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Simultaneous determination of bisphenol A and bisphenol B in beverages and powdered infant formula by dispersive liquid–liquid micro-extraction and heart-cutting multidimensional gas chromatography-mass spectrometry

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The purpose of this study was to establish a reliable, cost-effective, fast and simple method to quantify simultaneously both bisphenol A (BPA) and bisphenol B (BPB) in liquid food matrixes such as canned beverages (soft drinks and beers) and powdered infant formula using dispersive liquid–liquid micro-extraction (DLLME) with in-situ derivatisation coupled with heart-cutting gas chromatography-mass spectrometry (GC-MS). For the optimisation of the DLLME procedure different amounts of various extractive and dispersive solvents as well as different amounts of the derivative reagent were compared for their effects on extraction efficiency and yields. The optimised procedure consisted of the injection of a mixture containing tetrachloroethylene (extractant), acetonitrile (dispersant) and acetic anhydride (derivatising reagent) directly into an aliquot of beverage samples or into an aqueous extract of powdered milk samples obtained after a pretreatment of the samples. Given the compatibility of the solvents used, and the low volumes involved, the procedure was easily associated with GC-MS end-point determination, which was accomplished by means of an accurate GC dual column (heart-cutting) technique. Careful optimisation of heart-cutting GC-MS conditions, namely pressure of front and auxiliary inlets, have resulted in a good analytical performance. The linearity of the matrix-matched calibration curves was acceptable, with coefficients of determination ($r2$) always higher than 0.99. Average recoveries of the BPA and BPB spiked at two concentration levels into beverages and powdered infant formula ranged from 68% to 114% and the relative standard deviation (RSD) was $<15\%$. The limits of detection (LOD) in canned beverages were 5.0 and 2.0 $ng\,l^{-1}$ for BPA and BPB, respectively, whereas LOD in powdered infant formula were 60.0 and 30.0 $ng\,l^{-1}$, respectively. The limits of quantification (LOQ) in canned beverages were 10.0 and 7.0 ng l–1 for BPA and BPB, respectively, whereas LOQ in powdered infant formula were 200.0 and 100.0 $ng\,l^{-1}$, respectively. BPA was detected in 21 of 30 canned beverages (ranging from 0.03 to 4.70 $\mu g\,l^{-1}$) and in two of seven powdered infant formula samples (0.23 and 0.40 $\mu g\,l^{-1}$) collected in Portugal. BPB was only detected in canned beverages being positive in 15 of 30 samples analysed (ranging from 0.06 to 0.17 $\mu g\,l^{-1}$). This is the first report about the presence of BPA and BPB in canned beverages and powdered infant formula in the Portuguese market.

Keywords: gas chromatography/mass spectrometry; bisphenol A; canned beverages; infant formula; DLLME

Introduction

Packaging materials are used in food industry to protect foodstuff from microbiological, chemical and physical contamination during its storage and distribution. However, no food-contact material is completely inert and reactions with the food contained within (e.g. permeation, sorption and corrosion) may occur, leading to the release of some chemical packaging components, some of which can be hazardous to the food consumers (Sablani and Rahman 2007).

A compound that has given rise to many concerns in recent years is bisphenol A (2,2-*bis*(4-hydroxyphenil)propane; BPA) owing to systematic evidence about its presence in canned food products, due to their migration from packaging materials. BPA, first synthesised in 1891, was not extensively used until the early 1950s when a steady production increase was observed, which by 2006 reached approximately 3.8 million tons $year^{-1}$ (see www.bisphenol-a.org and www.bisphenol-a-europe.org). Most BPA is used as a monomer in the manufacture of polycarbonates employed in food-contact materials such as returnable beverage bottles, infant feeding bottles, tableware (plates and mugs) and storage containers, and in the production of epoxy resins used in surface coatings for food and beverage cans and vats (Chapin et al. 2008). Although less discussed, bisphenol B (2,2-*bis*(4-hydroxyphenil)butane; BPB), a congener of BPA used by the chemical industry in the manufacture of phenolic resins (Merck 2001), can also be considered a

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potential migrant and food contaminant (Grumetto et al. 2008).

Regardless of the ubiquitous presence of BPA in the environment, the primary source of exposure to the compound is through the diet as a result of the migration of the BPA monomer from the packaging (Vandenberg et al. 2007). Air, dust and water exposition (including skin contact during bathing and swimming) are other possible sources of exposure, as well as occupational exposure during the manufacture of BPA and BPA-containing products (Vandenberg et al. 2007; Chapin et al. 2008).

It is well known that BPA has an oestrogenic activity, demonstrated by *in vivo* laboratorial experiments in rats and mice with oral administration of BPA (Ashby and Odum 2004). *In vitro* human studies have shown that the oestrogenic effect is attained by binding to the nuclear oestrogen receptor (Nagel et al. 1998; Blair et al. 2000) whereas the conjugated metabolites are devoid of this activity (Matthews et al. 2001; Shimizu et al. 2002). To the congener BPB, endocrine-disruptive properties similar to those of BPA are endorsed (Yoshihara et al. 2004), specifically oestrogenic and anti-androgenic activities (Kitamura et al. 2005). It is believed that BPA could be associated with reproductive cancers (testicular, prostate, breast, uterine, ovarian, etc.), fertility problems (low sperm count, decreased sperm quality) and other endocrine-related endpoints (Vandenberg et al. 2007). Many of the oestrogenic effects caused by BPA have been reported to occur at concentrations below the recommended safe daily exposure (Melzer et al. 2010).

Despite ongoing controversy over exposure levels, a European Union risk assessment report dated from 2008 and reconfirmed in 2010 concluded that there is no evidence of an increased risk for humans by the present exposure due to BPA in the environment (European Union 2008, 2010). A similar report released by the US Food and Drug Administration (USFDA) also found no reason for major concern (USFDA 2008), although it was criticised for including mostly industry-funded studies and excluding newer data obtained in the National Toxicology Program report. More recently, in January 2010 the USFDA published recommendations for parents on the minimisation of children's exposure (USFDA 2010). Currently, the tolerable daily intake set by the European Commission and the reference dose established by the US Environmental Protection Agency (USEPA) is 0.05 mg kg^{-1} body weight day^{-1} (European Union 2010).

Several studies have reported BPA concentrations in foods, beverages and infant formula (reviewed in Ballesteros-Gómez et al. 2009). However, there is still a need to update exposure analysis including assessing and accounting for variability in the consumption of BPA, in particular any possible geographic and/or temporal deviations (Ackerman et al. 2010). The presence of BPB was reported by Grumetto et al. (2008) in canned peeled tomatoes with 21.4% of positive samples.

