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Occurrence of polycyclic aromatic hydrocarbons and their hydroxylated metabolites in infant foods

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ABSTRACT

Eleven polycyclic aromatic hydrocarbons (PAHs) have been analysed in commercial milk formulae and infant cereals. Two hydroxylated PAHs metabolites (1-OH-Pyr and 3-OH-B[a]P) and their conjugates were also analysed in milk samples. To determine the selected PAH metabolites, a simple, fast quantitative and economic method was developed. This method comprising ultrasound-assisted solvent extraction, enzymatic hydrolysis, solid-phase clean-up and detection by liquid chromatography with fluorescence detection (LC–FD) and liquid chromatography tandem mass spectrometry (LC–MS/MS) as confirmatory technique. The method was evaluated by constructing calibration curves, measurement of recovery, precision and the limits of detection. The purpose of this survey was to determine the selected analytes, to assess the exposure of babies and infants and to produce data for comparison with proposed limits that were being considered at the time of the survey. The results showed that not only no samples would have exceeded the limit for benzo[*a*]pyrene which is used as an indicator for the presence of PAHs, but also no hydroxy PAH metabolites have been detected.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemicals that are formed during the incomplete burning of coal, oil, gas, wood, garbage, or other organic substances, such as tobacco and food components. Fifteen of these compounds (benzo[*a*]anthracene, cyclopenta[*cd*]pyrene, chrysene, 5-methylchrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene, dibenzo[*ah*]anthracene, benzo[*g,h,i*]perylene, dibenzo[*a,l*]pyrene, dibenzo[*a,e*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,h*]pyrene) were recognised as clearly mutagenic and carcinogenic by the Scientific Committee on Food (Commission Regulation, 2006). According to this Committee, benzo[*a*]pyrene (B[*a*]P) can be used as a marker for the occurrence and effect of these carcinogenic PAHs in food. Nevertheless, further analyses of the relative proportions of these PAHs in foods would be necessary to inform about future review of the suitability of maintaining B[*a*]P as a marker (Commission Regulation, 2006).

The occurrence of PAHs in foods is due both to deposition from the air on the surface of plants, and the pollution resulting from manufacture processes such as drying, roasting, or smoking (Codex Alimentarius Commission, 2005; European Commission, 2002). Their presence in baby foods results in health risk to the infant, since they are more sensitive than adults to these contaminants. Since 1st April 2005 maxima residual limit (MRL) of 1.0 μg/Kg for B[*a*]P in foods for infants and young children, including cereal-based food and milk formulae, was set (Commission Regulation, 2006).

Once absorbed in humans and animals, PAHs are distributed by blood route to several tissues, especially to lipophylic tissues, due to their non polar character. Nevertheless, PAHs are metabolised to a complex mixture of quinines, phenols, dihydrodiols, triols and tetrols in the biological system. Pyrene and benzo[*a*]pyrene (B[*a*]P) are two of the best characterised PAHs and may be biotransformed in humans and animals to numerous phase 1 metabolites including 1-OH pyrene (1-OH-Pyr) and 3-OH benzo[*a*]pyrene (3-OH-B[a]P). These OH-metabolites may be biotransformed to phase 2 metabolites by conjugation with glutathione, glucuronide or sulphate (European Commission, 2002). The aim of the metabolisation is to increase their polarity to obtain hydrophilic substances in order to expedite their excretion. Though this mechanism produces a desintoxication, some PAHs are metabolised to active mutagen or carcinogen substances, which are capable of attacking cellular DNA. Moreover, some assays with animals have shown that some PAHs metabolites are suspected to be endocrine disruptors acting like hormones. Their effects occur mainly in infants of mothers exposed during pregnancy and lactation (Gozgit, Nestor, Fasco, Pentecost, & Arcaro, 2004).

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Though bile, faeces and urine are the principal elimination of PAHs and PAH metabolites, their transference to milk could be occurred (Grova, Feidt, Laurent, & Rychen, 2002; Grova, Rychen, Monteau, le Bizec, & Feidt, 2006; Lapole et al., 2007; Lutz et al., 2006). In this way Grova et al. (2006) and Lapole et al. (2007) found that the oral exposure of lactating goats to PAHs, resulted in a constant level of native PAH forms and in a significant increase in monohydroxylated metabolites in milk. The results were similar to those obtained by Lutz et al. (2006) when they determine the transfer kinetics of soil-bound PAHs to milk in lactating cows.

