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Analytical Methods

Simultaneous determination of bisphenol A, octylphenol, and nonylphenol by pressurised liquid extraction and liquid chromatography–tandem mass spectrometry in powdered milk and infant formulas

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ABSTRACT

A new analytical method, using pressurised liquid extraction (PLE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS), was developed for the simultaneous determination of bisphenol A (BPA), octylphenol (OP) and nonylphenol (NP) in powdered infant formulas (IF) and powdered skimmed milk (PM). The analytes were extracted by PLE, using this optimised conditions: ethyl acetate as solvent, 70 °C of temperature, reversed-phase silica C_{18} as dispersing agent and three cycles of extraction. The extracts were then injected in LC–MS/MS using a Gemini C_{18} column and a mixture of 5% water and 95% methanol/acetonitrile, both with 0.1% ammonia, as a mobile phase. Recoveries at different fortification levels (0.5 and 0.05 mg kg^{-1}), were between 89% and 92% for BPA, 84 and 98% for OP, and 93% and 101% for NP. The method was applied to the analysis of samples of PM and IF, bought in Italian and Spanish markets. In positive samples, phenols concentration ranged from 0.07 to 1.29 mg kg^{-1} for BPA, from 0.028 to 1.55 mg kg^{-1} for OP and from 0.026 to 1.47 mg kg^{-1} for NP.

1. Introduction

During the last decade there has been a worldwide scientific and public discussion about the potential consequences of long-term dietary exposure to endocrine disruptors compounds (EDCs). Even at low concentrations, chronic exposure to EDCs is of toxicological concern and this increased when humans are exposed to mixtures of similar acting EDCs and/or during sensitive windows of development (Klaassen, 2008). Many of the EDCs [i.e. bisphenol A (4,4′-(propane-2,2-diyl)diphenol) (BPA), octylphenol (OP) and nonylphenol (NP)] have estrogen-like effects and chronic toxicity causing, at low doses, a variety of adverse effects (Ferguson, Scallet, Flynn, Meredith, & Schwetz, 2000; Klaassen, 2008; Knepper, Barceló, & De Voogt, 2003).

Alkylphenols (APs), including OP and NP, are widely used as intermediates to produce surfactants and as stabilizers of resins, and esters (Knepper et al., 2003). These compounds are also found into the environment as metabolites of APs, mainly by biodegradation from sewage treatment plants. Some researchers have reported a wide occurrence of APs in some products for human consumption (Loyo-Rosales, Rosales-Rivera, Lynch, Rice, & Torrents, 2004; Smchmitz-Afonso, Loyo-Rosales, Aviles, Rattner, & Rice, 2003).

BPA is a synthetic substance widely used in industry in the production of epoxy resins and polycarbonates, and as an antioxidant in polyvinyl chloride (PVC) plastics (European Directive 2002/72/EC; Opinion of the Scientific Committee on Food on Bisphenol A, SCF/CS/PM/3936, 2002). Epoxy resins are used as inner surface coating of food and beverage cans. Polycarbonates are used in the manufacture of plastic food containers, such as infant feeding bottles and tableware. PVC is used in a variety of products that includes materials intended to come into contact with food. The migration of BPA from epoxycoated can surfaces (Kang & Kondo, 2002; Yoshida, Horie, Hoshino, & Nakazawa, 2001), polycarbonate plastics (Nerın, Fernandez, Domeno, & Salafranca, 2003), and PVC products (Lopez-Cervantes & Paseiro-Losada, 2003; Lopez-Cervantes, Sanchez-Machado, Paseiro-Losada, & Simal-Lozano, 2003) into food simulants and food has been reported.

The main sources of exposure to BPA and APs for newborns and infants derives from migration from the lining of cans into infant formulas (IF) and from the polycarbonate baby bottles (Muncke, 2009).

On the above, there is need for researching and for revising of existing methods in order to have reliable tools for risk assessment

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and control of human exposure to BPA, NP and OP. To the best of our knowledge, nowadays no method has been published for the simultaneous determination of NP, OP and BPA in IF.

For the determination of BPA, OP and/or NP in different food matrices, emphasis is placed on the main strategies developed for sample treatment, which usually consists of several laborious and time-consuming steps in order to achieve the required sensitivity and selectivity. Special treatments can be required depending on the matrix composition (Ballesteros-Gomez, Rubio, & Perez-Bendito, 2009). The pressurised liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction), offers the advantages of reducing solvent consumption and automating sample handling (Goodson, Summerfield, & Cooper, 2002). ASE has been applied (Shao et al., 2007a) for analysing BPA, OP and NP in meat by LC–MS/MS. Others authors (Tavazzi, Benfenati, & Barcelò, 2002) have extracted APs from fish liver using ASE and Florisil clean-up, comparing the efficiency of ASE with conventional Soxhlet extraction; others (Carabias-Martinez, Rodriguez-Gonzalo, & Revilla-Ruiz, 2006) have analysed the levels of BPA and NP in cereals by ASE and LC–MS.