Taking in account the low levels of BPA usually found, very sensitive methods must be selected to ensure a reliable determination of the compound in complex food matrixes. Over the last decade the development of new analytical equipment and novel extraction techniques has led to significant improvements in the sensitivity, selectivity, speed and cost of the analysis. Issues of green chemistry and environmental protection should also be taken into account in the evaluation of analytical methods. In the field of sample preparation solid-phase extraction (SPE) with different sorbents (Cao et al. 2009), pressurised liquid extraction (PLE) (Carabias-Martínez et al. 2006), solid-phase micro-extraction (SPME) (Nerín et al. 2002; Chang et al. 2005), matrix solid-phase dispersion (Shao et al. 2007), or dispersive liquid–liquid microextraction (DLLME) (Rezaee et al. 2009; Wang et al. 2009) have been proposed as alternatives to the classical liquid–liquid extraction procedures in BPA analysis. Among these procedures the most simple, rapid and environmentally friendly sample preparation proposal is most likely the DLLME procedure whose principles have been recently reviewed by Rezaee et al. (2010). Basically, DLLME consists in the formation of a cloudy solution promoted by the fast addition of a mixture of extractive and dispersive solvents to the aqueous sample. The tiny droplets formed and dispersed among the aqueous sample solution are further joined and sedimented in the bottom of a conical test tube by centrifugation, allowing great enrichment factors and good yields for a wide range of compounds. The resultant sedimented phase is usually directly analysed by gas chromatography (GC) or liquid–chromatography (LC) (Rezaee et al. 2010). This procedure has been successfully applied in the extraction of BPA from water samples (Rezaee et al. 2009; Wang et al. 2009), as well as in other different types of compounds such as multi-pesticide residues (Cunha et al. 2009), aromatic hydrocarbons (Rezaee et al. 2006), metals (Jahromi et al. 2007), and aromatic amines (Farajzadeh et al. 2007) from a wide variety of samples. To the best of our knowledge the use of a DLLME procedure for the extraction of BPA and congeners from food matrixes has not previously been reported.

The determination of BPA has been carried out by LC with fluorescence (Grumetto et al. 2008), ultraviolet (Rezaee et al. 2009), or mass spectrometry (MS) detection (Maragou et al. 2006; Shao et al. 2007). Alternatively, some authors make use of GC-MS, a technique that provides a higher resolution, although it has the inconvenience of requiring prior derivatisation of the analyte (Ballesteros-Gómez et al. 2009).

A noteworthy improvement for the association of DLLME extraction with GC determination of BPA was given by Wang et al. (2009) who proposed the simultaneous extraction/derivatisation of the analyte by adding acetic anhydride to the extractant/dispersant mixture used.

In the field of chromatographic analysis, a major advance in recent years entails the introduction of heart-cutting multidimensional GC coupled to mass spectrometry (MDGC-MS), which substantially increases the sample capacity, power of separation of trace compounds in complex matrixes, reliability and robustness compared with conventional GC-MS methods (Farajzadeh et al. 2007; Cunha and Fernandes 2010). The heart-cutting MDGC-MS is achieved with a Deans Switch device which allows the selection of sections of a primary column separation, based upon retention time, to pass to the start of a second column for MS analysis or to go to a restrictor column for waste.

The main objective of this study was to develop and validate a reliable and fast DLLME/heart-cutting MDGC-MS method for the determination of BPA and BPB in food matrixes, specifically canned soft drinks, beers and powdered infant formula. Special attention was given on the optimisation of the DLLME procedure by cautious evaluation of the nature and amount of extractive and dispersive solvents as well as the amount of derivative reagent. The developed method was used to assess the occurrence of BPA and BPB in canned soft drinks, beers and powdered infant formula samples commercialised in Portugal.

Materials and methods

Reagents and materials

Standards of bisphenol A (BPA; 99% purity) and bisphenol B (BPB; ≥98% purity) were purchased from Sigma (West Chester, PA, USA). Internal standard (IS) d_{16}-bisphenol A ($BPAd_{16,}$ 98 atom% D) was also purchased from Sigma. Derivatisation reagent acetic anhydride (AA; >99% purity) was purchased from Fluka (Neu-Ulm, Germany). Potassium carbonate (K_2CO_3) was analytical grade obtained from Merck (Darmstadt, Germany). Trichloroacetic acid (TCA, >99% purity) was obtained from Sigma. From the same company were obtained the pH test strips (0–14 pH resolution: 1.0 pH unit). Dispersive solvents acetonitrile (MeCN), acetone (AC) and methanol (MeOH) were high-purity grade solvents for pesticide residue analysis obtained all from Fluka. Extractive solvents tetrachloroethylene (T4CE), trichloroethylene (T3CE), carbon disulfide (CD), chlorobenzene (CB) and carbon tetrachloride (CTC) were high purity solvents for HPLC analysis obtained from Sigma.

Ultra-high-purity helium (99.999%) for GC-MS was obtained from Gasin (Maia, Portugal).

Conical plastic tubes of propylene (TPP; Techno Plastic Products, Trasadingen, Switzerland) were used in all the experiments performed in this study, due its lower price, availability, resistance and disposable nature. To evaluate the possible presence of BPA and BPB in the plastic tubes, for each batch of experiments a sample of blank water (free of BPA and BPB) was analysed. If BPA and/or BPB were detected, the tubes were replaced.

Standards

Individual stock solutions of BPA (20 mg l^{-1}) and BPB (20 mg l^{-1}) were prepared in MeOH. Standard working solutions containing both BPA and BPB in concentrations between 0.02 and 10 µg l^{-1} (depending on the experiment) were prepared in 0.5% potassium carbonate solution (0.5% K_2CO_3). An individual working solution of $BPAd_{16}$ (1 mg l^{-1}) was prepared in 0.5% K_2CO_3 from a stock solution of 20 mg l^{-1} in MeOH. All solutions were stored at −18°C when not in use. Matrix-matched calibration curves were achieved by analysis of blank samples (free of BPA and BPB) spiked with known amounts of working solution in order to obtain the desired concentration range. The matrixes were always spiked before pre-treatment or extraction. The concentration of analytes in the samples analysed was obtained by the IS method.