Metabolites are usually not included in the classical monitoring schemes of PAHs in milk samples (Aguinaga, Campilloa, Viñas, & Hernández-Córdoba, 2007; Kishikawaa, Wada, Kuroda, Akiyama, & Nakashima, 2003; Zanieri et al., 2007). Therefore, in the last years some works have been reported not only for determining native PAHs in milk samples, but also for determining their metabolites (Bulder et al., 2006; Grova et al., 2002; Grova et al., 2006; Lapole et al., 2007; Lutz et al., 2006).

The essential aim of this work was to check 11 mutagenic and carcinogenic PAHs, two hydroxylated PAH metabolites (1-OH-Pyr and 3-OH-B[a]P) and their conjugates, in commercial milk formulae and infant cereals. Selection of these two particular PAH metabolites was based on consideration of the importance as biomarker of PAHs exposure (1-OH-Pyr) and the importance as carcinogen (3-OH-B[a]P) (European Commission, 2002). To quantify native PAHs, a method developed by the present authors has been applied (Rey-Salgueiro, García-Falcón, Martínez-Carballo, & Simal-Gándara, 2008). To determine the selected PAH metabolites, a simple, fast quantitative and economic method was developed.

2. Materials and methods

2.1. Chemicals, solutions and materials

The eleven PAHs studied (benzo[*b*]fluoranthene (B[*b*]F, 98%), benzo[*k*]fluoranthene (B[*k*]F, 98%), benzo[*a*]pyrene (B[*a*]P, 97%), benzo[*ghi*]perylene (B[*ghi*]P, 98%), indeno[1,2,3-*cd*]pyrene (I[1,2,3-*cd*]P, 98%), benzo[*a*]anthracene (B[*a*]A, 98%), dibenzo[*ah*] anthracene (DB[*ah*]A, 97%), chrysene (Chr, 99%), 5-methylchrysene (5-Mch, 99%), dibenzo[*al*]pyrene (DB[*al*]P, 99%), benzo[*j*]fluoranthene (B[*j*]FA, 100%)), the two selected hydroxylated metabolites (1-hydroxy pyrene (1-OH-Pyr) and 3-hydroxy benzo[*a*]pyrene (3-OH-B[a]P)), the used antioxidants (*tert*-butyl hydroquinone (97%), ascorbic acid, butylated hydroxy anisole and butylated hydroxy toluene and galic acid) and also triton X-100 R were purchased from Sigma Aldrich (Madrid, Spain). These PAHs were used as markers for the 15 PAHs of toxicological significance. Ammonium acetate (97%), acetic acid (96%), HPLC grade acetonitrile, analytical grade dichloromethane, methanol, ethyl acetate and *n*-hexane were all supplied by Panreac (Madrid, Spain). ß-Glucuronidasa/arylsulphatase obtained from *Helix pomatia* was supplied by Roche (Madrid, Spain).

Individual 100 mg/L stock solutions of PAHs were prepared by dissolving about 0.010 g of product in a small amount of acetonitrile, hexane or acetonitrile:toluene (2:3) and diluting to 100 mL with the same solvent, which was selected depending on the solubility of the PAHs. From these solutions, solutions containing 10 and 0.10 mg/L concentrations of the different PAHs in *n*-hexane were prepared separately. From these diluted individual solutions, mixed solutions with PAHs ranging from 10 to 700 μg/L were prepared in acetonitrile following evaporation of the hexane. Working standard solutions used to construct the calibration curve were prepared in acetonitrile by dilution to reach concentrations between 0.020 and 40 μg/L.

To avoid losses of 1-hydroxy pyrene and 3-hydroxy benzo[*a*]-pyrene, due to their light-induced oxidation, individual 100 mg/L stock solutions were prepared in acetonitrile with 2.0 g/L *tert*-butyl hydroquinone. From these individual solutions, 100 μg/L mixed solutions were prepared in 2.0 g/L *tert*-butyl hydroquinone in acetonitrile. Working standard solutions used to construct the calibration curve were prepared in 2.0 g/L *tert*-butyl hydroquinone in acetonitrile by dilution to reach concentrations between 0.25 and 5.0 μg/L. These solutions were stored in amber flasks at 4 °C. PAHs solutions were stable for at least 6 months, hydroxylated PAHs only 1 month.