Separation, identification and quantitation of BPA and/or APs is today reliably made with mass spectrometric methods, namely liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS), and thus main attention is devoted to these techniques, but other methods using LC coupled to fluorescence or electrochemical detection, as well as immunochemical methods are also employed (Ballesteros-Gomez et al., 2009; Braunrath, Podlipna, Padlesak, & Cichna-Marka, 2005; Garcia-Prieto, Lunar, Rubio, & Perez-Bendito, 2008; Smchmitz-Afonso et al., 2003). Therefore, prior to GC–MS analysis, a specific derivatization step is necessary (Kuo & Ding, 2004). LC–MS/MS, applying APCI and ESI ionisation techniques, provides clear advantages in terms of achieving comparable sensitivity without tedious derivatization steps. Recent efforts have also been made to employ tandem-MS systems for structural identification of these compounds in food, including single quadrupole (Carabias-Martinez et al., 2006; Maragou, Lampi, Thomaidis, & Koupparis, 2006) from cereal or milk samples and triple quadrupole (Loyo-Rosales et al., 2004; Shao, Han, Tu, & Huang, 2007b; Shao et al., 2005, 2007a; Smchmitz-Afonso et al., 2003) in beverages, meat, eggs, fish or milk for BPA, OP and/or NP determination.

The aim of this work was to develop a new rapid, sensitive, and reproducible analytical strategy for determining BPA, OP and NP contents in powdered milk (PM) and infant formulas (IF) using pressurised liquid extraction (PLE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) with a triple quadrupole (QqQ) mass analyzer.

2. Experimental

2.1. Solvents and standards

BPA, OP and NP were provided by Aldrich (Madrid, Spain). Methanol (HPLC-grade), acetonitrile, *n*-hexane, acetone, ethyl acetate, dichloromethane and isopropanol were purchased from Merck (Darmstadt, Germany). Ammonia and ammonium formate (HCO_2NH_4, 97%) were supplied by Sigma–Aldrich (Madrid, Spain). Anhydrous sodium sulphate granular powder was provided by Scharlau (Barcelona, Spain). Reversed-phase silica C_{18} and C_8, silica and florisil (MFE-Pack 50 μm) were provided by Panreac, Barcelona, Spain. Deionized water (<8 MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

Individual stock solutions were prepared by dissolving 10 mg of each compound in 10 ml of methanol. They were stored in glass-stoppered bottles at 4 °C in the dark. Standard working solutions at various concentrations were daily prepared by appropriate dilution of aliquots of the stock solutions in methanol or in sample extract.

2.2. Sample collection

Ten samples of different PM and IF were purchased from different supermarkets in Camerino (Italy) and Valencia (Spain). The composition was: the PM 34% proteins, 52% carbohydrates and 0.9% lipids; and the IF (adapted and follow-up) composition were 11.6–16% proteins, 54–55%carbohydrates (lactose or lactose + maltodextrin), 24–28% lipids, and some were added of nucleotides, prebiotic and probiotic agents. All samples were added with different vitamins and minerals and were vacuum packed in commercial airtight canned containers in a N_2/CO_2 (<3% O_2) modified atmosphere. The samples differed in their composition, but they were subjected to the same thermal treatment. All milk samples were stored at room temperature and after their packages had been opened they were put into specific glass food containers at 4 °C and analysed within 3 days.

2.3. Sample preparation

Due to photosensibility of the analytes, all the procedure was developed in the darkness, by protecting the samples with an aluminium foil. PM or IF (10 g) was blended with 7 g of reversed-phase silica C_{18} and 5 g of anhydrous sodium sulphate for 5 min in a mortar using a pestle. This mixture was introduced into a stainless steel extraction cell (33 ml capacity), which was positioned in the ASE system connected to a four-bottle solvent controller, both from Dionex (Sunnyvale, CA, USA). Nitrogen, at a pressure of 1500 psi (1 psi = 6894.76 Pa), was supplied to assist the pneumatic system and to purge the extraction cells. The extraction cells were preheated for 2 min, and the analytes were extracted with ethyl acetate at 70 °C and 10.3×10^6 Pa for 10 min of static time, in three cycles, at 60% of flush; then, the extraction cells was purged for 90 s with nitrogen to eliminate any trace of the extraction solvent.

Each ASE extract (50 ± 3 ml) was concentrated to approximately 1 ml in a Büchi R200 (Labortechnik, Flawil, Switzerland) rotary evaporator, set at 30–35 °C and 25.0×10^6 Pa, in a 250 ml round-bottom flask. For the samples of PM, the extract was transferred to a 15 ml conical tube and the round-bottomed flask was rinsed with 1500 μl of a mixture methanol/acetonitrile 50/50 and evaporated to dryness at 30 °C using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the solution was reconstituted with 500 μl of methanol/acetonitrile 50/50 and filtered through 0.20 μm nylon filter prior the injection.

Due to the different composition between PM and IF (mainly for the extraction procedure: 0.9% lipids against 24–28% lipids, respectively), for the sample preparation of IF some variations have been necessary: after the concentration on the rotary evaporator, the extract was transferred to a 15 ml conical tube, the round-bottomed flask was rinsed with 2000 μl of isopropanol and 2000 μl of methanol and it was evaporated almost to dryness at 30 °C using a multi-sample Turbovap LV Evaporator. The extracts were reconstituted with 500 μl methanol and ultrasonicated for 40 min at room temperature. Two different phases appeared: the lipidic and the methanolic. The lipidic phase was discarded, and the methanolic phase was collected and filtered through 0.20 μm nylon filter prior the injection.