Sampling

A total of 22 samples of canned soft drinks comprising six brand colas, seven energetic drinks, six carbonated drinks and two no carbonated flavour drinks were randomly purchased in local supermarkets. A total of eight samples of canned beers were also purchased in local supermarkets. All samples were stored at room temperature (±20°C) protected from light and opened only at the moment of analysis.

A total of seven samples of canned powdered infant formula were also purchased in local supermarkets. The powdered infant formula were reconstituted with water at the ratio prescribed on the packaging (approximately 18 g of powdered milk in 120 ml of water) before the analysis.

Blank matrixes

A few samples of colas and beers in glass bottles were also acquired and analysed. Because they show no signals of BPA and BPB they were used as 'blank matrixes'. In the same way milk samples packed in glass bottles were used as 'blank matrixes' for BPA and BPB quantified in powdered infant formula.

Sample preparation

Soft drinks and beers

Approximately 10 ml (≈10 ± 0.2 g) of sample were placed into a 25 ml screw cap plastic tube with conical bottom, spiked with 100 µl of $BPAd_{16}$ solution (to reach 10 µg l^{-1}), and 5% K_2CO_3 solution was added until pH ≥ 10. A mixture of MeCN (440 µl), T4CE (30 µl) and AA (30 µl) was rapidly injected into the sample tube. The tube was closed and shaken gently by hand for 1 min. After that it was centrifuged at 11 000 rpm for 2 min and 20 µl of the sedimented phase were transferred for a vial provided with a 100 µl insert. Finally, 1 µl of the extract was injected in the MDGC-MS system.

Powdered infant formula

Approximately 10 ml (≈10 ± 1 g) of sample were placed into a 25 ml screw cap plastic tube, spiked with 100 µl of $BPAd_{16}$ solution (to reach 10 µg l^{-1}) and 5 ml of TCA solution (10% in MeOH) was added. After vortexing for 1 min and centrifuging at 11,000 rpm for 5 min the upper layer was transferred to another tube and 5% K_2CO_3 solution was added until pH ≥ 10. Then 10 ml of the extract were transferred to a tube with a conical bottom and a mixture of MeCN (420 µl), T4CE (50 µl) and AA (30 µl) was rapidly injected. The other steps of the procedure were the same as described above for soft drinks, providing a final volume of sedimented phase of 35 µl.

GC-MS equipment and conditions

The determination of BPA and BPB was performed on an Agilent (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port, a Deans Switch device, an inert 5975B mass selective detector with electron impact (EI) ionisation chamber, and a 7683B Series injector/autosampler.

Heart-cutting GC separation was performed with the columns set as follows: primary column, 5 m × 0.32 mm I.D. × 0.10 µm film DB-5HT (J&W Scientific, Folsom, CA, USA), secondary column, 15 m × 0.25 mm I.D. × 0.25 µm film thickness DB-5MS (J&W Scientific) and restrictor, 4.31 m × 0.18 mm I.D. deactivated empty capillary column. The front inlet pressure with electronics pneumatic controls (EPC), directly connected with the primary column, was set with a ramped pressure (169.2 kPa, 0.31 kPa min^{-1} to 172 kPa), and a separate pneumatic control module (PCM) with EPC was attached to an auxiliary inlet, directly connected with the Deans switch device, delivering helium at a constant pressure of 166.4 kPa. The injection was made in splitless mode (0.5 min) and the injector temperature was 280°C. For heart-cutting the analytes of interest, the Deans switch valve (solenoid valve) was initially switched off (following to restrictor column), switched on at 6.0 min to move all the analytes of interest to the MS detector and switched off again at 7.5 min until the end of run (Figure 1). The oven temperature programme was as follows: 140°C held for 1 min, ramped to 280°C at 40°C min^{-1} held for 3.3 min. Total run time was 9 min. The MS transfer line temperature was held at 280°C.

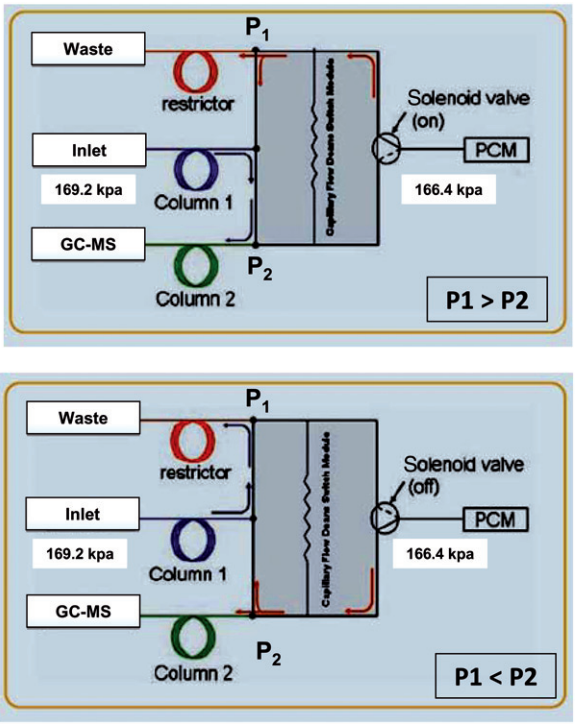

Figure 1. Deans switch GC-MS system used. (A) The solenoid valve is in the 'on' position allowing effluent to flow to the 2D GC separation column prior to MS detection. (B) The solenoid valve is in the 'off' position and effluent from the primary column is flowing to the exit gas line.

Mass spectrometric parameters were set as follows: electron impact ionisation with 70 eV energy; ion source temperature, 230°C; MS quadrupole temperature, 150°C and solvent delay 5 min. The MS system was routinely set in selective ion monitoring (SIM) mode and each analyte was quantified based on peak area using one target and three qualifier ion(s). Complete SIM parameters and retention times of the analytes are shown in Table 1. Agilent Chemstation was used for data collection/processing and GC-MS control.

Results and discussion

Optimisation of extraction and derivatisation conditions

In order to develop and optimise a DLLME procedure for an effective and reproducible extraction of low

Table 1. MS conditions for the heart-cutting GC-MS analysis of acetylated BPA, BPB and IS (time windows and ions selected in SIM mode, quantification ions are shown in bold).

