	n.d.	n.d.
Soft-drink	2.3±0.3	n.d.	n.d.	n.d.	n.d.
Canned food					
Sweet corn 1	369±18	40±1	3.4±0.7	n.d.	2.7±0.3
Sweet corn 2	252±19	37±6	4.4±0.8	n.d.	1.1±0.1
Pinapple 1	2.8±0.1	n.d.	n.d.	n.d.	n.d.
Pinapple 2	3.1±0.6	n.d.	n.d.	n.d.	0.9±0.1
Red pepper	157±25	35±7	4.7±1.0	n.d.	1.6±0.1
Asparagus	675±100	53±11	274±40	11±1.5	2.8±0.2

[a]SD: Standard deviation

BADGE, BFDGE, BFDGE·2H$_2$O and BFDGE·2HCl were not detected in the analyzed samples.

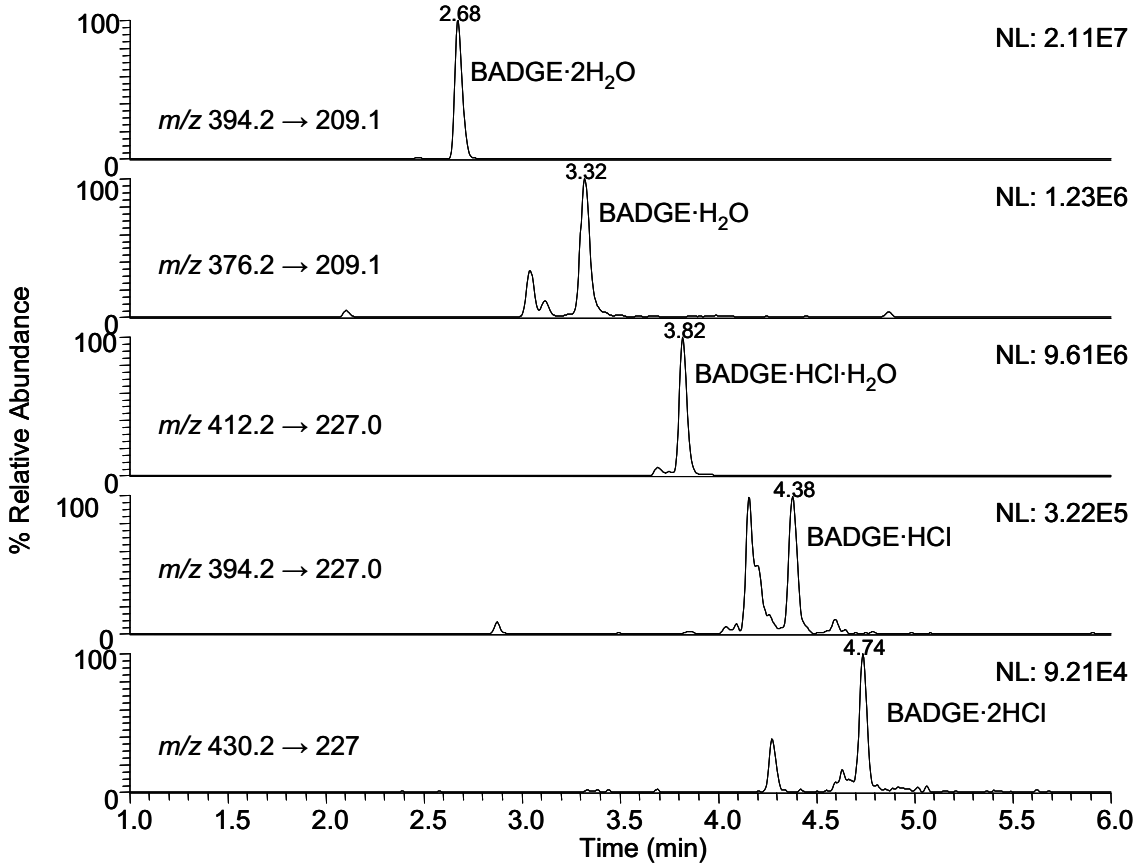

Figure 4. LC-MS/MS chromatogram of asparagus sample.

4. Conclusions

In this paper a fast liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is proposed for the simultaneous analysis of BADGEs and BFDGEs in canned food samples and soft drinks. Highly efficient separation, in less than 5 minutes, was obtained by using a Fused Core^{TM} column at 600 μL min^{-1}. Good limits of quantitation below 1.6 μg L^{-1} in soft drinks and below 4.0 μg kg^{-1} in canned food were obtained.

The LC-MS/MS method developed was used to analyze these compounds in several soft drinks and canned foods. BADGE·$2H_2O$ was always present in the analyzed samples at μg kg^{-1} level in soft drinks, whereas in canned food concentrations rose up 675 μg kg^{-1} (asparagus sample). Moreover, the other compounds BADGE·H_2O, BADGE·HCl·H_2O, BADGE·HCl and BADGE·2HCl were also detected in the canned food samples at concentrations lower than BADGE·$2H_2O$. The absence of both false positives and false negatives were confirmed by the use of H-SRM acquisition mode.

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3.2.2. ARTICLE CIENTÍFIC V.

Liquid chromatography/tandem mass spectrometry (highly selective selected reaction monitoring) for the analysis of isopropylthioxanthone in packaged food

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Liquid chromatography/tandem mass spectrometry (highly selective selected reaction monitoring) for the analysis of isopropylthioxanthone in packaged food

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ABSTRACT

Isopropylthioxanthone (ITX), usually applied as a mixture of 2- and 4-isomers, is a common photo-initiator in UV inks used in paper- or plastic-based packaging materials. In this work a pentafluorophenylpropyl column (HS F5) has been used to achieve the chromatographic separation of the two isomers. A gradient elution with acetonitrile and a 25 mM formic acid–ammonium formate at pH 3.75 are required to provide an R_s of 1.3 between the two compounds. The fragmentation pattern of ITX was studied using two mass analyzers, an ion trap (IT) (multi-stage fragmentation) and a triple quadrupole mass analyzer of hyperbolic rods (accurate mass (AM) measurement). The protonated molecule $[M+H]^+$ observed in the mass spectrometry (MS) spectrum lost an isopropyl group, $[M+H–C_3H_6]^+$. Later, this ion fragmented, yielding the radical ion $[M+H–C_3H_6–CHO]^{\bullet+}$. The elemental composition of these product ions was confirmed by AM measurement. Electrospray ionization (ESI) was used as an ionization source to couple liquid chromatography (LC) to MS. Instrumental quality parameters of three acquisition modes provided by the triple quadrupole mass analyzer were studied and good run-to-run precision (relative standard deviation, RSD, lower than 10%) and limits of detection (LODs) down to 0.8 pg injected in the LC–MS/MS system were obtained. Finally the LC–MS/MS method using H-SRM Q1 acquisition mode was used to analyze 2- and 4-ITX in a range of food samples. The use of highly selective selected reaction monitoring (H-SRM on Q1) resulted in improved selectivity without sensitivity loss.

1. Introduction

Isopropylthioxanthone (ITX), usually applied as a mixture of 2- and 4-isomers (Fig. 1), is a common photo-initiator in UV inks used in paper- or plastic-based packaging materials. Although the inks are applied to the external face, contamination can occur during the rolling procedure and during storage, when the external printed face comes into contact with the internal non-printed one [1]. Moreover, these compounds can also migrate directly through packaging material, if non-permeable barriers are used, and contaminate food. The first alert to the presence of this substance in several types of packed foods took place in September 2005, when the Italian authorities detected ITX in baby milk. Since then, ITX has been found in food samples in various European countries and the European Food Safety Authority (EFSA) was asked to advise the European Commission on the risk for human health of the use of ITX. The concentration levels reported for ITX in food ranged from 0.09 to 440 $\mu g\,L^{-1}$. For instance, 2-ITX has been found in milk and milk-based beverages at between 2.6 and 440 $\mu g\,L^{-1}$ (European Food Safety Authority) [2–5], in fruit juices at 0.09–357 $\mu g\,L^{-1}$ [1,4,5], in yoghurt at 6.2–512 $\mu g\,kg^{-1}$ [6], in wine at 6.2–512 $\mu g\,kg^{-1}$ [7] and in baby foods at 86–208 $\mu g\,kg^{-1}$ [5]. Moreover, the EFSA [8] indicated that infants exclusively fed with infant formulae packed in cartons printed with UV-cured inks are potentially more exposed to ITX than other population groups due to their high consumption of food per kg body weight. On the question of toxicity, the existing *in vivo* genotoxicity studies do not indicate a genotoxic potential for ITX and no other toxicity data on ITX are available.

So far, there are several methods available in literature for determining ITX in food but few methods are able to separate the two isomers. Traditionally, 2-ITX is analyzed by gas chromatography coupled to mass spectrometry (GC–MS) [3,9,10]. Liquid chromatography (LC) has also been used and most methods published in the literature for the determination of ITX in food samples used reversed-phase columns (C8 or C18) without achieving the chromatographic separation of the two ITX isomers (2-ITX and 4-ITX). Only Bagnati et al. [2] separated both ITX isomers, by using a zirconium column, but more than 30 min analysis time was required. Although detection systems such as spectrophotometry (UV) and fluorescence [5] have been used, nowadays mass spectrometry

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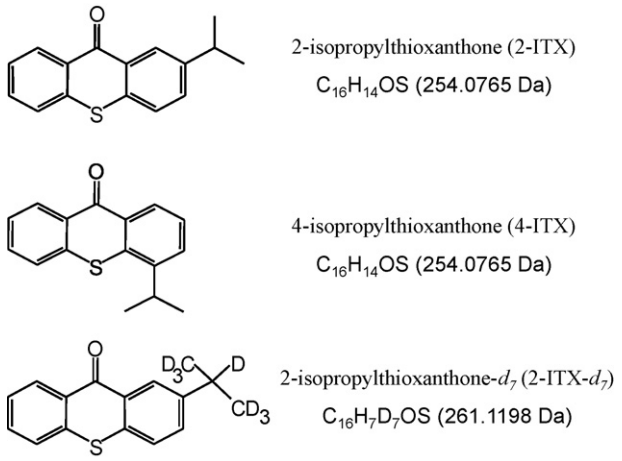

Fig. 1. Chemical structures of ITX (2- and 4-isomers) and ITX-d_7.

(MS) with different types of analyzers (single quadrupole, triple quadrupole, ion trap (IT) and Q-trap) [1–4,6,7] is used to increase sensitivity, selectivity and confirmation capabilities. Until recently, only low-resolution mass spectrometry had been used for the analysis of ITX in food samples, but very recently Morlock and Schwack [11–13] developed a rapid analytical screening method based on high-performance thin-layer chromatography coupled with mass spectrometry (HPTLC–MS), using Direct Analysis in Real Time (DART) and a TOF analyzer. Moreover, due to the complexity of food matrices and the low concentration levels of ITX, efficient pre-concentration and clean-up procedures are essential. Pressurized liquid extraction (PLE) [1,3,13], solid phase extraction (SPE) [4] and liquid–liquid extraction (L–L) [5] are commonly used for these purposes.

The aim of this paper is to report a liquid chromatography/tandem mass spectrometry method for the determination of both 2- and 4-ITX isomers in different foods, using highly selective selected reaction monitoring (H-SRM) to enhance the sensibility and sensitivity of the method. Moreover, the individual determination of both isomers could be important, due to the limited data available about the toxicity of this photo-initiator. Food samples were analyzed by the LC–MS/MS method developed after a simple and fast sample treatment procedure (acetonitrile extraction followed by an SPE clean-up).

2. Experimental

2.1. Chemicals and materials

Analytical standards of 2-isopropylthioxanthone (2-ITX), 4-isopropylthioxanthone (4-ITX) and deuterated 2-isopropylthioxanthone-d_7, diethylamine and bisphenol F-diglycidylether were purchased from Sigma–Aldrich (Steinheim, Germany). Mepiquat and chlormequat-d_4 (2-chloroethyl-d_4-trimethylammonium ion, CQ-d_4, 100 mg L^{-1}) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Difenzoquat was purchased from Chemservice (West Chester, PA, USA). Methanol (MeOH), acetonitrile (ACN) and water LC–MS grade were purchased from Riedel-de Haën (Seelze, Germany). Ammonium formate (≥99.0%) was obtained from Fluka (Steinheim, Sweden) and formic acid (98–100%) was purchased from Merck (Darmstadt, Germany). Stock standard solutions (200 mg kg^{-1}) were individually prepared by weight in methanol and stored at 4 °C. Intermediate solutions were prepared weekly from the stock standard solution by appropriate dilution in MeOH:water (1:1). Calibration standard solutions ranging from 10 to 10,000 pg g^{-1} of each ITX isomer were prepared daily with 1000 pg g^{-1} of the labelled compound (2-ITX-d_7). Mobile phases were filtered using 0.22 μm nylon filter (Whatman, Clifton, NJ, USA) and sample extract was filtered through 0.22 μm pore size Ultrafree-MC Centrifugal Filters (Millipore, Bedford, USA). OASIS HLB cartridges (60 mg) purchased from Waters (Mildford, MA, USA) were used for solid phase extraction.

Nitrogen (99.8% pure) supplied by Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the API source. High-purity Argon (Ar_1) and helium, purchased from Air Liquide (Madrid, Spain), were used as a collision-induced gas (CID gas) in the triple quadrupole and as a damper gas for the ion trap, respectively.

2.2. Instruments, LC and MS conditions

A liquid chromatograph (Accela system; Thermo Fisher Scientific, San José, CA, USA), equipped with a low-pressure quaternary pump, autosampler and column oven and coupled to a mass spectrometer, was used. For chromatographic separation, 2 columns were evaluated, a SunFire™ C18 (150 mm × 2.1 mm i.d., 3.5 μm particle size) from Waters (Mildford, MA, USA) and a Discovery® HS F5 (150 mm × 2.1 mm i.d., 3 μm particle size) from Supelco (Bellefonte, PA, USA). For chromatographic separation, a mobile phase composed of solvent A (acetonitrile) and solvent B (25 mM formic acid–ammonium formate buffer at pH 3.75) was used at 300 μL min^{-1}. The gradient elution program was: 50% acetonitrile for 1 min followed by a linear gradient up to 80% acetonitrile in 1 min. This percentage was then maintained for 4 min.

The liquid chromatography system was coupled to a triple quadrupole mass spectrometer TSQ Quantum Ultra AM (Thermo Fisher Scientific) equipped with electrospray ionization (ESI) source and hyperbolic quadrupoles that allows to work at high resolution and to performance accurate mass (AM) measurements with errors lower than 5 mDa. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow rates of 60, 20 and 40 a.u. (arbitrary units), respectively. Ion transfer tube temperature was set at 375 °C and electrospray voltage at 4 kV. Data were acquired in MS/MS with low-resolution selected reaction monitoring (SRM) and highly selective selected reaction monitoring modes. In SRM mode, a peak width of 0.7 Da full width half maximum (FWHM) on Q1 and Q3 and a scan width of 0.01 Da were used. In H-SRM mode, a peak width of 0.1 Da FWHM and a scan width of 0.01 Da on one of the quadrupoles were used, while the other one operated at low resolution. Argon was used as collision gas at 1.5 mtorr and the optimum collision energy (CE) for each transition was selected. Two transitions for each compound with a dwell time of 150 ms and 1 μscan were used, m/z 255 → 213 (quantification, CE: 22V) and m/z 255 → 184 (confirmation, CE: 40V) for 2- and 4-ITX, and m/z 262 → 214 (quantification, CE: 22V) and m/z 262 → 185 (confirmation, CE: 38V) for 2-ITX-d_7 used as internal standard for the isotope dilution method. The Xcalibur software version 2.0 (Thermo Fisher Scientific) was used to control the LC/MS system and to process data.

To optimize the source working conditions and to carry out the multi-stage mass spectrometry experiments, a 1 mg L^{-1} stock standard methanol solution of each compound was infused at a flow-rate of 3 μL min^{-1} by the syringe pump integrated into the TSQ instrument and mixed with the mobile phase (300 μL min^{-1}, ACN:formic acid–ammonium formate (70:30, v/v)), using a Valco zero dead volume tee piece (Supelco, Alcobendas, Spain).

For accurate mass measurements studies calibration in both high resolution and accurate mass modes was performance between m/z 74.0964 and 330.1700 using four calibration standards (diethylamine, mepiquat, difenzoquat and bisphenol

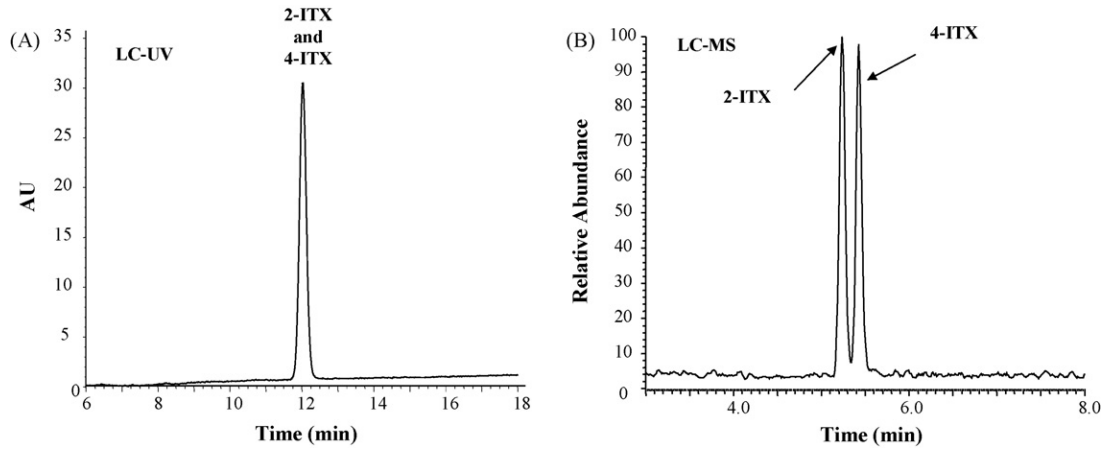

Fig. 2. Chromatograms of standard solution of ITX (2- and 4-isomer). (A) Separation obtained with a C18 column (SunFire C18, Waters). (B) Separation obtained with a pentafluorophenylpropyl column.

F diglycidylether). In addition, internal lock mass correction (chlormequat-d_4 and isopropylthioxanthone-d_7) was used to achieve accuracies below 5 mDa.

For fragmentation studies, a Classic LCQ instrument (ThermoFinnigan) equipped with an ion trap mass analyzer and ESI source was used. The ESI working conditions were: sheath gas and auxiliary gas (N_2) flow rates at 60 and 40 a.u., respectively; capillary heater temperature, 280 °C; and electrospray needle voltage, 4 kV. Product ion spectra from multi-stage mass spectrometry (MS^n) experiments were acquired (m/z 100–300) by profile mode. The $[M+H]^+$ ion was used as the precursor ion for tandem mass spectrometry experiments under the following working conditions: isolation width 1.5 m/z; 6 μscans; maximum injection time 200 ms; and activation time 30 ms. The trapping radio-frequency voltage (AQ) was set at 0.35 and the normalised collision energy (NCE%, amplitude of the voltage applied to end-cap electrode) was 30%.

2.3. Sample treatment

Four types of packaging foods (baby food, fruit juices, milk beverages and broth) were obtained from a local supermarket. Sample treatment is that published by Sun et al. [4] with slight modifications. An aliquot of 2.5 g of homogenised sample was weighed into a 15-mL centrifuge tube, and 10 μL of ITX-d_7 (90 μg/kg) and 10 mL of acetonitrile were added. The resulting mixture was shaken for 30 min on a rotating shaker and 1 mL of Carrez reagent 1 and 1 mL of Carrez reagent 2 were added. After this, the mixture was centrifuged at 3500 rpm for 15 min with a Selecta Centronic centrifuge (Selecta, Barcelona, Spain). Ten millilitres of the supernatant solution was diluted with 25 mL of LC–MS grade water and loaded into the SPE cartridge, which was previously conditioned with 6 mL of methanol and 6 mL of water. The analytes were eluted with 6 mL of acetonitrile. The collected fraction was evaporated to dryness under nitrogen stream and the extract was reconstituted with 500 μL of MeOH:water (1:1) and filtered through 0.22 μm-pore Ultrafree-MC Centrifugal Filters. Finally, 25 μL of this extract were injected into the LC–MS/MS system. Supelco Visisprep and Supelco Visidry SPE vacuum manifold (Supelco, Bellefonte, PA, USA) were used for SPE and solvent evaporation.

2-Isopropylthioxanthone was quantified by the isotope dilution method. The addition of a known level of deuterated (2-ITX-d_7) standard to the samples at the beginning of the sample treatment enabled the analyte to be quantified. This labelled compound was also used as internal standard for quantitation of 4-ITX.

3. Results and discussion

In this study, a fluorinated (pentafluorophenylpropyl) column (Discovery® HS F5) was evaluated to determine 2-ITX and 4-ITX. Various mobile phase compositions and gradient elution programs were tested to achieve the best separation of these isomers. A mobile phase consisting of acetonitrile and 25 mM formic acid–ammonium formate buffer at pH 3.75 provided an R_s of 1.3, in contrast with the total co-elution provided by a C18 column (Fig. 2), which is most frequently used to determine the total amount of both isomers. Moreover, the fluorinated column provided narrow peaks (peak width: 0.14 min), good peak shape (asymmetry factor: ~1.0) and good retention time repetitivity run-to-run (% relative standard deviation (RSD) < 0.15%, $n = 10$) and day-to-day (%RSD < 0.20%, $n = 10$ in 3 days). With the fluorinated column, only 6 min was required to separate the two isomers *versus* the 30 min needed with a zirconium column, which is the only method reported in the literature that can separate the isomers [2].

3.1. Liquid chromatography–mass spectrometry

In this research two analyzers were used: an ion trap for the fragmentation studies of ITX and a triple quadrupole for quantitative analysis, since this instrument provides high sensitivity and selectivity in dealing with complex matrices. Electrospray (ESI) in positive mode was the ionization source used to couple liquid chromatography and mass spectrometry. The ESI full-scan single MS spectrum obtained shows as base peak the protonated molecule $[M+H]^+$ (m/z 255) and no adducts were observed, although an ion at m/z 213 was also present, probably due to in-source fragmentation (Fig. 3). This ion can be assigned to the loss of the isopropyl moiety. Multi-step mass spectrometry (MS^n) in the ion trap mass analyzer was performed using $[M+H]^+$ (m/z 255) as precursor ion for MS/MS. For further MS^n experiments, the most abundant product ion was used as precursor ion. Both isomers showed the same fragmentation pattern. Fig. 3 shows the MS, MS/MS and MS^3 spectrum of 2-ITX as an example. The MS/MS spectrum of the protonated molecule indicated that the fragmentation started through the cleavage of the isopropyl group, yielding the ion $[M+H-C_3H_6]^+$ at m/z 213. Later, as can be seen in the MS^3 spectrum, this ion (m/z 213) fragmented, yielding the radical ion at m/z 184 $[M+H-C_3H_6-CHO]^{+\bullet}$, probably due to the cleavage of the alpha bond to the oxygen atom to produce the loss of a •CHO radical. These ions, originating in consecutive losses, can be seen in the MS/MS spectrum obtained with a triple quadrupole mass analyzer, due to multiple collisions through the

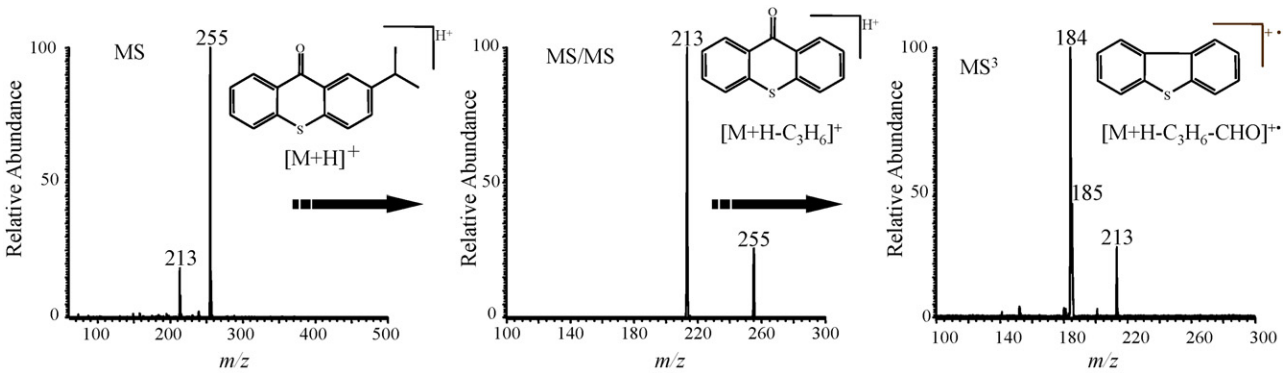

Fig. 3. MS, MS/MS and MS^3 spectra of 2-ITX obtained with the LCQ instrument (ion trap).

collision chamber. Fig. 4A) shows the protonated molecule isotopic cluster and the MS/MS spectra obtained using a triple quadrupole instrument with hyperbolic quadrupoles (TSQ). Two collision energies (40 and 22 eV) were used to maximize the intensity of both product ions used for quantitative and confirmatory purposes. In addition and as complementary data, to confirm the elemental composition of the product ions the accurate masses of each product ion were measured by means of the TSQ Quantum Ultra AM triple quadrupole. Accurate mass measurements were taken at peak width settings of 0.04 Da FWHM on Q3 and 0.7 Da FWHM on Q1. Two lock masses were needed to overcome the mass drift, *m*/*z* 126 (d_4-chlormequat) and *m*/*z* 262 (ITX-d_7) at low collision energy (CE: 5 eV), to avoid their fragmentation in the Q2 and to monitor the molecular ion and the protonated molecule, respectively. As can be seen in Fig. 4B), the mass measured for each product ion that differed by less than 0.6 ppm (±0.1 mDa) from the corresponding theoretical mass confirmed the elemental composition proposed.

3.2. Method performance

To verify the LC–MS/MS method performance, instrumental quality parameters such as limit of detection (LOD), run-to-run

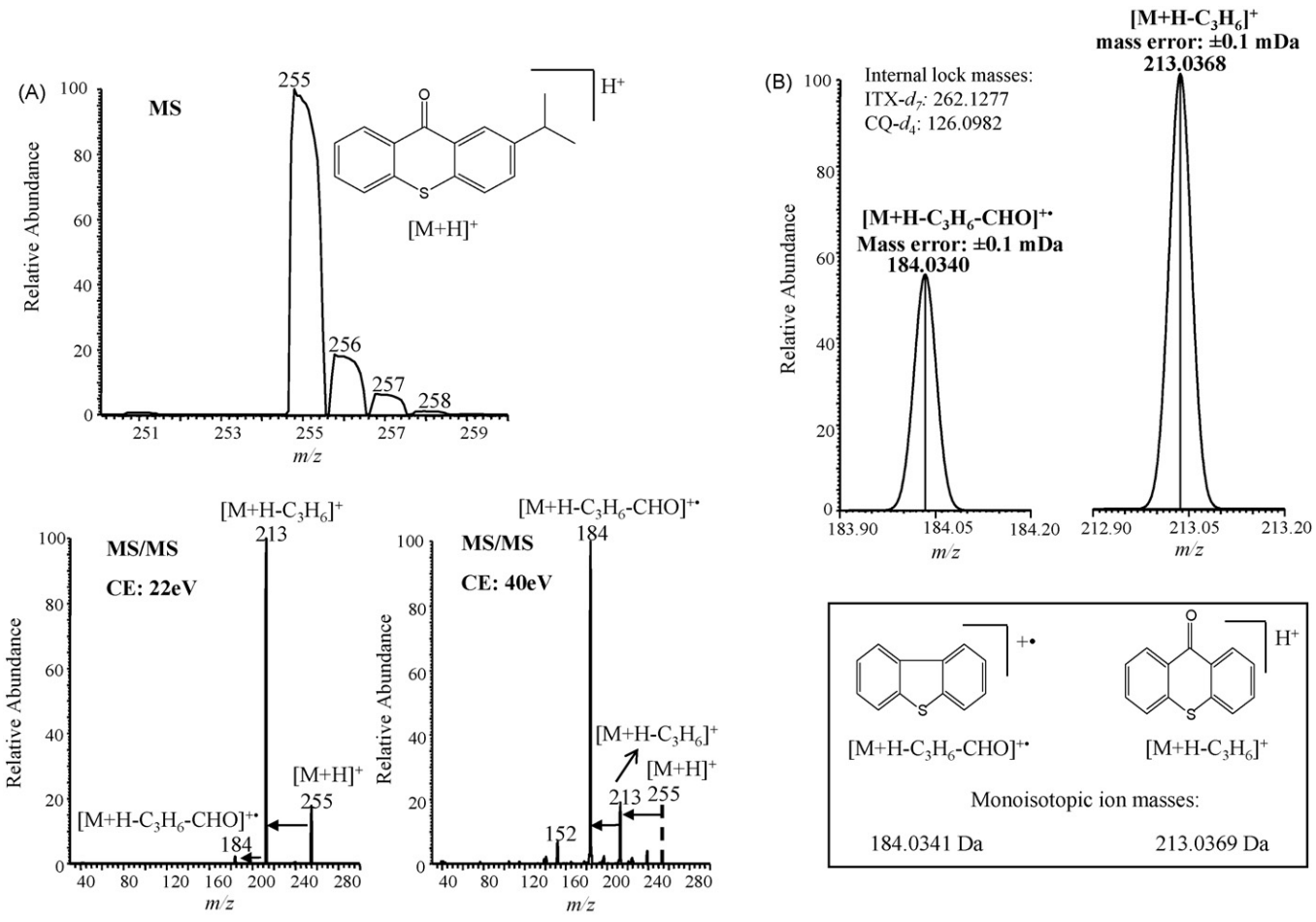

Fig. 4. (A) Low-resolution MS and MS/MS spectra at two collision energies. (B) Accurate mass measurement of product ions *m*/*z* 213.04 and 184.03. Spectra obtained with the TSQ Quantum Ultra.

precision, ion-ratio precision and linearity were calculated by both low-resolution selected reaction monitoring and highly selective selected reaction monitoring mode. At low resolution (SRM), both quadrupoles (Q1 and Q3) were set at 0.7 Da FWMH. Meanwhile, when working in H-SRM, two acquisition modes were tested. First, H-SRM (Q1) was used, which means working in Q1 with a peak width setting of 0.1 Da FWMH and Q3 at low resolution (0.7 Da FWHM). When using H-SRM (Q3) mode, Q1 operated at low resolution and Q3 with a peak width of 0.1 Da FWHM. Limits of detection, based on a signal-to-noise ratio of 3 (background noise was measured manually around the peaks corresponding to the compounds), were estimated by the injection of 25 μL of ITX standard solutions prepared at very low concentration levels (down to 10 pg g^{-1}) by dilution in mobile phase of the stock standard solution. No differences were found between LODs calculated using both SRM and H-SRM acquisition modes (0.6–0.8 pg injected) (Table 1), which demonstrates that H-SRM can be used to achieve higher selectivity without any significant loss in sensitivity.

To confirm the identity of an analyte, an error in the ion ratio between both quantitative and confirmatory transitions has to be lower than 20%. The ion ratio for 2- and 4-ITX was evaluated using the three acquisition modes (Table 1), ranging for both isomers from 1.9 to 2.3 with a relative standard deviation below 10% ($n = 5$). A similar ion ratio standard deviation (<10%, $n = 5$ in 3 days) was obtained on evaluating day-to-day precision.

Table 1
Instrumental quality parameters by LC–MS/MS

Parameter	2-ITX	4-ITX
SRM		
LODs pg injected	0.7	0.8
Run-to-run (%RSD, $n = 5$)	1.9	7.4
Run-to-run ion ratio ± SD[a] ($n = 5$)	2.1 ± 0.11	1.92 ± 0.09
Day-to-day ion ratio ± SD[a] ($n = 5$, 3 days)	2.07 ± 0.08	1.94 ± 0.08
H-SRM on Q1		
LODs pg injected	0.6	0.7
Run-to-run (%RSD, $n = 5$)	4.6	4.3
Run-to-run ion ratio ± SD[a] ($n = 5$)	2.19 ± 0.08	1.91 ± 0.05
Day-to-day ion ratio ± SD[a] ($n = 5$, 3 days)	2.15 ± 0.10	1.93 ± 0.06
H-SRM on Q3		
LODs pg injected	0.7	0.8
Run-to-run (%RSD, $n = 5$)	1.0	10.0
Run-to-run ion ratio ± SD[a] ($n = 5$)	2.38 ± 0.21	1.87 ± 0.20
Day-to-day ion ratio ± SD[a] ($n = 5$, 3 days)	2.32 ± 0.21	1.88 ± 0.17

Standard solution of 2- and 4-ITX at 220 pg g^{-1}.
[a] SD: Standard deviation.

Calibration curves based on the peak area ratio ($A_{compound}/A_{labelled\ compound}$) showed good linearity for all acquisition modes ($r^2 > 0.9996$, $r^2 > 0.9978$ and 0.9991 for SRM, H-SRM (Q1) and H-SRM (Q3), respectively). Five replicates of a 220 pg g^{-1} standard solution were injected in the LC–MS/MS system under the

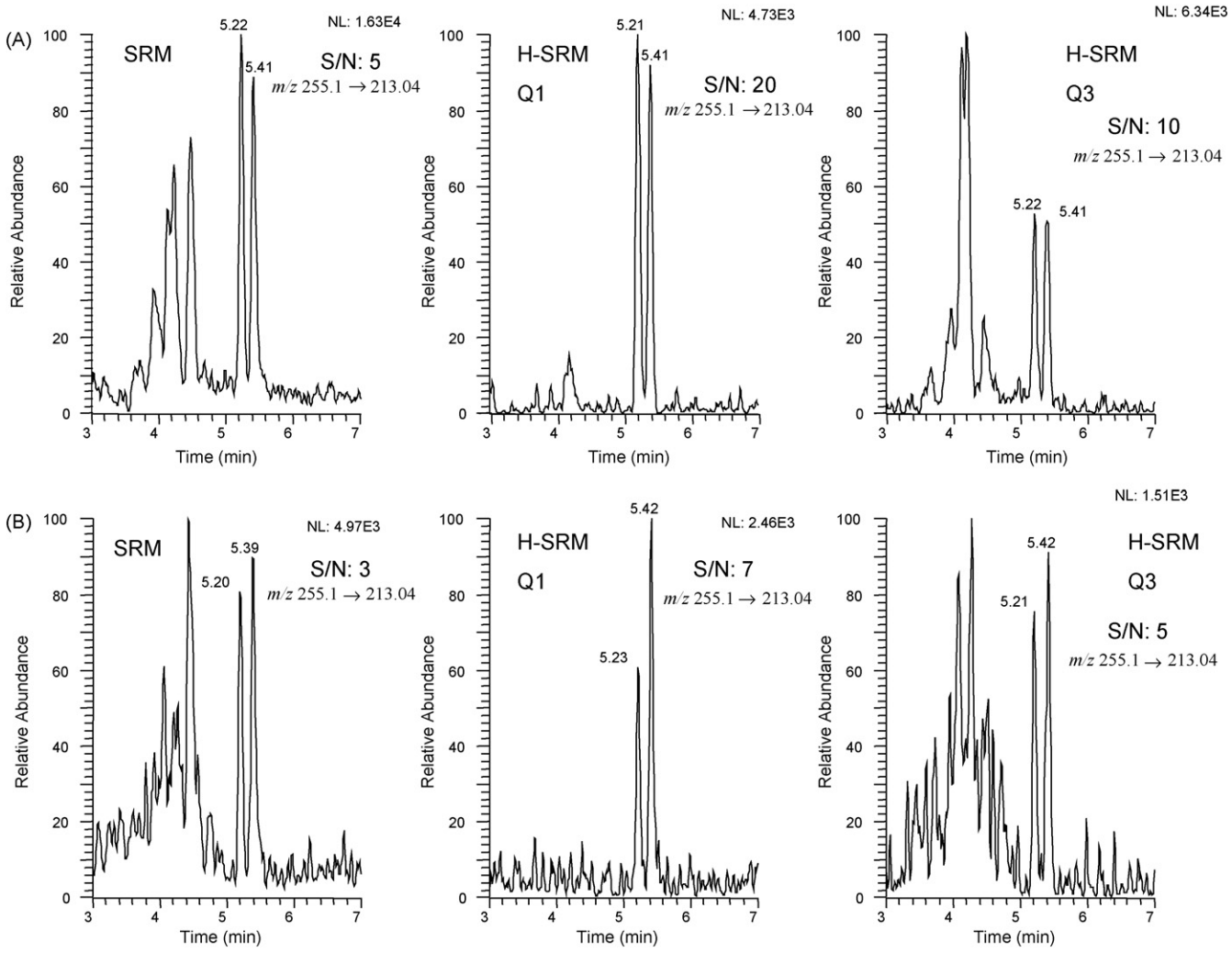

Fig. 5. LC–MS/MS chromatograms of spiked samples at 20 pg g^{-1} using three acquisitions modes (SRM, H-SRM Q1 and H-SRM Q3). (A) Multi-fruit purée; (B) Milk.