2.4. Liquid chromatography–tandem mass spectrometry

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, and a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini C_{18} (250 mm × 4.6 mm, 5 μm) analytical column supplied by Phenomenex (Barcelona, Spain), using an isocratic mobile phase consisting of 5% water and 95% methanol/acetonitrile (90/10 v/v), both with 0.1% ammonia at a flow rate of 0.4 ml min^{-1}. Parameters were optimised by continuous flow injection of a standard solution (1 μg ml^{-1}) via a syringe pump at a flow rate of 15 μl min^{-1}. The analysis was performed in negative ion mode.

The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens, 0.5 V; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen 99.99% purity) flow, 200 L h^{-1}. The analyser settings were as follows: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, −3 and 1; multiplier, 500; collision gas: argon (99.995%) at a pressure of 0.279 Pa; interchannel delay, 0.02 s; total scan time, 1.0 s. The mass spectrometer was operated in scan, product ion scan, and selected reaction monitoring (SRM) modes. The SRM transitions (precursor–product ion transitions) selected were *m/z* 227 → 212 for BPA, *m/z* 205 → 106 for OP and *m/z* 219 → 106 for NP, at a cone voltage of 40 V, at collision energy of 15 V and a dwell time of 0.2 s for all compounds. In-house method validation: the method was validated by analysing blank SM and IF fortified with BPA, OP and NP at concentrations of 0.5 and 0.05 mg L^{-1}. The fortifications were prepared by adding 100 or 50 μl (depending on the final concentration) of the different BPA, OP and NP standard solutions in methanol to 10 g of SM or IF. The samples were shaken and allowed to settle for 10 min before the extraction procedure.

3. Results and discussion

3.1. Sample preparation

The stability of BPA, OP and NP for the whole period of sample analysis was studied. In particular, various temperatures for the evaporation process of the solvent during sample preparation were studied, i.e. 30, 40 and 50 °C, winding the flasks with an aluminium foil to shelter it from light. Studying the pattern of degradation, and taking as 0% the situation at 30 °C, it was found that working at 40 °C there was a degradation of analytes of 10% for BPA, 8% for OP and 8% for NP, while at 50 °C the values were 40%, 50% and 60% for BPA, OP and NP, respectively. In addition, it was tested the temperature of 50 °C without adding the protection of aluminium foil, and we detected a percentage of degradation of analytes of 75% for BPA, 48% for OP and 64% for NP. These data were in agreement with the studies reported in literature by some authors (Hong-Mei & Nicell, 2008; Shao et al., 2005) who have observed that the rates of degradation of BPA tend to increase with temperature. Other researchers (Diepens & Gijsman, 2008) found that sunlight, humidity, and oxygen bring about BPA polycarbonate degradation in outdoor applications; our data are in strong agreement with those results. To increase BPA polycarbonate lifetime, this undesirable degradation process needs to be overcome. For the analysis, the temperature was set 30 °C for the evaporation process and all samples were protected from the light.

Moreover, during the extraction process by ASE, the sodium sulphate was useful to eliminate any residue of water that normally is contained in powdered milk (until 3% of water).

3.2. ASE optimisation

The extraction procedure was optimised by varying one parameter at time, starting from those of the ASE apparatus default method (pressure 10.3×10^6 Pa, 1500 psi, static time 10 min, flush volume 60%, purge time 90 s, one cycle). The preheat time (preheating the cell before filling with solvent), the purge time (length of time that nitrogen purged the cell after the extraction) and the static time, were maintained at 2 min, 90 s and 10 min, respectively. Different experiments established that preheating for 2 min and the static time for 10 min achieved the best recoveries, with all solvents and samples, and that a purge time of 90 s resulted in any remaining solvent from the extraction cycle being drawn out of the extraction cell.

Different solvents or mixtures of them were tested to establish which provided the best recoveries, because the nature of solvent is the most important parameter of the extraction. Methanol, dichloromethane, acetone, ethyl acetate and different mixtures of *n*-hexane/acetone (30/70, 70/30, and 50/50 v/v), were studied. The ASE temperatures (40–120 °C), number of cycles (1–5) and matrix dispersant agents (florisil, silica, reversed-phase silica C_{18} and C_8) were optimised to obtain maximum BPA, OP and NP recoveries. All experiments were carried out in triplicate using blank IF (24–28% fat content) spiked at 50 μg kg^{-1}. Graphics detailing the influence of the solvent, temperature, number of cycles and dispersing agent on the recoveries of BPA, OP and NP are provided in Fig. 1.

The tested solvent giving the best result was ethyl acetate (recovery 93% for BPA, 103% for OP and 101% for NP). The extract was clearer, the amount of fat in the extract was easy to eliminate (see Section 2.3) and the recoveries of the three compounds were the highest.