Waters Sep-Pak silica (690 mg) and Waters Sep-Pak Plus C18 cartridges were used as solid-phase extraction (SPE) minicolumns for purification and concentration. A Visiprep Solid Phase Extraction Vacuum Manifold was used to simultaneously process up to 24 SPE cartridges. Analytical grade C-45 nitrogen was supplied by Carburos Metálicos (Vigo, Spain). Additional equipment included a TurboVap evaporator (Caliper Life Sciences, Barcelona, Spain), an ultrasonic bath (P-Selecta, Barcelona, Spain), an oven (P-Selecta, Barcelona, Spain), an analytical precision scale (Sartorius, Madrid, Spain) and a vortex shaker (Heidolph, Barcelona, Spain). Disposables used were micropipettes (200–1000 μL) and injection vials (2 mL) furnished with screw caps and PTFE-lined butyl rubber septa and inserts (0.35 mL). Additional equipment included a rotary.

2.2. Samples

Nineteen samples of baby milk powder and 17 infant cereals were collected from different supermarkets. Samples of baby milk include: "infant milk" (6 samples), "follow-on milk" (8 samples) and "milk for special medical purposes" (5 samples). "Infant milk" means milk intended for particular nutritional use by infants during the first 4 to 6 months of life. "Follow-on milk" is only for babies over 6 months, as part of a mixed diet and should not be used as a breast milk substitute before 6 months (Commission Directive 91/321/EEC). Specialised formulae are for babies who have very special needs, such as premature babies or babies with metabolic or other disorders. These products differ in composition from formulas for healthy infants and are used under medical supervision only.

Samples of infant cereals cover a broad range of products: two "single grain cereals" (rice or oats), two "multigrain cereals" (combinations of two, five or eight grains, such as wheat, rice, barley, rye, corn, millet, sorghum or oats) and 13 "multigrain cereals with fruit, honey or cocoa".

2.3. Determination of native PAHs

The pre-analytical treatment was based on a procedure for the determination of PAHs in smoked foods, instant coffee or bread previously reported by the present authors (García Falcón, González Amigo, Lage Yusty, López de Alda Villaizán, & Simal Lozano, 1996; García-Falcón, Cancho-Grande, & Simal-Gándara, 2005; Rey-Salgueiro et al., 2008).

Sample (1.0 g) was subjected to ultrasound-assisted solvent extraction with 3 × 10 mL *n*-hexane for 10 min each in an EPA amber glass vial. The extract obtained was centrifuged (1000 U/min) during 5.0 min to facilitate separation of the liquid fraction. The ≈30 mL-extract was cleaned up directly with Sep-Pack silica plus cartridges (Waters, Spain), adding 10 mL *n*-hexane to avoid losses. The eluate was evaporated till dryness under a stream of nitrogen in a TurboVap LV Concentration Workstation (Caliper Life Sciences, Spain) and re-dissolved to a final volume 0.5 mL acetonitrile for HPLC–FD analysis.

Table 1
LC–FD conditions for the quantification of PAHs.

Chromatographic conditions		LC–FD parameters	
Solvent A		80% ACN	
Solvent B		20% H_2O	
Flow		1 mL/min	
Oven temperature		33 °C	
Injection volume		50 μL	
Solvent programming		80% A for 0 min 95% A in 40 min 95% A for 5 min 80% A in 1 min 80% A for 10 min	
Detection conditions	Time window (min)	λ_{exc}	λ_{em}
B[*a*]A	10–16	274	414
Chr			
5-Mch			
B[*j*]F	16–22	300	446
B[*b*]F			
B[*k*]F			
B[*a*]P			
D[*al*]P	22–30	296	406
D[*ah*]A		393	453
B[*ghi*]P	30–40	300	470
I[1,2,3-*cd*]P			

Chromatographic conditions were based on the method developed by Rey-Salgueiro et al. (2008), where the 11 selected PAHs in this work were detected in different bread samples. The liquid chromatographic system used was a Thermo Separation Products (TSP) P2000 binary pump equipped with a TSP AS1000 autosampler, a TSP SCM1000 vacuum membrane degasser and a Jasco FP-1520 fluorescence detector. PAHs separations were performed with a 25 cm × 4.6 mm (length × i.d.), 5.0 μm particle, Supelcosil LC-PAH obtained from Supelco. Chromatographic conditions, mobile phase, and FD operating conditions are given in Table 1.