Table 2
Quality parameters of the SPE LC–MS/MS method using H-SRM

Compound	Milk sample				Multi-fruit purée			
	LOD (pg g^{-1})	Run-to-run, %RSD		Ion ratio ± SD[a]	LOD (pg g^{-1})	Run-to-run, %RSD		Ion ratio ± SD[a]
		Medium[b] concentration	Low[c] concentration			Medium[b] concentration	Low[c] concentration	
2-ITX	12.0	9.7	14	2.06 ± 0.2	2.0	4.5	7.4	2.03 ± 0.07
4-ITX	13.0	10	10	1.90 ± 0.2	3.6	5.8	8.2	1.97 ± 0.07

[a] SD: Standard deviation.
[b] 1.5 ng g^{-1}.
[c] 0.14 ng g^{-1}.

three acquisition modes to determine run-to-run reproducibility. The relative standard deviations based on concentration showed no significant differences between acquisition modes (RSDs < 10%). Nevertheless, better results were obtained for 2-ITX (RSDs 1–5%) than for 4-ITX (RSDs 4.5–10%), probably due to the use of the 2-ITX-d_7 as internal standard for quantitation. Moreover, low errors in the concentration calculated, relative to the target value, were obtained, ranging from 1.0 to 5.0% for 2-ITX and 0.2 to 8.0% for 4-ITX. It can be concluded that H-SRM modes provide good sensitivity and are robust enough to be proposed as an acquisition mode for the determination of 2- and 4-ITX by LC–MS/MS.

3.3. Feasibility of the method

To explore the capabilities of the H-SRM acquisition mode and to evaluate possible matrix effects, two packaged foods, fat (milk) and non-fat (multi-fruit purée), free of ITX were analyzed. These samples were spiked with ITX isomers at a low concentration (20 pg g^{-1} for each one) and submitted to the sample treatment detailed in Section 2.3. After the clean-up, 25 μL of extract were injected into the LC–MS/MS system. Fig. 5 shows the LC–MS/MS chromatograms corresponding to these spiked samples obtained in SRM and H-SRM in Q1 and in Q3. The H-SRM on Q1 mode provided the greatest sensitivity. The signal-to-noise ratio (S/N) using this mode is 7–20 (milk/multi-fruit purée) *versus* 5–10 for H-SRM Q3 and 3–5 for SRM at low resolution. In addition, selectivity was greatly improved with H-SRM Q1, which provided cleaner chromatograms (Fig. 5). For these reasons, this acquisition mode was selected for future studies. Method LOD values calculated from these spiked samples in the H-SRM (Q1) mode at ppt levels (Table 2) were 2–5 times lower than those obtained with the low-resolution SRM mode (10 pg g^{-1} in multi-fruit and 22.0–26 pg g^{-1} in milk). Our LODs are up to 300-fold lower than those obtained by Bagnati et al. [2] for both isomers. This improvement is due to the pre-concentration step here applied and moreover to the higher sensitivity of the LC–MS/MS method used (our instrumental LODs are 10 times lower).

Absolute recoveries of the method were calculated analyzing (n = 6) by external calibration without internal standard samples free of ITX spiked at two concentration levels, one at 65 pg g^{-1} and the other at 600 pg g^{-1}. For multi-fruit purée high recoveries 85% (RSD 12%) were obtained and no matrix effects was observed since the same response for a standard solution and a sample spiked after the clean-up step was obtained. Milk sample provided lower recoveries (~30%) but quantitative results were not compromised since isotope dilution method was used and very low detection limits were achieved. For the other matrices, recoveries were estimated from the response of the labelled standard used for isotope dilution and high values similar to those found for multi-fruit purée sample, were obtained.

To evaluate run-to-run precision, six replicates of two spiked samples (milk and multi-fruit purée) at two concentration levels (0.14 and 1.5 ng g^{-1}) were analyzed. The relative standard deviations based on concentration ranged from 7.4 to 14% at low concentration and from 4.5 to 10% at medium concentration (Table 2). Ion ratios obtained at H-SRM on Q1 for the two spiked samples (multi-fruit and milk) are consistent with those obtained with standard solutions, 2.03 ± 0.1 for 2-ITX and 1.95 ± 0.2 for 4-ITX.

To evaluate the applicability of the LC–MS/MS method for the analysis of ITX in food samples, 18 packaged foods from Spanish supermarkets were analyzed. Samples were prepared as described in Section 2.3 and analyzed in triplicate to determine both 2- and 4-ITX. Samples covered different kind of matrices, including both fat (milk, poultry stock and cream vegetable soups) and non-fat (fruit commodities, water for infants and vegetable stock) products. As an example, Fig. 6 shows the H-SRM chromatograms corresponding to pear purée (Fig. 6A) and milk chocolate (Fig. 6B) samples. The 2-ITX isomer was detected and confirmed in both samples. The results obtained for all the samples analyzed are summarized in

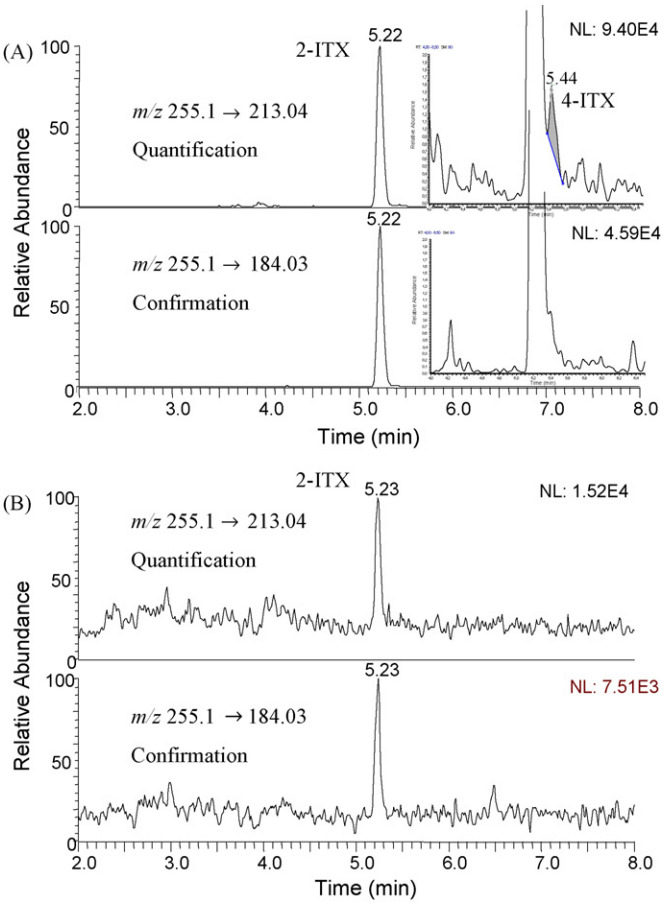

Fig. 6. LC–MS/MS (H-SRM on Q1) chromatograms of two packaging food samples: (A) pear purée; (B) milk chocolate.

Table 3
Packaged food analysis using a LC–MS/MS by H-SRM acquisition mode[a]

Sample type	2-ITX pg g^{-1} (%RSD)	4-ITX pg g^{-1} (%RSD)
Baby food (milk cereal)	n.d.	n.d.
Baby food (multi-fruit)	n.d.	n.d.
Baby food (multi-cereal)	<7	n.d.
Baby food (water)	<7	n.d.
Baby food (pear purée)	580 (7.0)	~4
Milk 1	n.d.	n.d.
Milk 2	n.d.	n.d.
Milk 3	n.d.	n.d.
Soy milk	n.d.	n.d.
Milk chocolate	13 (12.7)	n.d.
Vegetable cream	n.d.	n.d.
Vegetable cream	n.d.	n.d.
Broth (vegetables)	n.d.	n.d.
Broth (poultry)	n.d.	n.d.
Fruit juice (orange)	n.d.	n.d.
Fruit juice (pineapple)	n.d.	n.d.
Fruit juice (apple)	n.d.	n.d.
Fruit juice (peach)	n.d.	n.d.

n.d.: Not detected.
[a] $n = 3$.

Table 3; 2-ITX was detected in four samples (three baby foods and one milk chocolate) at concentrations ranging from 2 to 580 pg g^{-1}. 4-ITX was detected only in one sample (baby food pear purée) at a concentration close to the LOD (Fig. 6A). This sample was the one that contained the highest concentration of 2-ITX (Table 3).

4. Conclusions

This paper reports the development of an LC–MS/MS method for the analysis of 2- and 4-ITX. The isomers were separated ($R_s > 1.3$) in fewer than 6 min, with a pentafluorophenylpropyl column and acetonitrile:formic acid–ammonium formate used as mobile phase. Complementary structural information provided by ion trap multi-stage mass spectrometry and accurate mass measurement on a triple quadrupole of hyperbolic rods within errors lower than 1 ppm enabled the fragmentation pathway of ITX isomers to be established. Low-resolution SRM was compared for performance with H-SRM acquisition modes, with the conclusion that selectivity can be improved without compromising sensitivity when using H-SRM mode with a peak width setting of 0.1 Da FWHM in the hyperbolic quadrupoles.

H-SRM (Q1 at 0.1 Da FWHM) showed the best acquisition mode for the analysis of food samples, since it provides better signal-to-noise ratio and lower limits of detection than low-resolution SRM and H-SRM (Q3). On analysis of several food commodities (18) by the LC–MS/MS (H-SRM Q1) method, 2-ITX was detected in four samples, while 4-ITX was detected only in one sample at very low concentration. With its high selectivity, low limits of detection and good reproducibility, the LC–MS/MS (H-SRM on Q1) method is recommended for individual determination of ITX isomers in food samples.

Acknowledgements

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3.2.3. ARTICLE CIENTÍFIC VI.

Analysis of UV ink photoinitiators in packaged food by fast liquid chromatography at sub-ambient temperature coupled to tandem mass spectrometry

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Analysis of UV ink photoinitiators in packaged food by fast liquid chromatography at sub-ambient temperature coupled to tandem mass spectrometry

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Abstract

A fast method of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was developed for the analysis of eleven UV ink photoinitiators in packaged food. Chromatographic separation was achieved in a pentafluorophenylpropyl (PFPP) column at 5ºC and acetonitrile:25 mM formic acid-ammonium formate (pH 2.7) in gradient elution. To reduce sample treatment, a QuEChERS (quick, easy, cheap, effective, rugged and safe) method for the extraction and clean-up of UV photoinitiators in packaged foods was evaluated. Triple quadrupole working in H-SRM on Q1 mode was used for both quantitation and confirmation purposes and the most intense and

selective transitions were chosen. Quality parameters of the developed QuEChERS LC-MS/MS method were established and applied for the analysis of photoinitiators in food packaged at ng kg^{-1} levels.

Keywords: Pentafluorophenyl propyl (PFPP) column, sub-ambient temperature, Tandem mass spectrometry, UV ink photoinitiators, QuEChERS, packaged food.

Introduction

The alert for food contamination by UV ink photoinitiators arose in Europe in November 2005, when the Italian Food Control Authority detected that the photoinitiator 2-isopropylthioxanthone (2-ITX) migrated into baby milk at concentrations ranging from 120 to 300 μg L^{-1}, resulting in the withdrawal from the market of more than 30 million liters of milk [1]. Since then, residues of other photoinitiators such as 2-ethylhexyl-4-dimethylaminobenzoate (EHDAB) or benzophenone (BP) have also been found in packaged food [2,3]. Photoinitiators are used as starters in the polymerization process to cure the ink by UV radiation. UV inks are used to print packaging materials such as multilayer laminates, rigid plastic, cardboard and paper. Although intermediate aluminum layers are commonly used to prevent the migration of ink components into food products, the unintentional transfer of printing ink components from the outer printed surface onto the food contact surface can occur when the printed material is rolled on spools or stacked during storage. Nowadays, these compounds are not regulated by specific EU legislation and maximum residue levels (MRL) in food are not established, but according to the European Food Safety Authority (EFSA) [4] the presence of some of them could be considered

undesirable. Up to now, a maximum permitted amount for migration from packaging materials to packaged food has only been established for BP. This Specific Migration Limit (SML) was set at 600 μg L^{-1} for this photoinitiator [5].

In addition, the EU approved a Commission Regulation 2023/2006 [6], which sets out the rules for good manufacturing practice (GMP) for groups of materials and articles that are intended to come into contact with food. These materials should not transfer their constituents to food in quantities that might endanger human health or bring about unacceptable changes in the composition of foodstuffs. Information about UV ink photoinitiators is also included in this document.

So far, in the literature there are few methods for the simultaneous analysis of UV ink photoinitiators. For analytical procedures, gas chromatography coupled to mass spectrometry (GC-MS) is the technique most frequently used to analyze this family of compounds. For instance, 2-ITX has been determined in milk samples [3,12,13], although other UV ink photoinitiators such as EHDAB, BP, 4,4'-bis(diethylamino)-benzophenone (DEAB) and 1-hydroxycyclohexyl phenyl ketone (HCPK) have been found in beverages [3,7,8]. Liquid chromatography (LC) with UV detection has been used to study the migration of some photoinitiators from printed food-packaging materials into food simulants or powdered milk [9,10]. In addition, some methods for the analysis of ITX in food and food packaging materials by LC with fluorescence detection have also been reported [11,12]. However, liquid chromatography-tandem mass spectrometry (LC-MS/MS) [2,3,13-18] has become popular for the analysis of UV ink photoinitiators, in order to confirm the identity of the analytes in food samples, following directive 2002/657/EC [19]. In general, most of these LC-MS/MS methods

are devoted to the determination of ITX in food samples by reversed-phase liquid chromatography. The chromatographic separation of the two isomers (2-ITX and 4-ITX) can only be achieved by more selective columns such as a zirconium column and a pentafluorophenyl propyl (PFPP) column [15,17]. For the other UV ink photoinitiators, a few LC-MS/MS methods have been described using C18 columns [3,18], but with relatively long analysis times (above 20 min).

Due to the complexity of food matrices and the low concentration levels expected for UV ink photoinitiators in these samples, efficient preconcentration and clean-up procedures are usually needed. Liquid-liquid extraction (LLE) [2,3,9,12,20] using acetonitrile or hexane is commonly used for the analysis of photoinitiators in liquid and fatty food samples. To reduce solvent consumption and improve selectivity, solid phase extraction (SPE) [14,17,18] is used as an alternative to LLE. Other extraction techniques such as pressurized liquid extraction (PLE) [2,11,13] and solid phase microextraction (SPME) [21] have also been used for the analysis of these compounds. Nowadays, the QuEChERS method (***Qu****ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged and **S**afe*) is a frequent and attractive alternative method for sample preparation in food analysis. The QuEChERS method is particularly popular for determination of polar, middle polar and non-polar pesticide residues in various food matrices [22-27], because of its simplicity, low cost, suitability for high throughput and relatively high efficiency with a minimal number of steps.

The aim of this work is to develop a fast liquid chromatography-tandem mass spectrometry method using a QuEChERS extraction method for the simultaneous

determination of the most commonly employed UV ink photoinitiators in various packaging foods.

2. Experimental

2.1. Materials and chemicals

The UV ink photoinitators (Figure 1), all of them of analytical grade, ethyl 4-dimethylaminobenzoate (EDMAB, 99%, CAS No. 10287-53-3), benzophenone (BP, 99%, CAS No. 119-61-9), 4,4'-bis(diethylamino)-benzophenone (DEAB, 99%, CAS No. 90-93-7), 4-benzoylbiphenyl (PBZ, 99%, CAS No. 2128-93-0), 2,4-diethyl-9*H*-thioxanthen-9-one (DETX, 98%, CAS No. 82799-44-8), 1-hydroxycyclohexyl phenyl ketone (HCPK, 99%, CAS No. 947-19-3), 2-hydroxy-2-methylpropiophenone (HMPP, 97%, CAS No. 7473-98-5), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%, CAS No. 24650-42-8), 2-ethylhexyl 4-(dimethylamino)benzoate (EHDAB, 98%, CAS No. 21245-02-3), 2-isopropylthioxanthone (2-ITX, 99.7%, CAS No. 5495-84-1), 4-isopropylthioxanthone (4-ITX, 99.5%, CAS No. 83846-86-0) and 2-isopropyl-D7-thioxanthen-9-one (2-ITX-D_7 used as internal standard (I.S.), 99.5%, CAS No. 400-880-8822) were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (98-100%) was provided by Merck (Darmstadt, Germany). Anhydrous magnesium sulfate was obtained from Sigma (Steinheim, Germany), sodium chloride from Fluka (Steinheim, Sweden), and propylamino (PSA) bonded silica SPE bulk from Supelco (Gland, Switzerland). OASIS HLB cartridges (60 mg) purchased from Waters (Mildford, MA, US) were used for solid phase extraction. Supelco Visiprep and Supelco Visidry SPE vacuum manifold (Supelco) were used for SPE and solvent evaporation.

LC-MS grade methanol (MeOH), acetonitrile (ACN) and water were purchased from Riedel-de Haën (Seelze, Germany).

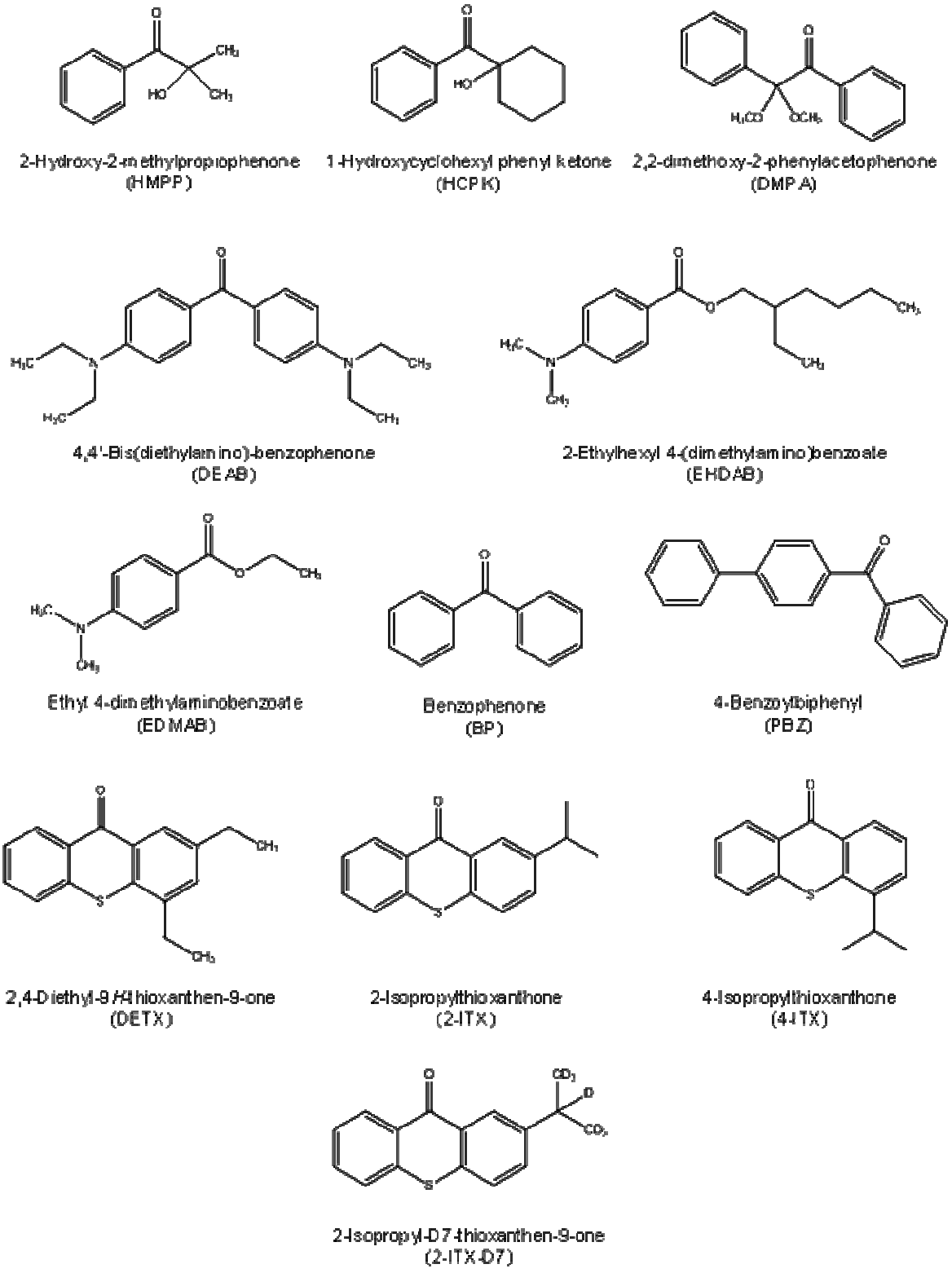

Figure 1. Chemical structures of photoinitiators.

Stock standard solutions of UV ink photoinitiators (1,000 mg kg^{-1}) were individually prepared by weight in methanol and stored at 4ºC. Working solutions were prepared weekly by appropriate dilution in acetonitrile:water (1:1) of the stock standard solution. Mobile phases were filtered using 0.22 μm nylon membrane filters (Whatman, Clifton, NJ, US) and sample extracts were filtered through 0.22 μm pore size Ultrafree-MC centrifuge filters (Millipore, Bedford, US).

Nitrogen (99.98% pure) supplied by Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the API source; and high-purity Argon (Ar1), purchased from Air Liquide (Madrid, Spain), was used as a collision-induced gas (CID gas) in the triple quadrupole instrument.

2.2. Instrumentation

A liquid chromatography system (Accela; Thermo Fisher Scientific, San José, CA, US), equipped with a low-pressure quaternary pump, autosampler and column oven, was used. The chromatographic separation was performed in a pentafluorophenyl propyl column, Discovery® HS F5 (150 mm x 2.1 mm i.d., 3 μm particle size), from Supelco (Bellefonte, PA, US), using a gradient elution of acetonitrile (solvent A) and 25 mM formic acid-ammonium formate buffer at pH 2.7 (solvent B): 50% solvent A for 0.5 min followed by a linear gradient up to 80% solvent A in 2.5 min and an isocratic step for 3 minutes at this latter percentage. The flow-rate was 450 μL min^{-1} and the column temperature was held at 5ºC, providing a back-pressure ≤ 350 bar.

The liquid chromatography system was coupled with a triple quadrupole mass spectrometer TSQ Quantum Ultra AM (Thermo Fisher Scientific), equipped with electrospray ionization (ESI) source and hyperbolic quadrupoles able to work in

enhanced mass resolution mode (mass resolution at 0.1 *m/z* FWHM, full - with half maximum). Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow-rates of 60, 20 and 40 a.u. (arbitrary units), respectively. The ion transfer tube temperature was set at 375^{o}C and electrospray voltage at +4 kV. Selected reaction monitoring (SRM) and highly-selective reaction monitoring (H-SRM) acquisition modes were used. In SRM mode, a mass resolution of 0.7 *m/z* FWHM on both Q1 and Q3 and a scan width of 0.01 *m/z* were used. In H-SRM mode, a mass resolution of 0.1 *m/z* FWHM on Q1 and a scan width of 0.01 *m/z* were employed, while the other quadrupole operated at low resolution (0.7 *m/z* FWHM). Argon was used as collision gas at 1.5 mtorr and the optimum collision energy (CE) for each transition monitored (quantifier and qualifier) is shown in Table 1. The chromatogram was segmented into two windows, and two transitions for each compound with a dwell time of 50 ms and 1 μscan were monitored (Table 1). The Xcalibur software version 2.0 (Thermo Fisher Scientific, San Jose, CA, US) was used to control the LC/MS system and to process data.

To optimize both the ESI source and tandem mass spectrometry working conditions, 1 mg L^{-1} stock standard methanol solution of each compound was infused at a flow-rate of 3 μL min^{-1} using the syringe pump integrated in the TSQ instrument and mixed with the mobile phase (450 μL min^{-1}, acetonitrile:formic acid-ammonium formate buffer (70:30, *v/v*)), by means of a Valco zero dead volume tee piece (Supelco).

Table 1. SRM acquisition parameters

Segment	Time (min)	Analyte	Precursor ions	Product ion Assigment (Quantifier/Qualifier)	Collision energy (CE, V)	Ion Ratio (%RSD)
1	0-3.7	HMPP	165.1 $[M+H]^+$	91.1 $[C_7H_7]^+$ 119.0 $[M+H-H_2O-C_2H_4]^+$	11 23	1.1 (10)
		HCPK	205.1 $[M+H]^+$	105.0 $[C_7H_5O]^+$ 187.1 $[M+H-H_2O]^+$	13 5	2.6 (9)
		EDMAB	194.1 $[M+H]^+$	151.1 $[M+H-CH_3-C_2H_4]^{+\bullet}$ 134.1 $[M+H-CH_3-C_2H_5O]^+$	23 31	1.4 (2)
		DMPA	225.1 $[M-CH_3O]^+$	197.1 $[M-CH_3O-CO]^+$ 105.0 $[C_7H_5O]^+$	14 23	1.8 (10)
		BP	183.1 $[M+H]^+$	105.0 $[C_7H_5O]^+$ 77.0 $[C_6H_5]^+$	15 34	1.3 (8)
2	3.7-6.0	PBZ	259.1 $[M+H]^+$	105.0 $[C_7H_5O]^+$ 181.1 $[M+H-C_6H_6]^+$	17 18	2.7 (2)
		DEAB	325.2 $[M+H]^+$	176.1 $[M+H-C_{10}H_{15}N]^+$ 281.2 $[M+H-C_2H_5-CH_3]^+$	28 27	2.6 (3)
		2-ITX / 4-ITX	255.1 $[M+H]^+$	213.0 $[M+H-C_3H_6]^+$ 184.0 $[M+H-C_3H_6-CHO]^{+\bullet}$	22 40	1.9 (4)
		2-ITX-D7	262.1 $[M+H]^+$	214.0 $[M+H-C_3D_6]^+$ 185.0 $[M+H-C_3D_6-CHO]^{+\bullet}$	23 42	1.8 (5)
		DETX	269.1 $[M+H]^+$	241.1 $[M+H-C_2H_4]^+$ 213.0 $[M+H-C_2H_4-C_2H_4]^+$	23 30	1.1 (3)
		EHDAB	278.2 $[M+H]^+$	151.1 $[M+H-CH_3-C_8H_{16}]^{+\bullet}$ 134.0 $[M+H-CH_3-C_8H_{17}O]^+$	23 27	4.4 (4)

2.3. Sample treatment

2.3.1. Packaged foods

(i) For the QuEChERS method, sub-samples of 2.5 g were weighed into a 50 mL PTFE centrifuge tube (Serviquimia, Barcelona, Spain). 5 µL of 2-ITX-D_7 used as a surrogate (100 µg kg^{-1}) and 12 mL of acetonitrile were added. Then the mixture was shaken vigorously for 1 min using a vortex (Stuart, Stone, UK). After this step, 1.5 g of NaCl and 4 g of $MgSO_4$ were added to the extract and then shaken again for 1 min. The extract was then centrifuged at 2,500 rpm for 1 min using a Selecta Centronic centrifuge (Selecta, Barcelona, Spain) and 10 mL of the supernatant were transferred into a 15 mL graduated centrifuge tube that contained 250 mg of PSA (propylamine bonded silica SPE bulk) and 750 mg of $MgSO_4$. The mixture was energetically shaken for 1 min in a vortex and centrifuged again at 3,700 rpm for 1 min. Finally, 8 mL of the supernatant were evaporated to dryness under a nitrogen stream and reconstituted in 500 µL acetonitrile:water (1:1, *v/v*). Prior to analysis, the extract was filtered through 0.22 µm-pore Ultrafree-MC centrifugal filters and transferred into an amber vial to prevent analyte photodegradation. Finally, 10 µL of this extract were injected into the LC-MS/MS system.

(ii) An SPE method previously described in our research group for the analysis of ITX was also used [17]. Briefly, an aliquot of 2.5 g of homogenized sample was weighed into a 15 mL centrifuge tube; and 5 µL 2-ITX-D_7 (surrogated, 100 µg/kg) and 10 mL of acetonitrile were added. The resulting mixture was shaken for 30 min in a rotating shaker (Breda Scientific, Breda, Netherlands) and 1 mL of Carrez reagent 1 and 1 mL of Carrez reagent 2 were added. Then, the mixture was centrifuged at 3,500 rpm for 15 min with a Selecta Centronic centrifuge and 10 mL of the supernatant solution

were diluted with 25 mL of LC-MS grade water and loaded into an OASIS® HLB (60 mg) SPE cartridge, which was previously conditioned with 6 mL of methanol and 6 mL of water. The analytes were eluted with 6 mL of acetonitrile. The collected fraction was evaporated to dryness under a nitrogen stream and was treated as described above for the QuEChERS method.

A total of 14 packaged food samples, including baby food, fruit juices, water, wine, two blank samples, a pineapple juice sample packaged in a plastic bottle and a baby food sample in a glass bottle obtained from local supermarkets (Barcelona, Spain), were analyzed. 2- and 4-ITX were quantified by isotope dilution using the deuterated standard (2-ITX-D_7), while the other photoinitiators were quantified by matrix matched calibration. In order to control possible contaminations method blank samples were analyzed.

2.3.2. Packaging materials in contact with food

Packaging materials in contact with food were processed by means of the method described by Sagratini *et al.* [3]. Briefly, the food carton was opened and the food content processed following the procedures described in Section 2.3.1., while the internal side of the packaging material was washed with LC-MS grade ultrapure water and then wiped. A 10 cm x 5 cm scrap of packaging polycoupled carton was cut into 1 cm^2 pieces, and then soaked in 50 mL of dichloromethane (amber glass bottle) for 24 h. After this, the organic solvent was collected and evaporated to 1 mL using nitrogen in a Turbovap® II Concentration Workstation (Zymark Corporation, Hopkinton, Massachusetts, USA), and finally evaporated to dryness using a Visidry vacuum manifold. The extract was reconstituted with 5 µL of 2-ITX-D_7 solution (100 µg kg^{-1})

and 495 µL of methanol:water 1:1 (*v/v*), filtered through 0.22 µm-pore Ultrafree-MC centrifugal filters and transferred into an amber injection vial. Finally, 10 µL of this extract were injected into the LC-MS/MS system.

3. Results and discussion

3.1. Chromatographic separation

In this study, the fluorinated (pentafluorophenylpropyl) column (Discovery® HS F5) proposed in a previous paper for the chromatographic separation of the two ITX isomers (2-ITX and 4-ITX) [17] was used to separate eleven photoinitiators currently used in food packaging [1], using gradient elution based on a mobile phase of acetonitrile/formic acid-ammonium formate buffer (25 mM, pH 2.7). First, the gradient elution was optimized and the best separation was obtained in 6 min using a linear gradient from 50% ACN to 80% in 2.5 min. However, under these conditions several co-elutions occurred: PBZ/DEAB, EDMAB/DMPA/BP and DETX/EHDAB. To improve the chromatographic separation, the effect of temperature was evaluated between 5ºC and 25ºC. As Figure 2 shows, chromatographic resolution improved significantly when temperature decreased and the best separation, especially for EDMAB/DMPA/BP, was at 5ºC (Figure 2C), providing resolutions better than 1.1 for these photoinitiators in less than 7 min, which led to the choice of this temperature for further studies. Temperatures below 5ºC were not evaluated because of the limitation on the minimum temperature allowed by the column oven controller (5ºC). To reduce the analysis time, flow-rate was increased up to 450 µL min^{-1} (Figure 2D). Under these

working conditions, there was good chromatographic separation of all compounds in less than 5 min analysis time, generating a low backpressure (< 350 bar).

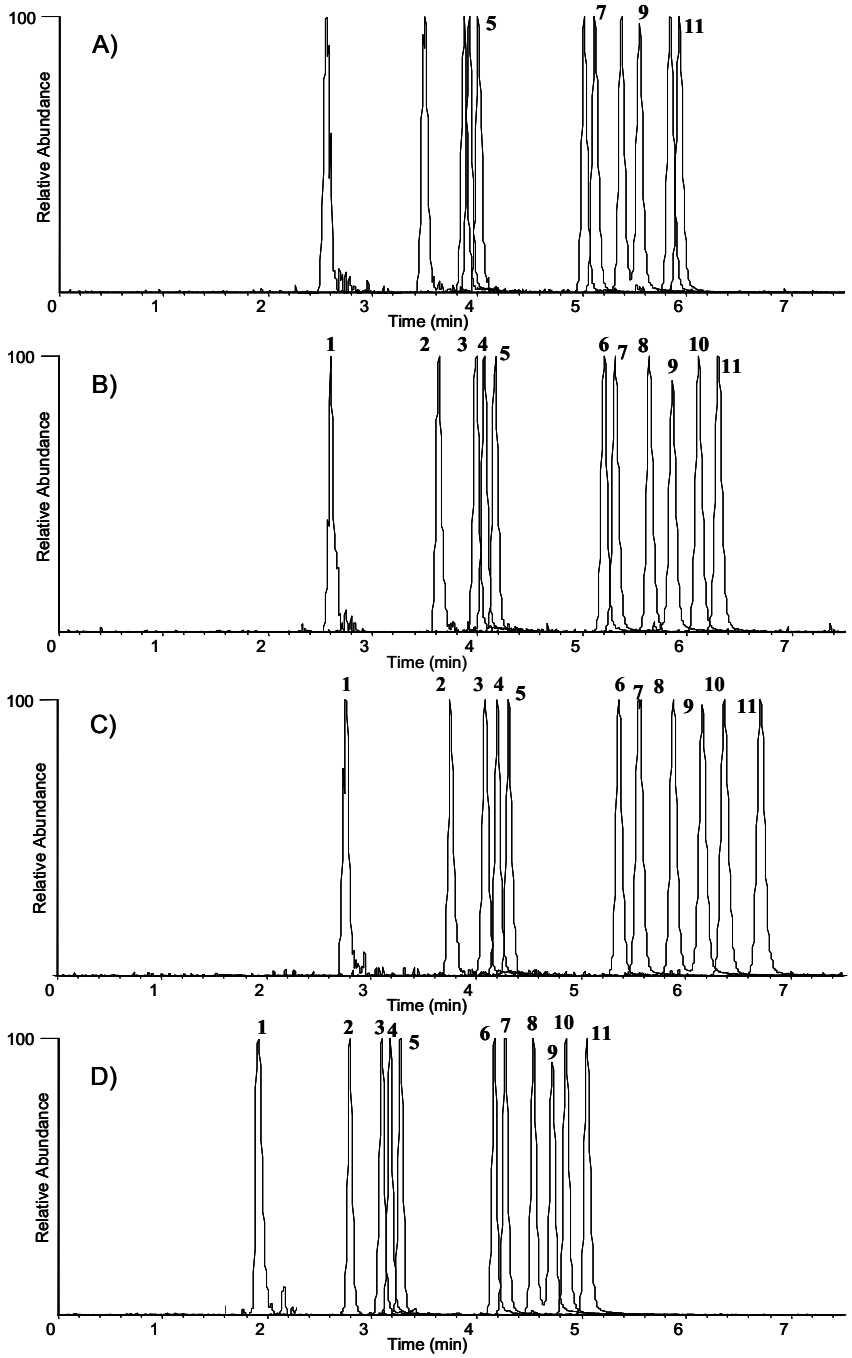

Figure 2. Effect of column temperature on the separation of the eleven UV Ink photoinitiators. LC-MS/MS reconstructed chromatograms at (A) 25°C, (B) 15°C, (C) 5°C at 300 μL min^{-1} and (D) 5ºC at 450 μL min^{-1}. Peak identification: 1, HMPP; 2, HCPK; 3, EDMAB; 4, DMPA; 5, BP; 6, PBZ; 7, DEAB; 8, 2-ITX; 9, 4-ITX; 10, EHDAB; 11, DETX.

3.2. Liquid chromatography-mass spectrometry

The liquid chromatographic system was coupled to a triple quadrupole mass spectrometer using an ESI source in positive mode. For most of these compounds, the ESI (positive) full scan MS spectrum showed only the isotopic cluster corresponding to the protonated molecule $[M+H]^+$. However, for some of them (HMPP, HCPK, DMPA, DEAB), ions originated by in-source fragmentation were also observed (Table 1). The in-source fragmentation was especially important for DMPA, whose mass spectrum showed the in-source loss of a methoxy group as base peak, yielding the ion at *m/z* 225 $[M-CH_3O]^+$. The significant differences between structures of some of these photoinitiators produced important differences in electrospray responses. Thioxanthone-based photoinitiators (2-ITX, 4-ITX and DETX) showed the highest response, followed by the alkyl-amino-based compounds (DEAB, EHDAB and EDMAB) (10 to 20 times lower) and the phenone-based compounds (BP, PBZ and DMPA) (20 to 200 times lower). HMPP and HCPK showed the lowest ionization efficiency.