Analyte $[M]^+$	t_R (min)	Time windows (min)	Data acquisition rate (scans/s)	SIM ions m/z (% abundance)
$BPAd^{16}$ (I.S.) $[MW+86]^+$	6.65	3.56–6.95	**3.26**	**224**(100), 242(20), 284(14), 326 (8)
BPA $[MW+86]^+$	6.68			**270**(14), 213(100), 228(20), 312(8)
BPB $[MW+86]^+$	7.39	6.95–9.0	**3.56**	**213**(100), 255 (40), 297 (24), 326 (2)

levels of BPA and BPB from complex food matrixes, several parameters such as the nature and volume of extractive and dispersive solvents and the amount of derivatising reagent were studied. Evaluation of these parameters involved, in most cases, percentage of recovery and enrichment factor (EF):

$$EF = [(\ \%\text{Recovery} \times (V_{aq}/V_{sed}))/100]$$

where V_{aq} and V_{sed} are, respectively, the volume of the sample aliquot and the volume of the final sedimented phase.

Selection of extractive and dispersive solvents and effect of it volumes

As referred above, the extraction of BPA by DLLME was performed in water samples in previous work (Rezaee et al. 2009; Wang et al. 2009). The study presented here intends to cover the quantification of BPA and BPB using $BPAd_{16}$ as IS, in high complex food matrixes such as soft drinks, beers and powdered infant formula. Initially the procedure was optimised in beverages (soft drinks and beers), being the best two combinations of extractive and dispersive solvents further evaluated in powdered infant formula (submitted to a previous pre-treatment), in order to obtain the most similar DLLME procedure for all matrixes in study.

The performance of the extractive and dispersive solvents was evaluated simultaneously. All the extractive solvents selected had: (1) higher density than water, (2) immiscibility with water, (3) good solubility for analytes, and (4) good chromatographic behaviour. Tetrachloroethylene, trichloroethylene, carbon disulfide, chlorobenzene and carbon tetrachloride were the extractive solvent evaluated. The dispersive solvents evaluated were methanol, acetonitrile and acetone. These solvents fulfil the requirements of being miscible with both aqueous sample and extractive solvent and to possess the capacity to decrease the interfacial tension of the extractive solvent, thus making the droplet size smaller and increasing the extraction efficiency.

The extractive/dispersive solvent pairs were chosen according to both extraction recovery obtained and the volume of sedimented phase. Equal amounts of each dispersive solvent (390 µl) containing 90 µl of T4CE, T3CE, or CTC or 80 µl of CB or 110 µl CD and 30 µl of AA were added into 10 ml of blank spiked sample (with $pH \geq 10$) and analysed; the results were compared with the results obtained from experiments made with equal amounts of analytes and the same mixture of solvents (dispersive, extractive and derivatising). The data presented in Figure 2(A)–(C) indicate that the best conditions in beverages were achieved using the combination MeCN/T4CE. This mixture, as well as the mixture MeCN/T3CE, was evaluated in the blank extract of spiked powdered infant formula. The results obtained were similar to those reported for beverages, residing the only disadvantage in the fact that the volume of sedimented phase was about 20% less than the volume obtained in the beverages due to the thin film (probably lipid and/or protein) formed between the aqueous and sedimented phase.

The next step performed in the optimisation was the evaluation of the extractive solvent volume. In this kind of extraction, it is known that lower volumes enhance the enrichment factor, although reducing the volume of sedimented phase. For the purpose of the present study two replicates were investigated using 0.5 ml of MeCN containing four different volumes of T4CE: 30, 50, 70 and 90 µl. Lower volumes were avoided due to the small volume of sedimented phase formed, with subsequent effects on reproducibility. The enhancement of the volume of T4CE from 30 to 90 µl resulted in an increased volume of sedimented phase from 20 to 70 µl in the beverages, and from 15 to 65 µl in the powdered infant formula. The best results in terms of enrichment factor and peak area were obtained with 30 µl of T4CE, thus it was selected as the optimum volume of extractive solvent for beverages. Experiments with powdered infant formula showed an optimum T4CE volume of 50 µl, providing a somewhat lower although very acceptable enrichment factor, as can be seen in Table 2. With the optimised volumes of extraction solvent it was possible to obtain a %RSD of the sedimented phase volume lower than 2% in all matrixes studied.

To study the effect of the dispersive solvent volume on extraction efficiency, different volumes of MeCN (from 0.5 to 2 ml with gaps of 0.5 ml) containing 30 µl