The best recoveries were obtained at the temperature range from 70 to 100 °C. The small variations between recovery values obtained for OP and NP at different temperatures are within the relative standard deviation, which indicates that there is no significant variation. On the contrary, the recoveries of BPA at temperatures above 70 °C were lower. A temperature of 120 °C gave the worst results, because an increase of the temperature resulted in a dirty extract with an increasing amount of material co-extracted from the matrix, that hampered the quantification of the compounds; therefore, 70 °C was used as operating temperature for extraction, which provided an average of recoveries of 92% for BPA, 95% for OP and 88% for NP.

In order to obtain maximum extraction efficiency the influence of the number of cycles was also explored (Fig. 1). The number of extraction cycles indicates the number of times that static heating and flushing steps are repeated. The best recoveries were reached in tree cycles, with an average recovery of 93% for BPA, 89% for OP and 92% for NP. Larger number of cycles did not result in better recoveries.

Another parameter investigated was the type of dispersing agent. Fig. 1 shows as the best dispersing agent for obtaining the best performances was reversed-phase silica C_{18}; in particular the recoveries were 99% for BPA, 99% for OP and 102% for NP. Florisil showed high recovery percentage for OP and NP (88% and 82%, respectively) but low for BPA (45%), while reversed-phase silica C_8 showed 60% and 72% for OP and NP, and 72% for BPA. The lowest recoveries were obtained with silica gel, that gave values lower than 50% for all compounds.

Hence, the optimised parameters for the extraction of BPA, OP, and NP from PM and IF samples, using ASE with ethyl acetate,

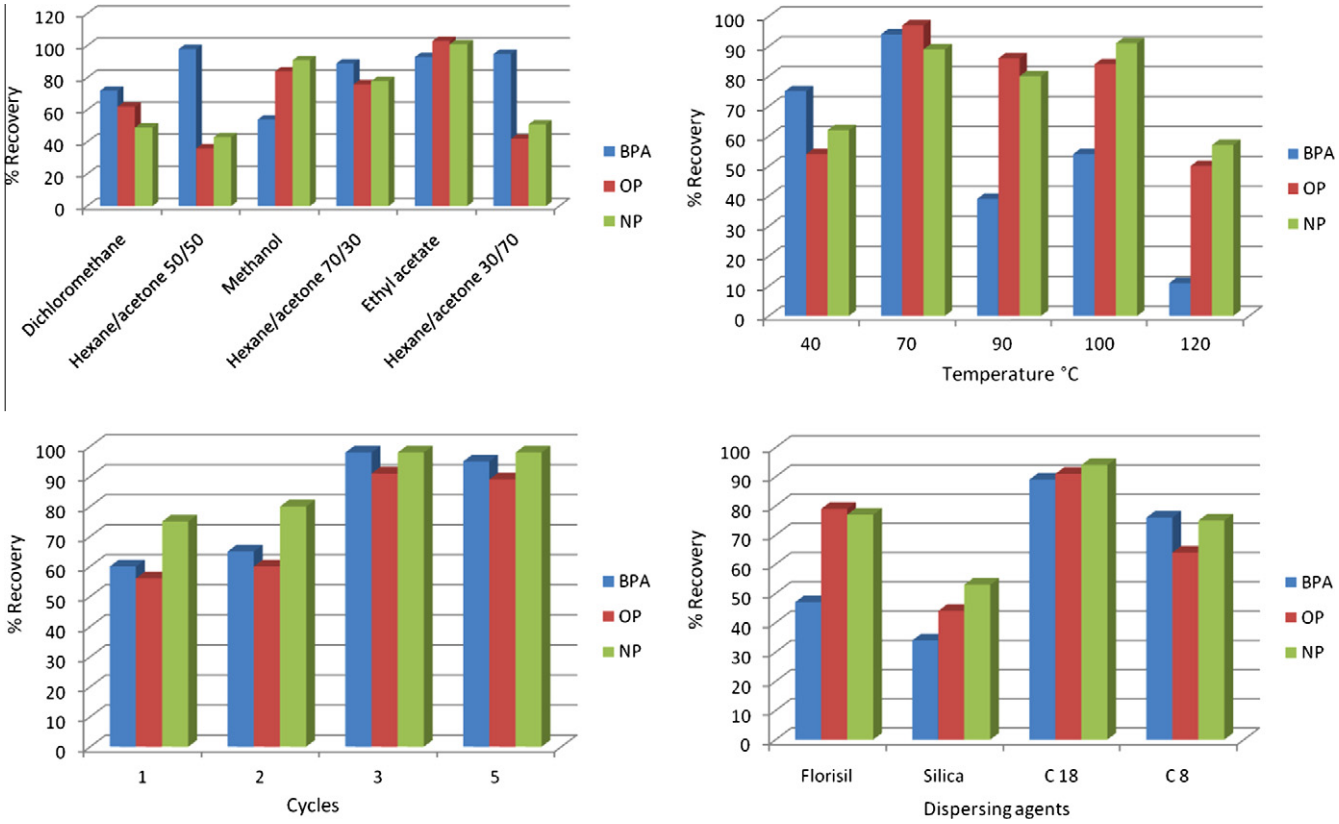

Fig. 1. Comparative study of extraction capacity in ASE experiments testing solvents, temperatures (°C), number of cycles and dispersing agents. All the analysis were performed in triplicate, with RSD values <15%.

were: 90% flush volume at 70 °C and 10.3×10^6 Pa for 10 min static time, running three cycles. These conditions provided an average recovery of 89% (85–99%) for BPA, 95% (87–103%) for OP and 100% (90–110%) for NP.