The fragmentation of these compounds under tandem mass spectrometry conditions in the triple quadrupole was studied and the most intense and characteristic transitions were selected for both quantitative and confirmation purposes. For the correct product ion assignment, collision energy curves (5-80 V) were studied. The assignments for both precursor and monitored product ions for each compound are given in Table 1, which also gives the selected transitions and the optimal collision energies. Due to the differences in chemical structure of the compounds studied, it was difficult to select common transitions for the whole family. For ITX isomers (2- and 4-ITX) and DETX the most intense product ions corresponded to the loss of the alkyl chains. For ITX the ion originated from the consecutive losses of the alkyl chain and the

CHO group (*m/z* 184) was also observed and selected as qualifier ion. The MS/MS spectrum of both BP and PBZ showed as a base peak the ion at *m/z* 105 corresponding to $[C_7H_5O]^+$ due to the α-cleavage of the carbonyl group. Another intense product ion corresponding to $[C_6H_5]^+$ was also observed and selected for confirmation. For compounds such as EHDAB and EDMAB, which contain both an amino and an ester group, the most intense product ions in the MS/MS spectra were generated by the consecutive losses of a methyl group and the alkyl chains of the ester group (*m/z* 151) and the methyl group together with the α-cleavage of the carbonyl group (*m/z* 134). The other photoinitiators, HCPK, HMPP and DMPA, showed a different fragmentation pattern because of the different functional groups in their structures. For HMPP, the base peak in the MS/MS spectrum was the product ion at *m/z* 119, probably due to the consecutive neutral losses of water and olefin (C_2H_4), and the product ion at *m/z* 91, corresponding to the tropylium ion often found for aromatic compounds containing a benzyl unit, while HCPK showed the ion at *m/z* 105 originated by the α-cleavage of the carbonyl group, as occurred for BP and PBZ, and the neutral loss of water (*m/z* 187). Finally, for DMPA two abundant product ions were obtained from the fragmentation of the in-source fragment ion, the characteristic ion at *m/z* 105 as at *m/z* 197, due to the loss of a CO group.

To evaluate the performance of the fast LC-MS/MS method developed, instrument quality parameters such as limits of quantitation (ILOQ), linearity and run-to-run precision at two concentration levels, a low level close to the limit of quantitation (LOQ) and a medium level (HMPP: 3 mg L^{-1}; HCPK: 300 μg L^{-1}; other ink photoinitiators: 50-100 μg L^{-1}), were evaluated using selected reaction monitoring (SRM) acquisition mode. ILOQs (Table 2), based on a signal-to-noise ratio of 10:1, were calculated by the injection of 10 μL of UV ink photoinitiator standard solutions

prepared at low concentration levels (background noise was determined manually around the compound retention time). Thioxanthone-based photoinitiators provided the lowest instrument ILOQs (0.06 to 0.09 μg L^{-1}), while compounds based on alkyl-amino groups (DEAB, EHDAB and EDMAB) and PBZ provided ten-times higher values (0.9 to 1.5 μg L^{-1}). Whereas phenones and HCPK showed ILOQ values between 15 and 30 μg L^{-1}, HMPP provided the highest ILOQ due to its lower ionization efficiency with ESI.

Table 2. Comparison of SPE and QuEChERS extraction and clean-up procedures using baby food sample matrix.

Compound	SRM ILOQ (pg)	SPE method			QuEChERS method		
		MLOQ (μg/kg)	Accuracy (%)**	run-to-run precision**	MLOQ (μg/kg)	Accuracy (%)**	run-to-run precision**
HMPP	12000	710	91	2.7	666	94	2.9
HCPK	600	500	89		500	87	2.6
EDMAB	30	0.5	90	2.8	0.5	81	4.5
DMPA	300	1.5	88	2.1	0.7	83	3.4
BP	300	2.0	92	4.3	2.3	97	5.1
PBZ	30	0.7	91	5.1	0.7	88	4.6
DEAB	15	0.3	89	4.9	0.7	98	5.0
2-ITX	1.5	0.2	90	3.3	0.2	93	3.3
4-ITX	1.5	0.2	92	2.7	0.2	95	3.4
DETX	1.5	0.3	91	3.3	0.3	95	4.3
EHDAB	15	0.7	90	4.2	1.0	86	4.4

*Injection volume: 10 μL
**Spiked concentrations (μg L^{-1}): HMPP (2530), HCBPK (800), EDMAB (0.3), DMPA (4), BP (80), PBZ (1.4), DEAB (0.3), 2-ITX (0.14), 4-ITX (0.14), DETX (0.14) and EHDAB (0.3)

Calibration curves based on the peak area ration ($A_{\text{compound}}/A_{\text{internal standard}}$) (2-ITX-$D_7$ as I.S.) showed good linearity (correlation coefficient, r^2: >0.995). Run-to-run

precision was also determined at two concentration levels (n=5) by LC-MS/MS (RSD < 6.6%).

3.3. Method performance

In this study we evaluated the applicability of a QuEChERS procedure for the analysis of UV ink photoinitiators in packaged foods. This method was compared with a SPE one previously applied for the analysis of ITX [17] in terms of sensitivity, accuracy and precision. For these purposes two blank samples (pineapple juice and baby food) were spiked and submitted to both sample treatments. The results obtained for the baby food sample are summarized in Table 2.

In general, similar MLQs were obtained using both sample treatments for both matrices providing values down to μg kg^{-1} or even ng kg^{-1} for ITX and DETX (5 ng kg^{-1}), with the sole exception of HMPP, which showed the highest MLOQ value (666 μg kg^{-1}). To evaluate the run-to-run precision, six replicates of a blank sample spiked at the concentrations from 0.14 μg L^{-1} to 800 μg L^{-1}, except for HMPP (2.5 mg L^{-1}), (Table 2) were analyzed using both sample treatments. Similar relative standard deviations (%RSD) based on concentration were obtained for both SPE and QuEChERS, with values ranging from 1.9 to 5.1%. Good quantitation results, with accuracies (defined as % relative error) in the 81-98% range, were achieved. In addition, a statistical paired-sample comparison analysis was performed, based on the quantitation results obtained in both SPE and QuEChERS procedures. For a 95% confidence level, the results were not significantly different (*p*-value of 0.33). Thus, the QuEChERS method provided similar results in terms of MLOQs, run-to-run precision, and quantitation to results obtained for SPE, but with the additional advantage of being

12 times faster (per sample). These results mean that this method can be proposed for the fast analysis of UV ink photoinitiators in packaged food.

In addition, to improve sensitivity by minimizing interferences and background noise, enhanced mass resolution on precursor ions (H-SRM on Q1) was evaluated. For this purpose two blank samples (baby food and fruit juice) were spiked at a low concentration level (close to the quantitation limit) and analyzed by the QuEChERS method. Table 3 summarizes the peak intensity normalized to that of SRM mode and the signal-to-noise ratio obtained for each compound in pineapple and baby food, using SRM and H-SRM acquisition modes. It can be observed that the intensity of the compounds decreased when mass resolution increased; although a higher signal-to-noise ratio (S/N) was obtained due to a significant reduction in the background noise. This obtained MLOQs that were 1.25 to 30 times lower.

Table 3. SRM vs H-SRM (Q1) in pineapple juice and baby food matrices.

Compound	Pineapple matrix				Baby food matrix			
	SRM		H-SRM (Q1)		SRM		H-SRM (Q1)	
	Peak Signal (%)	S/N ratio	Peak Signal (%)	S/N ratio	Peak Signal (%)	S/N ratio	Peak Signal (%)	S/N ratio
HMPP	100	12	44	20	100	15	51	100
HCPK	100	14	63	30	100	15	62	30
EDMAB	100	40	48	50	100	20	57	25
DMPA	100	30	45	60	100	20	50	100
BP	100	70	43	500	100	60	41	450
PBZ	100	10	25	300	100	10	26	110
DEAB	100	210	25	300	100	130	26	250
2-ITX	100	250	27	750	100	200	27	500
4-ITX	100	250	30	900	100	260	29	700
DETX	100	40	30	800	100	20	30	300
EHDAB	100	150	30	250	100	60	37	200

3.4. Application of the method

To evaluate the applicability of the QuEChERS LC-MS/MS method, 14 packaged foods (food commodities and baby foods) from Spanish supermarkets were analyzed. Their packaging materials were also analyzed in order to identify the UV ink photoinitiators used in the printing process, which might then be expected to be found in the packaged foods. Since BP can be used in the manufacture of plastic materials, analysis of blanks is relevant in order to detect contamination during the analytical procedure. In this study, no contamination was observed when analyzing method blank samples. The results obtained showed that all the packaging materials contained between 4 and 8 photoinitiators, among which BP was always present at high concentrations (between 2 and 350 ng cm^{-2}). DMPA and the tertiary amine EHDAB were also found in many of the cartons analyzed, the first one at relatively high concentrations (0.2 – 1 ng cm^{-2}). Other photoinitiators such as EDMAB and DEAB were detected in some of the packaging materials, but at lower concentrations (0.005 – 0.6 ng cm^{-2}). The photoinitiator 2-ITX (0.005 – 0.1 ng cm^{-2}) was also detected in all the analyzed samples, while 4-ITX was only found in 3 of the 14 samples, but at concentration levels similar to 2-ITX levels. Finally, PBZ and DETX were found in only a few samples, probably due to less use, while HCPK and HMPP were not detected in any of the cartons analyzed. These results corroborate those reported in the literature [3,10] about the presence of these compounds in packaging materials where BP was found at relatively high concentrations in almost all samples analyzed.

The results obtained in the analysis of the 14 packaged foods are summarized in Table 4. These results showed that only 1-4 of the photoinitiators identified previously in the food packaging materials were detected in the foodstuff, with BP being the most

abundant one, with concentrations ranging from 1.8 to 40 μg kg^{-1}. It must be pointed out that in two of the samples (baby food 3 and *gazpacho* 1) an important deviation (>42%) in the BP ion ratio was observed, which did not allow its confirmation in the samples (Directive 2002/657/EC) [19]. The presence of BP in all the samples could be due, not only to its use as a UV ink photoinitiator, but to its application in the production of polyethylene (PE) coating film [28], which is directly in contact with food. EDMAB and 2-ITX were also found in a relatively high number of samples (10 and 7 samples, respectively), but at lower concentrations (ng kg^{-1}) than BP. HMPP and HCPK were not detected in any sample, as expected from the results obtained in the analysis of the carton materials, while the other photoinitiators such as DETX and EHDAB were detected in just a few samples at low ng kg^{-1} levels. For example, Figure 3 shows the LC-MS/MS chromatogram obtained for a pineapple juice sample and the corresponding packaging material. Among the seven photoinitiators detected in the corresponding carton material, only four of them, BP, DEAB and both ITX isomers, were detected in the pineapple juice sample.

In addition, it should be pointed out that the greater sensitivity provided by the H-SRM in Q1 acquisition mode detected and identified some of the analyzed compounds, which could not be detected when low-resolution SRM acquisition mode was used. For instance, 4-ITX in *gazpacho* 1, DETX in fruit juice 1 and EHDAB in baby food 3 and fruit juice 2 were quantified at low concentration levels by H-SRM.

Table 4. Packaged food samples analyzed using QuEChERS LC-MS/MS method using H-SRM (μg kg^{-1}).

Sample type	Packaging volume (mL)	HMPP	HCPK	EDMAB	DMPA	BP	PBZ	DEAB	2-ITX	4-ITX	DETX	EHDAB
Baby food 1 (fruit and cereal)	250	n.d.	n.d.	~MLOD	n.d.	40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Baby food 2 (milk and cereal)	250	n.d.	n.d.	n.d.	n.d.	29	n.d.	n.d.	n.d.	n.d.	n.d.	~MLOD
Baby food 3 (milk, fruit, cereal)	250	n.d.	n.d.	~MLOD	~MLOD	n.c.*	n.d.	n.d.	0.8	n.d.	~MLOD	0.6
Baby food 4 (multi-fruit)	200	n.d.	n.d.	0.5	n.d.	3.0	n.d.	n.d.	0.4	n.d.	n.d.	n.d.
Fruit juice 1 (peach and grape)	200	n.d.	n.d.	n.d	n.d.	2.5	n.d.	n.d.	0.2	~MLOD	0.07	n.d.
Fruit juice 2 (orange)	200	n.d.	n.d.	n.d	n.d.	6.5	n.d.	n.d.	0.2	~MLOD	n.d.	0.6
Fruit juice 3 (pineapple)	200	n.d.	n.d.	n.d.	n.d.	2.8	n.d.	0.7	0.2	0.07	n.d.	n.d.
Gazpacho 1	1000	n.d.	n.d.	2.5	n.d.	n.c.*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gazpacho 2	1000	n.d.	n.d.	0.5	n.d.	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gazpacho 3	1000	n.d.	n.d.	1.6	n.d.	12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gazpacho 4	1000	n.d.	n.d.	0.5	n.d.	8.0	n.d.	n.d.	0.4	n.d.	n.d.	n.d.
White wine	1000	n.d.	n.d.	n.d.	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sangria	1000	n.d.	n.d.	n.d.	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Water	1000	n.d.	n.d.	n.d	n.d.	3.8	n.d.	n.d.	~MLOD	n.d.	n.d.	n.d.

n.d.: not detected.

*n.c.: not confirmed. Ion ratio error higher than 20%.

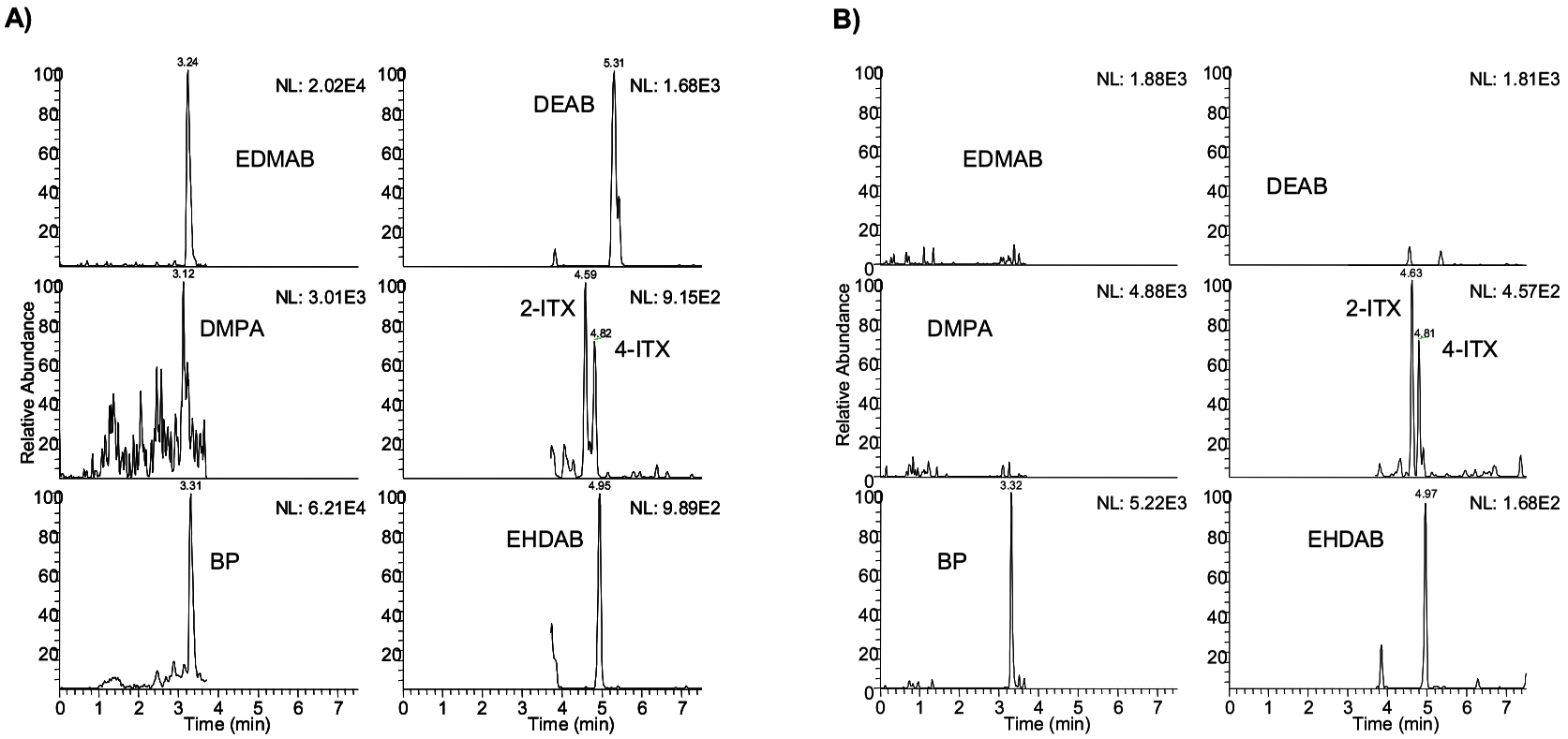

Figure 3. Analysis of (A) a packaging material containing a pineapple juice sample and (B) a pineapple juice sample. Conditions as indicated in the experimental section.

Conclusions

In this study, a fast LC-MS/MS method was developed for the analysis of UV ink photoinitiators in packaged food. Good chromatographic separation, including ITX isomers, was achieved by using a pentafluorophenyl propyl (PFPP) column and operating at low temperature (5ºC). A flow rate of 450 μL min^{-1} was used to reduce the analysis time below 5.5 min without compromising the chromatographic efficiency. To reduce the sample treatment time, a QuEChERS method is proposed for the extraction and clean-up of UV photoinitiators in packaged foods.

The ESI mass spectra of this family of compounds were generally dominated by the $[M+H]^+$, except for DMPA, which showed important in-source fragmentation. For this compound, $[M-CH_3O]^+$ was selected as a precursor ion in MS/MS. H-SRM on Q1 is proposed as acquisition mode, since an up-to-30-fold improvement in MLOQs was obtained.

Several photoinitiators, BP, PBZ, DEAB, 2-ITX, 4-ITX, DETX, EHDAB, DMPA and EDMPA, were detected in the packaging materials, with benzophenone always present and at the highest concentration level. This photoinitiator was also detected in all packaged food samples, while the other compounds were only found in a few samples at low ng kg^{-1} levels. These results allow us to propose the QuEChERS LC-MS/MS as a simple, fast, robust and reproducible method for the analysis of photoinitiators in packaged food.

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3.2.4. MESURES DE MASSA EXACTA EN LC-MS

Un aspecte important en l'anàlisi de contaminants en mostres ambientals i d'aliments és obtenir una correcta confirmació de la seva presència en les mostres. Segons la Directiva 96/23/EC per poder confirmar la presència d'un compost es necessiten com a mínim de 3 a 4 punts d'identificació. En LC-MS/MS amb un analitzador de triple quadrupol els quatre punts d'identificació es poden obtenir mitjançant la monitorització d'un mínim de dues transicions selectives, un ió precursor i dos ions producte (1,5 punts d'identificació per cada ió producte i 1 punt per ió precursor). Si es monitoritzen dos ions precursors i de cadascun d'ells un ió producte es poden aconseguir fins a cinc punts d'identificació. A més, per tal de confirmar la presència d'un compost també s'ha de complir que l'error de la relació d'intensitats entre l'ió producte de quantificació i el de confirmació ("*ion-ratio*") a la mostra no sigui superior al 20% de la relació obtinguda en un estàndard a més de que coincideixin els temps de retenció de l'anàlit i el patró. Tot i complir aquest criteris de confirmació es poden trobar casos de falsos negatius i falsos positius. Els falsos positius en l'anàlisi per LC-MS/MS amb un analitzador de triple quadrupol monitoritzant dues transicions per compost, poden ser deguts a la presència de compostos interferents que coeluexen total o parcialment amb l'anàlit i que presenten transicions isobàriques. Encara que aquest escenari es poc probable hi ha alguns casos recollits a la bibliografia que il·lustren la importància de realitzar una correcta selecció de les transicions a monitoritzar (Pozo i cols., 2006 i Schürmann i cols., 2009). Per evitar aquests problemes es recomana la monitorització de més de dues transicions selectives sempre que sigui possible per poder discernir entre el senyal de l'anàlit i el de la interferència o treballar en alta resolució en aquells casos en els que no és possible monitoritzar més de dues transicions. Pel que fa referència als falsos negatius, aquests es poden donar si la interferència afecta únicament a una de les transicions monitoritzades la qual cosa dóna lloc a un error en el *"ion-ratio"* superior al 20% (Pozo i cols., 2006). En aquest cas a part de monitoritzar més de dues transicions per anàlit com en el cas anterior, es recomanable treballar a una resolució més elevada, per fer més específiques les transicions i/o obtenir les mesures de massa exacta tant dels ions precursors com dels ions producte.

En aquesta Tesi tal i com hem comentat abastament s'ha emprat un analitzador de triple quadrupol treballant en el mode H-SRM degut a la bona sensibilitat i selectivitat que proporciona en la majoria de casos. Ara bé, per tal d'obtenir una confirmació addicional de la presència dels anàlits a les mostres analitzades s'ha avaluat la possibilitat de realitzar mesures de massa exacta en línia amb la cromatografia tant d'ions precursors com d'ions producte aprofitant la capacitat d'aquest instrument de treballar a una relativa alta resolució tant en el primer quadrupol (ions precursors) com en el tercer quadrupol (ions producte) i la possibilitat d'obtenir mesures de massa exacta amb una bona precisió i exactitud (característiques descrites al Capítol 2 d'aquesta memòria).

Per tal d'obtenir una bona precisió i exactitud en la mesura de la massa cal realitzar una acurada calibració de l'instrument, tal i com s'ha descrit al Capítol 2, i a més dur a terme una correcció de la mesura de *m/z* en temps real mitjançant la utilització de masses de referència (*lock masses)*. Atès que en el nostre cas es pretén realitzar mesures de massa exacta de compostos coneguts, prèviament identificats s'ha treballat a la màxima resolució possible i s'han realitzar les mesures de massa exacta durant tot el cromatograma, la qual cosa ha permès obtenir les traces dels pics cromatogràfics a la màxima resolució. S'ha emprat el mode H-SIM (Q1: 0,04 m/z FWHM) per realitzar mesures de massa exacta dels ions precursors i el mode H-SRM treballant a la màxima resolució en el Q3 en el cas de mesures de massa exacta dels ions producte. Com a calibrants interns s'han seleccionat el CQ-d_4 (*m/z* 126.0982, $[M]^{+\bullet}$), el 2-ITX-d_7 (*m/z* 262.1280, $[M+H]^+$) i el trímer de la tirosina (Tyr-Tyr-Tyr a *m/z* 508.2080, $[3M]^+$) per tal de tenir ben definit un interval de masses de treball adequat per als compostos a estudiar. Els calibrants es van infusionar a una concentració de 1 µg kg^{-1} (2 µL min^{-1}) per tal de minimitzar al màxim possibles efectes de supressió iònica dels anàlits. A la Figura 3.4A es mostra a mode d'exemple el cromatograma obtingut en el mode H-SIM d'una mostra d'aliments per a infants (580 ng kg^{-1}) (article científic V, Taula 3) on es pot confirmar la presència del 2-ITX amb un error en la mesura de la massa de 1 mDa. A la Figura 3.4B es mostra l'espectre de masses corresponent.

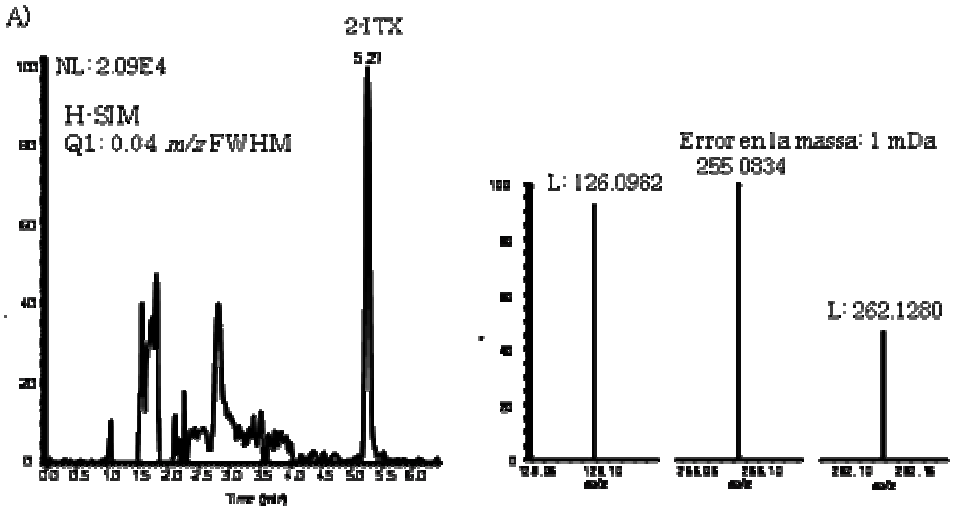

Figura 3.4. Mesura de massa exacta en mode H-SIM (Q1:0,04 unitats de *m/z*) en l'anàlisi de 2-ITX en una mostra d'un puré de fruites per infants (580 ng kg^{-1}). A) Cromatograma i B) mesura de massa exacta.

En l'anàlisi del BADGE·$2H_2O$ i BADGE·HCl·H_2O en diverses mostres d'aliments la mesura de la massa exacta de l'ió precursor amb errors inferiors als ±5 mDa (Taula 3.1) en el mode d'adquisició H-SIM també ha permès obtenir una confirmació addicional de la presència d'aquests compostos. En aquest cas a més s'ha avaluat la possibilitat de dur a terme mesures de massa exacta d'ions producte, ja que treballar en H-SRM pot ser d'interès en l'anàlisi de contaminants orgànics en mostres ambientals i d'aliments on se solen trobar a baixes concentracions atesa la major sensibilitat en aquest mode de treball. Ja que els BADGEs presenten un ió producte característic a *m/z* 135 assignat a $[C_9H_{11}O]^+$ s'ha obtingut la mesura de la massa exacta d'aquest ió treballant en el mode H-SRM augmentant la resolució en el tercer quadrupol (Q3: 0,04 *m/z* FWHM). Aquestes mesures al igual que en els casos anteriors també s'han realitzat amb calibratge intern. En aquest cas com a calibrants interns tant sols s'han emprat el CQ-d_4 (*m/z* 126.0982) i el 2-ITX-d_7 (*m/z* 262.1280) que cobreixen l'interval de masses a mesurar i s'ha treballat a una energia de col·lisió de 5V per tal de no fragmentar els ions precursors dels calibrants i mesurar la seva *m/z* en el tercer quadrupol. En canvi per als compostos d'interès s'ha seleccionat l'energia de col·lisió òptima en cada cas per obtenir la màxima intensitat de l'ió producte. Pel BADGE·$2H_2O$ en les diferents matrius analitzades s'han obtingut errors en la mesura de la massa del mateix ordre que els obtinguts en H-SIM, inferiors als ±5 mDa, en canvi pel

BADGE·HCl·H_2O els errors han estat més grans. Tot i això aquests errors permeten tenir una confirmació addicional de les mostres analitzades i confirmar els resultats obtinguts mitjançant SRM.

Taula 3.1. Mesures de massa exacta en H-SIM i H-SRM en l'anàlisi de BADGEs.

Mostra	Compost	Nivell (ng/g)	Error en la mesura massa (mDa)	
			Precursor	Producte (*m/z* 135)
Blat de moro	BADGE·2H_2O	383.3	-2.4	-0.3
	BADGE·HCl·H_2O	5.6	-0.2	-3.1
Pinya	BADGE·2H_2O	4.4	-2.2	1.0
Pebrot vermell	BADGE·2H_2O	249.9	1.9	-3.9
	BADGE·HCl·H_2O	17	2.7	-9.3
Espàrrecs	BADGE·2H_2O	1382	-3.4	3.6
	BADGE·HCl·H_2O	1337	2.5	-11.9

En l'anàlisi de fotoiniciadors per LC-MS/MS treballant en el mode d'adquisició H-SRM (Q1: 0,1 *m/z* FWHM) en diverses mostres d'aliments (Taula 4, article científic VI, apartat 3.2.3) la benzofenona (BP) no ha estat quantificada en dues de les mostres analitzades (mostra d'aliments per infants 3 i *gazpacho* 1) ja que l'error en el *ion-ratio* de la benzofenona (BP) superior al 20% (aliment per infants: 45% i *gazpacho* 1: 42%). Per tant, seguint els criteris establerts per la legislació no es pot confirmar la presència d'aquest compost i en conseqüència en el treball aquetes dues mostren es donen com negatives. Ara bé, l'error en el *ion-ratio* pot ser degut a la presència d'una interferència isobàrica en la transició de quantificació i/o de confirmació i per poder confirmar la presència d'aquest compost és necessari monitoritzar una tercera transició selectiva o treballar en alta resolució. En aquest cas la monitorització d'una tercera transició no es possible ja que la BP únicament presenta dos ions producte en l'espectre de MS/MS la qual cosa fa necessari treballar en alta resolució i obtenir la mesura de massa exacta de l'ió precursor i/o dels ions producte per tal de confirmar la seva presència. Les mesures de massa exacta realitzades amb l'instrument de triple quadrupol emprat en aquesta Tesi no van aportar suficient exactitud i precisió per resoldre el problema probablement degut a les limitacions que presenta aquest instrument pel que fa a resolució, ja que com a màxim pot treballar a una resolució de 0,04 *m/z* FWHM que per a un ió de *m/z* 183, com és cas de la BP, correspon a un poder de

resolució de 4575. Amb l'objectiu d'intentar confirmar la presència de la BP a les dues mostres aquestes s'han analitzat amb un instrument d'alta resolució un l'Orbitrap (Exactive Mass Spectrometer de Thermo Fisher Scientific) que permet treballar a un poder de resolució de fins a 100000. Amb aquest analitzador i treballant a un poder de resolució de 50000 en escombratge total d'ions (*full scan*) va ser possible separar espectromètricament l'anàlit de la interferència obtenint una molt bona exactitud en la mesura de la massa exacta amb un error de 1.3 mDa en ambdós casos. Aquest mètode ha permès quantificar i confirmar la presència de BP en aquestes dues mostres analitzades a una concentració de 2.0 μg kg^{-1} en la mostra d'aliments per infants i de 2.5 μg kg^{-1} en la mostra de *gazpacho*. A la Figura 3.5 es mostra a mode d'exemple el cromatograma obtingut en l'anàlisi de BP a la mostra d'aliment per infants així com l'espectre de masses del corresponent pic on es pot observar que encara que la concentració de BP a la mostra és molt inferior a la interferència isobàrica s'obté una bona exactitud en la mesura de la massa la qual cosa ens ha permès confirmar la presència d'aquest anàlit.

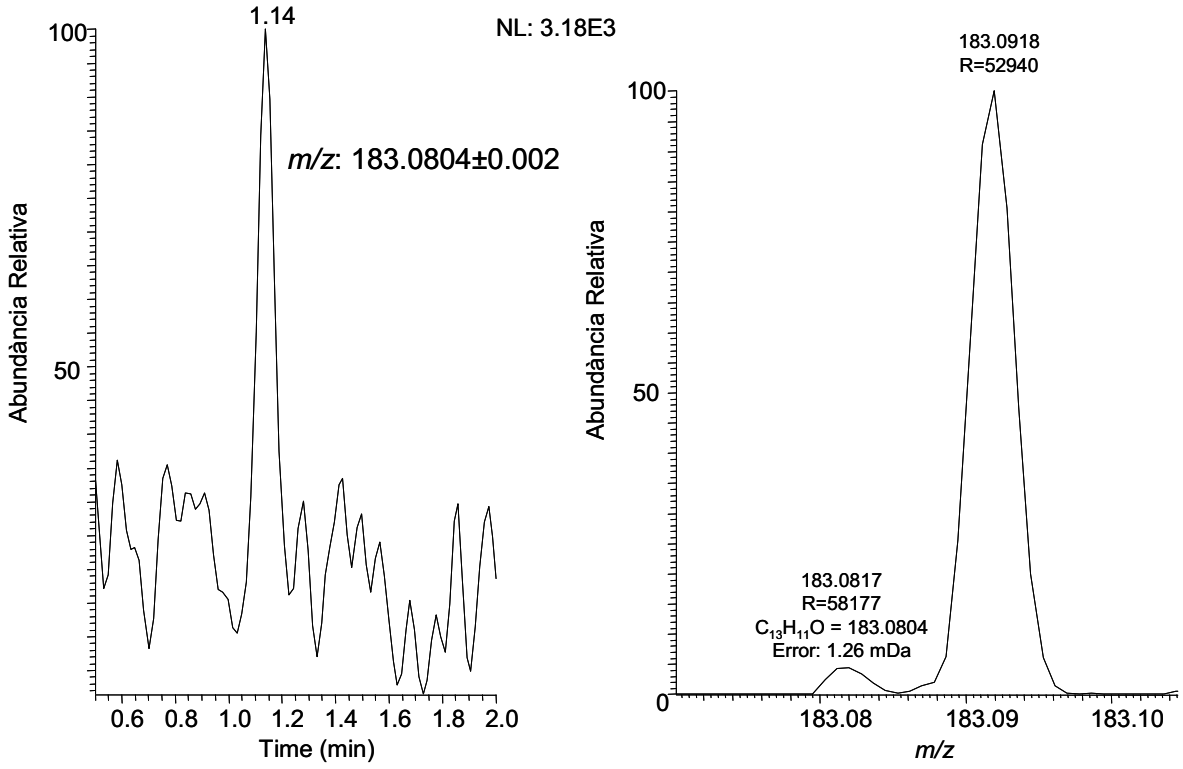

Figura 3.5. Anàlisi de BP en una mostra d'aliments per infants en LC-HRMS treballant amb un analitzador Orbitrap. Condicions de treball: *Full-scan* 50 – 1000 *m/z,* voltatge electroesprai 4000V, *sheath gas* i gas auxiliar 60 i 20 unitat arbitràries, respectivament, temperatura del capil·lar 375ºC.

3.3. DISCUSSIÓ DE RESULTATS

En aquest apartat de discussió de resultats es pretén presentar i discutir els resultats obtinguts en el desenvolupament de mètodes de cromatografia de líquids ràpida per a l'anàlisi dels BADGEs, BFDGEs i de fotoiniciadors en mostres d'aliments inclosos en aquest capítol.

3.3.1. CROMATOFRAGIA DE LÍQUIDS RÀPIDA

Tal i com hem comentat anteriorment a la introducció d'aquest capítol, les columnes de partícules semiporoses (2.7 μm diàmetre de partícula) poden arribar a presentar eficàcies comparables a les obtingudes amb les columnes sub-2 μm amb l'avantatge de requerir una menor pressió. En aquesta Tesi la utilització d'una d'aquestes columnes semiporoses de 5 cm de longitud (Ascentis Express Fused Core™ C18) ha permès obtenir una bona resolució en la separació cromatogràfica dels isòmers dels BFDGEs en menys de 4 minuts i amb eficàcies similars a les obtingudes amb una columna de mida de partícula inferior als 2 μm (Aquity BEH C18 de 1.7 μm de mida de partícula) (Article científic IV, Apartat 3.2.1). Això es pot observar a la Figura 3.6 on es mostra a mode d'exemple la separació obtinguda per als tres isòmers del BFDGE·$2H_2O$ en aquestes dues columnes. En ambdós casos l'amplada dels pics a mitja alçada és la mateixa i les eficàcies són similars. Pel que fa referència a la pressió a l'entrada, la columna semiporosa requereix 200 bar en front dels 513 bar de la columna sub-2 μm. Aquesta menor pressió possibilita augmentar la longitud de la columna fins als 15 cm sense superar la pressió màxima del sistema cromatogràfic la qual cosa ha permès augmentar el nombre de plats teòrics i obtenir una bona separació de tots els compostos estudiats, BADGEs i BFDGEs. Això ha permès l'anàlisi simultània d'aquests compostos en mostres d'aliments enllaunats en menys de 5 minuts (Article científic IV, Apartat 3.2.1) reduint de forma significativa el temps de cromatograma per aquesta família de compostos ja que la majoria de mètodes publicats a la literatura presenten uns temps d'anàlisi superiors als 15 min (Pardo i cols., 2006, Soeborg i cols., 2006, Sendón i cols., 2004 i Uematsu i cols.,2001). El temps d'anàlisi obtingut en aquesta memòria fins i tot és inferior a l'obtingut per Yonekubo i cols., 2008, emprant UHPLC amb una columna porosa sub-2 μm de 5 cm de longitud que és d'aproximadament 9 minuts.