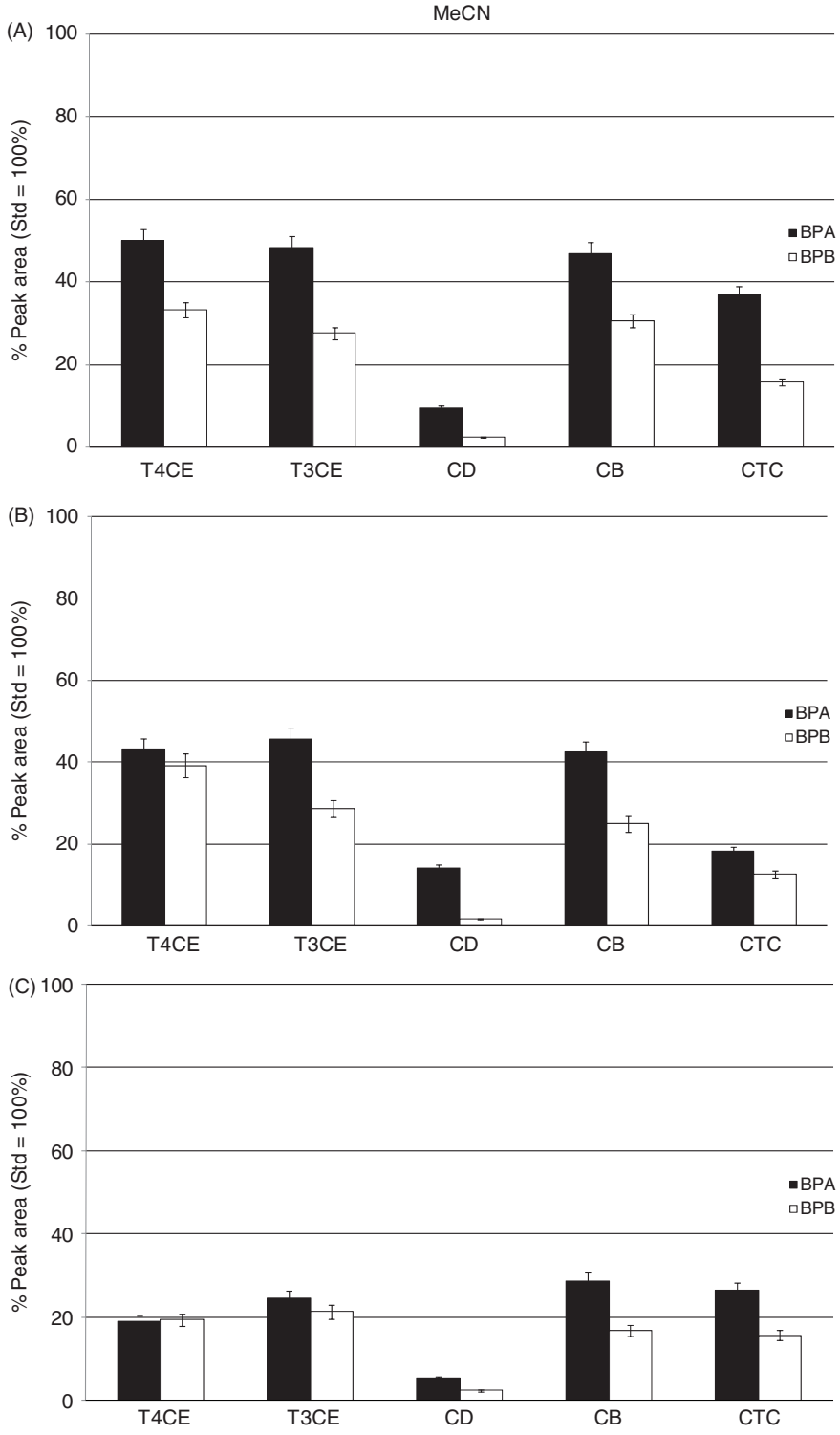

Figure 2. Comparison of average peak area response of different dispersive solvents (A) MeCN, (B) MeOH and (C) AC, contaning different extractive solvents (T4CE, T3CE, CD, CB, and CTC), normalised to the standard peak area (standard = 100%) ($n = 2$).

of AA and 30 µl of extractive solvent were tested in soft drinks. The volumes of sedimented phase obtained were quite similar (20 µl) during all experiments. However, increasing the volume of MeCN from 1 to 2 ml resulted in decreased extraction efficiency. From the obtained results no obvious variation on extraction efficiency can be seen when 0.5 or 1 ml of MeCN were used. However, taking into consideration

Table 2. Average recoveries (%, $n = 6$), repeatability (%RSD, $n = 6$), enrichment factor (EF), dynamic linear ranges ($\mu g\,l^{-1}$), LOQ ($\mu g\,l^{-1}$, $n = 10$) and LOD ($\mu g\,l^{-1}$, $n = 10$) for BPA and BPB obtained in spiked beverages (soft drinks) and powdered infant formula samples using DLLME procedure and heart-cutting GC-MS analysis.

	Beverages									Powdered								
	Concentration level									Concentration level								
	$0.05\,\mu g\,l^{-1}$			$0.2\,\mu g\,l^{-1}$						$0.05\,\mu g\,l^{-1}$			$0.2\,\mu g\,l^{-1}$					
Analyte	% Recovery	% RSD	EF	% Recovery	% RSD	EF	Linearity range ($\mu g\,l^{-1}$)	LOQ ($ng\,l^{-1}$)	LOD ($ng\,l^{-1}$)	% Recovery	% RSD	EF	% Recovery	% RSD	EF	Linearity range ($\mu g\,l^{-1}$)	LOQ ($ng\,l^{-1}$)	LOD ($ng\,l^{-1}$)
BPA	82	8	410	93	8	465	0.02–10	10.0	5.0	114	15	326	83	7	237	0.5–10	200.0	60.0
BPB	111	7	555	107	2	535	0.02–10	7.0	2.0	68	11	194	77	9	220	0.5–10	100.0	30.0

that lower volumes of dispersive solvent give enhancement of the enrichment factor, 0.5 ml of MeCN was chosen as the optimum volume for the dispersive solvent for all matrixes studied.

Selection of derivatising reagent, effects of its volume and reaction time

Among the derivatisation methods (e.g. trimethylsilytation and acetylation) used for BPA GC-MS determination, acetylation was chosen taking into account its availability, simplicity of use and time consumed. The selected derivatisation *in situ* with acetic anhydride in the presence of K_2CO_3 has previously been used for BPA analysis in water (Wang et al. 2009). In the present study, the simultaneous DLLME/acetylation procedure was evaluated for the first time in complex food matrixes, not only for BPA but also for BPB. The amount of acetic anhydride and the reaction time were investigated to achieve the highest derivatisation reaction yield. The influence of derivatising reagent volume was evaluated by using 500 µl of MeCN containing 30 µl of T4CE and different volumes of AA (20, 30, 40 and 50 µl) in beverage blank samples spiked with 10 or 20 $\mu g l^{-1}$ of BPA and BPB; the reaction time was 1 min. The highest derivatisation reaction yield was observed when volumes of 20 and 30 µl were used, thus 30 µl was chosen as a volume of derivatising reagent. The addition of K_2CO_3 solution to the sample allowed a $pH \geq 10$, assuring the neutralisation of acetic acid produced during the acetylation reaction.

Under the optimal conditions chosen, experiments were made with increasing reaction times, from 1 to 10 min. As previous reported in the work of Wang et al. (2009) no differences were observed in the peak area of the analytes, thus the reaction time used during the extraction procedure was fixed in 1 min. Next to the good yields obtained with acetic anhydride in the presence of K_2CO_3 solution, the main advantage of this procedure is the possibility to carry out simultaneously the extraction and the derivatisation procedures.

Pre-treatment of powdered infant formula

Due to the fact that powdered infant formula contains high amounts of proteins (10–15%) and fats (20–30%) a treatment of the samples before the DLLME was necessary. Most of the procedures reported for the analysis of BPA in powdered infant formula and breast milk involve deproteinisation with an organic solvent miscible with water such as methanol (Maragou et al. 2006; Shao et al. 2007) and 2-propanol (Ye et al. 2006). Combination of methanol with trichloroacetic acid has been found to be useful for protein precipitation