3.3. Optimisation of IF preparation conditions

For analysis of samples of animal origin, lipid may cause the main interference in the analysis of low polarity contaminants in biological materials. Thus, the presence of lipids in the extracts must be avoided or reduced as much as possible, in order to extend the column lifetime and to improve detection and quantification limits (Ballesteros-Gomez et al., 2009; Shao et al., 2007b). The principal difference between the IF and the PM is the lipid content: for the first one, the percentage of lipids are in the range 24–28%, while it is 0.9% for the second. In fact, for this reason, the ASE of the IF was reconstituted with a mixture of isopropanol/methanol 50/50 instead of acetonitrile/methanol 50/50, taking into account the higher lipophilicity and capacity of dissolve fats of isopropanol with respect to acetonitrile. After the evaporation process, methanol was added to residue that was sonicated to dissolve analytes; two non mixable phases were formed, the upper (methanolic) was taken, filtered and injected in LC–MS/MS. Unlike authors (Kuo & Ding, 2004) analysed samples with similar lipid content and need derivation process for the GC–MS BPA determination.

3.4. LC–MS/MS

Before optimising the MS/MS conditions, full scans and daughter scans under positive and negative mode for each compound were acquired to select the most abundant mass-to-charge ratio (m/z) for further studies by direct infusion. The results indicated that the negative mode full scan showed the deprotonated molecule $[M-H]^-$ to be the most abundant ion. Fig. 2 shows the product ion scan of BPA, OP and NP using LC–MS/MS. Although the relative abundance of the product ions depends on the collision energy used, other authors (Maragou et al., 2006; Shao et al., 2005) (to monitor BPA), choose the same transitions to monitor these compounds, and, explaining that the BPA fragment ion at m/z 212 is related to the loss of the CH_3 group from the deprotonated molecule, and the OP and NP fragment ion at m/z 106 indicated the loss of C_7H_{16} (for OP) and C_8H_{18} (for NP) groups from the deprotonated molecule. To detect the trace levels of these compounds, multiple-reaction monitoring (MRM) mode has been used. In addition, each compound was also characterised by its retention time. The criteria adopted for accepting the analysis were a retention time deviation of less than 10%.

The ionisation of ESI source occurs in the solution state; therefore, the mobile phase composition may affect the sensitivity of the analyte. To achieve the highest sensitivity, different solvent of mobile compositions were studied: water–methanol, water/acetonitrile and water/methanol/acetonitrile, all at different ratios and flows. The additives assayed were ammonia and ammonium formate at different concentrations for levels of pH from 6.3 to 10.8. Commonly, the addition of these additives in the negative mode has often been used to increase the response of target compounds. In this study, it was observed for the ammonia but not for ammonium formate. Therefore, 0.1% ammonia was added to the mobile phase. The final parameters are described in the Section 2.4.

Fig. 3 shows the chromatograms for each selected precursor → product ion transition obtained from (A) a methanolic solution standard of BPA, OP and NP, (B) an extract of the IF blank sample obtained after spiking it with 0.05 mg kg^{-1} of each compound, and (C) an extract of a naturally contaminated IF containing