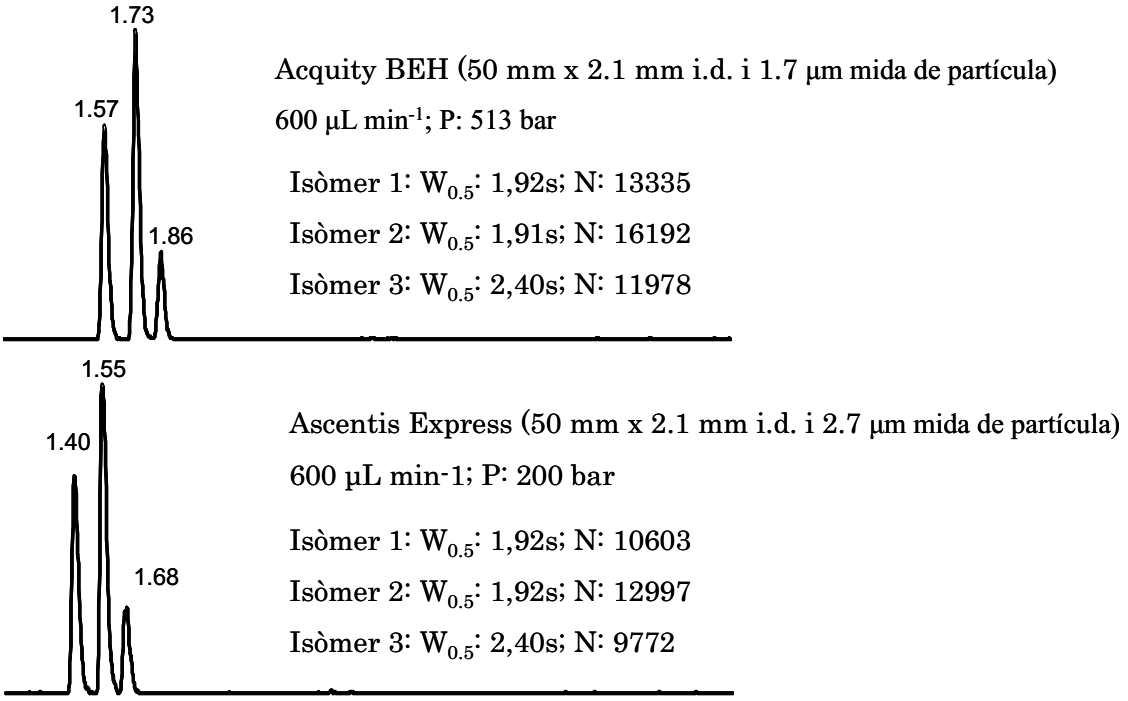

Figura 3.6. Separació cromatogràfica obtinguda per als isòmers del BFDGE·$2H_2O$ amb una columna 1.7 μm i una columna de partícules semiporoses (2.7 μm).

Amb la finalitat de desenvolupar un mètode per de l'ITX per LC-MS es va optimitzar la separació cromatogràfica dels dos isòmers (Article científic V, Apartat 3.3.2). Aquests dos isòmers, el 2-ITX i el 4-ITX, coelueixen en les columnes de fase invertida (C8 o C18) i a més presenten la mateixa fragmentació la qual cosa impossibilita la seva separació per espectrometria de masses. En aquesta Tesi es proposa la utilització d'una columna de fase estacionària pentafluorofenil propil (PFPP) que permet (Figura 2 de l'article científic V) separar els dos isòmers amb una bona resolució (Rs = 1.3) en menys de 6 minuts. A més aquest mètode presenta una molt bona sensibilitat treballant en el mode H-SRM en el primer quadrupol (Q1) amb uns límits de quantificació (7 – 43 ng kg^{-1}), un ordre de magnitud inferiors als obtinguts per Bagnati i cols., 2007 els únics autors que proposen un mètode per separar els dos isòmers.

Aquesta fase estacionaria PFPP també ha estat emprada per desenvolupar un mètode d'anàlisi per a la determinació de 11 fotoiniciadors, DETX, BP, PBZ, HMPP, HCPK, DMPA, DEAB, EHDAB i EDMAB, i els dos isòmers de l'ITX mitjançant LC-MS/MS (Article científic VI, Apartat 3.2.3) ja que a la literatura la

majoria dels mètodes publicats tant sols analitzen alguns d'aquests compostos. En aquest treball s'ha estudiat l'efecte de la temperatura de la columna en la separació per tal de millorar la resolució cromatogràfica i evitar coelucions entre els diferents compostos analitzats. Es proposa treballar a 5ºC condicions en les quals a més, s'obté una millora significativa en la resolució (Rs>1.3) entre els dos isòmers de l'ITX. Aquesta millora és probablement deguda a un canvi conformacional amb la temperatura tant de la fase estacionaria i com dels anàlits que permet accentuar les diferencies en l'interacció dels dos isòmers amb la fase estacionaria. A temperatures inferiors a l'ambient aquesta fase estacionaria es troba en estat sòlid (Cole i cols., 1992; Morel i cols., 1981; Liu i cols., 2005) la qual cosa pot explicar la major interacció dels anàlits i un augment de la selectivitat, mentre que a temperatures més elevades la retenció es menor i les diferencies en les interaccions desapareixen donant com a conseqüència la coelució dels dos isòmers. El mètode proposat, columna PFPP de 15 cm i 3 μm de mida de partícula a una temperatura de treball de 5ºC un cabal de fase mòbil de 450 $\mu L\ min^{-1}$ ha permès obtenir un temps d'anàlisi inferior als 5.5 minuts a una pressió ($P \leq 350$ bar) que es troba per sota de les màximes permeses per la columna. El temps d'anàlisi cromatogràfica és inferior al d'altres mètodes publicats a la literatura (Shen i cols., 2009, Sagratini i cols., 2008, Sun i col., 2007 i Gil-Vergara i cols., 2007) els quals presenten temps d'anàlisi en tots els casos superiors als 10 min. Pel que fa referència als límits de quantificació en mostres d'aliments aquests són semblants als publicats a la literatura tant per GC-MS com per LC-MS/MS (Van Hoeck i cols., 2010, Shen i cols., 2009, Sagratini i cols., 2008, Sun i col., 2007 i Gil-Vergara i cols., 2007) trobant-se entre 0,2 i 2 $\mu g\ kg^{-1}$ per a la majoria de compostos excepte la HMPP i la HCPK que no presenten una bona sensibilitat degut a la baixa eficàcia de ionització que presenten en ESI (Article científic VI, apartat 3.2.3).

3.3.2. ANÀLISIS DE MOSTRES

Els mètodes de cromatografia ràpida acoblada a l'espectrometria de masses en tàndem desenvolupats en aquest capítol han estat aplicats a la determinació dels BADGEs i els BFDGEs en mostres d'aliments de base aquosa i begudes refrescants enllaunades i de fotoiniciadors en aliments envasats amb cartró. En l'anàlisi d'aliments és ben conegut que la matriu afecta de manera important la

ionització quan s'utilitza l'ESI com a font d'ionització. A més, les noves tendències en l'anàlisi de mostres d'aliments per LC-MS/MS es dirigeixen cada vegada més a la utilització de mètodes de tractament de mostra ràpids, senzills i poc selectius com poden ser l'extracció líquid-líquid, QuEChERS i SPE amb fases poc selectives i d'elevada retenció que poden donar lloc a l'aparició d'interferències. En aquesta Tesi per tal de dur a terme una correcta quantificació i reduir al màxim aquests problemes s'han utilitzat diferents mètodes de quantificació. Així, per a l'anàlisi de l'ITX atès que es disposa comercialment de l'estàndard marcat isotòpicament (2-ITX-d_7) s'ha utilitzat la dil·lució isotòpica, mètode que permet corregir els efectes matriu tant en la etapa de tractament de mostra com en la ionització. Per a la resta dels compostos estudiats en aquesta Tesi, no es disposa d'estàndards marcats isotòpicament i en conseqüència s'han implementat altres procediments. En primer lloc s'ha avaluat la possibilitat d'utilitzar rectes de calibració externa preparades en dissolvent. En el cas dels BADGEs i BFDGEs s'ha observat que les rectes de calibració preparades en matriu utilitzant dos matrius blanques diferents, una beguda de cola i pebrot vermell envasats tots dos en vidre i la calibració externa coincideixen tal i com es pot observar a la Figura 3.7 on es mostra a mode d'exemple les rectes de calibració del BADGE·$2H_2O$ preparades en solvent (MeOH:aigua) i en les dues matrius. Aquests resultats han permès proposar la calibració externa per a l'anàlisi dels BADGEs i els BFDGEs en mostres d'aliments enllaunats i en begudes refrescants. En canvi per als fotoiniciadors els resultat obtinguts en preparar la recta de calibració en matriu blanca addicionada, un suc de pinya i un aliment per infants, les quals se sotmeten a tot el tractament de mostra i la calibració externa han posat de manifest un important efecte matriu la qual cosa impossibilita la utilització de la calibració externa. En conseqüència les mostres d'aliments per infants han estat quantificades emprant la recta preparada en l'aliment per infants mentre que la resta de mostres han estat quantificades emprant el suc de pinya.

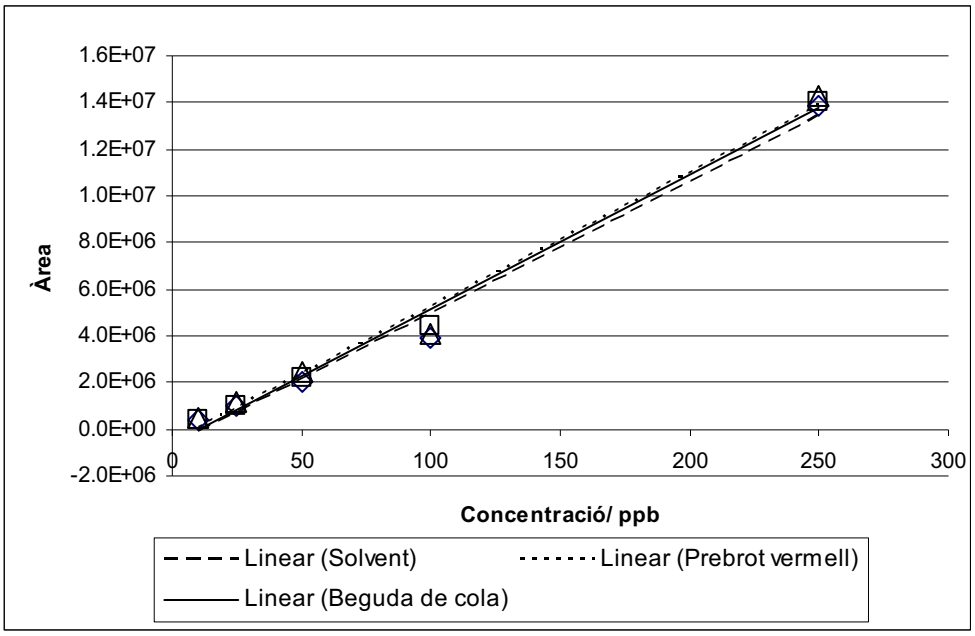

Figura 3.7. Rectes de calibració pel BADGE·2H_2O preparades en solvent i matrix matched en una beguda de cola i pebrot vermell.

En aquesta Tesi i per tal de disminuir l'efecte matriu en la detecció les mostres han estat també analitzades emprant el mode *highly-selective selected reaction monitoring* (H-SRM) que treballa augmentant la resolució dels quadrupols fins a 0,1 *m/z* a FWHM, tant en els ions precursors (Q1) com en els ions producte (Q3). Aquest mode d'adquisició permet augmentar l'especificitat del mètode alhora que millora la sensibilitat ja que es produeix una disminució del soroll químic sense produir una pèrdua significativa del senyal de l'anàlit tal i com ha quedat demostrat al capítol 2 d'aquesta memòria. Aquesta millora en la sensibilitat en treballar amb el mode H-SRM augmentant la resolució en el Q1 s'ha observat tant en l'anàlisi de BADGEs i BFDGEs en mostres d'aliments enllaunats (Taula 3 article IV, apartat 3.2.1) com en l'anàlisi de fotoiniciadors en mostres d'aliments envasats amb cartró (Figura 5 article V apartat 3.2.2 i Taula 2 article VI apartat 3.2.3) on per exemple ha permès detectar i quantificar la presència d'alguns fotoiniciadors en les mostres d'aliments analitzades les quals serien considerades negatives utilitzant el mode SRM. Aquest instrument tal i com s'ha comentat anteriorment permet treballar a una resolució de 0,04 unitats de *m/z* a FWHM i realitzar amb una bona precisió i exactitud mesures de massa exacta tant d'ions precursors com d'ions producte la qual cosa ha permès obtenir en alguns casos una confirmació extra de la presència dels anàlits a les mostres analitzades. L'única excepció ha estat l'anàlisi de la BP dues mostres on ha estat necessari recórrer a

l'espectrometria de masses d'alta resolució utilitzant un Orbitrap a un poder de resolució de 50.000 com analitzador per tal de poder identificar i confirmar la presència d'aquest compost.

Pel que fa referència a la presència de BADGEs i BFDGEs a les mostres estudiades (Article científic IV (apartat 3.2.1) tan sols es van detectar els derivats hidrolitzats dels BADGEs, fet que es pot explicar si es té en compte que aquestes mostres són totes de base aquosa i el medi és àcid, com per exemple en la majoria de les begudes refrescants, condicions que afavoreixen la hidròlisis dels grups epòxid. Per altra banda, a cap de les mostres es va detectar la presència dels BFDGEs, la qual cosa demostra que s'està aplicant correctament la legislació vigent que prohibeix el seu ús en materials que han d'estar en contacte amb aliments. A la Figura 3.8 es representa en forma de diagrama de barres el número de mostres analitzades i els compostos detectats tant en mostres d'aliments com en begudes refrescants. Aquests resultats posen de manifest que el BADGE·$2H_2O$ és present a totes les mostres analitzades, trobant-se a concentracions entre 2.1 μg L^{-1} i 5.1 μg L^{-1} a les mostres de begudes refrescants i entre 157 μg kg^{-1} i 675 μg kg^{-1} als aliments enllaunats, encara que a la pinya les concentracions trobades van ser inferiors i similars a les de les begudes refrescants. A les mostres de begudes refrescants els altres BADGEs no van ser detectats, però en canvi a les mostres d'aliments el BADGE·2HCl es va trobar en un 83% de les mostres analitzades i el BADGE·H_2O i el BADGE·HCl·H_2O en un 67%, mentre que el BADGE·HCl únicament va ser detectat en un 17% de les mostres. En tots els casos els nivells de concentració d'aquests BADGEs van ser inferiors als trobats pel BADGE·$2H_2O$. Aquests resultats coincideixen amb els publicats a la literatura on generalment a les mostres de verdures i fruites de base aquosa es troben els derivats hidrolitzats del BADGE a concentracions que van des de 1 μg kg^{-1} fins a 860 μg kg^{-1} (Berger i cols., 2001, Yonekubo i cols., 2008 & 2009).

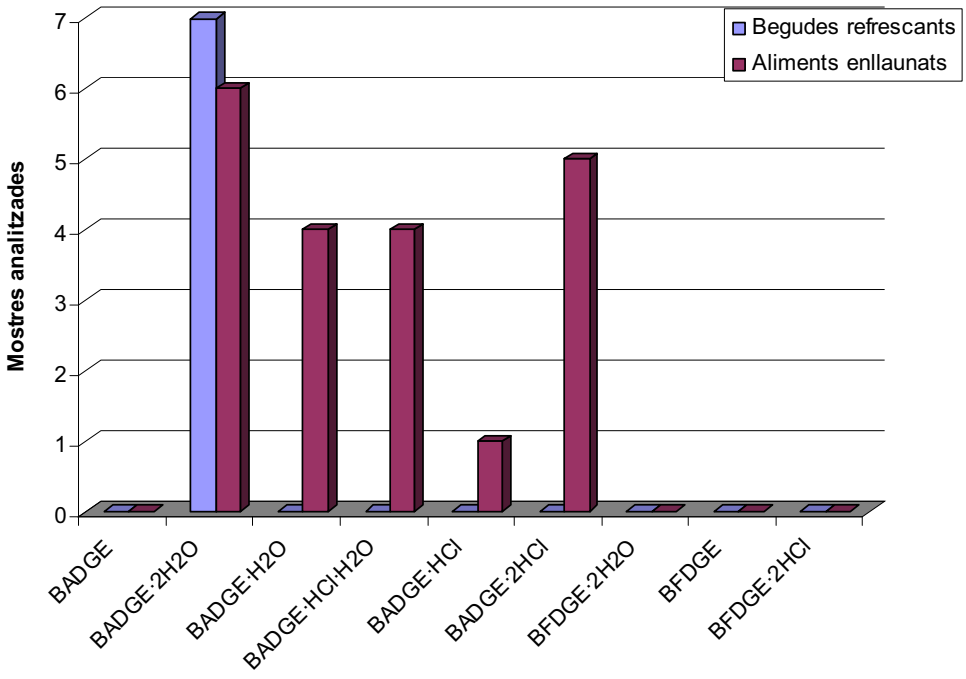

Figura 3.8. Distribució de BADGEs i BFDGEs a les mostres de begudes refrescants i aliments enllaunats analitzats.

El mètode de cromatografia de líquids emprant una columna de fase estacionaria PFPP ha estat utilitzat en l'anàlisi de mostres d'aliments envasats amb cartró així com en els envasos corresponents per tal de determinar diferents fotoionitzadors (Article científic VI, apartat 3.2.3). Dels resultats podem concloure que el 2-ITX, la BP, la DMPA i la EHDAB són els fotoiniciadors més utilitzats en l'impressió dels cartrons ja que tal i com es mostra a la Figura 3.9, on es representa el nombre de mostres positives de cadascun dels compostos en els cartrons analitzats aquests compostos han estat detectats en la gran majoria. Un altre fotoiniciador detectat en un important nombre de mostres ha estat el DEAB (71%). Mentre que altres com el DETX, 4-ITX i PBZ són utilitzats en menor grau. El HMPP i el HCPK no han estat detectats a cap de les mostres analitzades, aquest fet pot ser degut a que per aquests compostos el límit de detecció obtingut és molt superior atesa la menor eficàcia en la ionització en ESI d'aquests dos compostos. El nombre de mostres positives, així com també les concentracions dels fotoiniciadors, han estat inferiors en els aliments analitzats.

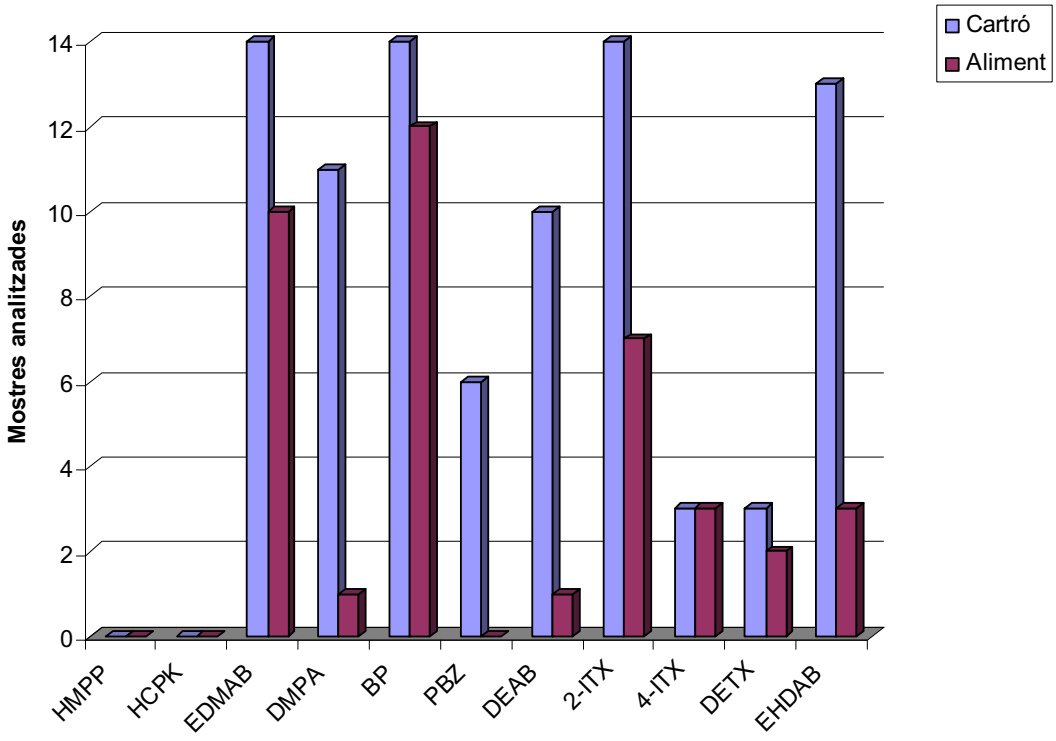

Figura 3.9. Distribució dels fotoiniciadors en els envasos i mostres d'aliments envasats amb cartró analitzats.

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La BP va ser detectada en el 86% de les mostres analitzades a concentracions entre 1.8 μg L^{-1} i 40 μg L^{-1}, la qual cosa pot ser deguda a que aquest compost també s'utilitza en el film de les capes interiors dels empaquetaments multi-capes i per tant pot migrar cap a l'aliment per contacte directe de la mostra amb el film a més de la migració deguda al contacte entre les capes externes dels empaquetaments i les capes internes tal i com succeeix amb els altres fotoiniciadors. Aquests resultats posen de manifest que per a l'ITX dels dos isòmers possibles, el 2-ITX és el que es detecta en un major nombre de mostres (100% dels cartons i 50% dels aliments) a concentracions entre 0.2 μg L^{-1} i 0.8 μg L^{-1} en aliments, mentre que l'isòmer 4-ITX únicament ha estat detectat en el 21% dels cartrons i aliments analitzats i sempre a concentracions inferiors (entre ~MLOD i 0.07 μg L^{-1}). Altres fotoiniciadors detectats als aliments analitzats han estat el EDMAB (50%), EHDAB (21%), DETX (14%), DMPA (7%) i el DEAB (7%), mentre que la PBZ que ha estat detectada en 6 de les 14 mostres de cartrons no s'ha identificat en cap dels aliments analitzats. Cal també destacar que el HMPP i el HCPK no s'han detectat en cap de les mostres d'aliments, tal i com era d'esperar ja

que aquests compostos no havien estat identificats a cap dels envasos. Ara bé cal remarcar que això pot ser degut als relativament elevats límits de quantificació (LOQ) d'aquests compostos, 666 μg L^{-1} i 500 μg L^{-1} respectivament, en front dels poc μg L^{-1} obtinguts per a la resta de compostos. A més els resultats obtinguts de l'anàlisi dels fotoiniciadors en els cartrons i en els aliments posa de manifest que en els processos d'impressió d'aquestes tintes UV generalment es fan servir mescles de fotoiniciadors i que aquests migren cap als aliments.

3.4. CONCLUSIONS

El treball experimental inclòs en el Capítol 3 de la memòria en el que s'han establert mètodes de LC-MS/MS per a l'anàlisi de BADGEs, BFDGEs i fotoiniciadors en aliments ha permès arribar a les següents conclusions:

- L'elevada eficàcia de les columnes de partícules superficialment poroses de 2.7 µm de mida de partícula ha permès proposar un mètode de UHPLC per a l'anàlisi dels diglicidil èters del bisfenols. S'ha aconseguit una bona separació cromatogràfica dels BADGEs, BFDGEs i dels seus derivats hidrolitzats i halogenats en menys de 5 minuts emprant MeOH:àcid fòrmic/formiat d'amoni com a fase mòbil. Per obtenir una adequada separació dels isòmers del BFDGEs es proposa substituir el MeOH per ACN. Aquest mètode acoblat a l'espectrometria de masses en tàndem s'ha utilitzat en l'anàlisi dels BADGEs i dels BFDGEs en mostres d'aliments obtenint un límits de quantificació entre 0,13 µg L^{-1} i 1,6 µg L^{-1} en begudes refrescants i d'entre 1,0 µg L^{-1} i 4,0 µg kg^{-1} en mostres d'aliments enllaunats.

- Es proposa utilitzar una columna de fase estacionaria pentafluorofenil propil per separar (Rs: 1,3) els dos isòmers de l'ITX (2- i 4-ITX). Per analitzar conjuntament l'ITX i altres 9 fotoiniciadors es proposa emprar aquesta mateixa columna a una temperatura de 5ºC i un cabal de 450 µL min^{-1}, condicions que permeten separar tots els compostos en menys de 5.5 minuts. Aquest mètode ha permès obtenir uns límits de quantificació en SRM entre 0,2 µg kg^{-1} i 2,3 µg kg^{-1} en mostres d'aliments envasats amb cartró excepte per a la HMPP i la HCPK que augmenten fins a aproximadament 200 vegades degut a la baixa ionització que presenten en electroesprai.

- Per tal d'incrementar la selectivitat i la sensibilitat dels mètodes es proposa emprar el mode d'adquisició H-SRM augmentant la resolució del primer quadrupol fins a 0,1 *m/z* FWHM. Aquest mode d'adquisició ha permès detectar la presència d'alguns fotoiniciadors a nivells per sota dels límits de detecció del mètode SRM.

- Es recomana emprar la capacitat de mesura de massa exacta del triple quadrupol TSQ Quantum Ultra treballant a una resolució de 0,04 *m/z* FWHM per obtenir informació addicional sobre els compostos presents a les mostres. Els errors en la massa mesurada inferiors als 4 mDa per als ions precursors (H-SIM) i als 12 mDa per als ions producte (H-SRM) han permès confirmar la presència de ITX, BADGE·2H_2O i BADGE·HCl·H_2O als aliments analitzats. El limitat poder de resolució d'aquest instrument (poder de resolució de 4575 per a un *m/z* 183) ha obligat a emprar un Orbitrap (treballant a un poder de resolució de 50000) per confirmar la presència de BP en mostres on coelueix amb una interferència.

- Els resultats obtinguts en l'anàlisi de BADGEs i BFDGEs posen de manifest que el BADGE·2H_2O és present a totes les mostres analitzades degut a que en ser de base aquosa i àcida s'afavoreix la hidròlisi dels grups epòxid. Les menors concentracions trobades en les mostres de begudes refrescants es pot explicar per un efecte de dilució (major volum per unitat de superfície). Els altres BADGEs hidrolitzats detectats (BADGE·H_2O, BADGE·HCl·H_2O i BADGE·2HCl) únicament s'han trobat a les mostres d'aliments enllaunats i a concentracions inferiors que les del BADGE·2H_2O.

- L'anàlisi de fotoiniciadors en envasos ha posat de manifest que els més emprats són la BP, el 2-ITX, la DMPA i el EHDAB i a més, que sempre es troben al menys quatre dels fotoiniciadors estudiats en aquesta Tesi. A les mostres d'aliments el nombre de compostos detectats ha estat inferior als presents en els envasos, excepte la BP que va ser detectada en gairebé totes les mostres analitzades a concentracions entre 1.8 μg kg^{-1} i 40 μg kg^{-1}. Els altres fotoiniciadors (2-ITX, 4-ITX, EDMAB, EHDAB, DETX, DMPA, DEAB i PBZ) han estat detectats en un menor nombre de mostres i menor concentració (entre 0,07 μg kg^{-1} i 2,5 μg kg^{-1}).

CAPÍTOL 4

PRECONCENTRACIÓ EN FASE SÒLIDA EN LÍNIA

4.1. INTRODUCCIÓ

Les baixes concentracions a les que es troba el BPA en mostres ambientals i d'aliments obliga a un exhaustiu tractament de mostra previ a la determinació per cromatografia de gasos o per cromatografia de líquids que en general inclou etapes de filtració, extracció, preconcentració, fraccionament i neteja (*clean-up*). La major part dels mètodes proposats a la literatura per a l'anàlisi de BPA utilitzen extracció en fase sòlida. Ara bé, aquest procediment comporta una sèrie de dificultats i problemes que provenen principalment del fet que el BPA és àmpliament utilitzat a la industria dels plàstics, la qual cosa dóna lloc amb freqüència a la contaminació de les mostres durant el tractament, atès que la majoria dels materials emprats avui dia en els laboratoris de rutina són de plàstic. Un dels objectius d'aquesta memòria és el desenvolupament de mètodes d'anàlisi que permetin minimitzar les possibles fonts de contaminació durant el tractament de mostra. Amb aquesta finalitat s'ha acoblat l'extracció en fase sòlida en línia amb la cromatografia de líquids i s'ha avaluat aquest procediment per analitzar el BPA i altres compostos relacionats en mostres líquides, en concret, aigua i begudes refrescants.

En aquest apartat s'inclou en primer lloc l'article VII intitulat "*Pitfalls in the analysis of bisphenol A: sources and solutions*" on es discuteixen de forma exhaustiva les diferents fonts de contaminació i els problemes que sorgeixen en l'anàlisi de BPA tant aquells que provenen del tractament de mostra com els relacionats amb el procediment d'anàlisi instrumental utilitzat, LC-MS o GC-MS. Aquest article també fa referència a les estratègies a seguir per tal d'obtenir bons resultats en l'anàlisi quantitativa. La segona part de la introducció (apartat 4.1.2) està dedicada a comparar els avantatges i inconvenients dels sistemes de preconcentració en línia (*on-line*) en front dels sistemes fora de línia (*off-line*). En aquest apartat es comenten diferents configuracions de sistemes en línia i a més, atesa la importància que te avui dia reduir el temps d'anàlisi, en aquest apartat també s'ha avaluat la possibilitat de treballar amb cromatografia de líquids ràpida i d'elevada eficàcia (<5 min, $W_{1/2}$< 5s) acoblada a sistemes de preconcentració en línia.

4.1.1. ARTICLE CIENTÍFIC VII.

Pitfalls in the analysis of Bisphenol A: Sources and Solutions

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Chapter 9

PITFALLS IN THE ANALYSIS OF BISPHENOL A: SOURCES AND SOLUTIONS

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1. INTRODUCTION

Due to the widespread use and manufacture of polycarbonate plastics and epoxy resins, Bisphenol A (BPA) (2,2-bis[4-hydroxyphenyl] propane) is frequently found at low concentration levels in environment matrices such as water, river sediments and sewage sludge. Analysis of this compound requires extensive sample treatment, filtration, extraction, pre-concentration and clean-up. Subsequently, highly efficient separation techniques, such as gas chromatography (GC) and liquid chromatography (LC), mainly coupled to mass spectrometry (MS), are generally used for its identification and quantification. In this paper the key problems encountered in the analysis of BPA, the symptoms and remedies are discussed. Accurate determination of BPA at ultra-low concentrations is frequently hindered by many factors mainly attributable to its ubiquity, such as contamination from glassware, syringes, and other materials. Furthermore, water purification systems may contaminate with BPA the purified water. In addition, problems arising from the instrumental analysis when using liquid chromatography and gas chromatography coupled to mass spectrometry (LC-MS and GC-MS) mainly related to derivatization in GC-MS, to low ionization efficiency and ion suppression effects in LC-MS and to fragmentation in tandem mass spectrometry (MS/MS) are also discussed.

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2. SAMPLE TREATMENT

There are a number of critical factors that may all contribute to obtain poor results in the analysis of BPA. Some of these critical factors are associated to manipulations during sample preparation that bring a risk of analyte loss or sample contamination. For instance, the analysis of water samples begins with the preservation, storage and filtration of the samples, steps that may lead to errors in the analysis. When samples are stored the stability of the analyte in the sample matrix should be preserved again losses and degradation. Water samples are generally acidified and methanol (up to 10-20%) is usually added to prevent BPA adsorption in the glass containers and onto particulate matter. In order to prevent BPA degradation by bacterial activity during sample storage ethylenediaminetetraacetic acid (EDTA) up to 10 mM [1] or formaldehyde up to 1% are frequently added [2]. For solid samples refrigeration or storage at -18ºC is recommended to preserve the sample. Filtration is usually used as preliminary step in water analysis and also as the last step before injecting extracts in the GC-MS or LC-MS systems. Particular care must be taken during the filtration since errors can occur when membrane filters are employed. For instance, it has been shown that up to 90% of BPA can be adsorbed onto nylon filters having a significant effect on the reliability of the analytical results. The presence of small amounts of an organic solvent such as methanol (10%) in the water sample helps to prevent this adsorption [3]. Filters of other type of material such as regenerate cellulose do not present this adverse effect, but unfortunately such filters can release compounds which may chromatographically interfere with BPA. One way to minimize the problem of the interferents is to increase the resolving power of both the chromatographic and mass spectrometric systems but it is not always possible. The simplest option to prevent both adsorption of BPA and the introduction of interferents in the sample is to use ultra-centrifugation instead of filtration [3], since this procedure efficiently remove the particulate matter guarantying a high recovery of BPA and avoiding interferents.

Another problem is that BPA is inherently ubiquitous in the laboratory environment, so samples can easily be contaminated during sample treatment. Background contamination of BPA can occur at the ng L^{-1} level, mainly from solvents, SPE cartridges, glassware, plastic ware and other reagents and laboratory tools. Moreover the determination of BPA in environmental samples requires a sample treatment that involves multiple re-dissolving or desorption steps using organic solvents and that makes easier BPA to be leached from different contamination sources. Additionally, in solid phase extraction (SPE), BPA can be introduced from plastic components of the SPE cartridges (cartridges and frits) generally employed to extract and to pre-concentrate BPA in water analysis and to clean-up sediment and sludge extracts. The release of BPA from two of the most common cartridges used for BPA analysis, OASIS HLB and Bond Elute C18, has been studied by Inoue et al., [4], who showed that blank samples analyzed using these cartridges contained concentrations ca. 13 ng L^{-1} of BPA. These authors suggested washing the SPE cartridges with at least 15 mL of methanol to remove this contamination.

Particular care should be taken with the quality of the "purified" water used in the different steps of the analysis such as SPE cartridge conditioning, standard preparation, mobile phase preparation, etc., especially when using high sensitive analytical methods. BPA has been found at concentrations ranging from 20 to 200 ng L^{-1} in ultra high quality (UHQ)

water leached from the plastics and epoxy-resins used in the water purifying equipment. There is also the added difficulty that this water contamination is far too varied making difficult the use of blanks to overcome the problem. As an example, Figure 1 shows the chromatograms of UHQ water obtained with a Milli-Q system early in the morning (Figure 1A) and after the production of ~5L UHQ water (Figure 1B). A decrease in the concentration of BPA (from 200 ng L^{-1} to 25 ng L^{-1}) is observed throughout the day as UHQ water is produced. One way to solve this problem is to remove BPA by passing the UHQ water obtained from the water purifying system through hydrophobic membrane filters where BPA is efficiently retained. This procedure is recommended by Watabe et al., [5] who used C18 filters to obtain BPA-free water. Figure 2 shows both the chromatogram of UHQ water in which BPA was detected, and that obtained when analyzing water filtered through C18 membrane filters. BPA contamination was reduced to concentration levels below the detection limit. UHQ water of LC-MS quality that may be BPA-free is commercially available but always water blanks should be analyzed in order to guarantee that no BPA is present.

Potential leaching of BPA during sample treatment can also occur in the analysis of solid samples as happens for instance, when using pressurized liquid extraction (PLE), technique which is nowadays widely applied to analyze BPA in solid environmental samples such as soils and sludges. Particular care must be taken rinsing stainless-steel cells and filter discs with BPA-free UHQ water and several mixtures of solvents of different polarity such as, dichloromethane/hexane and methanol/acetone before the cells and discs are used to prevent BPA contamination. Furthermore, other laboratory tools such as the syringes used for both sample treatment and LC injection can also yield to BPA contamination. As an example, Figure 3 shows the chromatogram obtained injecting 50 µL of mobile-phase (acetonitrile/phosphate buffer) using a syringe with a cemented needle, where a concentration of BPA of 5 µg L^{-1} was detected [5]. The authors even found this contamination in newly purchased syringes and in syringes washed with methanol, acetonitrile, hexane and chloroform. They suggest that BPA contamination might have come from the adhesive epoxy-resins or resinous parts of the cemented needle.