In the present work PAHs recoveries were determined by spiking blank of cereals and milk powder ($n = 6$). After spiking, samples were stored at refrigeration in the dark for 24 h to facilitate equilibration with the sample matrix. The set of samples analysed was processed together with a reagent blank to test for contamination in the extraction process. Spiking levels were selected in accordance with the PAHs levels typically found in this type of samples. As Table 2 shows, the selected method was robust enough to quantify PAHs in commercial infant cereals and milk formula.

Detection and quantification limits (LODs and LOQs) were evaluated on the basis of the noise obtained with the analysis of unfortified blank samples ($n = 6$). LOD and LOQ were defined as the concentration of the analyte that produced a signal-to-noise ratio of 3 and 10, respectively (ACS, 1980).

2.4. Determination of hydroxylated PAHs and their conjugated forms

Milk sample (1.0 g) was subjected to three consecutive ultrasound-assisted solvent extractions with 9.0 × 6.0 × 5.0 mL acetonitrile:ethylacetate (70:30) with 0.8 g/L *tert*-butyl hydroquinone for 10 min each, in an EPA amber glass vial. The extract obtained was centrifuged (1000 U/min) during 5.0 min to facilitate separation of the liquid fraction. The eluate was evaporated till dryness under a stream of nitrogen in a TurboVap LV Concentration Workstation (Caliper Life Sciences, Spain) and re-dissolved to a final volume of 50 mL NH_4Ac/HAc pH 5.5 buffer. For the hydrolysis of the conjugated PAH metabolites, this mixture was incubated during 2 h at 37 °C with 20 μL β-glucuronidasa/arylsulphatase before incubation step, oxygen was removed with a nitrogen stream. Afterwards 10 mL acetonitrile was added and the mixture was loaded onto a C18 sep-pack cartridge (previously activated with 5 mL acetonitrile followed by 10 mL H_2O). Before elution step with 10 mL methanol:ethyl acetate (50:50), minicolumns were drying during 15 min under nitrogen stream at 15 bars. This eluate was again evaporated to dryness and the residue was re-dissolved in 0.5 mL acetonitrile for LC analysis.

PAHs metabolites separations were performed with a 250 × 4.6 mm (length × i.d.), 5.0 μm particle, Supelcosil LC-PAH obtained from Supelco. Chromatographic conditions, mobile phase, and FD operating conditions are given in Table 3.

LC–MS/MS was used as confirmatory technique. Analysis were performed on the Finnigan Surveyor HPLC System (Thermo), using a 150 × 2.0 mm (length × i.d.), 5.0 μm particle, Luna C8 analytical column obtained from Phenomenex (Madrid, Spain) and a 4 × 2 mm i.d., 5 μm particle, guard column containing the same packing material. The temperature of the HPLC column was kept constant at 40 °C. Mobile phase A was acetonitrile, mobile phase B was water. Analytes eluted using a linear gradient as Table 1 shows. A TSQ Quantum Discovery triple-stage quadrupole mass spectrometer (ThermoElectron, San Jose, CA, USA) was used as mass spectrometer. PAH metabolites were detected in negative electrospray ionisation (ESI) in selected reaction monitoring (SRM) acquisition mode. The spray voltage was set at 3.5 kV, with the sheath and auxiliary gas pressures (nitrogen) set at 35 and 5.0 psi, respectively, with a capillary temperature of 320 °C, and Tube lens voltage of 99 V. Collision energies were optimised for each transition reaction with an argon gas pressure of 1.5 m Torr

Table 2
Recoveries, repeatabilities, instrument linear dynamic ranges and limits of detection (LOD) and quantification (LOQ) in μg/Kg ($n = 6$) PAH and metabolites.