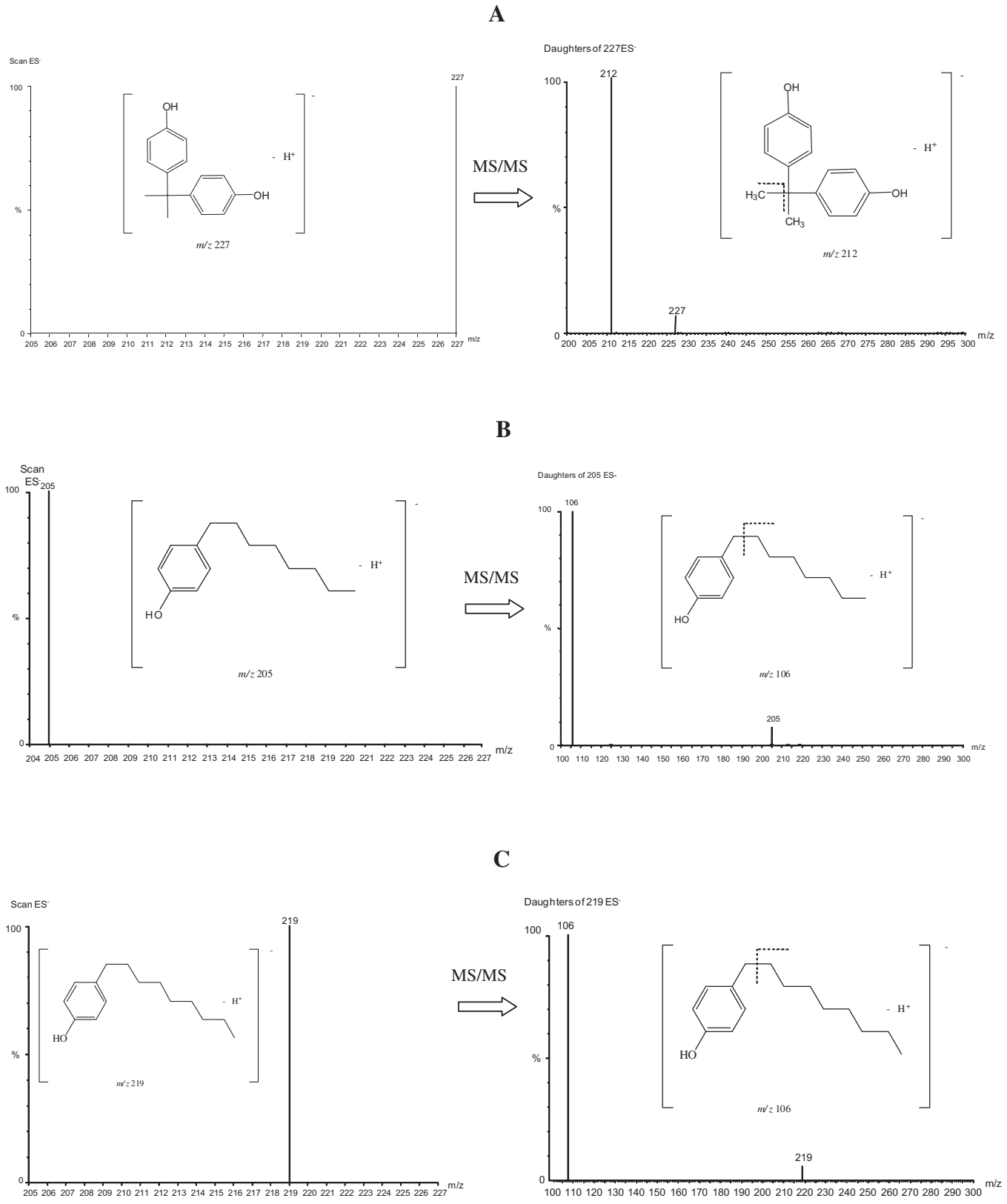

Fig. 2. Daughter scan MS/MS spectra obtained in flow injection analysis (FIA) of standard analytes at concentration of 1 mg L^{-1}: (A) BPA, (B) OP, (C) NP.

1.29 mg kg^{-1} of BPA, 1.55 mg kg^{-1} of OP and 0.69 mg kg^{-1} of NP. The chromatograms corresponding to the sample that contains the compounds shows a good relation with the peak areas of selected transitions.

3.5. Validation of the method

The PLE method, followed by LC–MS/MS, was tested for linearity, matrix effect, recoveries, repeatability, LODs and LOQs.