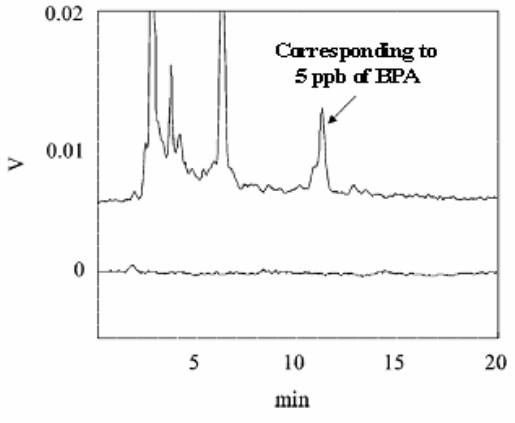

Figure 1. On-line column switching LC-MS/MS chromatograms of BPA in UHQ water (1 mL loaded). A) UHQ water collected in the morning (0.2 ng injected). B) UHQ water collected after 5 L of produced water (0.025 ng injected)

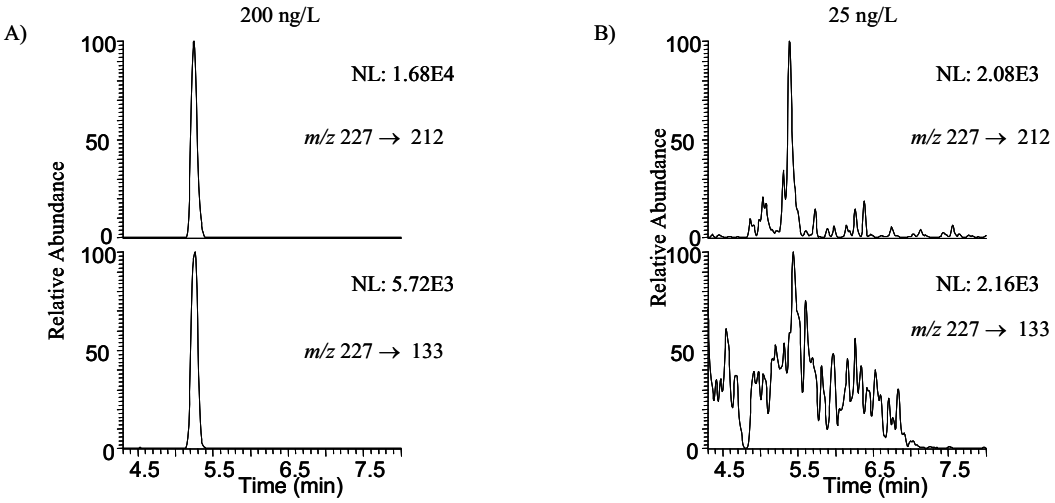

Figure 2. On-line column switching LC-electrochemical detection (50 mL loaded) of UHQ water (0.15 ng injected) and UHQ water filtered through C18 membrane (not detected). Reproduced from the publication of Watabe et al., [5], with the permission from the American Chemical Society, Copyright 2002.

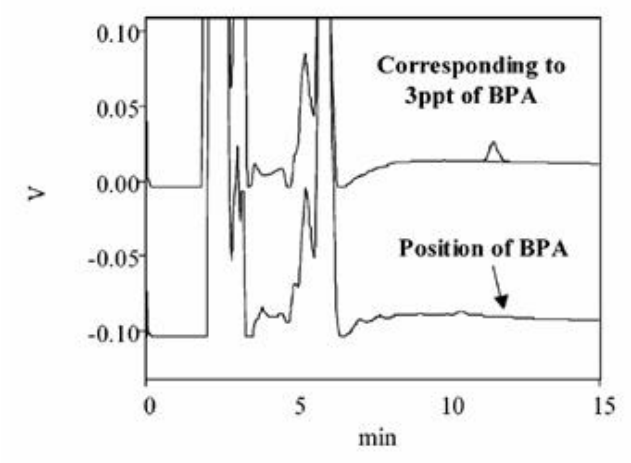

Figure 3. LC-Fluorescence detection chromatograms of mobile phase (50 µL) injected with a cemented syringe (upper) and syringe without cemented needle (lower). Mobile phase, 20 mM sodium phosphate buffer (pH 7.0)-acetonitrile (60:40 8v/v)). Reproduced from the publication of Watabe et al., [5], with the permission from the American Chemical Society, Copyright 2002.

Since various steps of the sample treatment are potential sources of BPA contamination, procedural blanks must be performed for each batch of samples to take the contamination into account. However, there are multiple sources of contamination that are difficult to control and that affect the robustness of the method and frequently to subtract blank responses is not enough for an accurate quantification. For this reason special care should be taken to guarantee the minimal contamination of BPA from the known sources such as solvents, water, laboratory tools, extraction and clean-up procedures, etc. Procedures such as ultra-centrifugation and on-line pre-concentration and/or on-line clean-up protocols coupled to the

LC-MS determination can help to reduce sample manipulation and possible contamination. Thus its use is encouraged whenever it is possible.

3. CHROMATOGRAPHY-MASS SPECTROMETRY

Highly efficient separation techniques, such as gas chromatography and liquid chromatography, mainly coupled to mass spectrometry, are used to identify and quantify BPA in environmental samples [6]. Gas chromatography/mass spectrometry can provide good chromatographic performance and peak shape. However, in order to achieve high sensitivity a derivatization step is frequently used to analyze this compound at low concentrations in environmental samples. For instance, LODs can be decreased up to 200 times as reported by Stuart et al., [7] if derivatization with phenyltrimethylammonium hydroxide (PTA-OH) is performed instead of analyzing BPA directly by GC-MS. Among the derivatization reagents currently used, the silylation reagent N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) is the most popular, especially when electron ionization (EI) is used. The main problem with the derivatization of compounds with more than one hydroxyl group such as BPA, is the formation of several derivatives in varying proportions [8] which hinders the analysis. To guarantee that only both hydroxyl groups are, the addition of trimethylchlorosilane (TMCS) as a catalyst is recommended [9,10]. When negative ion chemical ionization (NICI) is applied, pentafluoro reagents such as pentafluorobenzoyl bromide (PFBBr) and pentafluoropropionic anhydride (PFPA) are used. The fully derivatized compound is quantitatively obtained with both reagents. NICI spectrum of PFB-BPA derivative showed an abundant characteristic ion resulting from the loss of one pentafluorobenzyl radical [11,12]. In contrast, the NICI spectrum of the PFPA derivative is dominated by non-specific fragment ions, which affects the selectivity of the method [13].

Nowadays BPA is more commonly analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) [6]. This technique offers advantages such as simplicity of sample treatment without the requirement of derivatization steps, low detection limit and good selectivity when dealing with complex matrixes. However, problems and difficulties can emerge in the analysis of BPA. Electrospray (ESI) in negative mode is the ionization source most currently used since atmospheric pressure chemical ionization (APCI) is less sensitive for BPA analysis than ESI. At least 4 times higher LODs are obtained in APCI. This can be due to several factors, first to in-source fragmentation of molecular ion (loss of $\bullet CH_3$). This fragmentation takes place even under mild temperature conditions, and it increases at high vaporizer and heated-capillary temperatures [14]. Moreover, proton affinity, which plays an important role in ionization efficiency in APCI, can explain the low ionization efficiency of BPA. The components commonly used in mobile phases for BPA analysis have lower proton affinity than BPA being easily deprotonated.

However, BPA also shows some ionization problems in ESI that make the analysis difficult. Due to the low acidity of the phenolic groups (pKa_1=9.8 and pKa_2=11.3), deprotonated BPA is practically absent in the frequently used methanol/water or acetonitrile/water mobile phases. This is one of the main reasons for the low efficiency in the ESI, since this technique requires target analytes to be ionized in the liquid phase. Some strategies have been used to improve ESI efficiency and thus the sensitivity of the LC-MS

methods. Addition of buffer to the mobile phase (ammonium acetate) and post-column addition of basic solutions (ammonia) have been evaluated in order to increase deprotonated BPA in the liquid phase but in both cases the BPA response could not be improved [14]. Furthermore, mobile phases with high pH and high ionic strength are not recommended in ESI since they increase the electrospray current, thus generating electric problems. On the other hand, one of the main problems in the LC-MS analysis of BPA is ion suppression when using ESI. Ion suppression mainly occurs because of the competition of $[M-H]^-$ with other ions present in the mobile phase during ion evaporation. Buffer additives, sample matrix components and poor chromatographic separation all enhance BPA ion suppression. For instance, the addition of ammonium acetate and ammonium formate to the mobile phase strongly decreases the responses of BPA and its halogenated derivatives when ESI is used in negative mode in combination with methanol as an organic modifier [14-17]. This decrease in response occurs because acetate and formate ions compete with the deprotonated BPA ion. Additionally, methanol generally produces higher BPA responses than acetonitrile, which may be attributable to both the higher pKa values in acetonitrile and to the lower surface tension of methanol, which favors ion evaporation.

Regarding the ion suppression caused by matrix effect, the co-elution of matrix components influences the analyte signal intensity. This effect is especially important for BPA because of the problems in the ionization and ion suppression mentioned above. Improved sample preparation and chromatographic selectivity are the two most effective ways to circumvent this problem. As an example, when analyzing BPA and its halogenated derivatives in surface water, the signals of BPA and monochloro-BPA (MCBPA) were improved by modifying the mobile phase gradient to increase the retention of the analytes and to force their elution into a cleaner chromatographic area. This prevented co-elution with matrix components at the eluting front [18]. This effect is clearly shown in Figure 3, which shows the LC/MS chromatograms corresponding to the analysis of a water sample, using two different gradient elution programs. Nevertheless, it is not always possible to separate the analytes from chromatographic interferences. In such cases, sample treatment must be improved to overcome matrix effects. For instance, the use of an on-line column-switching system with restricted access material (RAM) has been proposed to remove humic acids and other macromolecular matrix components from sediment extracts [19]. Moreover, RAM materials have also been used for on-line LC-MS determination of BPA and 4-octylphenol in human serum samples to reduce the influence of the matrix [20].

In LC-MS, tandem mass spectrometry (MS/MS) is generally proposed to improve selectivity and sensitivity in the analysis of BPA in complex matrices at low concentration. Triple quadrupole mass analyzers (QqQ) are the most popular instruments, due to their selectivity and sensitivity in selected reaction monitoring (SRM) acquisition mode. Nevertheless, other mass analyzers such as ion trap (IT) and hybrid analyzers have also been used in LC-MS/MS to analyze BPA. However, BPA is not well fragmented in tandem mass spectrometry due to the poor fragmentation efficiency in collision induced dissociation (CID) and only two relatively weak product ions are usually obtained. This is illustrated in Figure 5, which shows the MS spectrum of BPA and the tandem mass spectrum at the optimum collision energy. The product ion scan shows one product ion due to the loss of a methyl group $[M-H-CH_3]^{-?}$ (*m/z* 212) and another product ion resulting from the cleavage of the hydroxy-benzyl group $[M-H-C_6H_5OH]^-$ (*m/z* 133). These two ions are frequently monitored for quantification and confirmatory purposes [6] and they are also observed in the MS/MS

spectra obtained with other analyzers such as IT and hybrid instruments (QqTOF or QTrap). Generally, tandem mass spectrometry offers high sensitivity and selectivity, but for BPA, due to the poor MS/MS fragmentation, similar sensitivity can be obtained by monitoring either the precursor ion in SIM mode, or two transitions in SRM mode. Additionally, tandem mass spectra obtained with IT mass analyzers could show product ions at *m/z* higher than the molecular ion (*m/z* 227) due to ion-molecule reactions. For instance, the product ion at *m/z* 244 due to the interaction between the product ion originated by the loss of the methyl group (*m/z* 212) and the methanol used in the mobile phase that remains in the ion trap, has been observed [14].

In summary, ESI in negative mode as ionization source, no additives in the mobile phase and the use of methanol instead of acetonitrile are the recommendations to be followed for the analysis of BPA by LC-MS. In addition, to overcome ion suppression due to matrix components it is recommended to improve the chromatographic separation and also the sample treatment using on-line pre-concentration systems. Finally, to improve the selectivity of the method and to confirm the identity of BPA in environmental samples LC-MS/MS, monitoring two transitions is recommended. Moreover, following the Commission decision 2002/657/EC [21], to confirm the identity of an analyte, an error in the ion ratio between quantitative and confirmatory transitions must be lower than 20%.

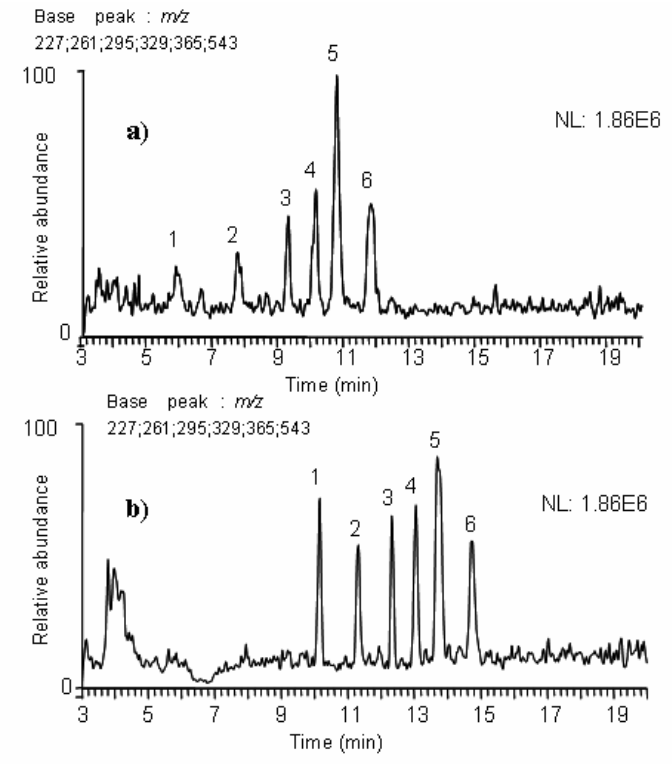

Figure 4. Full Scan LC-MS chromatogram of BPA and its halogenated derivatives using two gradient elution programs: A) Gradient 1 and B) Gradient 2. Peak number: 1. BPA, 2. MCBPA, 3. DCBPA, 4. TCBPA, 5. TeCBPA and 6. TBBPA.. Reproduced from the publication of Gallart-Ayala et al., [14], with permission from John Wiley & Sons, Ltd., Copyright 2007.

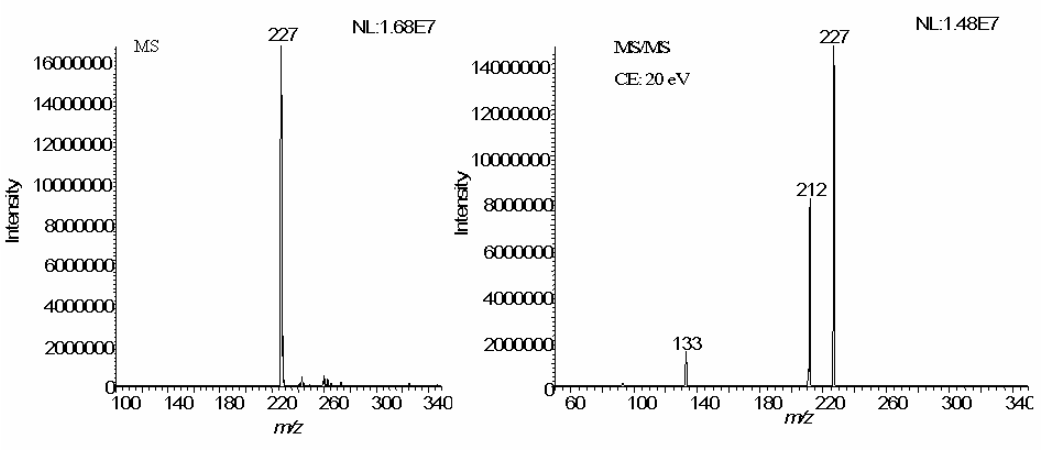

Figure 5. MS and MS/MS spectra of BPA obtained with a triple quadrupole instrument (TSQ Quantum Ultra AM).

4. QUANTITATION

A key prerequisite for accurate analysis is the availability of pure synthetic reference compounds for calibration. In this case, BPA and some labelled compounds are available commercially (BPA-d_{16}, BPA-d_6 and BPA-$^{13}C_{12}$). However, standards may not be available for metabolites or transformation products of BPA. In such cases, the standards must be synthesized. For instance, chlorinated and brominated derivatives of BPA have been synthesized by various laboratories [18,22-25], because only tetrachlorobisphenol A (TeCBPA) and tetrabromobisphenol A (TBBPA) are available commercially.

To overcome matrix effects and problems with recoveries in sample treatment, the use of surrogates and isotope dilution as quantitation method is recommended. This approach allows matrix effects and recoveries to be corrected, since both labelled and native compounds will show the same behaviour. BPA-d_{16} is commonly used to determine BPA in environmental samples by isotope dilution for both LC-MS and GC-MS. Furthermore, BPA-$^{13}C_{12}$ has been proposed as a surrogate in LC-MS for the analysis of water samples [26]. However, in LC-MS (ESI in negative mode) especial care must be taken when selecting the precursor ion of BPA-d_{16} (MW 244 Da) since instead of the de-deuterated molecular ion $[M\text{-}D]^-$ (*m/z* 242) the base peak of the mass spectrum is the ion at *m/z* 241 corresponding to $[(M\text{-}2D+2H)\text{-}H]^-$ due to deuterium/hydrogen exchange when BPA-d_{16} is dissolved in protic solvents such as methanol or water [14,27]. In GC-MS non-protic solvents are generally used to prepare standard solutions. However, this deuterium/proton exchange was also observed when BPA-d_{16} was used as labelled compound in the analysis of BPA without derivatization. Varelis et al., [28] attributed this phenomenon to an electrophilic exchange of deuterium atoms with active hydrogen of the internal surface of the columns. When BPA-d_{16} is derivatized the deuterium/proton exchange does not take place because the deuterium atoms of the hydroxyl groups are replaced by the derivatization reagent [28]. When no labelled standards are available, an alternative quantitation method to correct matrix effects and recoveries is standard addition although it is unsuitable for a large number of samples and so it is not used

frequently. For instance, this procedure has been used for the analysis of halogenated derivatives of BPA in water with LC-MS/MS providing quantitative results at low μg L^{-1} levels [18]. External calibration seems to be the least suitable quantification method, because errors due to recoveries and MS response are not controlled. However, external calibration is frequently used to analyze BPA in different matrices, mainly when a large number of compounds and samples are to be analyzed. In this case special care must be taken to include the analysis of blank samples to monitor the various sources of BPA contamination mentioned above.

5. CONCLUDING REMARKS

The determination of BPA in complex environmental matrices at very low concentration levels (down to ng L^{-1}) poses challenges to analytical chemists. Special care must be taken into account during the analysis to improve the accuracy of the results and to prevent false positives or false negatives. Bacterial degradation during storage must be prevented and in the analysis of water samples, ultracentrifugation is recommended to avoid BPA adsorption on the membrane filters and the introduction of new interfering compounds. Measures such as a thorough washing of cartridges used for both preconcentration and clean-up, must be adopted in order to prevent contamination by the BPA leaching from plastic materials. Moreover, the use of BPA-free water must be guaranteed and the filtration of UHQ water through a C18 membrane filters is recommended when contamination is detected. A good alternative to traditional sample treatment that reduces sample manipulation and prevents sample contamination is the use of on-line systems. Even though so, in the analysis of BPA, blank samples should be performed more frequently than usually to guarantee the absence of contamination.

For routine analysis of BPA, LC-MS/MS is recommended in order to avoid derivatization steps, to reduce sample manipulation and to reduce analysis time. ESI in negative mode provides the highest sensitivity, although no additives should be added to the mobile phase, in order to prevent ion-suppression and to improve ionization efficiency. The LC separation must be improved to prevent the co-elution of BPA with both the elution front and other interfering compounds, since ion suppression can occur and confirmation might be compromised. An improvement of chromatographic resolution with the advantage of short analysis time can be achieved using sub-2μm particle columns. Tandem mass spectrometry with low resolution analyzers provides good selectivity when dealing with complex environmental samples, although the use of mass spectrometric systems of higher resolution can help to avoid false positives. In spite of the poor CID fragmentation efficiency of BPA the sensitivity of tandem mass spectrometry is good enough for the analysis of this compound in environmental samples. Finally, quantitation should be performed by isotope dilution to correct recoveries and to overcome matrix effects.

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4.1.2. SISTEMES DE PRECONCENTRACIÓ EN LÍNIA

Un dels principals inconvenients dels mètodes de preconcentració és que generalment són llargs, lents i tediosos ja que requereixen una sèrie d'etapes abans d'obtenir un extracte adequat per a l'anàlisi instrumental. A més, tal i com s'indica a l'apartat anterior aquests mètodes poden provocar contaminacions i interferències que acaben afectant els resultats de l'anàlisi. Si es comparen els sistemes de preconcentració convencionals fora de línia amb els sistemes en línia, aquests últims presenten una sèrie d'avantatges entre les quals cal esmentar la facilitat per desenvolupar mètodes ràpids i augmentar la productivitat. Això és possible atès que les etapes de condicionament, neteja i elució es poden automatitzar i realitzar de forma simultània amb la determinació cromatogràfica d'una altra mostra. Altres avantatges dels sistemes de preconcentració en línia són la minimització del risc de contaminació de les mostres durant el tractament de mostra i de les pèrdues dels anàlits en l'etapa d'evaporació i/o extracció o les degudes a possibles degradacions durant el tractament de mostra, així com la millora de la precisió i exactitud dels mètodes. La major sensibilitat dels mètodes en línia es deu a la transferència total a la columna analítica de l'anàlit extret, en contraposició als mètodes fora de línia on únicament una alíquota de l'extracte final és analitzada cromatogràficament. Aquesta millora en la sensibilitat dels mètodes de preconcentració en línia permet reduir per una banda la quantitat de mostra a tractar i per l'altra el consum de solvents orgànics .

D'entre les possibles configuracions en línia que es poden emprar per acoblar un sistema de preconcentració a la cromatografia de líquids, en aquesta memòria hem utilitzat l'anomenat "*column switching*". En aquesta configuració s'insereix una petita columna (columna de càrrega) en el bucle de càrrega d'una vàlvula d'injecció de 6–10 ports on s'introdueix un determinat volum de mostra després de les etapes prèvies de condicionament i neteja de la columna de càrrega. Posteriorment, mitjançant la commutació de la vàlvula d'injecció es connecta aquesta columna a la columna analítica i s'elueixen els anàlits transferint-los a la columna analítica mitjançant el gradient d'elució emprat per a la separació analítica. Un dels problemes que presenten els mètodes de preconcentració en línia és el deteriorament de les columnes de càrrega amb el temps, aquest deteriorament pot arribar a afectar d'una manera important la seva selectivitat i capacitat d'extracció. Aquest fet en combinació amb la conveniència de reutilitzar aquestes

columnes, fa necessari dur a terme etapes de regeneració i neteja adequades després de cada anàlisi per tal d'evitar problemes de contaminacions creuades (*"cross contamination"*) entre mostres analitzades consecutivament.

El BPA és generalment analitzat mitjançant mètodes de preconcentració fora de línia, però tal i com hem comentat a l'apartat anterior aquests procediments presenten freqüentment problemes de contaminacions i interferències. Per això es possible trobar a la bibliografia alguns procediments de preconcentració i neteja en línia per a l'anàlisi de BPA en mostres d'aigua (Ou i cols., 2006), llet (Yan i cols., 2009; Ye i cols., 2008) i fluids biològics (Ye i cols., 2008a; Yan i cols., 2009 i Ye i cols., 2008b) que permeten minimitzar aquests problemes i augmentar la sensibilitat dels mètodes.

Un aspecte a destacar és que fins fa ben poc els sistemes de preconcentració en línia disponibles comercialment, com per exemple el sistema PROSPEKT (Programmable On-line Solid Phase Extraction Technique, Spark Holland), el LiChrograph OSP-2 (Merck), el Microlab SPE (Hamilton) o l'ASPEC XL (Gilson) entre d'altres, no podien ser acoblats a sistemes cromatogràfics que treballesin en condicions d'UHPLC a pressions ultra elevades (>6000psi) el quals normalment utilitzen columnes de rebliment de mida de partícula inferior als 2 μm. Aquest fet era degut a que els sistemes convencionals de preconcentració en línia que normalment treballen a pressions inferiors als 6000 psi provoquen una despressurització del sistema d'UHPLC en l'etapa de transferència dels anàlits des de la columna de càrrega a la columna analítica que produeix una pèrdua significativa d'eficàcia i la distorsió dels pics cromatogràfics la qual cosa dificulta de manera important l'acoblament directe entre els dos sistemes. Recentment la empresa Waters (Mallet i cols., 2010) ha introduït al mercat un sistema compacte d'UHPLC amb preconcentració en línia que permet treballar a una pressió total del sistema de 15000 psi tant en la zona de preconcentració com en la de separació cromatogràfica, la qual cosa possibilita l'acoblament directe de l'etapa de preconcentració amb la cromatografia d'elevada eficàcia a ultra elevades pressions encara que implica la utilització de columnes de càrrega especialment dissenyades per suportar aquestes elevades pressions. Cal destacar però que fins a la data d'escriptura d'aquesta memòria no hi ha cap publicació científica que utilitzi i/o avaluï l'aplicabilitat d'aquest nou sistema.

En aquesta Tesis s'ha estudiat l'acoblament directe de sistemes de preconcentració en línia amb la cromatografia de líquids utilitzant columnes amb partícules semiporoses (*Fused core o Core shell*) les quals tal i com hem indicat anteriorment al capítol 3 (apartat 3.2.1) ens aporten la possibilitat de realitzar separacions ràpides d'elevada eficàcia a pressions moderades (<6000 psi). Això permet acoblar de manera fàcil els sistemes de preconcentració i en línia amb la cromatografia ràpida treballant a elevats cabals de fase mòbil.

4.2. TREBALL EXPERIMENTAL

El desenvolupament dels sistemes en línia inclosos en aquest capítol ha implicat en tots els casos una primera etapa d'optimització de la separació cromatogràfica directa sense l'etapa de preconcentració emprant una columna *Fused-Core™* C18 (50 mm x 2.1 mm I.D. i 2.7 μm de mida de partícula) que treballa a una relativa baixa pressió (<6000 psi) per tal d'obtenir una separació cromatogràfica ràpida, amb una bona resolució entre els anàlits estudiats. A més ha calgut optimitzar les condicions de càrrega i elució dels anàlits de la columna de SPE amb la finalitat d'obtenir la major selectivitat i sensibilitat possible. Posteriorment s'ha procedit a acoblar al sistema de preconcentració en línia al sistema cromatogràfic, la qual cosa ha implicat en alguns casos realitzar petits ajustos de les condicions cromatogràfiques finals. En l'etapa de preconcentració en línia s'han optimitzat els paràmetres que influeixen tant en l'extracció com en la transferència dels anàlits a la columna analítica i que afecten les recuperacions i la sensibilitat del mètode. Així, s'han ajustat el cabal i la composició del solvent de la mostra, el volum de mostra a carregar, el temps de transferència, el solvent emprat en aquesta etapa, així com les condicions de treball en les etapes de neteja i condicionament prèvies a l'anàlisi a fi i efecte d'evitar les contaminacions creuades i afavorir la reproductibilitat entre les anàlisis. Les característiques i condicions de treball dels sistemes en línia optimitzats es troben descrites als articles científics VIII i IX inclosos en aquest capítol intitulats *"On-line solid phase extraction fast liquid chromatography-tandem mass spectrometry for the analysis of Bisphenol A and its chlorinated derivatives in water samples"* (artícle VIII) i "*Analysis of bisphenols in soft-drinks by on-line solid phase extraction liquid chromatography-tandem mass spectrometry*" (article IX) dels apartats 4.2.1 i 4.2.2.

Aquests mètodes de preconcentració en línia i cromatografia de líquids ràpida i d'elevada eficàcia han estat acoblats a l'espectròmetre de masses de triple quadrupol TSQ Quantum Ultra AM descrit al Capítol 2 d'aquesta memòria. La font d'ionització emprada en aquests treballs ha estat el *Heated-Electrospray* (H-ESI) que permet treballar en el mode d'ESI convencional, però que a més permet aplicar una temperatura elevada en el vaporitzador per escalfar el gas auxiliar si és necessari (H-ESI). El primer autor en descriure l'avantatge d'augmentar la temperatura en electroesprai fou Ikonomu l'any 1994 (Ikonomu i col., 1994) que va observar un augment en l'abundància dels ions generats per electroesprai quan s'escalfava l'esprai de micro gotes mitjançant un flux de gas calent coaxial a la direcció de l'esprai. Aquest flux de gas coaxial i co-direccional afavoreix, per convecció, el transport de les gotes carregades i dels ions en fase gas cap a l'entrada de l'espectròmetre de masses. A més, l'elevada temperatura del gas facilita l'evaporació del solvent, sobretot de l'aigua, i afavoreix la fissió de les gotes produint gotes carregades més petites. Per altra banda aquest gas auxiliar calent també produeix un augment de l'angle del conus de Taylor fent possible la disminució del voltatge necessari per a la formació de l'esprai. Aquests fets van donar lloc al desenvolupament de la font H-ESI *(heated-electrospray source ionitzation).* Entre les avantatges del H-ESI hom pot citar la millora de la relació senyal/soroll (S/N), l'augment de l'estabilitat de l'esprai, i una considerable millora de l'exactitud i la precisió en l'anàlisi de mostres complexes, ja que la major desolvatació i l'augment de l'eficàcia de la ionització permet minimitzar els efectes de la matriu. A la literatura s'ha publicat algunes aplicacions d'utilització d'aquesta font d'ionització tant en mostres biològiques (Mirza i col., 1993; Ikonomou i col., 1994; Mangrum i col., 2002; Rychlovsky i col., 2002; Benesh i col., 2003 i Daneshfar i col., 2004; Vandenbroucker i cols., 2008; Damen i cols., 2009; Chen i cols., 2009 i Van der Broek i cols., 2010), com en mostres d'aliments (Esparza i cols., 2009; Martínez-Villalba i cols., 2010). En aquesta Tesi s'ha emprat el H-ESI per a l'anàlisi dels bisfenols en mostres de begudes refrescants per tal de reduir l'efecte matriu i millorar la sensibilitat del mètode.

Finalment, amb l'objectiu de millorar la selectivitat i la sensibilitat en la detecció es va treballar amb l'analitzador de triple quadrupol en el mode d'adquisició H-SRM que permet una filtració més selectiva i efectiva dels ions reduint considerablement el soroll de fons sense que es vegi afectada la intensitat

del senyal de l'anàlit obtenint com a conseqüència un important increment de la relació senyal/soroll (S/N). En aquests treballs es van optimitzar les condicions experimentals en espectrometria de masses i es van seleccionar les transicions (ió precursor → ió producte) més abundants i més selectives per tal de realitzar l'anàlisi quantitativa i permetre la confirmació de la presència dels anàlits a les mostres. El mode d'adquisició a baixa resolució SRM (0,7 unitats de *m/z* a FWHM) es va comparar amb els modes de H-SRM on el primer quadrupol (Q1) i/o el tercer quadrupol (Q3) treballen a una resolució de 0,1 unitats de *m/z* a FWHM. Aquest treball experimental es troba recollit als articles científics VIII i IX inclosos en aquest capítol.

4.2.1. ARTICLE CIENTÍFIC VIII.

On-line solid phase extraction fast liquid chromatography-tandem mass spectrometry for the analysis of Bisphenol A and its chlorinated derivatives in water samples

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On-line solid phase extraction fast liquid chromatography–tandem mass spectrometry for the analysis of bisphenol A and its chlorinated derivatives in water samples

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ABSTRACT

In this study an on-line column-switching fast LC–MS/MS method was developed to analyze bisphenol A (BPA) and its chlorinated derivatives in water. Fast liquid chromatographic separation was performed on a C18 reversed phase column based on fused-core particle technology (2.7 μm particle size) providing analysis times shorter than 3 min and high peak efficiencies. The main benefit of this LC system is that it can easily be hyphenated to a conventional on-line preconcentration device allowing the direct analysis of water samples without any pretreatment at concentrations levels down to 60 ng L^{-1} and preventing contaminations frequently reported in the analysis of BPA. This on-line SPE fast LC system was coupled to a triple quadrupole mass spectrometer operating in enhanced mass resolution mode (Q1 FWHM = 0.7 Th, Q3 FWHM = 0.1 Th) in order to minimize interferences and chemical noise. This highly sensitive and selective method was successfully employed to analyze BPA and its chlorinated derivatives in water samples.

1. Introduction

Bisphenol A (BPA) (2,2-bis[4-hydroxyphenyl] propane) is widely used in the production of polycarbonate plastics and phenolic–epoxy resins, which have a variety of applications, such as plastic food containers and epoxy food-can coatings. Additional applications of BPA include printed circuit boards, composites, adhesives, and tooling. Due to the continuous release of BPA into the environment, in comparison to plasticizers such as phthalates, BPA is commonly detected in aquatic ecosystems. In addition, BPA derivatives are found in the environment. These include some halogenated derivatives of BPA, such as tetrachlorobisphenol A (TeCBPA) (commonly used as flame retardants in polymers), and other chlorinated derivatives, such as monochloro-, dichloro- and trichloro-BPA, which are generated by the chlorination of BPA by the residual chlorine used both as a disinfectant in water treatment plants and as a bleaching agent in paper recycling plants. For instance, these chlorinated derivatives have been found at low μg L^{-1} concentrations in effluents from paper recycling plants [1,2].

Due to the large volumes of BPA produced and the corresponding threat of pollution in the aquatic environment, its toxicity has been intensively studied over the last 20 years. This research has shown that it is not genotoxic or carcinogenic, but only slightly toxic [3]. In contrast, the toxicity of halogenated derivatives of BPA, such as tetrabromobisphenol A (TBBPA) and TeCBPA, is greater than that of BPA. This indicates that halogen atoms may play a role in the toxicity of these derivatives [4]. However, the main environmental concern of BPA and its chlorinated derivatives is not their toxicity, but rather their estrogenic potential, which has been confirmed by numerous studies *in vitro* and *in vivo* [5–9]. Because of these characteristics, BPA is known as an endocrine disruptor [10], and is almost ubiquitous in the environment. In addition the European Commission places BPA in the list of priority substances and has registered it in line with the EU legislation for the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).

Studies have been conducted to monitor and quantify low levels of BPA and its chlorinated derivatives in water. Gas chromatography coupled to mass spectrometry (GC–MS) with and without derivatization and/or liquid chromatography coupled to mass spectrometry (LC–MS) are commonly used to analyze these compounds [11]. More recently, LC–MS has become a more popular method since it offers advantages such as simple sample treatment without the need for derivatization steps, low detection limits, and good selectivity when dealing with complex matrixes. In LC–MS, tandem mass spectrometry (MS/MS)—mainly using triple quadrupole mass analyzers (QqQ) in selected reaction monitoring (SRM) acquisition mode—is commonly used to improve selectivity and sensitivity in the analysis of BPA and its halogenated derivatives in complex matrices at low concentrations. Nevertheless, other mass analyz-

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ers such as ion trap (IT) and hybrid analyzers have also been used [12–14].

Since BPA and its chlorinated derivatives are found in the environment at very low concentration levels (ng L^{-1}) preconcentration and clean-up procedures are mandatory. Off-line solid phase extraction (SPE) is commonly used for the analysis of these compounds in water samples and large-sample volumes followed by solvent evaporation steps are always required [15,16]. Most of these procedures are time-consuming and error-prone, for instance Inoue et al. [17] reported that the leaching of BPA from the cartridges used in off-line SPE is similar to the BPA concentration in water environmental samples. Developing on-line sample preparation procedures is a good way to reduce procedural errors, contamination, and analysis time. With such benefits, several papers have been published using on-line SPE coupled to conventional LC (5 μm particle size columns) to analyze BPA in environmental samples (water and sediments) and biological fluids (milk and urine) [18–23] with analysis times higher than 10 min.

To reduce analysis time, the integration of high speed LC columns into on-line SPE-LC systems is generally recommended. Until now, on-line SPE has been hyphenated to short analytical LC columns to analyze water samples [24]. Turbulent-flow chromatography (TFC) and short monolithic columns have also been used on-line with fast chromatographic separation to analyze priority pesticides and emerging contaminants in surface and drinking water [25,26].

Despite the common application of these methods, there is a clear attraction to develop methods that couple on-line SPE systems to ultra high performance liquid chromatography (U-HPLC), which would provide fast and ultra fast run times simultaneous with ultra high efficient chromatographic separations. This would then give high sample throughputs. However, the direct hyphenation of an SPE sample device with a U-HPLC system using a sub-2 μm particle column is challenging. This is mainly due to two factors [27]: firstly, since U-HPLC uses sub-2 μm particle columns combined with high flow rates (up to 1.0 mL min^{-1} with 2.1 mm i.d. columns), high backpressures of up to 1000 bar—which are not directly compatible with the commercially available on-line extraction systems that operate at backpressures <400 bar—can be generated. This is relevant because switching from a low pressure to a high pressure system can produce band broadening and distorted peaks. Secondly, large amounts (several mL) of a strong solvent such as methanol, typically used in the SPE elution step, cannot be directly introduced into the U-HPLC systems without producing band broadening and interfering with retention.