PAH	LOD	LOQ	Instrument linearity		Recovery ± RSD (%)	
	μg/Kg		Standards concentration range (μg/L ACN)	Added (μg/Kg ACN)	Infant cereals	Infant milk formulae
1-OH-P	0.15	0.50	1.0–8.0	2.5		102[a] ± 6.5/79[b] ± 4.0
3-OH-B[a]P	0.10	0.25	0.50–6.0	1.0		79[a] ± 2.3/70[b] ± 5.3
B[*a*]A	0.040	0.10	0.020–3.4	0.80	82 ± 3.0	116 ± 6.2
Chr	0.25	0.75	0.12–20	5.0	85 ± 2.0	104 ± 4.9
5-Mch	0.12	0.35	0.12–20	5.0	102 ± 2.0	102 ± 5.9
B[*j*]F	0.65	1.7	0.30–60	14	95 ± 4.0	102 ± 4.6
B[*b*]F	0.10	0.25	0.040–6.0	1.5	92 ± 6.0	107 ± 7.4
B[*k*]F	0.010	0.025	0.010–1.0	0.20	102 ± 7.0	113 ± 6.2
B[*a*]P	0.025	0.075	0.010–2.0	0.50	103 ± 5.0	101 ± 8.7
D[*al*]P	0.16	0.50	0.15–25	6.0	90 ± 3.0	107 ± 12
D[*ah*]A	0.10	0.25	0.040–7.0	2.0	97 ± 4.0	100 ± 6.3
B[*ghi*]P	0.70	1.7	0.30–60	14	98 ± 3.0	103 ± 6.3
I[1,2,3-*cd*]P	0.35	1.0	0.20–30	7.0	89 ± 3.0	100 ± 5.6

[a] Without enzymatic step.
[b] With enzymatic step.

Table 3
LC–MS/MS and LC–FD conditions for the confirmation and quantification of PAH metabolites.

Chromatographic conditions	LC–MS/MS parameters	LC–FD parameters
Solvent A	40% ACN	50% ACN
Solvent B	60% H_2O	50% H_2O
Flow	0.25 mL/min	1 mL/min
Oven temperature	40 °C	40 °C
Injection volume	20 μL	50 μL
Solvent programming	40% A for 2 min	50% A for 3 min
	75% A in 2 min	70% A in 3 min
	75% A for 4 min	70% A for 4 min
	98% A in 2 min	95% A in 2 min
	98% A for 5 min	95% A for 6 min
	40% A in 0.1 min	50% A in 0.1 min
	40% A for 10 min	50% A for 10 min

Detection conditions	Transition reactions (m/z)	Collision energy (eV)	Time window (min)	λ_{exc}	λ_{em}
1-OH-P	217 → 189	35	0–12	346	389
3-OH-B[a]P	267 → 239	42	12–25	308	432

(1 Torr¼ 133.3 Pa) (Table 3). Data processing was performed using Xcalibur software.

3. Results and discussion

Most of the analytical methods for determining PAH metabolites were developed in urine samples (Simon, Lafontaine, Delsaut, Morele, & Nicot, 2000; Simon, Morele, Delsaut, & Nicot, 1999; Smith et al., 2002; Wang et al., 2005; Whinton, Witherspoon, & Buckley, 1995), faeces (van Schooten, Moonen, van der Wal, Levels, & Kleinjans, 1997), blood and other biological fluids as fish bile (Mazéas & Budzinski, 2005; Richardson, Gubbins, Davies, Moffat, & Pollard, 2004). Though some analytical methods have been optimised for the determination of hydroxylated PAHs in milk (Grova et al. 2006; Lutz et al. 2006) to our knowledge no extraction procedures have been previously reported for their determination in powder milk.

3.1. Stability of PAH metabolites

Though several authors have reported the determination of PAH metabolites in different matrices, only few of them have mentioned the use of antioxidants or surfactants to avoid their oxidation losses. In this way Simon et al. (1999, 2000) used triton X-100 R to improve the recoveries of 1-OHP and 3-OH-BaP from urine samples. Mazéas and Budzinski (2005) and Richardson et al. (2004) added 2-mercaptoethanol and 4% ascorbic acid, respectively, to avoid oxidation phenomena for the quantification of PAH metabolites in bile.

In the present work not only losses during the analytical protocol, but also during standard storage of the analytes in acetonitrile, even at 4.0 °C, were observed. In this way, it was also verified by some assays, that degradation of PAHs metabolites increases with high temperature, under oxygen atmosphere and in aqueous media. Due to its high solubility in acetonitrile, *tert*-butyl hydroquinone was employed to obtain standard stability (2.0 g/L) and to avoid losses during sample treatment (0.8 g/L). This antioxidant does not lead chromatographic interference with the selected PAHs metabolites.