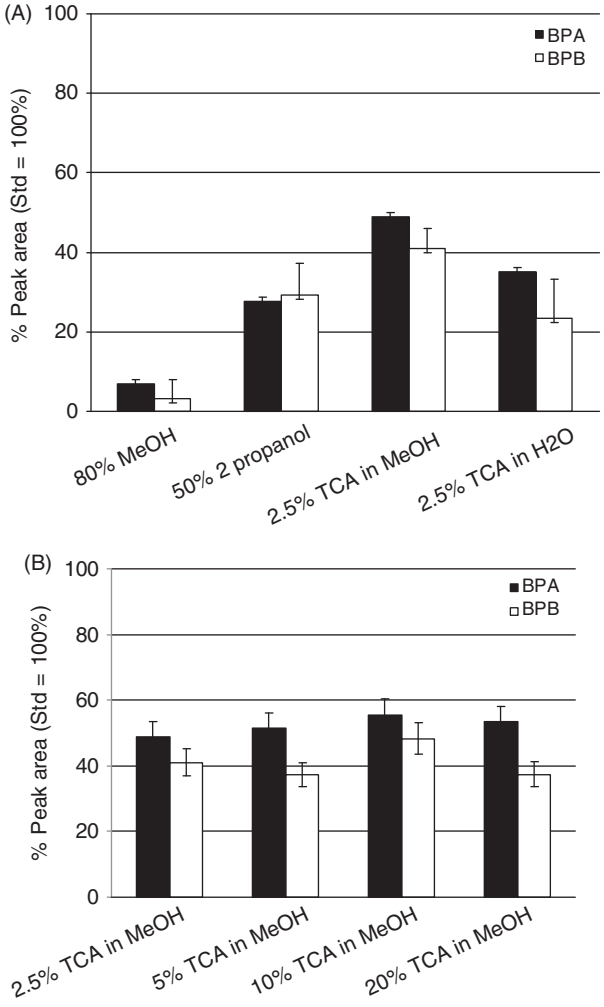

Figure 3. Comparison of the average of peak area response using: (A) different deproteinisation solutions (normalised to the standard peak area – standard = 100%) and (B) different concentration of TCA solution in methanol (normalised to the standard peak area – standard = 100%) ($n = 3$).

without lost of BPA in milk samples (Braunrath et al. 2005). In this study, blank milk samples (free of BPA and BPB) were spiked with an appropriate volume of standard solutions to achieve 20 $\mu g l^{-1}$ of both BPA and BPB, and then treated with different solutions namely: 80% methanol, 50% 2-propanol, 2.5% TCA in methanol, and 2.5% TCA in water. The extracts obtained after this treatment were spiked with BPA_{d16} (to reach 10 $\mu g l^{-1}$), submitted to the DLLME procedure and compared with a solution of 0.5% K_2CO_3 containing the same amount of all the analytes. The percentage of peak areas obtained is presented in Figure 3(A). The best results were obtained with 2.5% TCA in methanol. However, the chromatograms achieved with this deproteinisation step had high number of interferences, making difficult the proper quantification of the analytes of interest. Thus, higher concentrations of TCA in methanol (e.g. 5%, 10% and 20%) were tested for deproteinisation. As can be seen

in Figure 3(B) the percentage of peak areas obtained were similar. However, the use of 10% of TCA in methanol shows a slightly higher area, especially in the case of BPB, all together with a minor number of interferences. By taking into account these factors 10% TCA in methanol was selected.

Optimisation of chromatographic conditions

To increase sample throughput during GC analysis, which would consequently reduce the laboratory operating costs, a multidimensional GC system with Deans switch device was used. This system allows one to transfer portions of effluent from the first column to the second column for MS analysis while fractions without analytical interest could be directly diverted for waste via a restrictor column (Figure 1) (Cunha et al. 2009; Cunha and Fernandes 2010; Donato et al. 2007). In this study a short wide-bore High Temperature DB-5 capillary column (HTDB-5) with low film thickness (5 m × 0.32 mm × 0.10 μm) was combined with a second narrower chromatographic DB-5 capillary column (15 m × 0.25 mm × 0.25 μm). This set of columns requires a 4.31 m × 0.18 mm I.D. inert column as a restrictor column, according to the Deans switch calculator software. Difficulties encountered in Deans switch operation are generally due to control pressure differences during the chromatographic run. Since column flow rate is inversely proportional to temperature, to maintain the same flow in the columns 1.0 ml min^{-1} in the first and 2.0 ml min^{-1} in the second, a ramped pressure was adjusted. With the optimised conditions, consisting in a pressure from 162.2 kPa to 172.0 kPa in the front inlet, and a constant pressure in the auxiliary inlet, it was possible to avoid vacuum levels higher than 10^{-5} Torr, which ensures the proper functioning of the MS detector. The Deans switch was operated in mode 'on' between 6.0 and 7.5 min, to allow the determination of all the studied analytes, and in mode 'off' in the beginning and at the end of the chromatographic run. This set-up not only protects the second column and the MS detector from all the interfering substances that elutes before and after the compounds of interest, improving the reliability and robustness of the whole GC/MS system, but also allows faster analytical work, because there are no need to wait for the complete elution of the latter compounds (retention time > 7.5 min), which are immediately diverted to waste via the restrictor column (Figure 4).

In terms of data acquisition both factors (1) number of ions in the given time window, and (2) dwell time (time spent monitoring a single ion) were optimised in SIM mode to achieve the best selectivity and to maximise sensitivity. In this study four ions were selected for each analyte, with a dwell time between 30 and 80 ms, resulting in a data acquisition rate higher than 3.26 data points s^{-1}. These conditions allow the attainment of seven to ten points across a peak, which permitted to reach quantitative needs (Poole 2003).

It is should be noticed that there are interferences (almost with the same retention time) from all beer samples when ions m/z 213 and also m/z 228 were used, avoiding the attainment of the same ion ratios as in the standards. Thus, the optimisation of SIM conditions was achieved with matrix-matched standards, being the ion 270 used for quantification of the derivatised BPA due it is high selectivity (Cao et al. 2010). For quantification of acetyl BPB and acetyl $BPAd_{16}$ the respective base peaks were used (Table 1).

Quality parameters

The analytical method was validated in what respects linearity, recovery, precision, limits of detection (LOD) and quantification (LOQ), in the different matrixes studied.

Linearity

It is well known that the matrix effect can strongly affect the chromatographic performance. Thus, in a preliminary experiment, the slopes of the calibration curves from standard solutions were compared with those obtained from matrix-matched standards. The results obtained showed a slight enhancement of response for BPA and BPB in all matrixes under study. This effect, previously observed with other analytes (Cunha et al. 2009; Cunha and Fernandes 2010), could be related to the properties of the analyte itself or to the presence of other ionisable compounds in the extract. Thus, the linearity was established using seven concentration levels equal for BPA and BPB in blank extract samples (standards added to blank samples of each matrix studied). The standard concentration ranged from 0.02 to 10 μg l^{-1} in the soft drinks and from 0.5 to 10 μg l^{-1} in the powdered infant formula (Table 2). Soft drinks and beers showed similar behaviour in terms of slopes, thus allowing the use of the same calibration curve for both kind of samples. Calibration curves were constructed by plotting the analyte/IS ratio obtained against the concentration of each analyte. The results obtained demonstrated a good linearity within the tested interval, with determination coefficients (R^2) always higher than 0.998 in the two distinct matrixes studied.