The aim of this research was to evaluate the capacity of an on-line column-switching fast LC–MS/MS method used to analyze BPA and its chlorinated derivatives in water samples. We achieved this using a chromatographic column, based on fused-core technology. These columns provide fast and highly efficient separations under standard LC backpressures (<400 bar). This is because the particles with a 0.5 μm radius shell of porous stationary phase surrounding a 1.7 μm non-porous core exhibit reduced diffusion mass transfer, which allows for high mobile phase flow rates with a similar efficiency and peak capacity to that achieved in columns with sub-2 μm porous particles [28]. The on-line SPE LC–MS/MS method developed in this study was used to analyze BPA and its halogenated derivatives at low concentrations in water samples.

2. Experimental

2.1. Chemicals and consumables

Bisphenol A, 2,2-bis(4-hydroxyphenyl)propane (BPA), bisphenol A-d_{16} (BPA-d_{16}), and tetrachlorobisphenol A, 2,2-bis(3,5-dichloro-4-hydroxyphenyl)propane (TeCBPA) were obtained from Sigma–Aldrich (Steinheim, Germany). Monochlorobisphenol A (MCBPA), dichlorobisphenol A (DCBPA), and trichlorobisphenol A (TCBPA) were synthesized and purified in our laboratory [2]. Methanol (MeOH), acetonitrile (ACN), and water grade LC–MS were purchased from Riedel-de Haën (Seelze, Germany). Ultra high quality (UHQ) water was obtained by purification in an Elix-Milli-Q system (Millipore Corp. Bedford, MA). Stock standard solutions of individual compounds and BPA-d_{16} (10 mg L^{-1}) were prepared in methanol and stored at 4 °C. Intermediate solutions were prepared weekly from the stock standard solutions by appropriate dilution in MeOH:water (10:90). Calibration standard solutions prepared in methanol:water (10:90) ranging from 50 ng L^{-1} to 1000 ng L^{-1} of each compound were prepared daily with 200 ng L^{-1} of the labelled compound BPA-d_{16}. Mobile phases were filtered using 0.45 μm nylon filters (Whatman, Clifton, NJ, USA). In addition, to analyze these compounds precautions had to be taken during the sample treatment to prevent and minimize sample contamination and/or to avoid the loss of the analytes during treatment.

Nitrogen (99.8% pure) supplied by a Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the MS atmospheric pressure ionization (API) source. High-purity argon (Ar_1) purchased from Air Liquide (Madrid, Spain) was used as a collision-induced gas (CID gas) in the triple quadrupole mass spectrometer.

In this study, two columns were used to perform the chromatographic separation of BPA and its chlorinated derivatives: an Ascentis Express C18 (fused-core) column (50 mm × 2.1 mm, 2.7-μm particle size) (Supelco, Sigma–Aldrich) and an Aquity BEH C18 column (50 mm × 2.1 mm, 1.7-μm particle size) (Waters Corp.).

2.2. On-line and chromatographic conditions

A Summit® x2 Dual-Gradient System (Dionex, Sunnyvale, CA) equipped with a Summit P680 dual ternary gradient pump, a TCC-100 Thermostatted Column compartment with a 10-port switching valve and an ASI-100T automated sample injector with a 2500-μL injection loop was used for both on-line preconcentration and chromatographic separation. Chromatographic separation was performed on the Ascentis Express C18 (fused-core) column at 30 °C using a ternary mobile phase (ACN/MeOH/water) and gradient elution mode.

A Hypersil Gold C18 column (20 mm × 2.1 mm, 12 μm particle diameter, 175 Å pore size) (Thermo Fisher Scientific, Whatman, MA) was used for the fully automated SPE on-line trace enrichment procedure. The optimal on-line preconcentration conditions were as follows: 1 mL of sample was loaded onto the SPE column, which had been previously preconditioned with MeOH:water (5:95, v/v), at a flow rate 1 mL min^{-1}; the analytical column was equilibrated to the initial conditions of the chromatographic separation 30:20:50 (acetonitrile:methanol:water) at a flow rate of 600 μL min^{-1}; the SPE column was then washed with 10:90 (v/v) MeOH:water and after 4.3 min the analytes were eluted into the analytical column in back-flush mode at 600 μL min^{-1} after the gradient elution program had started (at 0 min, 30:20:50; from 0 min to 1 min, a linear gradient elution up to 80:20:0 and finally an isocratic step of 5 min at these conditions); finally, 5 min after the back-flush elution of the analytes, the switching valve was switched to the inject position and the SPE column was equilibrated with MeOH:water (5:95, v/v) while the chromatographic analysis was running. A general scheme of the whole procedure is detailed in Fig. 1.

2.3. Mass spectrometry conditions

The on-line solid phase extraction (SPE) liquid chromatography system was coupled to a triple quadrupole mass spectrometer TSQ Quantum Ultra AM (Thermo Fisher Scientific) equipped with

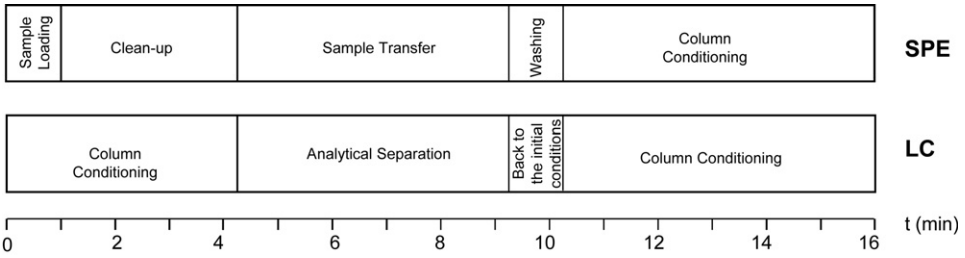

Fig. 1. Time schedule of the on-line SPE column switching LC procedure.

Table 1
Tandem mass spectrometry transitions for the acquisition mode.

Compound	Precursor ion (m/z)[a] $[M-H]^-$	Quantitation		Confirmation		Ion ratio ± SD[d] ($n=5$)
		Product ion (m/z)[b]	CE[c] (V)	Product ion (m/z)[b]	CE[c] (V)	
Bisphenol A	227.1	212.08	20	133.07	26	3.2 ± 0.08
Bisphenol A-d_{16}	241.2	223.10	22	142.00	25	2.8 ± 0.10
Monochlorobisphenol A	261.1	210.07	26	182.07	30	0.83 ± 0.11
Dichlorobisphenol A	295.0	244.03	25	216.04	32	1.0 ± 0.07
Trichlorobisphenol A	328.9	277.99	33	249.99	26	1.9 ± 0.15
Tetrachlorobisphenol A	364.9	313.95	28	285.96	33	1.3 ± 0.1

[a] Mass resolution: 0.7 Th (FWHM).
[b] Mass resolution: 0.1 Th (FWHM).
[c] Collision energy.
[d] Standard deviation.

hyperbolic quadrupoles and an electrospray ionization source (ESI) operating in negative mode. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas, and auxiliary gas at flow rates of 60 a.u., 20 a.u., and 40 a.u. (arbitrary units), respectively. The ion transfer tube temperature was set at 375 °C and the electrospray voltage was −4 kV. Tandem mass spectrometry data were acquired in high-selective selected reaction monitoring (H-SRM) mode using the deprotonated molecule $[M-H]^-$ as a precursor ion.

Two transitions for each compound were monitored using a dwell time of 20 ms and 1 μscan (Table 1). For the low resolution method, both Q1 and Q3 operated at 0.7 Th FWHM (full width half maximum). For the enhanced mass resolution method on Q1, Q1 operated at 0.1 Th FWHM and Q3 at 0.7 Th, while for enhanced mass resolution on Q3, Q1 operated at 0.7 Th FWHM and Q3 at 0.1 Th FWHM. Argon was used as a collision-induced-disociation (CID) gas at 1.5 mTorr and the optimum collision energy (CE) for each transition was optimized. The results are summarized in Table 1. The Xcalibur software version 2.0 (Thermo Fisher Scientific) was used to control the LC/MS system and to process data.

To optimize the source working conditions and to perform the tandem mass spectrometry experiments, 1 mg L^{-1} standard solutions prepared in methanol were infused at a flow rate of 3 μL min^{-1} using the syringe pump integrated into the TSQ Quantum Ultra AM instrument, and mixed with the mobile phase (600 μL min^{-1}, ACN:MeOH:water (50:20:30)) using a Valco zero dead volume tee piece (Supelco, Alcobendas, Spain).

2.4. Samples

Several water samples from different sources were analyzed. These sources included a paper recycling plant (effluent), a wastewater treatment plant (WWTP) from an important industrial area close to Barcelona (influent), a river water and a drinking water treatment plant (DWTP) (influent and samples collected at different points during the water treatment taking into account the hydraulic retention times, HRT) sited in the river Llobregat (Catalonia, Spain). The treatment at the DWTP consisted of the following: prechlorination to break-point, the addition of coagulants and flocculants, sand filtration, and dilution with groundwater in variable amounts to improve water quality. Following this, ozonation, granular activated carbon (GAC) filtration, and a final post-chlorination took place, to ensure the chlorine residual was applied. Water samples were collected in 1-L glass bottles and 1 mL of ascorbic acid (0.1 M) was added to avoid chlorination from the residual chlorine during storage (4 °C). Prior to analysis, 1 mL of MeOH was added to the water samples (1 L) and the particulate matter was removed by centrifugation at 4000 r.p.m.

Bisphenol A was determined using the isotope dilution method. The deuterated standard (BPA-d_{16}) was added at the beginning of the sample treatment. BPA-d_{16} was also used as an internal standard to quantify the chlorinated derivatives since no labelled standards are actually available for these chlorinated compounds. Nevertheless, matrix-matched calibration using surface water free of BPA and its chlorinated derivatives spiked at different concentration levels were used to quantify the samples. The results were calculated including the expanded uncertainty within a 95% of level of confidence.

3. Results and discussion

3.1. Liquid chromatography–tandem mass spectrometry

To analyze BPA and its chlorinated derivatives with LC–MS a reversed phase column (SunFire C18 column, 150 mm × 2.1 mm ID and 3.5 μm particle size, Waters) and a MeOH:water in gradient elution mode (300 μL min^{-1}) were used. Methanol was selected since it provides higher BPA responses in ESI than acetonitrile [2,29,30]. Moreover, any buffer was added to the mobile phase since it has been reported that additives produce ion suppression of BPA and its chlorinated derivatives when ESI in negative mode is used [2,14,29,31,32].

This liquid chromatography separation was coupled to the triple quadrupole mass spectrometer using an ESI source in negative mode. The ESI (negative) full-scan MS spectrum of BPA and MCBPA showed only the isotopic cluster corresponding to the deprotonated molecule $[M-H]^-$ as had been observed previously [2]. Nevertheless, the spectra of the highly chlorinated BPAs showed a double-charged ion at lower m/z values corresponding to the dou-

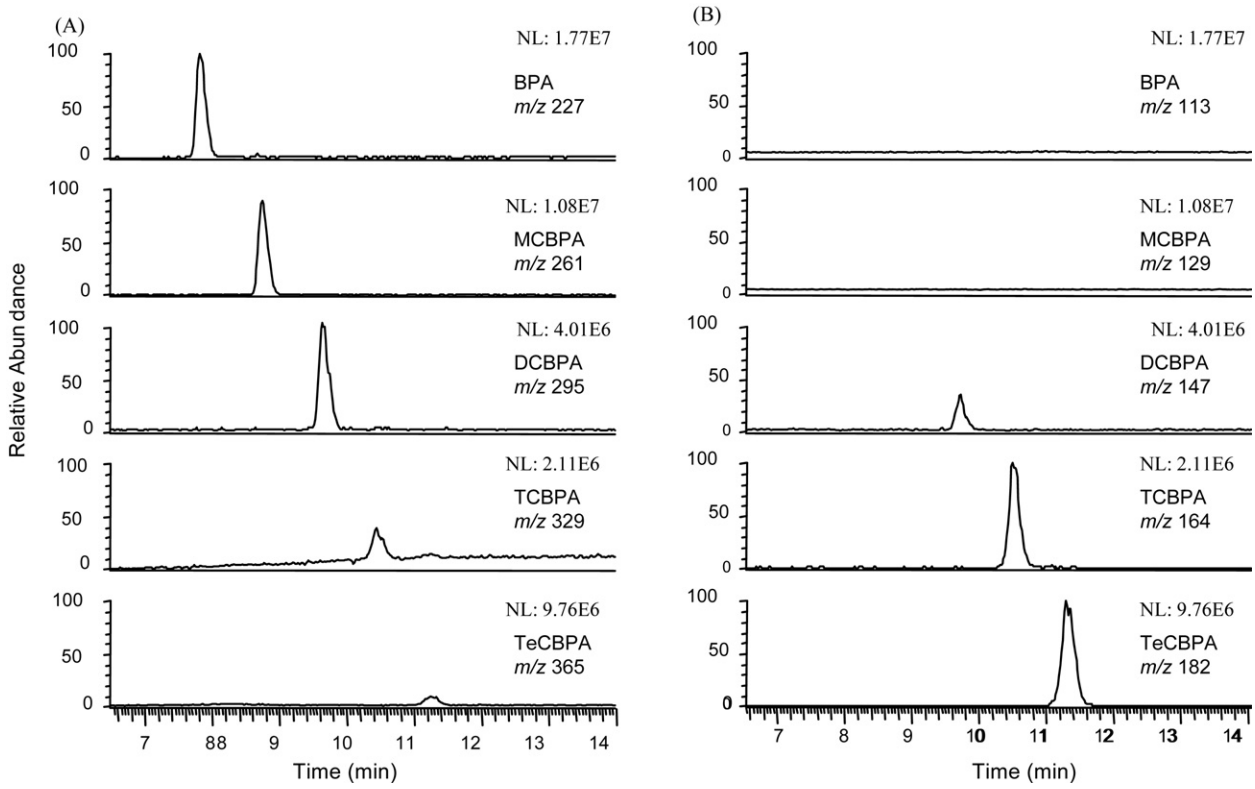

Fig. 2. Full-scan LC–MS chromatograms of BPA and its chlorinated derivatives using a SunFire C18 (150 mm × 2.1 mm ID and 3.5 μm particle size) (Waters) and MeOH:water (60:40) as a mobile phase at flow rate of 300 μL min^{-1}. Ion monitored (A) $[M-H]^-$ and (B) $[M-2H]^{2-}$.

ble deprotonation of both hydroxyl groups $[M-2H]^{2-}$. The relative abundance between the mono-charged and double-charged ions depended on the chlorination degree. An increase on the number of chlorine atoms produced an increase on the signal of the double-charged ion, probably favoured by the lower p*K*a values of the highly chlorinated derivatives of BPA that made easy the double deprotonation in the liquid phase. Fig. 2 shows the LC–MS chromatogram acquired in full-scan mode using a MeOH:water mobile phase. As Fig. 2 shows poor responses were obtained for the chlorinated derivatives of BPA when the mono-charged ion $[M-H]^-$ was monitored. In contrast, responses were more prominent for the highest chlorinated derivatives when the double-charged ions $[M-2H]^{2-}$ were recorded. Furthermore, when using ACN instead of MeOH only the mono-charged ions were observed while the responses for BPA and MCBPA decreased (from 1.5 to 2 times). Therefore, to obtain the best responses for BPA and MCBPA and to avoid the formation of the double-charged ions for the highly halogenated derivatives, ternary mobile phases ACN:MeOH:water were tested and the best separation and detection was obtained using a mobile phase of 30:20:50. In order to improve the efficiency of the separation and reduce the analysis time two columns were evaluated, one based on fused-core particle technology (Ascentis Express C18) and the other a sub-2 μm particle size column (Aquity BEH C18). Both provided a base line separation for all the chromatographic peaks with high efficiencies, which were similar or slightly better for the fused-core column with the additional advantage of lower backpressure (300 bar in front of 725 bar) and obtaining in both cases the separation in less than 3 min at a flow rate of 600 μL min^{-1}.

Under these conditions, tandem mass spectrometry conditions were optimized (collision energies, CID gas pressure, and spray voltage) using the highest ion on the isotopic cluster as a precursor ion. The product ion scan spectrum of BPA showed two abundant ions, one at *m*/*z* 212—the base peak—and the other at *m*/*z* 133 corresponding to the fragments $[M-H-CH_3]^-$ and $[M-H-C_6H_6O]^-$, respectively. For the chlorinated derivatives of BPA (MCBPA, DCBPA, TCBPA, and TeCBPA) the two most abundant product ions resulted from the loss of a CH_4Cl and from the loss of C_2H_4OCl, in agreement with those observed in an ion trap mass analyzer [2]. These product ions were selected for quantification and confirmation of the chlorinated BPA derivatives in water samples (Table 1).

3.2. On-line SPE procedure

There are several critical factors that may contribute to poor results in the analysis of BPA. Special care must be taken into account during sample manipulation due to background contamination at ng L^{-1} mainly coming from solvents, SPE cartridges and plastic ware [17,20]. In this work, in order to minimize sample manipulation and to prevent sample contamination an on-line solid phase extraction liquid chromatography method was developed. For this purpose the two fast LC separations commented above in Section 3.1 have been coupled to an on-line SPE method. The coupling of the sub-2 μm particle size column to the SPE was not possible due to the big difference between the backpressure of both systems (<400 bar for the SPE and >750 bar for the LC). In contrast the lower backpressure obtained with the fused-core column (300 bar) allowed the direct hyphenation, So this last method has been used for further studies.

To achieve the highest level of sensitivity and maximize recoveries we ensured optimal working conditions (e.g. sample load flow rate, loaded sample volume, washing solvent) during the preconcentration step. Sample load flow rate is limited by the highest pressure admissible by the SPE column (130 bar) and by the lowest flow rate required to ensure the equilibration of the enrichment column, improving the capacity for retaining analytes. Based on these factors, a flow rate of 1 mL min^{-1} was selected to load the water

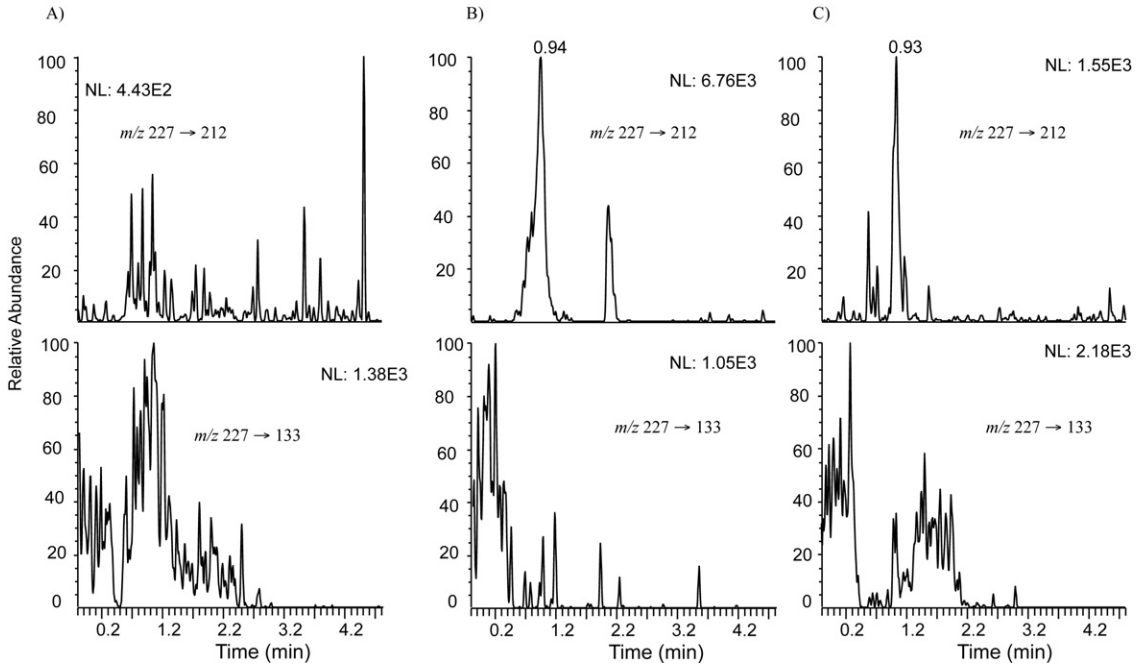

Fig. 3. On-line SPE LC–MS/MS of: (A) UHQ LC–MS water, (B) UHQ LC–MS water filtered through a nylon filter, and (C) UHQ LC–MS water filtered through a regenerate cellulose filter.

samples into the SPE-column. The sample volume to be loaded was also limited by the volume of the vials (4 mL) and the loop volume (2.5 mL). A sample load of 1 mL was enough to ensure sufficient sensitivity to enable the detection of these compounds at concentrations as low as ng L^{-1} and over a reasonable sample loading time.

When analyzing water samples, we aimed to reduce the interfering compounds that could affect both the selectivity and sensitivity of the method, by adding a washing step after sample loading and before the introduction of the analytes into the analytical column. Several water:MeOH mixtures were tested to evaluate the effect of MeOH concentration in the washing solvent. For this reason a sample of surface water from a mountain river (river Garonne, Vall d'Aran, Spain) free from the studied compounds was used and was spiked with 200 ng L^{-1}. The best solvent composition was 10:90 MeOH:water at which level we were able to ensure a balance between the reduction of interferences and the recovery of analytes. Finally, on-line SPE recoveries were estimated by comparing the signal obtained by the direct injection into the analytical column (10 μL) of the surface mountain river water spiked at 10 μg L^{-1} (100 pg injected) with the signal obtained after loading 1 mL of the same sample spiked at 100 ng L^{-1} in order to load the same absolute amount (100 pg injected) into the SPE column. The recoveries ranged from 85% to 100%. Sample carry over from sample-to-sample is a major problem in on-line processes and in order to prevent it in the on-line SPE system, the system was flushed with 100% MeOH (5 min) after the complete elution of the analytes and again after the SPE column was conditioned with 5:95 MeOH:water. Analyses of blank samples were performed for every batch of samples to control carry over and background contamination. No significant signals were observed in these blank samples after a full working day.

BPA can be found at ng L^{-1} in (ultra high quality) UHQ water leached from plastic and epoxy resins used in the purifying equipments [20]. Therefore, to guarantee no BPA contamination in the UHQ water used as a solvent in this method, purified water samples obtained with an Elix-Milli-Q system and an LC–MS grade water samples were analyzed. BPA was found at concentrations ranging from 20 ng L^{-1} to 200 ng L^{-1} in the UHQ water obtained from a Milli-Q system. This range of contamination is particularly varied, which makes it difficult to use blanks to overcome the problem. For instance, a decrease in the concentration of BPA (from 200 ng L^{-1} to 25 ng L^{-1}) was observed throughout the day as UHQ water was produced. Since BPA was not detected in the LC–MS grade water, the UHQ water was used as a solvent to develop the method.

Filtration is currently used as preliminary step in water analysis to eliminate particulate matter. Since the BPA analysis revealed problems of background contamination due to the ubiquity of this compound in the laboratory environment, this step was also evaluated. Two types of membrane filters with a pore size of 0.45 μm were tested, one made of nylon and the other of regenerated cellulose. UHQ water samples free from BPA and spiked at 500 ng L^{-1} with the analytes were filtered using both types of membrane filters and then analyzed.

When nylon membrane filters were used up to 90% of BPA and its chlorinated derivatives disappeared, probably by adsorption onto the membranes. The addition of small amounts of an organic solvent such as methanol (10%) to the water sample before the filtration step prevented this adsorption. Regenerated cellulose membrane filters did not produce this adverse effect, but unfortunately such filters, as also occurred with the nylon ones, released some compounds that interfered chromatographically with BPA making BPA quantification difficult. Fig. 3 shows the chromatogram corresponding to the analysis of 1 mL of UHQ LC–MS water blank (Fig. 3(A)) and the same water sample (5 mL) after being filtered through the nylon filter (Fig. 3(B)) and the regenerated cellulose filter (Fig. 3(C)). In both cases, impurities were eluted at the same retention time as the BPA, and as a result could not be determined. To avoid adsorptions and to prevent the any interference, 10% of MeOH was added to the water sample and to eliminate the particulate matter, the water samples were centrifuged at 4000 r.p.m. rather than filtered.

3.3. On-line SPE fast liquid chromatography–enhanced mass spectrometry

It is well known that in the analysis of complex matrices, increasing the mass resolution power reduces interferences. Nev-

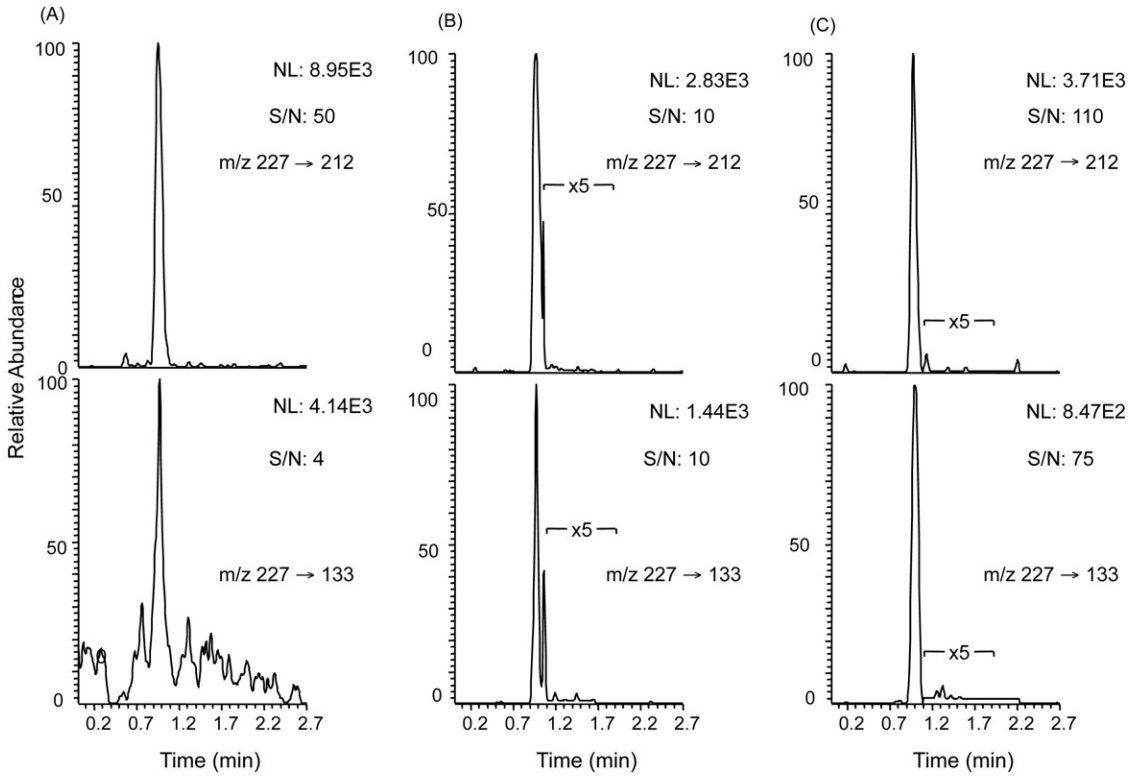

Fig. 4. On-line SPE LC–MS/MS chromatogram of a surface water sample spiked with bisphenol A at 100 ng L^{-1}. Acquisition mode: (A) SRM, (B) H-SRM on Q1, and (C) H-SRM on Q3.

ertheless, this approach forces the use of high resolution mass spectrometry systems such as time-of-flight analyzers but sacrificing sensitivity and linearity. In this study enhanced mass resolution capabilities of a hyperbolic triple quadrupole were used to determine BPA and its derivatives in water samples. To evaluate the performance of the enhanced mass resolution mode, a surface water sample free from the studied compounds spiked at 100 ng L^{-1} was analyzed using the enhanced mode on Q1 (Q1 FWHM = 0.1 Th, Q3 FWHM = 0.7 Th) and on Q3 (Q1 FWHM = 0.7 Th, Q3 FWHM = 0.1 Th). The results were compared with those obtained at low resolution (Q1 FWHM = 0.7 Th, Q3 FWHM = 0.7 Th). When the m/z window was reduced to 0.1 Th FWHM the mass resolving power increased to *ca.* 3.000 for this family of compounds (MW ≈ 300 Da) and was enough in most cases to avoid potential interferences with endogenous compounds in the water samples.

As can be seen in Fig. 4 where the on-line SPE LC–MS/MS chromatograms using the three acquisition modes are shown, the cleanest chromatograms and the best signal-to-noise ratio (S/N) were obtained for the H-SRM mode with enhanced mass resolution in Q3. Enhanced mass resolution on the precursor ions (Q1 at 0.1 Th FWHM) has been generally used [33–37] showing a good performance, but in our case enhanced mass resolution on Q3 showed the best selectivity and sensitivity for BPA (Fig. 4(C)) and it was selected as acquisition mode. Nevertheless, for the chlorinated derivatives of BPA no differences using enhanced mass resolution on Q1 or on Q3 were observed.

To evaluate the performance of the on-line SPE fast LC–MS/MS (H-SRM) method, quality parameters such as limit of quantitation (LOQ), run-to-run precision, ion-ratio precision, and linearity were studied. To estimate the method limits of quantitation (MLOQs) based on a signal-to-noise ratio of 10, UHQ LC–MS water free from BPA was spiked at very low level (down to 10 ng L^{-1}) and 10% MeOH was also added to each sample before the analysis. The values obtained are summarized in Table 2. The developed method provided similar MLOQs for all the compounds ranging from 57 ng L^{-1} to 60 ng L^{-1}, which was enough to determine the presence of BPA and its chlorinated derivatives in water samples.

Calibration standards between 50 ng L^{-1} and 1000 ng L^{-1} were prepared by diluting the stock standard solutions in water and adding 10% MeOH to each calibration standard before analysis using on-line SPE LC–MS/MS (H-SRM). The calibration curves based on the peak area ratio ($A_{compound}/A_{labelled\ compound}$) showed good linearity for all compounds ($r^2 > 0.9996$) in the working range. Run-to-run precision was estimated from the data obtained when

Table 2
Method limits of quantitation and repetitivity of the on-line column switching LC–MS/MS.

Compound	Method limits of quantitation (ng L^{-1})			Run to run, %RSD ($n = 5$)	
	LC–MS water	Surface water	Wastewater	Low concentration[a]	Medium concentration[b]
BPA	57	57	115	13	11
MCBPA	57	57	176	13	5
DCBPA	60	60	183	11	8
TCBPA	60	60	180	14	3
TeCBPA	57	57	140	14	3

[a] Surface water spiked at 50 ng L^{-1}.
[b] Surface water spiked at 300 ng L^{-1}.

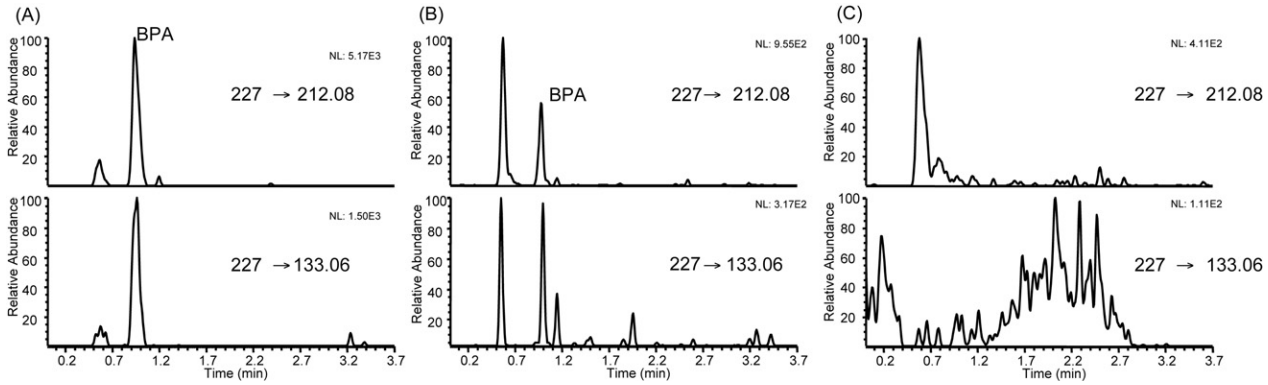

Fig. 5. On-line SPE LC–MS/MS chromatogram of water samples: (A) river water sample, (B) water sample after prechlorination step, and (C) water sample after sand filtration.

analyzing five replicates of a surface water sample spiked at two concentration levels, 100 ng L^{-1} and 500 ng L^{-1}. Precision values expressed as relative standard deviations (RSDs) based on concentration were slightly higher at low concentration levels (50 ng L^{-1}) but always below 15% (Table 2).

To confirm the identity of an analyte, an error in the ion ratio between both quantitative and confirmatory transitions had to be lower than 20%. The ion ratio for bisphenols was evaluated (Table 1). The results ranged from 0.83 to 3.2 with a relative standard deviation below 10% ($n = 5$).

3.4. Analysis of water samples

To evaluate the feasibility of the method for the analysis of BPA and its chlorinated derivatives in water, a surface water sample and a wastewater sample, were analyzed. The surface water was collected from the Garonne River (Vall d'Aran, Spain) and the wastewater was from the influent of a wastewater treatment plant (WWTP). Both samples were analyzed and no BPA or chlorinated derivatives were detected. They were therefore used to estimate the method limit of quantitation (MLOQs) by spiking these blank samples at very low concentration levels. Surface water provided MLOQs ranging from 57 ng L^{-1} to 60 ng L^{-1}, as the instrumental ones estimated using UHQ LC–MS water (Table 2). However, MLOQs estimated from the wastewater sample (115–183 ng L^{-1}) were only 2–3 times higher than those obtained for surface water. This is probably due to the slight matrix components that affected the electrospray ionization efficiency.

Finally, the developed method was used to analyze BPA and its chlorinated derivatives in several water samples: river water, samples from a drinking water treatment plant (DWTP) collected at different sampling points during the water treatment process and an effluent from a paper recycling plant. BPA was detected in two river water samples at 101 ± 22 ng L^{-1} (October 2008) and 322 ± 70 ng L^{-1} (September 2008) collected at the entrance of the DWTP. To study the effectiveness of the treatments performed in the DWTP, several sampling points after each step of the water treatment were collected and analyzed. The majority of BPA was eliminated (>85%) during the prechlorination step due to its high reactivity with chlorine. This is in agreement with the results

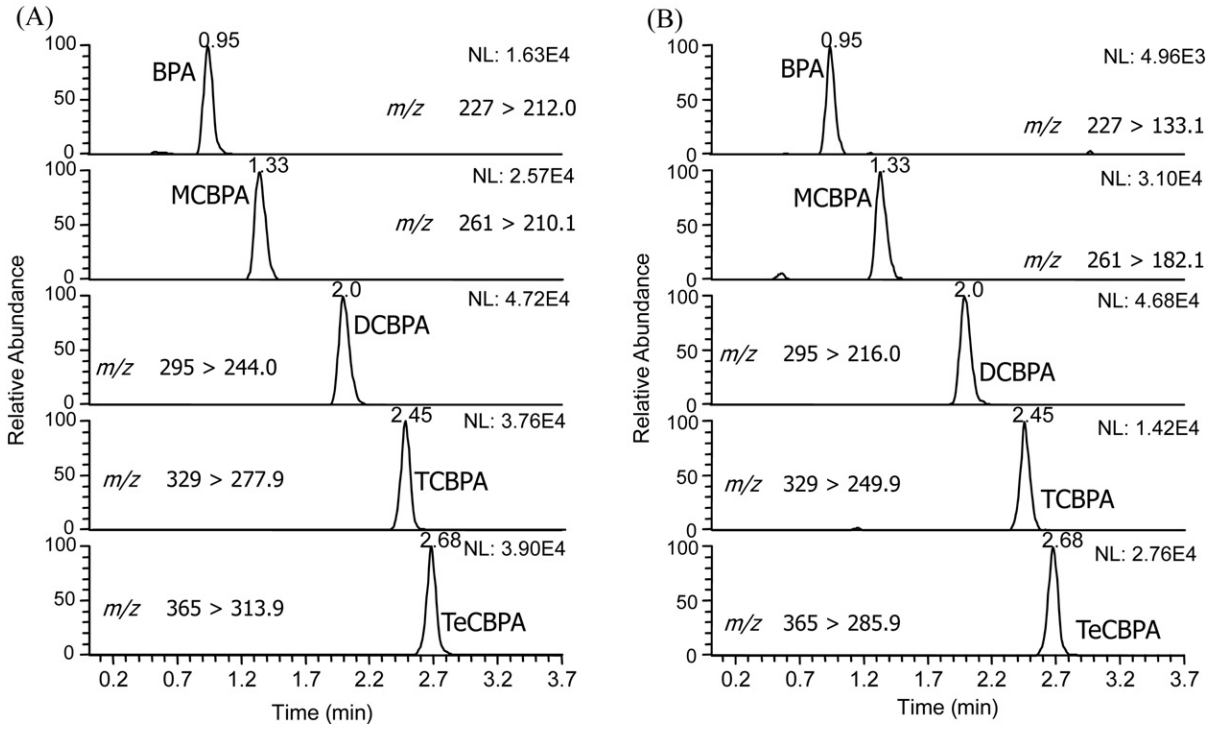

Fig. 6. On-line SPE LC–MS/MS chromatogram of an effluent wastewater sample from a paper recycling plant: (A) quantitation transitions and (B) confirmation transitions.

obtained by Gallard et al. [38], who studied BPA degradation by chlorination in water containing humic substances and observed that it was eliminated due to the formation of its chlorinated derivatives. Chlorinated derivatives were not detected in the samples collected in the DWTP, probably because of their low concentration. The residual BPA was eliminated during the sand filtration step. For example, Fig. 5 shows the BPA extracted chromatograms of river water (Fig. 5(A))—where BPA was detected at $322 \pm 70\ ng\ L^{-1}$—and also that of the same water collected after the prechlorination (Fig. 5(B)) and sand filtration steps (Fig. 5(C)).