3.2. Performance of the PAHs metabolites determination

3.2.1. Analytical system

In order to select the most appropriate detection wavelengths for the selected hydroxylated PAHs, excitation and emission fluorescence spectra were recorded and 1-OH-Pyr and 3-OH-B[a]P were detected by fluorescence with emission and excitation wavelengths of 346 and 389 nm, and of 308 and 432 nm respectively. Mass spectrometric detection was selected as confirmation technique. In recent years, liquid chromatography/mass spectrometry (LC/MS) instruments have become available in many laboratories. With LC coupled to triple-stage quadrupole tandem mass spectrometers (LC/MS/MS), a wide range of substances in complex biological matrixes can be quantified at low levels and with high specificity. Despite this, relatively few LC/MS methods have been reported for determination of hydroxylated metabolites of PAHs. Ferrari, Mandel, and Berset (2002) employed HPLC with mass spectrometric detection to determine urinary concentrations of 1-OH-Pyr. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has been used by Xu, Zhang, Zhang, Liu, and Weisel (2004) to determine 13 monohydroxylated PAH metabolites.

Using negative ion electrospray ionisation, triple quadrupole mass spectrometer parameters were optimised with 1-OH-Pyr and 3-OH-B[a]P standards. The collision-induced dissociation of the analytes was studied by flow injection to establish the most sensitive transitions to be used for the quantitative determination in the multiple reaction monitoring mode (MRM).

3.2.2. Preanalytical treatment

In order to optimise extraction, enzymatic deconjugation and purification/concentration steps, milk extracts from samples without PAHs metabolites were used. They were spiked with 1-OH-P (2.5 μg/Kg) and 3-OH-B[a]P (1.0 μg/Kg) and were employed to select conditions providing high recovery and sensitivity.

a. Extraction: Due to the ionic character of the selected hydroxylated PAHs, methanol is the solvent recommended by other authors to their extraction from biological samples such as faeces (van Schooten et al., 1997). In the present work, a mixture between acetonitrile and ethyl acetate (70:30) was used instead methanol as extraction solvent in three consecutive steps (9.0 × 6.0 × 5.0 mL), because of their higher volatility. As it was mentioned previously, small loses of these metabolites were observed during extraction procedure and 0.8 g/L *tert*-butyl hydroquinone was added to the solvent mixture extraction.

b. Enzymatic deconjugation: Metabolites of phase 2, such as glucuronide or sulphate conjugate metabolites could be found in milk samples. In this way, enzymatic hydrolysis by a mixture of β-glucuronidasa/arylsulphatase would be necessary to obtain free 1-OH-Pyr and 3-OH-B[a]P (Mazéas & Budzinski, 2005; Richardson et al., 2004; Simon et al., 2000; van Schooten et al., 1997; Wang et al., 2005; Whinton et al., 1995). Since these enzymes are supposed to work in aqueous media, extraction organic solvent (20 mL of acetonitrile:ethyl acetate) must be evaporated till dryness. It was observed that the temperature applied during evaporation step may limit the recoveries of hydroxylated metabolites. Therefore and in order to decrease exposure time of the hydroxylated PAHs at high temperatures, small volumes of solvent extraction should be used. It was found that extraction volumes higher than 20 mL produced degradation losses and therefore, 20 mL were set to extract the analytes from milk powder samples.

After solvent evaporation, the extract is re-dissolved in 50 mL of buffer (NH_4Ac/HAc pH 5.5) and the mixture of β-glucuronidasa/arylsulphatase is added to deconjugate the PAH metabolites. Incubation times reported in the bibliography are very different: 1 h (Richardson et al., 2004; Simon et al., 1999, 2000), 3 h (Smith et al., 2002), 4 h (Wang et al., 2005), 16 h (Lutz et al. 2006) and 20 h (Mazéas & Budzinski, 2005). Since no commercial standards of PAH metabolites of phase 2 are available, optimisation of this enzymatic hydrolysis step must be carried out directly in samples with conjugate forms, but no conjugates of the selected hydroxylated PAHs were detected in the milk samples.