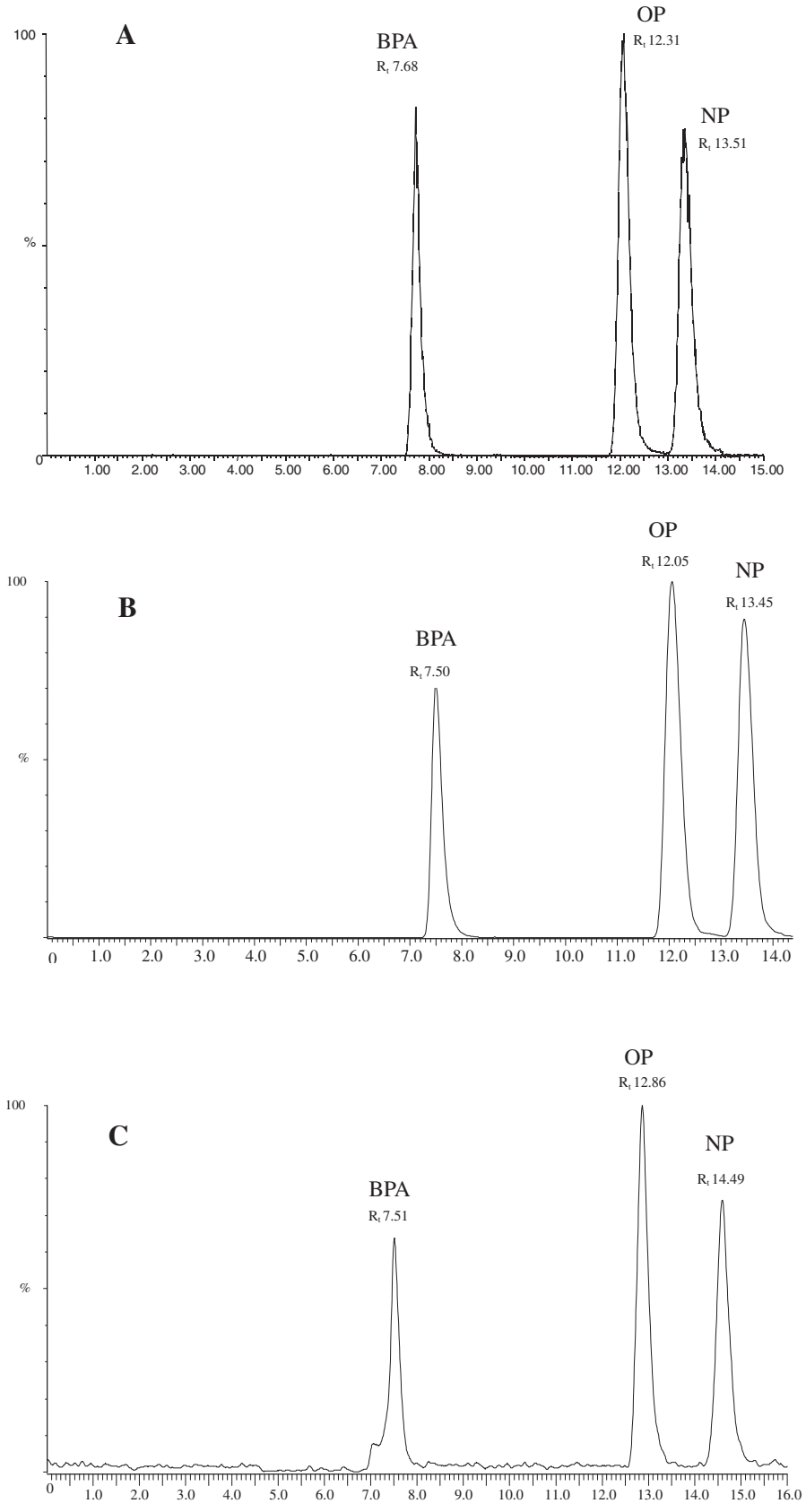

Fig. 3. Chromatograms for each selected precursor → product ion transition obtained from (A) a methanolic solution standard of BPA, OP and NP, (B) an extract of the IF blank sample obtained after spiking it with 0.05 mg kg^{-1} of each compound, and (C) an extract of a naturally contaminated IF (sample 9-S, see Table 2) containing 1.29 mg kg^{-1} of BPA, 1.55 mg kg^{-1} of OP and 0.69 mg kg^{-1} of NP.

Regression data of standards prepared in methanol, standards in extracts of IF and spiked standard samples of the same IF as extracted following the PLE procedure were studied.

The standards were prepared at six concentration levels; each level was prepared in triplicate and each calibration point was analysed twice. Calibration curves were constructed using the analyte peak area versus the concentration of the analyte. Linear calibration plots were obtained over a concentration range of three orders of LOQ. Good linearity was obtained for all analytes, with correlation coefficients of $r > 0.997$.

The need to use matrix matched standard curves was demonstrated by evaluating calibration curves based on standards diluted in methanol or in matrix extracts. These calibration curves presented good linearity (r value of all curves were >0.997), but with different slopes. The data show that suppression was higher than 68% for BPA, 25% for OP and 37% for NP, requiring the use of a matrix matched standard to perform a correct quantification. These results are in agreement with those previously reported (Shao et al., 2007a, 2007b) in eggs, meat and milk samples. The matrix effect was well compensated by the use of these matrix-matched standards, as demonstrated by the similarity between the slope obtained for the standard prepared in powdered milk extracts and that obtained for the standards spiked in the powdered milk samples and extracted throughout the procedure.

The analyte recovery of this procedure was evaluated by spiking different levels of standard analyte to samples at two levels in replicates of five. The results of recovery are listed in Table 1. The average recovery for each compound ranged from 89.01% to 92.25% for BPA, 83.51% to 98.45% for OP and 92.89% to 100.64% for NP. The reproducibility of this method was represented by the percent relative standard deviation (RSD) at each fortification level for each compound, and these values are also summarised in Table 1.

Six replicates of blank samples were extracted a level of 0.05 mg kg^{-1} and the run-to-run precision of each compound in PM and IF ranged from 4.6% to 7% for BPA, 8.4% to 10.1% for OP and 3.4% to 8.5% for NP and the day-to-day precision in PM and IF ranged from 9.0% to 11.4% for BPA, 11.3 to 14.03% for OP and 10.1% to 14.4%.

The LODs of the method, calculated using a signal-to-noise ratio of 3, were 0.005 mg kg^{-1} for BPA and NP and 0.003 mg kg^{-1} for OP; LOQs, using a signal-to-noise ratio of 10, were 0.016 mg kg^{-1} for BPA and NP and 0.010 mg kg^{-1} for OP. The LOQ was far below the current specific migration limit (SML) set for BPA by the EU Commission (600 ng g^{-1}) (Commission Directive 2004/19/EC, 2004).

Table 1
Percent recovery and repeatability of powdered skimmed milk and infant formulas at two fortification levels of 0.5 and 0.05 mg kg^{-1} ($n = 5$).