The chlorinated derivatives of BPA were detected in the effluent wastewater of the paper recycling plant (Fig. 6) at concentrations of MCBPA $739 \pm 80\ ng\ L^{-1}$, DCBPA $836 \pm 134\ ng\ L^{-1}$, TCBPA $460 \pm 90\ ng\ L^{-1}$, and TeCBPA $530 \pm 72\ ng\ L^{-1}$. These results are in agreement with those reported by other authors in studies of water samples from paper recycling plants in Japan [39], in which BPA was also detected at $679 \pm 100\ ng\ L^{-1}$.

4. Conclusions

In this study an on-line SPE fast LC–MS/MS method was developed for the analysis of BPA and its chlorinated derivatives in water. The low backpressure provided by the use of a fused-core column in the chromatographic separation allowed the direct hyphenation of a conventional on-line SPE system with U-HPLC obtaining high peak efficiencies and base line separation of the studied compounds in less than 3 min. Additionally, the use of enhanced mass spectrometry (H-SRM) working at 0.1 Th FWHM on Q3 provided good sensitivity and selectivity for the analysis of these compounds at a concentration of $ng\ L^{-1}$ in water samples.

With this methodology we did not experience problems of contamination caused by the release of BPA during sample treatment and by the presence of interfering compounds. It can therefore be considered a valuable and attractive tool for use in the routine monitoring of these compounds in water. The method developed was used to the analysis BPA and its chlorinated derivatives in different types of water samples. BPA was detected at low concentrations in river water at the entrance of a DWTP but was eliminated during the two first steps of the treatment (prechlorination and sand filtration). The chlorinated derivatives of BPA, however, were detected in the effluent wastewater of a paper recycling plant.

Acknowledgements

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4.2.2. ARTICLE CIENTÍFIC IX.

Analysis of bisphenols in soft-drinks by on-line solid phase extraction liquid chromatography-tandem mass spectrometry

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Analysis of bisphenols in soft drinks by on-line solid phase extraction fast liquid chromatography-tandem mass spectrometry

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Abstract

In this study, an automated on-line solid-phase extraction coupled to fast liquid chromatography-tandem mass spectrometry (on-line SPE fast LC-MS/MS) method was developed for the simultaneous analysis of bisphenol A (BPA), bisphenol F (BPF), bisphenol E (BPE), bisphenol B (BPB) and bisphenol S (BPS) in canned soft drinks without any previous sample treatment. A C18 (12 μm particle size) loading column was used for the SPE on-line preconcentration before the liquid chromatography baseline separation of bisphenol compounds using a C18 Fused Core™ (50 x 2.1 mm i.d.) column, which took less than 3 minutes. Gradient elution and heated electrospray were used to reduce matrix effect and improve ionization efficiency. To select the most intense and selective transitions, fragmentation studies were performed by multiple-stage mass spectrometry in an ion trap mass analyzer and tandem mass spectrometry in a triple quadrupole instrument, this latter instrument being used for quantitation in SRM mode. Quality parameters of the method were established and we obtained a simple, fast, reproducible (RSD values lower than 10%) and accurate (precision higher than

93%) method for the analysis of bisphenols in canned soft drinks at the ng L^{-1} level using matrix-matched calibration.

Keywords: Bisphenols, soft drink beverages, Fused-Core^{TM} column, on-line SPE.

1. Introduction

Food and beverage cans are internally coated with epoxy-based lacquers to prevent food coming into direct contact with the metal of the can. Bisphenol A (BPA) is currently used in the synthesis of these epoxy resins. Therefore, foodstuffs may be expected to contain residual BPA due to migration from the epoxy resin coatings. European legislation has established 0.6 mg kg^{-1} as the migration limit for BPA in articles intended to come into contact with foodstuffs [1]. Both the tolerable daily intake (TDI) set by the EU Commission [2] and the reference dose (RfD) established by the U.S. Environmental Protection Agency (US EPA) [3] are 0.05 mg BPA/kg body weight/day, whereas Health Canada established a provisional TDI for BPA at 25 μg kg^{-1} of body weight/day [4]. Due to these restrictions, other bisphenol compounds such as bisphenol F (BPF), bisphenol B (BPB), bisphenol E (BPE) and bisphenol S (BPS), considered as substitutes for BPA in industrial applications [5], are starting to be used for the production of epoxy resins. However, no maximum residue levels or migration limits have been established to date for these compounds in food. As regards toxicity, abundant data for BPA are available, although less information has been published on the other compounds. BPF, BPE and BPB have shown moderate to slight acute toxicity and an estrogenic activity similar to BPA [5], whilst BPS exhibited higher estrogenic activity, probably due to its polarity and the presence of sulfur in the structure [6].

Bisphenol A has been detected in canned food at relatively high concentrations ranging from 95 μg kg^{-1} to 842 μg kg^{-1} [7-14], as well as in lower amounts in canned soft drinks (0.032 – 4.5 μg L^{-1}) [13,15]. In contrast, there are few data on the concentrations of the other BP compounds in canned food; Grumetto et al.,[16] determined BPB (27.1-85.7 μg kg^{-1}) in canned peeled tomatoes and Viñas et al., [17] analyzed BPS in canned food, 11.5 – 175 μg L^{-1} in the supernatant and <LOD – 36.1 μg kg^{-1} in the food.

For the determination of BPs in foodstuffs, liquid chromatography or gas chromatography coupled to mass spectrometry (LC-MS and GC-MS) is generally used. When these compounds are analyzed by GC-MS, a derivatization step is recommended in order to increase the volatility of the compounds and to improve sensitivity in mass spectrometry. Since derivatization in GC-MS requires additional sample manipulation, thus increasing analysis time and reducing reproducibility, LC-MS has been used as an alternative technique in recent years for the analysis of this compound [18,19]. Until now, no more than two BPs have been analyzed simultaneously, BPA and BPB by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [16] and BPA and BPS by GC-MS [17].

Methods for analyzing BPA in food samples have been reviewed recently [18], showing that liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the most common sample preparation treatments. However, background contamination by BPA released from laboratory plasticware can generate significant errors in analyses at very low concentration levels [20,21]. A good alternative method which avoids this problem and minimizes sample manipulation is the use of on-line SPE.

The aim of this study was the simultaneous analysis of five bisphenolic compounds (BPA, BPF, BPE, BPS and BPB) in beverages by updating the SPE fast LC-MS/MS

method recently developed by our research group for the analysis of BPA and its halogenated derivatives in water [22]. Matrix effects were evaluated in order to propose a method for the routine analysis of these compounds in canned soft drinks.

2. Experimental

2.1. Chemicals and reagents

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA), bisphenol A-d_{16} (BPA-d_{16}), bisphenol S (bis-(4- hydroxyphenyl)sulfone; BPS), bisphenol F (bis(4-hydroxyophenyl)methane, BPF) and bisphenol E (bis(4-hydroxyophenyl)ethane, BPE) were obtained from Sigma-Aldrich (Steinheim, Germany). Meanwhile, bisphenol B (bis(4-hydroxyphenyl)butane, BPB) was purchased from TCI Europe (Zwijndrecht, Belgium). The structures of the studied compounds are given in Figure 1.

Methanol (MeOH), acetonitrile (ACN) and water LC-MS grade were purchased from Riedel-de Haën (Seelze, Germany). Stock standard solutions (10 mg kg^{-1}) were individually prepared by weight in methanol and stored at 4ºC. Intermediate solutions were prepared weekly from the stock standard solution by appropriate dilution in water. Calibration standard solutions ranging from 50 ng L^{-1} to 10 μg L^{-1} of each bisphenol compound were prepared daily containing 400 ng L^{-1} of the internal standard (BPA-d_{16}). Mobile phases were filtered using 0.22 μm membrane nylon filters (Whatman, Clifton, NJ, USA) and samples were centrifuged at 4000 r.p.m before analysis by LC-MS/MS.

Nitrogen (99.98% pure) supplied by Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the API source. High-purity Argon (Ar_1) purchased from Air

Liquide (Madrid, Spain), was used as a collision-induced-dissociation gas (CID gas) in the triple quadrupole instrument.

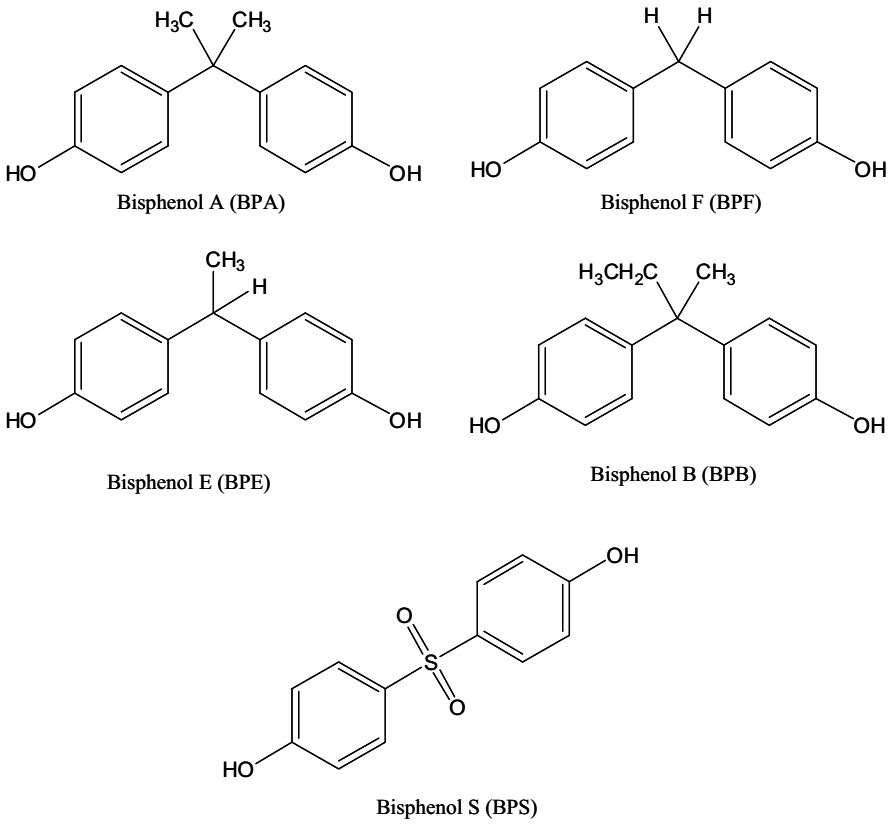

Figure 1. Chemical structures of bisphenols (BPs).

2.2. On-line SPE and chromatographic conditions

The system used for both on-line preconcentration and chromatographic separation was a Summit® x2 Dual-Gradient System (Dionex, Sunnyvale, CA) equipped with a Summit P680 dual ternary gradient pump and a TCC-100 thermostated column compartment that includes a 10-port switching valve and an ASI-100T autosampler with a 2.5-mL injection loop. The chromatographic separation was performed on a Supelco Ascentis Express C18 (Fused-Core™) column (Sigma-Aldrich) of 50 mm x 2.1 mm i.d. and 2.7 μm particle size, at 50ºC column temperature using a MeOH/water gradient

elution. For the fully automated on-line trace enrichment a Hypersil Gold C18 column (20 mm x 2.1 mm, 12 μm particle diameter, 175 Å pore size) (Thermo Fisher Scientific, Whatman, MA) was used. This column was previously conditioned with MeOH:water (5:95 v/v) at 1 mL min^{-1}. During this step, the analytical column was equilibrated at initial conditions of the chromatographic separation 15:85 (MeOH:water). The soft drink sample (1 mL) was then preconcentrated on-line in the SPE column using MeOH:water (5:95) (1 mL min^{-1}) as loading solvent. Then it was sequentially washed with 2 mL MeOH:water (15:85 v/v). After washing, the analytes were backflushed and transferred to the analytical column to perform the chromatographic separation using the following gradient elution program: 0 min 15% MeOH; from 0 to 3 min a linear gradient elution up to 80% MeOH followed by a isocratic step of 3.5 min. Standards prepared in cola matrix used for calibration were also preconcentrated on-line by the same SPE-LC procedure as for samples.

In order to prevent sample carry-over from sample-to-sample, which is a major problem with the on-line procedure, 100% MeOH (600 μL min^{-1}, 5 min) was used to flush the on-line SPE system after a complete analyte elution. Moreover, water blank samples were injected between sample batches to control carry-over and background contamination. No significant signals were observed in blank samples after a full working day.

2.3. Mass Spectrometry Conditions

The on-line solid phase extraction (SPE) liquid chromatography system was coupled to a triple quadrupole mass spectrometer (TSQ Quantum Ultra AM, Thermo

Fisher Scientific, San Jose, CA) equipped with a heated electrospray ionization source (H-ESI I) operating in negative mode (-4 kV) and vaporizer temperature of 300ºC. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow rates of 60, 20 and 40 a.u. (arbitrary units), respectively, and the ion transfer tube temperature was set to 375ºC. For tandem mass spectrometry, the deprotonated molecule $[M-H]^-$ was used as precursor ion. Two transitions for each compound were monitored using a dwell time of 20 ms and 1 μscan/scan (Table 1). When working at SRM mode (low resolution), both Q1 and Q3 operated at 0.7 *m/z* FWHM, whilst highly-selective selected reaction monitoring (H-SRM) mode on Q3, Q1 operated at 0.7 *m/z* FWHM and Q3 at 0.1 *m/z* FWHM. Argon was used as collision-induced-dissociation (CID) gas at 1.5 mTorr and the collision energy (CE) for each transition was optimized (Table 1). The Xcalibur software version 2.0 (Thermo Fisher Scientific) was used to control the LC/MS system and to process data.

To optimize the source working conditions and to carry out the tandem mass spectrometry experiments, a 1 mg L^{-1} stock standard methanol solution of each compound was infused at a flow-rate of 3μL min^{-1} using the syringe pump integrated into the TSQ instrument and a Valco zero dead volume tee piece (Supelco, Alcobendas, Spain) to mix the standard solution with the mobile phase (600 μL min^{-1}, 60:40 MeOH:water). Tandem mass spectrometry spectra were acquired between *m/z* 30 and *m/z* 300 in enhanced mass resolution mode on Q3 (0.1 *m/z* FWHM) in profile mode selecting the $[M-H]^-$ as precursor ion.

Table 1. Tandem mass spectrometry transitions for the acquisition mode.

Compound	Precursor (*m/z*), $[M-H]^-$	Quantitation		Confirmation		Ion Ratio ± SD[b]
		Product (*m/z*)	CE[a] (V)	Product (*m/z*)	CE[a] (V)	
Bisphenol A	227	212	20	133	26	3.2 ± 0.08
Bisphenol A-d_{16}	241	223	22	142	25	2.8 ± 0.10
Bisphenol F	199	93	23	105	22	1.3 ± 0.11
Bisphenol E	213	198	20	197	29	1.8 ± 0.07
Bisphenol B	241	212	19	226	28	12.5 ± 0.15
Bisphenol S	249	108	27	155	23	1.9 ± 0.01

[a]CE: collision energy
[b]SD: Standard deviation (n :5)

2.4. Soft drink samples

Eleven canned soft drinks including soda, beer, cola, tea and energy drinks were collected in Barcelona supermarkets. All samples were carbonated drinks except the tea drink products. The samples were stored unopened until analysis at 4 ºC. Twenty milliliter aliquots of carbonated soft drink samples were degassed by sonication for 20 minutes and centrifuged at 4000 r.p.m. Then, 1 mL was loaded into the on-line SPE LC-MS/MS system for analysis.

Bisphenol A was quantified using isotope dilution method adding the deuterated standard (BPA-d_{16}) before the centrifugation step. Matrix-matched calibration using a cola blank sample was used for quantitation of the other bisphenols, since isotopically labeled compounds were not commercially available and matrix effects were observed. Results include expanded uncertainty within a 95% confidence level.

3. Results and discussion

3.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Only BPA has been extensively studied by LC-MS/MS, whereas few data are available about the behavior of the other BPs (BPF, BPE, BPB and BPS). In this study, ESI was used to ionize BPs in negative mode using MeOH:water 50:50 (v/v) as mobile phase. The $[M-H]^-$, originated from the deprotonation of one hydroxyl group, was the only ion observed in the full scan mass spectrum of all the studied compounds. This ion was fragmented in a triple quadrupole instrument in order to identify the main product ions that can yield the most intense and the most characteristic transitions for both quantitative and confirmatory purposes.

The MS/MS spectra of bisphenols that contain an alkyl chain in the central carbon (BPA, BPE and BPB) showed the product ion originated from the radical loss of an alkyl group ($[M-H-CH_3]^{-\bullet}$ for BPA and BPE (Figure 2 A, C) and $[M-H-C_2H_5]^{-\bullet}$ for BPB (Figure 2D)), as a base peak. Furthermore, the loss of a methyl was also observed in the MS/MS spectrum of BPB (Figure 2D), since this compound has two different alkyl chains, methyl and ethyl, at the central carbon. Additionally, in the MS/MS spectra of bisphenols the characteristic product ion resulting from the cleavage of the hydroxyl-benzyl group, $[M-H-C_6H_6O]^-$ for BPA, BPF, BPE and BPB and $[M-H-C_6H_5O]^{-\bullet}$ for BPS (Figure 2 A-E) were also observed. Moreover, some of these compounds showed a characteristic product ion due to the cleavage of the hydroxyphenyl-alkyl bond that yields the ion at *m/z* 93 $[C_6H_5O]^-$ for BPF and the product ion at *m/z* 92, $[C_6H_4O]^{-\bullet}$ for BPS. For this last compound a product ion at *m/z* 184 that can be related to the loss of $\bullet SO_2H$ (Figure 2E) and additionally a characteristic abundant product ion at *m/z* 108, due to the consecutive losses of the hydroxyphenyl group and the sulfur oxide group $[M-H-C_6H_5O-SO_2]^{-\bullet}$ were observed. These consecutive losses were confirmed by multiple-stage mass spectrometry on an ion-trap (IT) mass analyzer. Table 1 summarizes the two transitions selected for each compound and the

optimum collision energies that maximize the intensity of the product ions used for both quantitative and confirmatory purposes.

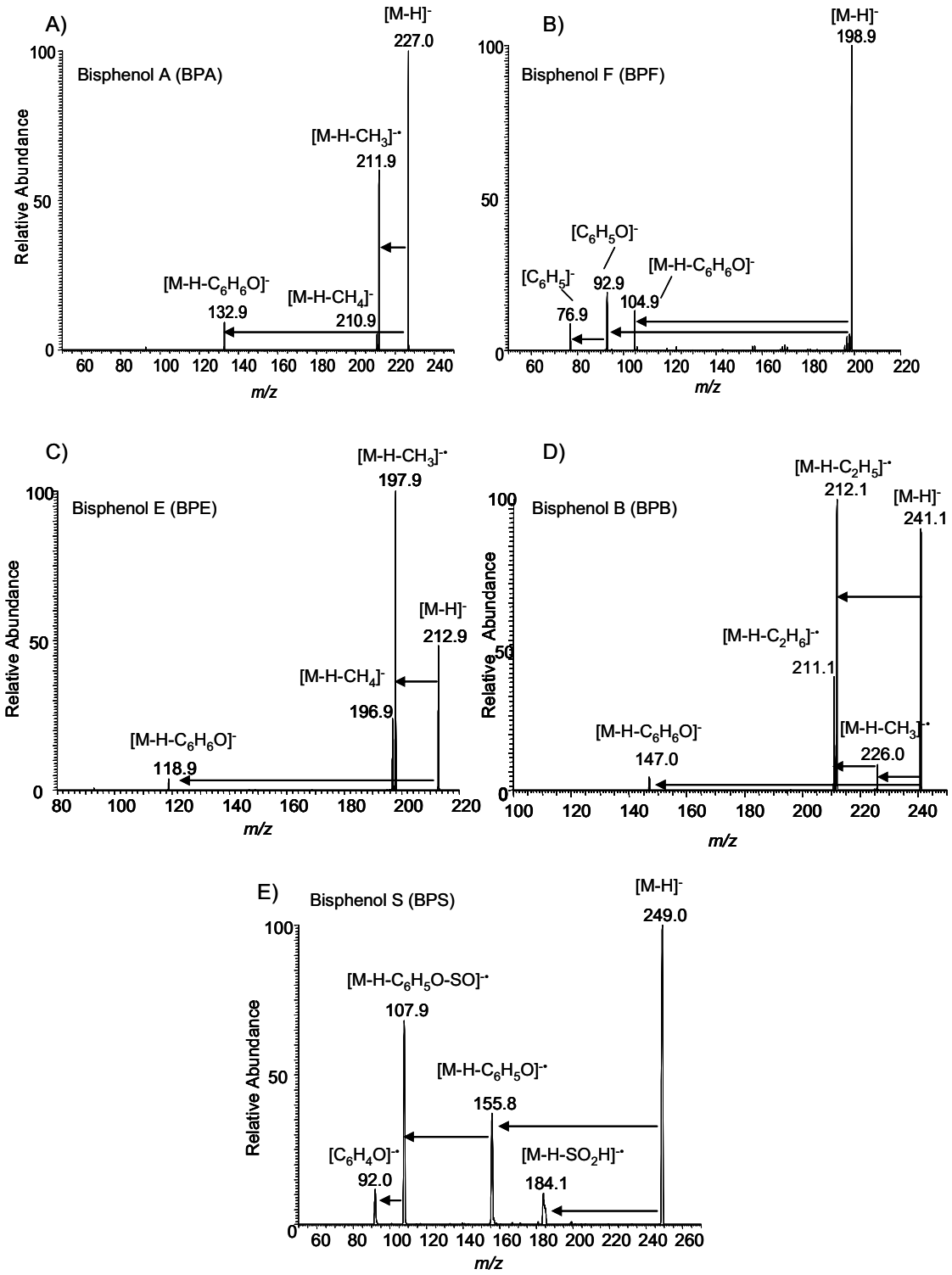

Figure 2. MS/MS spectra of bisphenols. Operating conditions: 0.7 *m/z* FWHM on Q1 and 0.1 *m/z* FWHM on Q3. A) BPA. B) BPF, C) BPE, D) BPB and E) BPS.

3.2. On-line SPE LC-MS/MS method

The use of a C18 (Fused-Core™) column provided a highly efficient chromatographic separation of BPs with a rapid analysis time (less than 3 min) working at low backpressure (< 400 bar), conditions which are compatible with the on-line SPE system. A base line chromatographic separation was achieved using MeOH:water in gradient elution mode at 600 µL min^{-1} and 50ºC. As an example, Figure 3 shows an on-line SPE LC-MS/MS chromatogram of a standard solution (1 mL, 10 µg L^{-1}).

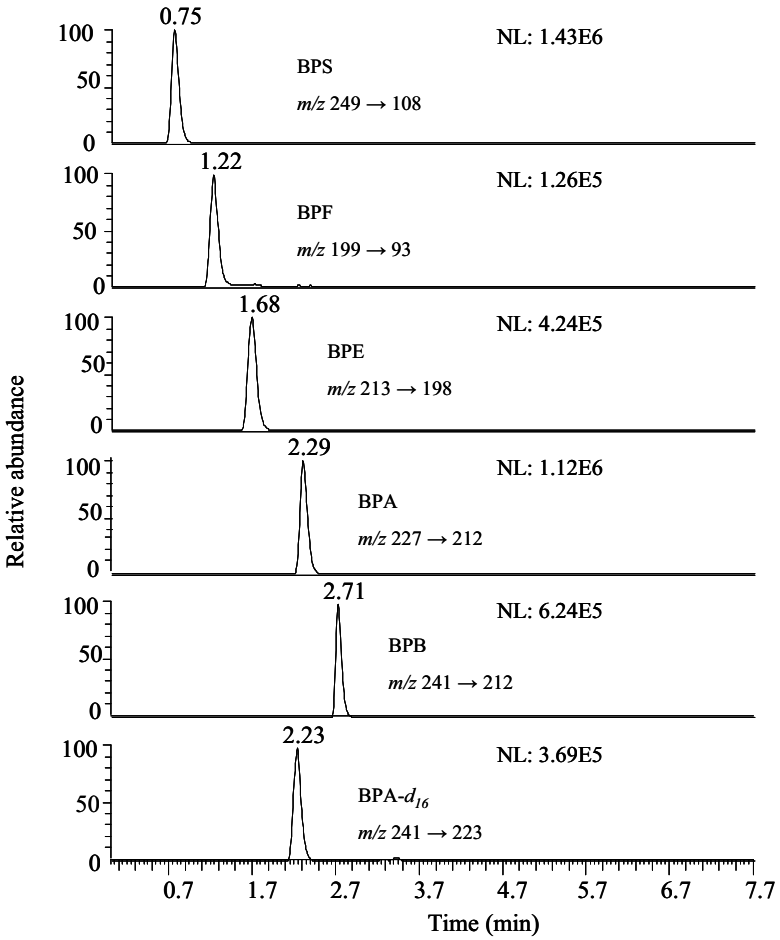

Figure 3. On-line SPE LC-MS/MS chromatograms of a standard solution (1 mL, 10 µg L^{-1}). Working conditions as indicated in experimental section.

For matrix effect evaluation, different soft drink samples including cola, lemon soda and tonic contained in glass bottles and free of BPs were spiked with BPs (10 μg L^{-1}) and analyzed by on-line SPE LC-MS/MS using ESI in negative mode as the ionization source. For these preliminary analyses, samples were loaded using MeOH:water (5:95) and the analytes were transferred in backflush mode by gradient elution (MeOH:water (50:50) and a linear gradient up to 100% of MeOH in 1 min). Under these conditions, the responses observed were 80-95% lower than those obtained for a standard solution at the same concentration level, probably due to the presence of matrix components that cause ion suppression in the ESI source. As an example of co-elution of matrix components with analytes, Figure 4A shows the chromatogram obtained for a cola sample acquired in SRM mode and also the UV (228 nm) chromatogram, where it can be observed that BPs eluted in a dirty area of the chromatogram. Several strategies were evaluated to reduce the matrix effect observed in the analysis of beverages and to improve both the selectivity and sensitivity of the method. First a clean-up step was added before transference of the analytes from the SPE column to the analytical column, using 15:85 MeOH:water to remove interferences. In addition, ionization efficiency was improved by increasing ESI temperature to 300ºC, providing extra desolvation. Under these conditions, the responses of BPs increased ~2.5 times. Since matrix effect was still observed, gradient elution was modified by reducing the amount of organic solvent and the gradient slope in order to increase retention of the analytes and to force their elution into a cleaner chromatographic area, thus minimizing the co-elution with matrix components in the eluting front. Figure 4B shows the chromatograms obtained for the cola sample spiked at 10 μg L^{-1} analysed using heated ESI and the new gradient conditions. An important reduction of matrix effect was observed, improving the responses by up to ~7 times.

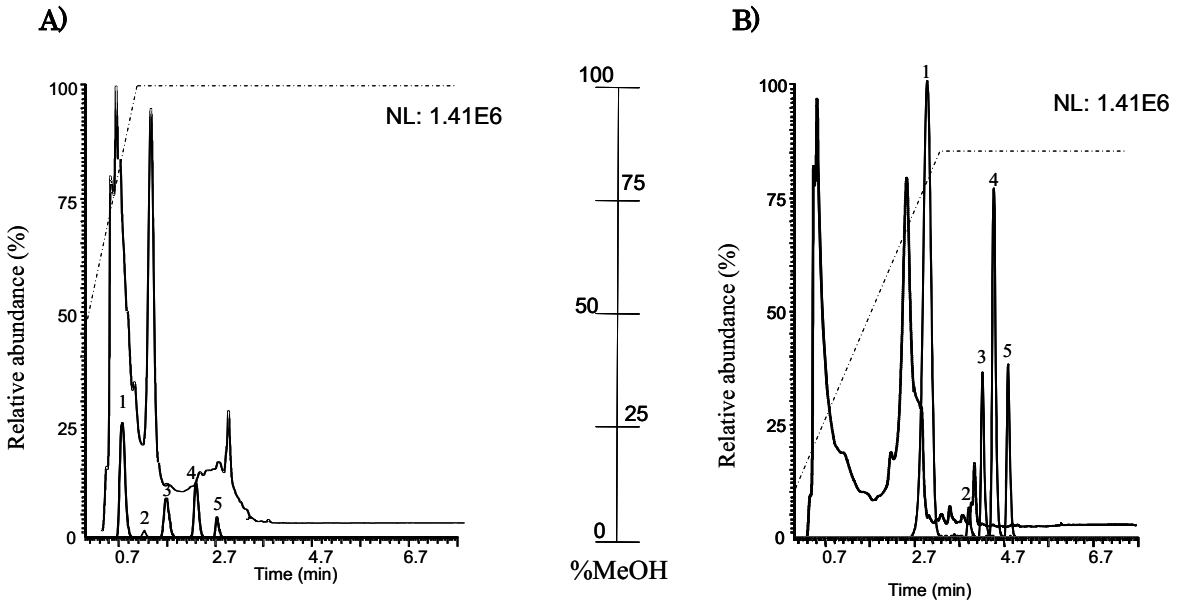

Figure 4. On-line SPE LC-MS/MS and LC-UV at 228 nm chromatograms of a glass cola sample spiked at 10 μg L^{-1} A) ESI at ambient temperature, gradient elution 0 min, 50:50 MeOH:water; from 0 to 1 min, linear gradient up to 100% MeOH and B) H-ESI at 300ºC, gradient elution 0 min 15% MeOH; from 0 to 3 min a linear gradient elution up to 80% MeOH, isocratic step (3.5 min). Compounds: 1. BPS, 2. BPF, 3. BPE, 4. BPA and 5. BPB.

The best way to compensate matrix effects in quantitative analysis is the use of isotope-labeled internal standards; however, for most of the bisphenols analyzed in this study (BPF, BPE, BPB and BPS), these standards were not available. For this reason matrix-matched calibration was evaluated for the analysis of BPs in canned soft drink beverages. Calibration curves, obtained from matrix-matched standards prepared for three soft-drink beverages (cola, lemon soda and tonic) were established for all the studied compounds. These curves displayed good linearity over the selected concentration range (50 ng L^{-1} – 10 μg L^{-1}) with linear regression correlation coefficients better than 0.996. Moreover, a statistical paired-sample comparison analysis

was performed using the slopes of the matrix-matched calibration curves prepared for the three soft drink beverages. For a 95% confidence level, the results were not significantly different (*p*-value of 0.23), showing that these matrices would provide similar quantitation results. In this study, for a routine analysis, cola from a glass bottle was used for matrix-matched calibration. For this matrix, on-line SPE recoveries were estimated by comparing the signal obtained by direct injection into the analytical column (10 μL) of a cola sample spiked at 25 μg L^{-1} (250 pg injected) with the signal obtained after loading 1 mL of beverage spiked at 250 ng L^{-1} in order to load the same absolute amount (250 pg injected) into the SPE column. Good recoveries from 85-100% were obtained for the studied compounds.

To evaluate the performance of the on-line SPE LC-MS/MS method for the analysis of bisphenols in canned soft drinks, quality parameters such as limit of detection (MLD), limit of quantitation (MLQ), run-to-run precision and ion ratio precision were studied in three soft drink beverages (cola, lemon soda and tonic). To estimate the limits of detection (MLDs) and limits of quantitation (MLQ), based on a signal-to-noise ratio of 3 and 10, respectively, the three soft drink beverages were spiked at a very low concentration level (down to 200 ng L^{-1}). Similar MLDs and MLQs were observed for most of the compounds in the three matrices (Table 2), showing that the method proposed is sensitive enough to quantify bisphenols in canned soft drinks down to ng L^{-1} (60-167 ng L^{-1}). Only for BPS in the tonic sample were these values lower. This can be explained by the fact that this compound eluted with the lowest retention time and could be sensitive to matrix changes. To obtain accurate quantitation measurements for this compound at concentrations lower than the cola LDQ (84 ng L^{-1}), matrix-match with the self-matrix is recommended. Bias and precisions were estimated from the data obtained from analyzing five replicates of each sample at two

concentration levels, 200 ng L^{-1} and 500 ng L^{-1}. Precision values expressed as relative standard deviations (RSDs) based on concentration were always better than 10% for both concentration levels (Table 2) and the bias values expressed as relative error were higher than 93 % at both low and medium concentration level (Table 2). To confirm the identity of the analyte, the ion ratio (quantitation-to-confirmation) for bisphenols was also evaluated (Table 1), obtaining an error below 10% for the three samples at the two concentration levels.

Table 2. Method limits of detection (LMDs), method limits of quantitation (MLQs) and run-to-run precision of the developed on-line SPE LC-MS/MS.

Compound	Matrix sample																	
	Cola[a]						Lemon soda[a]						Tonic[a]					
			Concentration level						Concentration level						Concentration level			
			Medium[b]		Low[c]				Medium[b]		Low[c]				Medium[b]		Low[c]	
	MLD (ng L^{-1})	MLQ (ng L^{-1})	%RSD (n:5)	Bias (%)	%RSD (n:5)	Bias (%)	MLD (ng L^{-1})	MLQ (ng L^{-1})	%RSD (n:5)	Bias (%)	%RSD (n:5)	Bias (ng L^{-1})	MLD (ng L^{-1})	MLQ (ng L^{-1})	%RSD (n:5)	Bias (%)	%RSD (n:5)	Bias (%)
BPS	25	84	4.5	97	3	97	15	50	3.5	97	4	96	5	15	2.5	97	2.5	98
BPF	50	167	8	96	10	93	32	106	5	96	8	94	42	140	4	96	5	96
BPE	25	84	6	97	6	94	12	40	5	95	5	97	18	60	6	95	9	93
BPA	25	85	2.5	98	3	98	15	50	4	97	5	96	25	85	3.5	97	5	94
BPB	50	167	3	97	5	96	40	132	5	95	10	95	50	167	7	96	10	93

[a] Glass beverage soft-drink

[b] Glass beverage soft-drink spiked at 500 ng L^{-1}

[c] Glass beverage soft-drink spiked at 200 ng L^{-1}

3.3. Sample analysis

The on-line SPE LC-MS/MS method was used for the simultaneous analysis of bisphenols (BPA, BPF, BPE, BPB and BPS) in eleven canned soft drinks and the results obtained are summarized in Table 3. Bisphenol A was detected in most of the analyzed samples at concentrations ranging from 44 ng L^{-1} to 607 ng L^{-1}. These values are consistent with the results published by Cao et al., [13]. Bisphenol F (BPF) was detected in only two samples, orange and lemon soda at a concentration of 218 ng L^{-1} and 141 ng L^{-1}, respectively, whilst other BPs were not detected in the analyzed samples. As an example, Figure 5 shows the LC-MS/MS chromatogram corresponding to the lemon soda (Figure 5A) where BPF and BPA were detected and the energy drink beverage where only BPA was identified (Figure 5B).

Table 3. Canned soft-drinks analysis using a LC-MS/MS.

	Concentration (ng L^{-1} ± tSD)				
Sample	**BPS**	**BPF**	**BPE**	**BPA**	**BPB**
Orange soda	n.d.	218±15	n.d.	607±25	n.d.
Lemon soda 1	n.d.	141±11	n.d.	433±13	n.d.
Lemon soda 2	n.d.	n.d.	n.d.	232±17	n.d.
Energy drink 1	n.d.	n.d.	n.d.	561±22	n.d.
Energy drink 2	n.d.	n.d.	n.d.	MLD*	n.d.
Tonic	n.d.	n.d.	n.d.	44±2	n.d.
Tea lemon	n.d.	n.d.	n.d.	MLD*	n.d.
Apple soda	n.d.	n.d.	n.d.	503±19	n.d.
Soda	n.d.	n.d.	n.d.	MLD*	n.d.
Cola	n.d.	n.d.	n.d.	522±22	n.d.
Beer	n.d.	n.d.	n.d.	n.d.	n.d.

*Detected using H-SRM on Q3 acquisition mode (MLD for BPA 5 ng L^{-1}).
n.d. not detected using the H-SRM on Q3 acquisition mode.