Nevertheless, it was possible to confirm that enzymatic hydrolysis was the most important step to obtain quantitative recoveries of the hydroxylated metabolites. This fact could be explained because of the lability of 3-OH-B[a]P in aqueous media, even with *tert*-butyl hydroquinone, and also because of the degradation products generated during incubation, which coeluted with 1-OH-Pyr. To verify this process, 50 mL buffer were added to some extracts of milk samples and they were spiked with 1.0 μg/Kg 3-OH-B[a]P and 2.5 μg/Kg 1-OH-Pyr. Twenty microlitre β-glucuronidasa/arylsulphatase were added and they were analysed after 30 min, 2 h and 8 h of incubation at 37 °C (Table 4). After 30 min incubation time, degradation of 3-OH-B[a]P was about 40%. Some antioxidants (ascorbic acid, galic acid, butylated hydroxy anisole and butylated hydroxy toluene) and also triton X-100R were tested in this step to avoid oxidations but no improvement was observed.

Since oxidation of hydroxy metabolites is the main source of degradation, before incubation step, oxygen was removed with a nitrogen stream. The recovery of 3-OH-B[a]P increased from 46% to 70% at 2 h incubation. Although 1-OH-P enjoys lower oxidation character than 3-OH-B[a]P, presents some chromatographic interferences when the extract is incubated more than 2 h. Recoveries about 150% were obtained at 8 h of incubation.

As the other authors reported (Wang et al., 2005) glucuronide hydrolysis was completed in less than 3 h. In this way and taking into account all the cited disadvantages, 2 h were selected as incubation time.

c. C18 SPE: To reach lower quantification limits and also to clean-up the extract, solid phase extraction (SPE) was carried out after buffer incubation. Sep-Pack C18 cartridges (Waters, Santiago de Compostela, Spain) were selected as it was often reported (Lutz et al., 2006; Mazéas & Budzinski, 2005; Simon et al., 1999; Wang et al., 2005). Experiments were performed to optimise the different steps of SPE extraction (loading and elution) of the hydroxy PAHs.

To increase the solubility of the PAH metabolites and to avoid the sorption to wall material, an organic solvent (acetonitrile) was added to buffer solution before SPE step. This solvent also facilitates movement of metabolites along the cartridge and the subsequent elution with low volumes of organic solvent. Acetonitrile/buffer solutions spiked with hydroxy PAHs were tested (Fig. 1), and the best recoveries were obtained with 20% acetonitrile. Therefore, 10 mL acetonitrile were added to 50 mL buffer after incubation.

The following solvents were evaluated during elution step: acetonitrile, hexane, dichloromethane, and mixtures between acetonitrile/dichloromethane (50/50), acetonitrile/dichloromethane (67/33) and methanol/ethyl acetate (50/50). Though by acetonitrile/dichloromethane (50/50) mixtures recoveries between 95% and 100% were obtained, they were rejected due to the carcinogenic character of dichloromethane. Recoveries about 95% were also observed with methanol/ethyl acetate (50/50) and this mixture was selected as elution solvent.

3.3. Characterisation

To validate the analytical method for determining hydroxylated (1-OH-Pyr and 3-OH-B[a]P) and total (hydroxylated PAHs and their conjugates) PAH metabolites, two isolated procedures should be studied. When free 1-OH-Pyr and 3-OH-B[a]P are determined, the analytical procedure would be carried out without enzymatic hydrolysis. When total 1-OH-Pyr and 3-OH-B[a]P are analysed, the whole procedure with enzymatic hydrolysis would be applied. To evaluate the method performance, spiked samples of commercial infant formula were processed. Table 2 shows the obtained results.

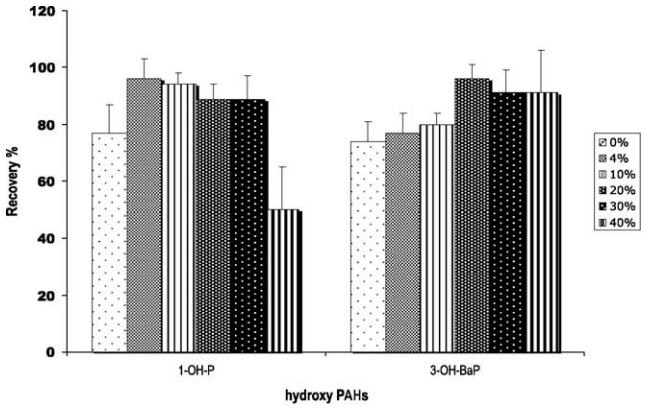

Fig. 1. Influence of acetonitrile (0%, 4%, 10%, 20%, 30%, and 40%; average ± standard deviation) added to the buffer on hydroxylated PAHs recovery obtained by SPE.