Recovery

The optimised DLLME procedure includes a simultaneous derivatisation which makes the comparison between pre- and post-extraction spiked

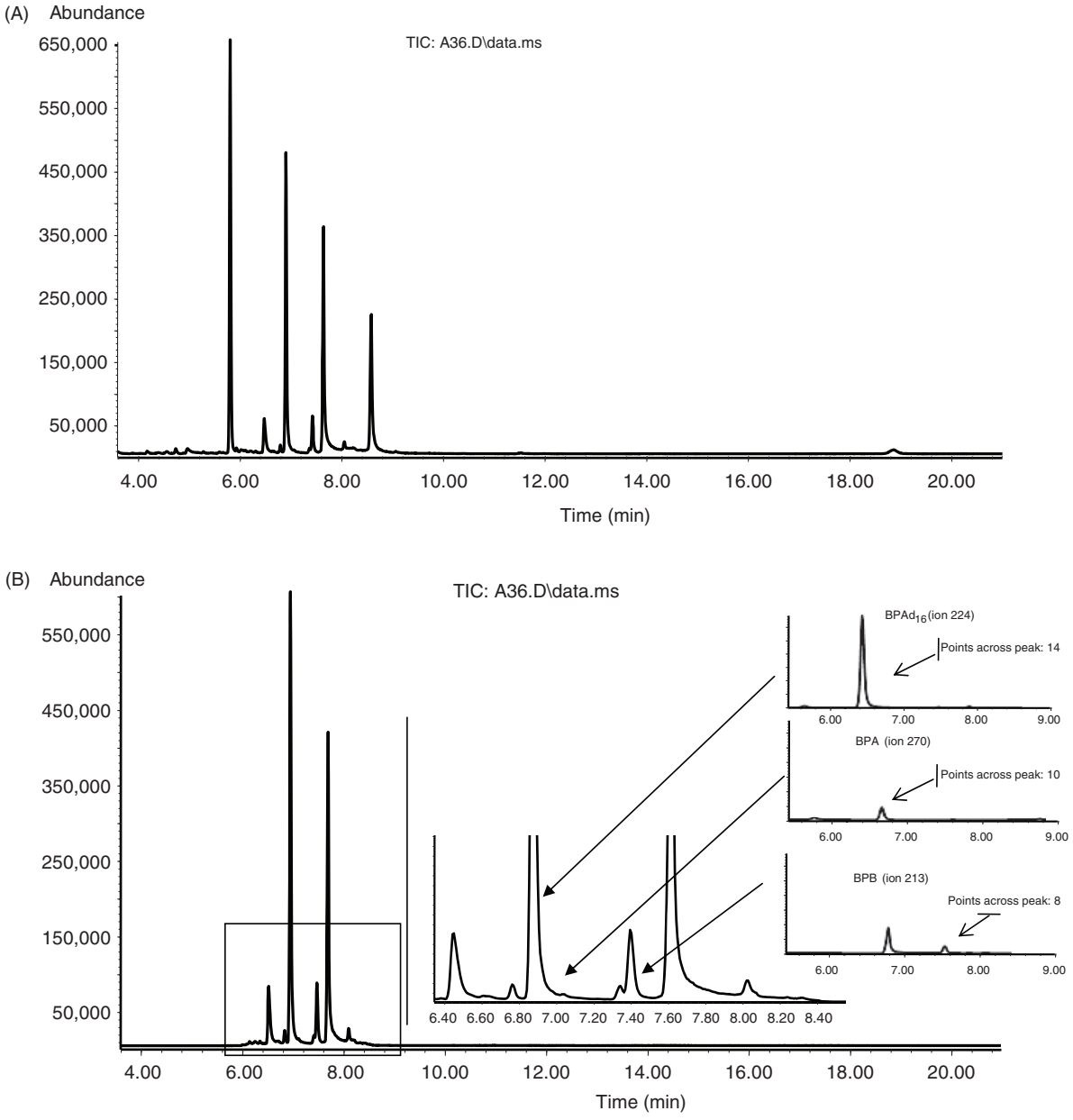

Figure 4. Total ion chromatogram of a soft drink sample with 2, 0.15 and 0.10 $\mu g l^{-1}$ of $BPAd_{16}$, BPA and BPB, respectivley, using (A) conventional mode GC-MS system and (B) Deans switch GC-MS system; and ion chromatogram in SIM mode for BPAd16 (ion 224), BPA (ion 270) and BPB (ion 213).

samples unworkable. Thus, a relative recovery was determined by comparing unspiked samples to the spiked samples at two concentration levels, being each level performed six times. For the spiked samples the analytes were spiked into the sample immediately before the extraction process. In the soft drinks the mean recovery values ranged from 82% to 111% and from 68% to 114% in the powdered infant formula, as can be seen in Table 2.

Repeatability

The relative standard deviation (RSD) was calculated from six spiked replicates of a soft drink and six spiked replicated of a powdered infant formula (both free of BPA and BPB) at two concentration levels. The RSD ranged from 2% to 8% in the soft drink, and from 7% to 15% in the milk sample (Table 2).

Limits of detection (LODs) and limits of quantification (LOQs)

The LODs of the proposed method were determined by successive analyses of chromatographic extracts of blank samples spiked with decreasing amounts of the analytes until a signal-to-noise ratio of 3:1 was reached, whereas the LOQs were determined

Table 3. BPA and BPB levels found in canned soft drinks and beers (average and relative standard deviation %RSD, $n = 2$).