Compound	Concentration (mg kg^{-1})	% Recovery		% RSDs	
		Powder milk	Infant formulas	Powder milk	Infant formulas
BPA	0.5	90.02	89.01	12.01	14.36
	0.05	92.25	91.23	14.22	13.59
OP	0.5	83.51	95.55	7.14	8.11
	0.05	88.26	98.45	11.16	10.75
NP	0.5	92.89	100.64	12.54	10.16
	0.05	95.14	97.12	15.47	13.46

Table 2
Concentrations of BPA, OP and NP in Italian (I) and Spanish (S) powdered milks.

Sample	Type	BPA mg kg^{-1}	OP mg kg^{-1}	NP mg kg^{-1}
1-I	Infant formula	0.07	nd[a]	nd[a]
2-I	Infant formula	nd[a]	0.036	0.058
3-I	Infant formula	nd[a]	nd[a]	0.026
4-I	Infant formula	nd[a]	0.042	0.042
5-I	Infant formula	0.09	nd[a]	nd[a]
6-S	Infant formula	1.10	nd[a]	nd[a]
7-S	Skimmed milk	0.80	1.27	1.47
8-S	Skimmed milk	nd[a]	0.028	0.065
9-S	Infant formula	1.29	1.55	0.69
10-S	Infant formula	nd[a]	nd[a]	nd[a]

[a] nd = not detectable, <LOD.

3.6. Application to real samples

Ten commercial samples of powdered milk, five from Italian and five from Spanish markets, were analysed; among them, two PM and eight IF. The results are shown in Table 2. In all analysed samples the contamination for at least one compound was observed. BPA (levels from 0.07 to 1.29 mg kg^{-1}) was present in four IF and in one PM. OP was present in five samples, three IF and two PM, in the range 0.028–1.55 mg kg^{-1}. NP was present in six samples, four IF and two PM, at levels from 0.026 to 1.47 mg kg^{-1}. In two samples (one IF and one PM) all three BPA, OP and NP were present. The results obtained were in the order with previous studies, related to meat, eggs and milk, and different canned foods. Investigation on commercial samples indicated that APs were ubiquitous in milk (Goodson et al., 2002; Shao et al., 2007a, 2007b).

With respect to IF, no data for OP and NP are available in literature, while for BPA the results of our analysis were lower that those obtained by others (Kuo & Ding, 2004) in IF from Taiwan markets and are in agreement with other previous studies that reported BPA concentration in IF and powdered milk (Casajuana & Lacorte, 2004; Maragou et al., 2006).

The results obtained showed that the BPA, OP and NP were present in PM and IF, but levels were in almost all cases below concern. This is not tranquillizing enough, considering the presence of BPA in the infant food packages, the long shelf-life of IF, the high lipid content of IF, which increases the migration from packaging toward IF, the possible chronic exposure, the potential toxic effects of these compounds (which behave as endocrine disruptors, also at low doses), the simultaneous presence of more than one of them in IF, and that IF is the main nourishment of babies. Giving all the above, there is, undoubtably, a strong need for specific studies on the levels of migration of these compounds in IF. At the present, only for BPA a specific limit of migration was given (Commission Directive 2004/19/EC, 2004). The BPA concentration found in one IF exceeded its specific limit of migration.

The European Food Safety Authority (EFSA) (EFSA-Q-2005-100, 2006) establishes the Tolerable Daily Intake (TDI) for BPA at 0.05 mg kg^{-1} body weight and a specific level of migration of BPA from the packages at 0.6 mg kg^{-1} (2004/19/CE). In April 2008, the Government of Canada proposed the reduction of the exposure to BPA in children and newborn, prohibiting its use in baby's bottles. Health Canada also underlined the need for new analytical methods to quantify the migration of the compound from the packaging to the IF; also, alternatives to BPA in material of packaging were desirable. EFSA in 2008 (EFSA-Q-2008-382, 2008) reviewed studies on BPA toxicity and maintains the TDI, although it established the need for further studies regarding toxicity and monitoring the levels of BPA in food.

4. Conclusions

A new analytical method for the simultaneous determination of BPA, OP and NP in powdered skimmed milk and infant formulas by LC–MS/MS has been developed.

The LOQs were 0.016 mg kg^{-1} for BPA and NP and 0.010 mg kg^{-1} for OP below the current specific migration limit (SML) set for BPA by the EU Commission.

The entire method, ASE using ethyl acetate as a solvent and a reversed-phase silica C_{18} as a dispersing agent followed by LC–MS/MS, has been shown to be suitable for milk samples with recoveries at μg kg^{-1}.

Coupled to any of the techniques, the advantages of the PLE in terms of the quality of the results (recovery, repeatability and intermediate precision and focused analytical scope) and the practical aspects (low cost, labour, waste, glassware, and space and high sample throughput) make it a powerful approach to simultaneous BPA, OP and NP analysis of all types of milk samples.

All analysed samples contained at least one of the three analytes, which demonstrate that these compounds are ubiquitous in milk. Therefore, the content of these compounds must be monitored to meet consumer food safety concern.

Possible future investigations could be directed to study the degradation of these analytes, producing molecules as catechols that can interact with the catecholamine metabolism via catechol-*O*-methyl transferase or can bind to proteins or DNA, producing allergies or mutations.

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