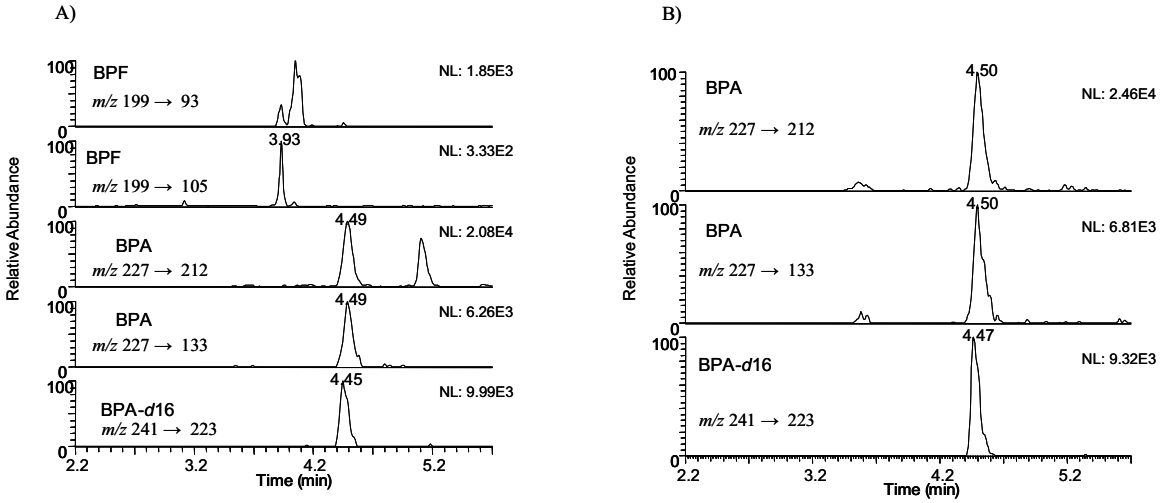

Figure 5. LC-MS/MS chromatograms of two canned soft-drink beverages: A) Lemon soda 1, B) Energy drink 1. Working conditions as indicated in experimental section.

Additionally, in this study enhanced mass resolution was used to minimize interferences and background noise when dealing with complex matrices. H-SRM on Q3 (Q1 0.7 *m/z* FWHM, Q3 0.1 *m/z* FWHM) provided lower limits of detection (5 to 10 times lower) than those obtained using SRM acquisition mode. This fact allowed us to confirm the presence of BPA at MLD level (5 ng L^{-1}) in three samples (energy drink 2, tea lemon and soda) that were considered to be negative under SRM mode working conditions. Moreover this acquisition mode in combination with the ion ratio confirmed the results obtained for the positive samples analyzed using SRM mode and prevented false positives.

4. Conclusions

This paper reports the development of an on-line SPE fast LC-MS/MS method for the simultaneous and direct analysis of bisphenols (BPA, BPF, BPE, BPB and BPS)

in canned soft drinks. Good chromatographic separation in less than 5 minutes was obtained using a Fused Core^{TM} particle column at 600 μL min^{-1} at low backpressure that enabled the on-line SPE system to be coupled with LC-MS/MS. In tandem mass spectrometry most of these compounds showed the loss of the alkyl group from the central carbon atom and the cleavage of the hydroxyl-phenyl alkyl bond. Only BPS showed different behavior related to the SO_2 group in the molecule.

The use of a clean-up step in the on-line SPE preconcentration in combination with a gradient elution that forced the compounds to elute in a cleaner chromatographic area and the use of heated electrospray (300ºC) enabled matrix effects to be minimized. Under these conditions, BPs can be analyzed at concentrations as low as 100 ng L^{-1} using matrix matched calibration. Selectivity and sensitivity can be improved using H-SRM with the advantage of preventing false negatives. This fast, robust, sensitive and selective method can be proposed for routine analysis of BPs in soft-drinks.

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4.3. DISCUSSIÓ DE RESULTATS

Com hem comentat anteriorment a la introducció d'aquest capítol i com hem posat de manifest a l'article VII (apartat 4.1.1), existeixen una sèrie de problemes i inconvenients en l'anàlisi de BPA que poden portar a errors en la seva determinació. En els treballs inclosos en aquest capítol a més de disminuir el temps d'anàlisi mitjançant l'ús de sistemes de cromatografia de líquids ràpida i d'elevada eficàcia ens vam plantejar minimitzar la introducció de contaminacions i interferències durant el tractament de mostra, així com disminuir i eliminar alguns dels problemes que es presenten en l'etapa de tractament de mostra per a aquesta anàlisi. En aquest apartat es discutiran tant les contaminacions que més freqüentment es poden trobar com altres problemes que es presenten durant el tractament de mostra i que dificulten tant l'anàlisi qualitativa com quantitativa, així com els diferents problemes que es presenten en l'anàlisi per LC-MS i es comentaran i es discutiran les solucions proposades en aquesta Tesis per tal de resoldre els problemes observats.

Entre els problemes que presenta l'anàlisi per LC-MS del BPA, dels seus derivats halogenats així com els altres compostos bisfenòlics tractats en aquest capítol cal esmentar en primer lloc la baixa eficàcia de la ionització i la supressió iònica produïda tant per la matriu com per la presència d'additius a la fase mòbil. Aquest efecte va ser observat en primer lloc en el desenvolupament d'un mètode per LC-MS per analitzar BPA juntament amb els seus derivats halogenats utilitzant un instrument de trampa d'ions equipat amb una font d'ionització ESI de geometria *on-axis* (article científic II, apartat 2.2.1.1 del capítol 2). Per una banda es va observar que l'addició d'àcids a la fase mòbil produïa una important disminució del senyal dels compostos que presentaven un major valor de pK_a (BPA i MCBPA). En aquest cas degut a que enregistràvem les molècules desprotonades $[M\text{-}H]^-$ la disminució de la intensitat d'aquests ions en addicionar un àcid a la fase mòbil es pot explicar per un desplaçament dels equilibris àcid-base cap a les especies àcides, la qual cosa disminueix el nombre d'ions en fase líquida. Així, és lògic que els compostos més afectats siguin els que presenten valors de pK_a més elevats. Ara bé, en realitzar l'addició post columna de solucions bàsiques per tal d'afavorir la desprotonació d'aquests compostos també es produeix una disminució en la intensitat del $[M\text{-}H]^-$ que en aquest cas pot ser deguda a un efecte de

supressió iònica. Això, pot explicar-se pel fet que la evaporació iònica dels ions negatius corresponents a la solució bàsica és més eficaç que la dels ions fenolat i per tant es veu afavorida. Aquests dos efectes comporten que les millors respostes s'obtinguin en no afegir additius a la fase mòbil. Per altra banda, s'ha observat que el tipus de solvent orgànic té un efecte significatiu en el senyal i per tant, en l'eficàcia de la ionització d'aquests compostos. Per exemple, s'observa una major resposta si s'utilitza MeOH en lloc de ACN. Això pot explicar-se per la menor tensió superficial del MeOH que afavoreix la desolvatació proporcionant una millor eficàcia en la ionització. Per aquesta raó s'ha seleccionat MeOH:aigua com a fase mòbil per a l'anàlisi per LC-MS del BPA i dels seus derivats halogenats. Posteriorment, en traspassar el mètode a l'instrument de triple quadrupol (TSQ Quantum Ultra AM), equipat amb una font ESI (geometria en angle), amb l'objectiu de disminuir els límits de detecció del mètode (article VIII, apartat 4.2.1). es va observar que els derivats halogenats del BPA amb un menor valor de pK_a donen lloc a ions doblement carregats. Aquest fet es pot explicar perquè el disseny de la font d'ionització (Ion Max de ThermoFisher Scientific), més energètica que l'emprada anteriorment, afavoreix la desolvatació i en conseqüència es produeix una desprotonació més efectiva dels grups hidroxil, que dòna lloc als ions de doble càrrega de les especies més àcides. A la Figura 4.1 es mostren els cromatogrames dels derivats clorats del BPA (DCBPA, TCBPA i TeCBPA) utilitzant MeOH:aigua a la fase mòbil quan es monitoritzen els *m/z* corresponents a les molècules desprotonades $[M–H]^-$ i les molècules doblement desprotonades $[M–2H]^{2-}$. La formació de dobles càrregues no s'observa en substituir el MeOH per ACN, però amb aquest últim solvent es produeix una disminució en la resposta tant pel BPA com pel MCBPA. Aquesta és la raó per la qual en aquesta memòria es proposen unes condicions de treball de compromís emprant gradient d'elució i una mescla ternària ACN:MeOH:aigua que proporciona la millor separació cromatogràfica possible en combinació amb la millor resposta possible en electroesprai per a tots els anàlits estudiats, en afavorir la formació dels ions monocarregats $[M–H]^-$. Tant per al BPA com per als altres bisfenols F, E, S i B (article IX, apartat 4.2.2) no s'ha observat la formació de dobles càrregues, probablement degut a que el pK_a d'aquestes substancies (entre 7.8 i 9.81) no és suficientment baix com per generar els ions doblement desprotonats. Aquest fet ens ha permès treballar amb una mescla MeOH:aigua com a fase mòbil que és la que proporciona la major eficàcia de ionització i la millor resposta en la determinació per LC–MS/MS.

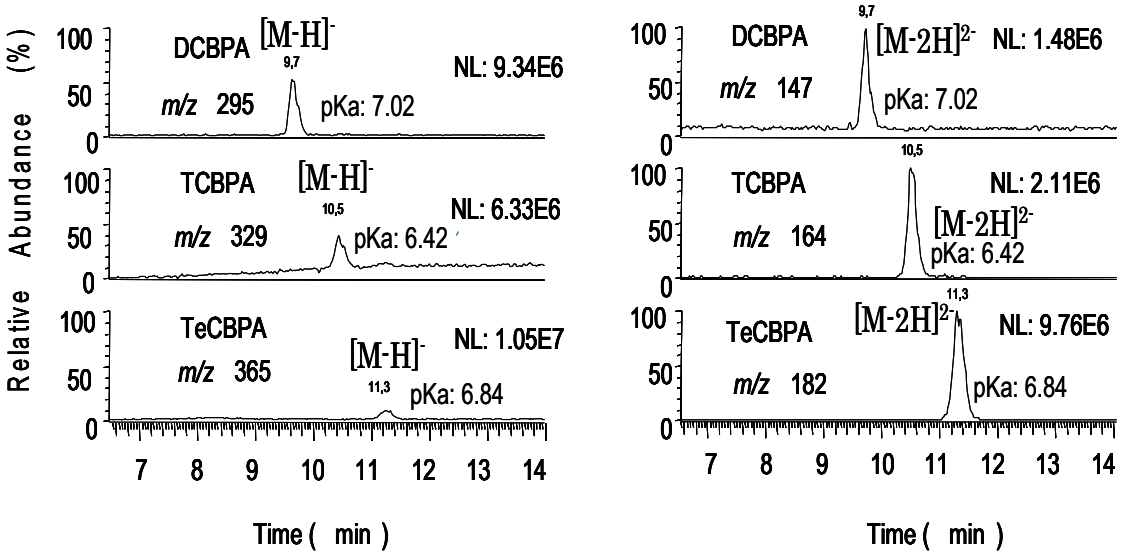

Figura 4.1. Cromatogrames LC-MS utilitzant MeOH:aigua com a fase mòbil d'una solució patró (100 μg L^{-1}) on s'han monitoritzat eks ions $[M\text{-}H]^-$ i $[M\text{-}2H]^{2-}$ pels derivats clorats del BPA (DCBPA, TCBPA i TeCBPA) emprant l'instrument de triple quadrupol i la font ESI (Ion Max).

A més dels efectes dels additius de la fase mòbil en la intensitat dels ions precursors $[M–H]^–$, els bisfenols també presenten una important supressió de la ionització deguda als components de la matriu de la mostra. Aquest efecte s'ha observat en l'anàlisi per LC–MS/MS del BPA i dels seus derivats halogenats en aigües superficials (article VII, apartat 4.1.1) utilitzant l'instrument de trampa d'ions amb una font *on-axis* ESI. A la Figura 4.2 es mostra el cromatograma obtingut per a una mostra d'aigua de muntanya addicionada a un nivell de concentració de 100 μg L^{-1} i analitzada emprant dos gradients d'elució diferents. Amb el gradient d'elució 1 (Figura 4.2A), encara que proporciona un menor temps d'anàlisi, s'observa una important supressió del senyal dels anàlits que elueixen a temps de retenció menors (BPA i MCBPA). Aquest efecte es va minimitza en modificar el gradient d'elució per tal de forçar la separació dels anàlits del front d'elució, on elueixen els components polars de la matriu. Això permet recuperar el senyal tal i com es pot observar a la Figura 4.2B) on es mostra el cromatograma obtingut per a la mateixa mostra en les noves condicions de treball (gradient 2). Aquest efecte matriu no s'ha observat en analitzar les mostres d'aigua superficial mitjançant el mètode de preconcentració en línia descrit en aquest capítol (apartat

4.2.1) i en el que s'utilitza l'instrument de triple quadrupol, probablement degut a que part dels compostos polars han estat eliminats en la etapa de preconcentració i neteja prèvia i a que la font ESI d'aquest instrument proporciona una millor desolvatació i eficàcia en la ionització.

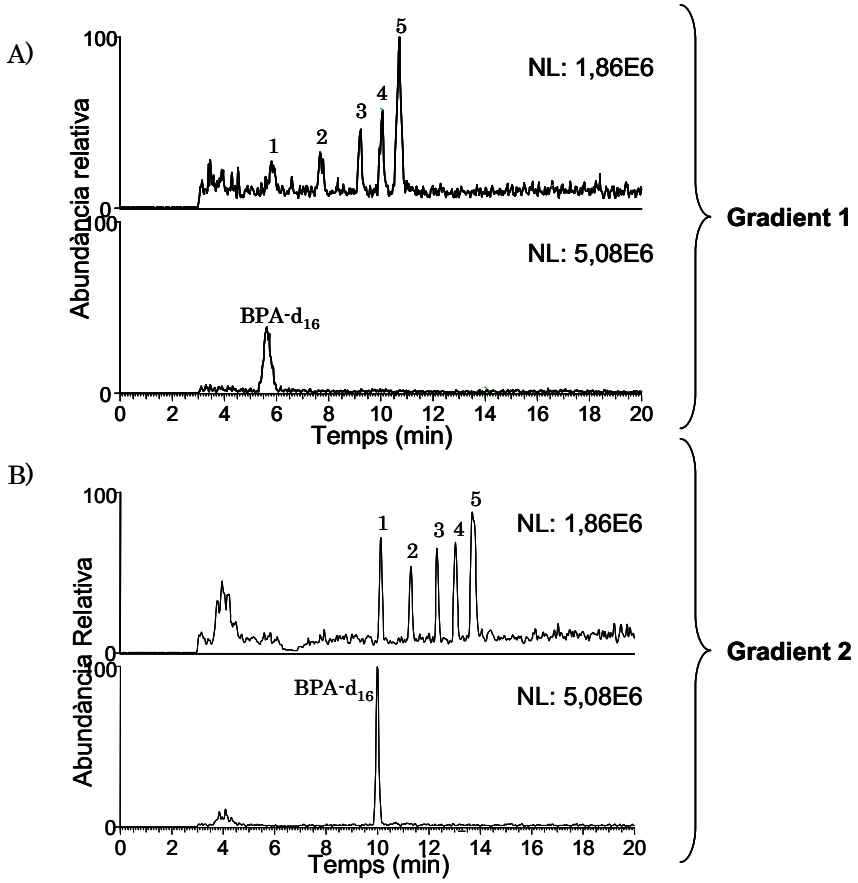

Figura 4.2. Cromatogrames LC-MS d'una mostra d'aigua de muntanya addicionada (200 μg L^{-1}) i analitzada emprant dos gradients d'elució: A) Gradient 1: 65% MeOH, 5 min 85% MeOH, 14 min 85% MeOH i B) Gradient 2: 50% MeOH, 0.5 min 65% MeOH, 5.5 min 85 MeOH i 13.5 min 85% MeOH.
1. BPA, 2. MCBPA, 3. DCBPA, 4.TCBPA, 5. TeCBPA.

Ara bé, en l'anàlisi de mostres de begudes refrescants (apartat 4.2.2) es torna a observar un efecte matriu important encara que s'utilitzi el sistema en línia i l'instrument de triple quadrupol amb la font ESI, com s'observa a la Figura 4 de l'article IX (apartat 4.2.2) on es mostra el cromatograma de LC-MS obtingut per una mostra de cola addicionada amb els bisfenols estudiats. En aquesta mateixa figura també s'inclou el cromatograma LC–UV que permet mostrar com els anàlits

coelueixen amb altres compostos de la matriu. En aquest cas per millorar la resposta i eliminar l'efecte matriu en primer lloc es va augmentar la temperatura de la font d'electroesprai fins a 300ºC la qual cosa va permetre disminuir en part l'efecte matriu (~2.5 vegades). Ara bé, per obtenir una reducció més significativa d'aquest efecte, ha estat necessari modificar de nou el gradient d'elució i separar els anàlits del front d'elució encara més. Com es pot apreciar a la Figura 4 de l'article científic IX (apartat 4.2.2) en aquestes noves condicions els anàlits elueixen més tard i en una zona més neta del cromatograma la qual cosa fa possible recuperar el senyal en un 50%.

Com hem comentat anteriorment a la introducció d'aquest capítol en l'anàlisi de BPA apareixen una sèrie de problemes deguts a la ubiqüitat d'aquest compost en el material de laboratori utilitzat en el tractament de mostra que donen lloc a contaminacions de les mostres. En els estudis realitzats en aquesta tesis s'ha posat de manifest que l'aigua purificada amb un sistema de purificació Milli-Q Elix conté BPA a concentracions que varien en funció de la quantitat d'aigua produïda (entre 20 ng L^{-1} i 200 ng L^{-1} al llarg del dia) la qual cosa impossibilita corregir la presència de BPA duent a terme una anàlisi de blancs i restant el senyal en les mostres analitzades. Per aquesta raó es proposa utilitzar aigua purificada de qualitat LC-MS tant a la fase mòbil com en la preparació de patrons ja que no s'ha detectat la presència de BPA en aquest tipus d'aigua.

Per altra banda, s'ha observat l'aparició d'interferències isobàriques que augmenten el soroll de fons en zones properes a la d'elució dels anàlits i que afecten d'una manera significativa a l'anàlisi quantitativa del BPA. L'estudi de les diferents etapes del mètode permet concloure que aquestes interferències, tal i com s'indica a l'article científic VIII (apartat 4.3.1), són degudes a l'etapa de filtració de les mostres. Després de la filtració apareixen en el cromatograma corresponent a la monitorització de la transició de quantificació del BPA (*m/z* 227 → 212) uns pics molt a prop del temps de retenció del BPA que dificulten la seva anàlisi. Tant els filtres de Nylon com els de cel·lulosa regenerada produeixen aquestes interferències. Finalment, com a solució de compromís es proposa centrifugar les mostres en lloc de filtrar-les.

Atesa la problemàtica del tractament de mostra en l'anàlisi de BPA, en aquesta Tesis es va decidir desenvolupar un mètode de preconcentració i neteja en línia per a la determinació conjunta del BPA i els derivats clorats en mostres

d'aigua, ja que permet minimitzar la manipulació de les mostres i evitar així la introducció de contaminacions i interferències. El mètode de preconcentració en línia s'acobla a la cromatografia de líquids d'elevada eficàcia utilitzant una columna de partícules semiporoses. La comparació de les eficàcies obtingudes emprant aquesta columna i una de partícules inferiors a 2 μm (Aquity BEH 50 mm x 2.1 mm i.d. i 1.7 μm de mida de partícula) posa de manifest tal i com es mostra a la taula 4.1 que s'obtenen eficàcies similars. Ara bé, la columna semiporosa presenta l'avantatge de generar una menor pressió (300 bar en front dels 725 bar), la qual cosa ha permès dur a terme l'acoblament dels dos sistemes (SPE i LC-MS/MS) de forma fàcil i directa.

Taula 4.1. Eficàcia de separació del BPA i derivats clorats i pressió del sistema cromatogràfic.

Compost	Eficàcia	
	[a]Ascentis Express C18 (2.7 μm)	[b]Acquity BEH C18 (1.7 μm)
BPA	22847	15045
MCBPA	16925	18553
DCBPA	19704	19281
TCBPA	23540	23282
TeCBPA	29093	27010

[a]Caiguda de pressió: 300 bar

[b] Caiguda de pressió: 725 bar

El mètode de SPE en línia en combinació amb el sistema de cromatografia de líquids ràpida i d'elevada eficàcia s'ha acoblat a l'espectrometria de masses en tàndem. Aquest mètode d'anàlisi ens ha permès determinar amb una bona fiabilitat tant la presència de BPA i dels seus derivats clorats en mostres d'aigües superficials a nivells de concentració de ng L^{-1}, tal i com s'indica a l'article científic VIII, com l'anàlisi de BPA junt amb altres bisfenols (BPF, BPE, BPB i BPS) en mostres de begudes refrescants tal i com s'indica a l'article científic IX. En aquests estudis, aprofitant les capacitats d'aquest instrument, s'ha avaluat l'avantatge de treballar a un poder de resolució major (resolució fins a 0,1 unitats de *m/z* a FWHM) per augmentar la selectivitat de l'anàlisi i la sensibilitat. Es va provar de treballar tant amb el Q1 com amb el Q3 a una resolució elevada i pel cas del BPA, tal i com es pot observar a la Figura 4 de l'article científic VIII (Apartat 4.2.1), la millor selectivitat i sensibilitat es va obtenir en augmentar la resolució en la

selecció dels ions producte (Q3). Aquest mode d'adquisició va ser utilitzat tant en l'anàlisi de BPA i els seus derivats clorats en mostres d'aigua com en l'anàlisi de bisfenols en begudes enllaunades per tal d'evitar tant falsos negatius com falsos positius en les mostres analitzades.

4.4. CONCLUSIONS

El treball experimental inclòs en el Capítol 4 de la memòria en el que s'han establert metodologies d'extracció en fase sòlida en línia amb la separació per cromatografia de líquids ha permès arribar a les següents conclusions:

- El mètode de preconcentració en línia acoblat al sistema de cromatografia de líquids d'ultra elevada eficàcia emprant una columna de partícules superficialment poroses desenvolupat per a l'anàlisi de bisfenol A en mostres d'aigua superficials i begudes refrescants ha permès minimitzar els problemes de contaminacions i pèrdues d'anàlit derivats del tractament de mostra.

- Per tal d'obtenir la millor sensibilitat en electrosprai i evitar la formació dels anions doblement carregats dels compostos amb un pK_a<7 (DCBPA, TCBPA i TeCBPA) es proposa la utilització d'una fase mòbil ternària ACN:MeOH:aigua. En canvi per a la determinació individual de BPA es recomana la utilització d'una fase mòbil MeOH:aigua, condicions a les quals aquest compost presenta la major eficàcia de ionització.

- En aplicar el mètode en línia per a l'anàlisi de BPA i altres bisfenols (BPF, BPE, BPB i BPS) en mostres de begudes refrescants enllaunades s'ha observat un important efecte matriu. Per afavorir la desolvatació es proposa augmentar la temperatura de la font d'electroesprai (H-ESI) a 300ºC la qual cosa millora la resposta unes 2.5 vegades. Per tal de disminuir encara més l'efecte matriu i augmentar la resposta es proposa modificar el gradient d'elució de manera que els anàlits s'elueixin en una zona més neta del cromatograma separada del front d'elució.

- El BPA ha estat detectat al riu del Llobregat a la entrada de la planta de tractament d'aigua potable (DWTP) de Sant Joan Despí a concentracions entre 101 ng L^{-1} i 322 ng L^{-1}. L'estudi del comportament del BPA en la planta de tractament d'aigua ha posat de manifest que aquest compost és eliminat en gran part durant l'etapa de precloració encara que els corresponents derivats clorats no han estat detectats possiblement degut a les baixes concentracions i a

les possibles reaccions d'oxidació col·laterals. Els derivats clorats si que han estat detectats en una mostra d'aigua residual recollida a la sortida d'una planta de reciclatge de paper a concentracions de: MCBPA 739 ng L^{-1}, DCBPA 836 ng L^{-1}, TCBPA 460 ng L^{-1} i TeCBPA 530 ng L^{-1}.

- L'anàlisi de mostres de begudes refrescants ha posat de manifest la presència de BPA en la majoria de les mostres a concentracions entre 18 ng L^{-1} i 607 ng L^{-1}, i de BPF en dues mostres, taronjada i llimonada, a concentracions de 218 ng L^{-1} i 141 ng L^{-1}, respectivament. Els altres bisfenols estudiats no han estat detectats en cap de les mostres analitzades.

- La utilització del mode *highly-selective selected reaction monitoring* (H-SRM) treballant a una resolució de 0,1 *m/z* FWHM en el tercer quadrupol (Q3) ha permès millorar la selectivitat i la sensibilitat del mètode d'anàlisi obtenint uns límits de quantificació al voltant dels 60 ng L^{-1} en aigües superficials, lleugerament superiors en aigües residuals (~ 170 ng L^{-1}) i d'entre 5 ng L^{-1} i 40 ng L^{-1} en begudes refrescants. Aquest mode d'adquisició ha permès identificar la presència de BPA en mostres a les quals no havia estat detectat utilitzant SRM.

CONCLUSIONS GENERALS

Del treball realitzat en aquesta Tesi Doctoral es poden extreure les següents conclusions globals:

En relació als estudis d'espectrometria de masses.

- La interpretació de la informació obtinguda amb dos analitzadors que operen en dos modes diferents d'espectrometria de masses en tàndem (en el temps i en l'espai) ha permès proposar una estratègia de treball per als estudis de fragmentació en LC-MS. A més les mesures de massa exacta tant d'ions precursors com d'ions producte obtingudes amb l'analitzador de triple quadrupol han estat decisives per a l'assignació d'algunes composicions elementals dels ions observats. Aquesta estratègia de treball ha estat utilitzada en els estudis de fragmentació de tres famílies de compostos, bisfenols, bisfenol diglicidil èters i fotoiniciadors establint les rutes de fragmentació de cadascuna.

- L'informació obtinguda en aquests estudis de fragmentació ha permès seleccionar les transicions adequades en cada cas per tal de dur a terme la quantificació i confirmació d'aquests compostos per LC-MS/MS.

- Els estudis de fragmentació duts a terme en l'analitzador de trampa d'ions en combinació amb la informació obtinguda amb ressonància magnètica nuclear de protó (^{1}H-NMR) ha possibilitat la caracterització dels derivats clorats del BPA (MCBPA, DCBPA i TCBPA) sintetitzats en aquesta tesi.

- L'estudi dels espectres obtinguts emprant espectrometria de masses en etapes successives acoblada a cromatografia de líquids en fase invertida ha permès identificar i caracteritzar cadascun dels isòmers del BFDGE (*orto,orto-, orto,para- i para,para*-BFDGE) i proposar un ordre d'elució en la separació cromatogràfica.

- L'augment de la resolució en espectrometria de masses treballant amb l'instrument de triple quadrupol en els modes H-SIM (Q1 0,04 *m/z* FWHM) i H-SRM (Q3 0,04 *m/z* FWHM) ha permès emprar aquest analitzador per

realitzar mesures de massa exacta tant d'ions precursors com d'ions producte amb una bona exactitud i precisió. Aquestes mesures de massa exacta han estat emprades per tal d'assignar correctament la composició elemental dels ions producte en els estudis de fragmentació així com per obtenir una confirmació extra de la presència d'alguns dels compostos estudiats en aquesta Tesi en les mostres analitzades. Per altra banda l'augment de la resolució del quadrupol fins a 0,1 *m/z* FWHM ha permès millorar la sensibilitat dels mètodes LC-MS/MS desenvolupats.

En relació a la metodologia analítica.

- La utilització d'una columna de fase invertida (C18) de partícules semiporoses ha permès posar a punt un mètode de cromatografia de UHPLC acoblat a l'espectrometria de masses en tàndem (UHPLC-MS/MS) per a l'anàlisi de bisfenol A diglicidil èter, bisfenol F diglicidil èter i dels seus derivats hidrolitzats en menys de 5 minuts.

- Per a l'anàlisi per LC-MS/MS dels fotoiniciadors es proposa emprar una columna de fase estacionaria pentafluorofenil propil (PFPP) que ha permès la separació cromatogràfica dels dos isòmers de l'ITX (2- i 4-ITX) amb una bona resolució (Rs: 1,3). Es recomana utilitzar una temperatura de 5ºC per resoldre cromatogràficament la separació d'onze fotoiniciadors.

- Es proposa la utilització de l'extracció en fase sòlida en línia amb la cromatografia de líquids UHPLC emprant una columna de partícules semiporoses acoblada a l'espectrometria de masses en tàndem per a l'anàlisi de bisfenol A, derivats clorats del BPA i altres bisfenols. Aquest mètode ha permès analitzar el BPA i els seus derivats clorats en mostres d'aigua a concentracions de ng L^{-1} en menys de 3 minuts i el BPA i altres bisfenols en mostres de begudes refrescants en menys de 5 minuts també a concentracions de ng L^{-1}. Aquests mètodes permeten minimitzar el tractament de mostra i reduir els problemes de contaminacions i d'introducció d'interferències que generalment s'observen en l'anàlisi de BPA.

En relació a les mostres analitzades.

- El mètode de SPE en línia amb LC-MS/MS ha permès determinar la presència de BPA en mostres d'aigua de riu a concentracions entre 101 – 322 ng L^{-1} mentre que els derivats clorats de BPA (MCBPA, DCBPA, TCBPA i TeCBPA) tant sols han estat detectats en una mostra d'aigua residual d'una planta de reciclatge de paper a concentracions entre 460 – 836 ng L^{-1}. Per altra banda el BPA i el BPF han estat identificats en mostres de begudes refrescants enllaunades a concentracions entre 18 – 607 ng L^{-1} i 141 – 218 ng L^{-1}, respectivament.

- En l'anàlisi dels BADGEs i BFDGEs en mostres d'aliments i begudes refrescants enllaunades únicament s'han detectat els BADGEs hidrolitzats probablement degut a que en aquestes mostres de base aquosa i/o àcides es facilita la hidròlisi dels grups epòxid. El BADGE·$2H_2O$ ha estat trobat a totes les mostres analitzades a concentracions entre 2,8 – 675 µg kg^{-1} (aliments enllaunats) i 2,1 – 5,1 µg L^{-1} (begudes refrescants). Els altres BADGEs hidrolitzats (BADGE·H_2O, BADGE·HCl·H_2O i BADGE·2HCl) únicament han estat detectats a les mostres d'aliments i a concentracions baixes (0,9 – 11 µg kg^{-1}).

- Pel que referència a l'anàlisi de fotoiniciadors el mètode desenvolupat ha estat aplicat a l'anàlisi de mostres d'aliments envasats en cartró observant la presència de BP a totes les mostres analitzades a concentracions entre entre1.8 µg kg^{-1} i 40 µg kg^{-1} degut a l'elevat ús d'aquest compost com a fotoiniciador de les tintes d'impressió UV i a la seva utilització en l'obtenció de pel·lícules de polietilè utilitzades en els envasos multi-capes. Els altres fotoiniciadors (2-ITX, 4-ITX, EDMAB, EHDAB, DETX, DMPA, DEAB i PBZ) analitzats han estat detectats en un menor nombre de mostres i a concentracions entre entre 0,07 µg kg^{-1} i 2,5 µg kg^{-1}.

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ABREVIATURES I ACRÒNIMS

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ABS	*Acrylonitrile Butadiene Styrene*
AcET	Acetat d'etil
ACN	Acetonitril
AM	Massa exacta (*Accurate mass)*
APCI	Ionització química a pressió atmosfèrica (*Atmospheric pressure chemical ionization)*
API	Ionització a pressió atmosfèrica (*Atmospheric pressure ionization)*
BADGEs	Bisfenol A diglicidil èters
BFDGEs	Bisfenol F diglicidil èters
BFRs	Retardants de flama bromats (*Brominated flame retardants)*
BPs	Bisfenols
C18	Octadecil sílica
C8	Octil sílica
DART	Anàlisi directe en temps real (*Direct analysis real time)*
DWTP	Planta de tractament d'aigua potable (*Drinking water treatment plant)*
EC	Compost endocrí (*Endocrine compound)*
EDCs	Compostos disruptors endocrins (*Endocrine-disrupting compounds)*
EDs	Disruptors endocrins (*Endocrine disruptors)*
EFSA	Autoritat europea de seguretat alimentària (*European food safety authority)*
EI	Ionització electrònica (*Electron ionization)*
EPA	Agència de protecció medi ambiental (*Environmental protection agency)*
ESI	Electroesprai *(Electrospray)*
EtOH	Etanol
EU	Unió Europea *(European Union)*
FDA	Administració d'aliments i fàrmacs (*Food and drug administration)*
FT-ICR	Resonància ciclotrònica d'ions amb transformada de Fourier *(Fourier transform ion cyclotron resonante)*
FWHM	*Full width at half maximum*
GC	Cromatografia de gasos (*Gas chromatography)*
GC-MS	Cromatografia de gasos-espectrometria de masses *(Gas chromatography-Mass spectrometry)*
GC-MS/MS	Cromatografia de gasos-espectrometria de masses en tàndem *(Gas chromatography-tandem mass sepectrometry)*
H-ESI	Electroesprai escalfat *(Heated electrospray source ionization)*
HILIC	Cromatografia líquida d'interacció hidrofílica *(Hydrophilic interaction liquid chromatography)*
HIPS	Poliestirè d'elevada densitat *(High Impact Polystyrene)*
HLB	Balanç hidrofílic lipofílic *(Hydrophilic lipofilic balance)*
HPTLC-MS	Cromatografia en capa fina d'elevada eficàcia-espectrometria de masses

	(High performance thin layer chromatography-mass spectrometry)
H-SIM	Monitorització d'ions selectius d'elevada selectivitat *(Highly-selective selected ion monitoring)*
H-SRM	Monitorització de reaccions selective d'elevada selectivitat *(Highly-selective selected reaction monitoring)*
IT	Trampa d'ions *(Ion trap)*
LC	Cromatografia de líquids *(Liquid chromatography)*
LC-MS	Cromatografia de líquids-espectrometria de masses *(Liquid chromatography-mass spectrometry)*
LC-MS/MS	Cromatografia de liquids-espectrometria de masses en tàndem *(Liquid chromatography-tandem mass spectrometry)*
LC-MSn	Cromatografia de liquids-espectrometria de masses en etapes succesives *(Liquid chromatography-multiple-stage mass spectrometry)*
LC-UV	Cromatografia de líquids amb detecció UV
LLE	Extracció líquid-líquid
MeOH	Metanol
MLOD	Límit de detecció de mètode (*Method limit of detection*)
MS	Espectrometria de masses (*Mass spectrometry*)
MS/MS	Espectrometria de masses en tàndem (*Tandem mass spectrometry*)
MSn	Espectrometria de masses en etapes succesives *(Multiple-stage mass spectrometry)*
NMR	Resonància magnètica nuclear *(Nuclear magnetic resonante)*
OctaBDE	Octabromo difenil éter (*Octabromodiphenyl ether)*
P&C	Paper i Cartró
PFPP	Pentafluorofenil propil *(Pentafluorophenyl propyl)*
PLE	Extracció líquida presuritzada *(Pressurized liquid extraction)*
PVC	Policloruro de vinilo
Q	Quadrupol
QqQ	Triple quadrupol
Q-TOF	Quadrupol temps de vol *(Quadrupol time of flight)*
Q-Trap	Quadrupol tampa linial *(Quadrupol linear trap)*
QuEChERS	*Quick, Easy, Cheap, Effective, Rugged, and Safe*
REACH	*Registration, Evaluation, Authorisation and Restriction of Chemical substances*
SD	Desviació estàndard *(Standard deviation)*
SIM	Monitorització selectiva d'ions *(Selected ion monitoring)*
SML	Nivell específic de migració *(Specific migration level)*
SPE	Extracció en fase sòlida *(Solid phase extraction)*
SPE-LC-MS/MS	Extracció en fase sòlida-cromatografia de liquids-espectrometria de masses *(Solid phase extraction-liquid chromatography-tandem mass spectrometry)*
SPME	Microextracció en fase sòlida *(Solid phase micro-extraction)*
SRM	Monitorització de reaccions selectives *(Selected reaction monitoring)*
TDI	Quantitat diària tolerable *(Tolerable daily intake)*
TOF	Temps de vol *(Time of flight)*

UE	Unió Europea
UHPLC	Cromatografia de líquids d'ultra elevada eficàcia *(Ultra high performance liquid chromatography)*
WEE	Residus d'equipament elèctric i electrònic *(Waste from Electrical and Electronic Equipment)*
WWTP	Planta de tractament d'aigües residuals *(Waste water treatment plant)*