Sample identification		Can type	BPA $\mu g\,l^{-1}$ % RSD	BPB $\mu g\,l^{-1}$ % RSD
Soft drinks	Brand cola		0.07(7)	0.11(4)
			0.21(5)	0.09(5)
			0.18(3)	0.16(8)
			0.15(9)	0.10(4)
			0.08(7)	0.08(8)
			0.40(5)	0.09(8)
	Carbonated drink	Two piece	0.17(4)	0.09(6)
			0.23(9)	n.d
			n.d.	n.d
			4.02(10)	n.d.
			1.22(8)	n.d.
			n.d.	n.d.
			n.d.	n.d.
	Energetic drink		3.24(3)	n.d.
			0.46(9)	0.08(6)
			n.d.	n.d.
			0.15(4)	0.08(5)
			n.d.	n.d.
			n.d.	n.d.
			n.d.	0.08(9)
	No carbonated drink		0.03(2)	0.07(4)
			0.07(5)	0.08(2)
Beers	Ales-stout	Two piece	n.d.	n.d.
			n.d.	n.d.
			0.65(7)	0.08(3)
			1.80(6)	n.d.
			3.24(9)	n.d.
			0.29(8)	0.08(8)
	Larger dark		4.70(9)	n.d.
			0.98(8)	0.06(8)

Note: n.d., not detected

considering a signal-to-noise ratio of 10:1. The LODs in beverages were 5.0 and 2.0 $ng\,l^{-1}$ for BPA and BPB, respectively, whereas LODs in powdered infant formula were 60.0 and 30.0 $ng\,l^{-1}$, respectively. The LOQs in beverages were 10.0 and 7.0 $ng\,l^{-1}$ for BPA and BPB, respectively, whereas LOQs in powdered infant formula were 200.0 and 100.0 $ng\,l^{-1}$, respectively (Table 2). The LODs values are lower than those reported in the previous papers for BPA in whole milk 100.0 ng kg^{-1} (Shao et al. 2007), powdered infant formula 1700.0 $ng\,l^{-1}$ (Maragou et al. 2006) and breast milk 280.0 $ng\,l^{-1}$ (Ye et al. 2006). Similar behaviour was observed in canned beverages, with levels of LODs herein reported better than those described by other authors, e.g. 45.0 $ng\,l^{-1}$ (Cao et al. 2009), 2000 $ng\,l^{-1}$ (Goodson et al. 2002) or 100 to 900 $ng\,l^{-1}$ (Braunrath et al. 2005).

Analysis of BPA and BPB in canned samples

The method was applied to the quantification of BPA and BPB in commercial canned soft drinks and beer samples as well as in powdered infant formula samples from the retail market in Portugal.

The results obtained for canned soft drinks and beers are shown in Table 3. BPA was found in 15 of 22 samples of soft drinks, with an average concentration of 0.71 $\mu g\,l^{-1}$ with levels found being extremely variable, ranging from 0.03 to 4.02 $\mu g\,l^{-1}$. These levels are in agreement with those reported by Geens et al. (2010), who found an average concentration of 1.0 $\mu g\,l^{-1}$ (ranging from 0.06 to 8.10 $\mu g\,l^{-1}$) in 45 canned beverages from Belgium, by Cao et al. (2009), who found an average concentration of 0.57 $\mu g\,l^{-1}$ (ranging from 0.13 to 4.5 $\mu g\,l^{-1}$) in 72 samples from Canada, and by Braunrath et al. (2005), which reported an average level of 1.0 $\mu g\,l^{-1}$ (ranging from 0.1 to 3.4 $\mu g\,l^{-1}$) in six soft drinks analysed from Austria. In contrast, Goodson et al. (2002) found no positive sample from seven soft drinks analysed, probably due to the high LOD of the method (2 $\mu g\,l^{-1}$).

In beers, BPA was detected in six of eight samples, with an average level higher than that obtained in soft drinks, 1.94 $\mu g\,l^{-1}$ (ranging from 0.29 to 4.70 $\mu g\,l^{-1}$), which is in agreement with the amount reported by Braunrath et al. (2005), 1.5 $\mu g\,l^{-1}$, for one sample.

Table 4. BPA and BPB levels found in canned powdered infant formula (average and relative standard deviation %RSD, $n = 2$).

Sample identification	Can type	BPA $\mu g\,l^{-1}$ % RSD	BPB $\mu g\,l^{-1}$ % RSD
Milk infant formula	Two piece	n.d.	n.d.
		n.d.	n.d.
		n.d.	n.d.
		n.d.	n.d.
		n.d.	n.d.
	Three piece	0.40(10)	n.d.
		0.23(8)	n.d.

Note: n.d., not detected.

BPB was found in 12 of 22 soft drinks samples analysed, with levels ranging from 0.07 to 0.16 $\mu g\,l^{-1}$. The highest levels were found in the brand colas samples, all of which were positive. In beers BPB was found in three of eight samples, with levels ranging from 0.06 to 0.08 $\mu g\,l^{-1}$. The levels obtained for BPB are considerable lower than those achieved for BPA, which could be explained probably by the minor use of this compound by the industry. However, the BPB levels reported here, for the first time in this kind of samples as far as we know, confirm the importance of monitoring this contaminant in a variety of canned foodstuff, besides the BPA.

The results found for BPA and BPB in powdered infant formula samples are summarised in Table 4. BPA was the only analyte detected; it was present in two samples of three pieces can, with a mean level of 0.32 $\mu g\,l^{-1}$. The concentrations here detected are similar to those reported by Shao et al. (2007), which found one positive sample from ten powdered infant formula samples analysed, with a level of 0.49 $\mu g\,kg^{-1}$. However, other authors reported distinct levels, i.e. not detected (Goodson et al. 2002), from 1.60 to 2.60 $\mu g\ kg^{-1}$ (Liu et al. 2008), lower than 1.70 $\mu g\ kg^{-1}$ (Maragou et al. 2006). This discrepancy may be partly attributed to the origin and number of the samples analysed, or more likely to the type of method used in the determination.

Conclusions

Considering the demands of fast, cost-effective and reliable analytical methods, a DLLME sample preparation procedure coupled with GC-MS analysis was developed and validated for the simultaneous quantification of BPA and BPB in food matrixes such as soft drinks, beers and powdered infant formula. Good results were obtained using a mixture of tetrachloroethylene as extractive solvent, acetonitrile as dispersive solvent and acetic anhydride as derivatising reagent. The proposed procedure takes advantages of the efficiency, ease of use, low cost and high enrichment factor provided by the DLLME and the high yields of the *in situ* acetylation procedure. Moreover, the use of deuterated BPA as IS increased the reliability of the results found in complex food matrixes. The optimised method allowed an appreciable decrease in the LODs and LOQs when compared with previous methods reported in the literature for similar matrixes. The use of a dual GC column system (a short wide-bore column connected to a longer and narrower second column) permitted a high sample capacity and increased robustness of the whole system. The applicability of the method was demonstrated in the determination of BPA and BPB in samples of canned soft drinks, canned beers, and powdered infant formula commercialised in Portugal.

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