Detection and quantification limits (LODs and LOQs) were evaluated on the basis of the noise obtained with the analysis of unfortified blank samples ($n = 6$). LOD and LOQ were defined as the concentration of the analyte that produced a signal-to-noise ratio of 3 and 10, respectively (ACS, 1980) and were then tested experimentally by spiking blank samples at such levels. External standard calibration was used to quantify the samples by LC–FD technique using multicomponent standards (Table 2). Linear calibration plots – verified by the Mandel fitting test ($P = 99\%$) (Mandel, 1964) – were obtained over a concentration range of two or three orders of magnitude, depending on the compound.

4. Determination of native PAHs and PAH metabolites in infant foods

Nineteen samples of infant formula and 17 infant cereals were collected and evaluated with the proposed methods. In only two samples, one milk and one cereal samples, B[*k*]F was found at levels of 0.10 and 0.30 μg/Kg, respectively. No metabolites were found in the selected samples. The analysed samples fulfil the quality EU criteria.

Few works have determined PAHs in infant foods (Aguinaga et al., 2007; FSA, 2006; Kishikawaa et al., 2003). In general the results obtained by these authors are similar to our results. The food standards agency (FSA) determined 15 PAHS in 97 samples of infant formulae milk obtained from across the UK (FSA, 2006). B[*a*]P was detected in 39 samples in concentrations levels lower than 1.0 μg/Kg. Kishikawaa et al. (2003) analysed three infant formulae samples, finding total levels of PAHs about 2.0 ± 0.30 μg/kg,

Table 4
Comparison of recoveries obtained with or without nitrogen stream during enzymatic deconjugation ($n = 3$).

Incubation time	Without nitrogen stream		Under nitrogen stream	
	% Recovery 1–OH–P (% RSD)	% Recovery 3–OH–B(a) P (% RSD)	% Recovery 1–OH–P (% RSD)	% Recovery 3–OH–B(a) P (% RSD)
30 min	106 (5.0)	60 (10)	104 (5.2)	85 (5.0)
2 h	75 (8.0)	46 (5.0)	79 (4.0)	70 (5.3)
8 h	150 (8.0)	44 (7.0)	145 (3.3)	76 (7.1)

ranging B[*a*]P concentration between 0.27 and 0.39 μg/kg . Aguinaga et al. (2007) determined 8 of the 11 selected PAHs in this work in one infant formulae. PAHs were not detected.

Other reports have been focused to the analysis of another source of milk. Zanieri et al. (2007) determined PAHs in breast milk samples from 32 smoking and no-smoking mothers. The results obtained have shown the impact of tobacco in milk pollution. In this way, PAH levels of the 11 breast milk samples from smoking mothers were about 11 μg/Kg for naphthalene and acenaphthene, 0.10 μg/Kg for anthracene and benzo(k)fluoranthene. About 0.70 μg/Kg B[*a*]P was detected in six milk samples. In no-smoking mothers no quantifiable B[*a*]P levels were detected. Kishikawaa et al. (2003) determined also human milk and total PAH levels ranging from 0.19 and 2.15 μg/kg.

Grova et al. (2002) assessed milk PAH contamination sources of milk samples collected from the tank milk at farms located near potential contaminating emission sources such as cementworks, steelworks, and motorways. For all potential contaminating sources, these eight PAHs were detected with similar profiles and at low concentrations except for fluorene and naphthalene. These results motivated later analysis to verify the metabolisation PAHs and their migration to milk (Grova et al., 2006).

5. Conclusions

The proposed methods help to cover the needs to asses the state of powder milk pollution. Detection and quantification limits of the analytical protocol proposed for the PAHs determination in infant foods, were found to be satisfactory and much lower than the restrictions given in proposals of EU Regulation for B[*a*]P (1.0 μg/kg). As far as we know, there are no published methodologies to determine PAH metabolites in powder milk formulae. The lability of these compounds, especially in aqueous medium, limits the quantitative analysis. Since oxidation of hydroxy metabolites is the main source of degradation, the addition of an antioxidant in the solvent extraction and the elimination of oxygen of aqueous solution during enzymatic deconjugation, improves recovery rates and precisions (79 ± 4.0 and 70 ± 5.3 for 1-OH-P and 3-OH-B[a]P, respectively). The analytical methodology developed could eventually be used for studies of these PAHs metabolites in other food of animal origin.

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