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Simultaneous determination of bisphenol A and bisphenol B in beverages and powdered infant formulae by dispersive liquid-liquid microextraction and heart-cutting multidimensional gas chromatography-mass spectrometry

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Abstract

The purpose of this study was to establish a reliable, cost-effective, fast and simple method to simultaneously quantify both bisphenol A (BPA) and bisphenol B (BPB) in liquid food matrixes such as canned beverages (soft drinks and beers) and powdered infant formulae using dispersive liquid-liquid microextraction (DLLME) with *in situ* derivatization coupled with heart-cutting gas chromatography-mass spectrometry (GC-MS). For the optimization 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 in the injection of a mixture containing tetrachloroethylene (extractant), acetonitrile (dispersant) and acetic anhydride (derivatizing 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 optimization 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 (R^2) always higher than 0.99. Average recoveries of the BPA and BPB spiked at two concentration levels into beverages and powdered infant formulae 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 formulae 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 formulae 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 µg L⁻¹) and in 2 of 7 powdered infant formulae samples (0.23 and 0.40 µg 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 µg L⁻¹). This is the first report about the presence of BPA and BPB in canned beverages and powdered infant formulae in the Portuguese market.

Key Words: DLLME (dispersive liquid-liquid microextraction), BPA (bisphenol A), BPB, (bisphenol B), powdered infant formulae, canned beverages, heart-cutting, gas chromatography-mass spectrometry (GC-MS), Deans switch

52 **Introduction**

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

61 A compound that has given rise to many concerns in recent years, is bisphenol A (2,2-bis(4-
62 hydroxyphenyl)propane; BPA) owing to systematic evidence about its presence in canned
63 food products, due to their migration from packaging materials. BPA, first synthesized in
64 1891, was not extensively used until early 1950s, when a steady production increase was
65 observed, which reached by 2006 a value of app. 3.8 million tons/year ([www.bisphenol-](http://www.bisphenol-a.org)
66 [a.org](http://www.bisphenol-a-europe.org); www.bisphenol-a-europe.org). Most of BPA is used as a monomer in the manufacture
67 of polycarbonates employed in food contact materials such as returnable beverage bottles,
68 infant feeding bottles, tableware (plates and mugs) and storage containers, and in the
69 production of epoxy resins used in surface coatings for food and beverage cans and vats
70 (Chapin et al., 2008). Although less discussed, bisphenol B (2,2-bis(4-
71 hydroxyphenyl)butane; BPB), a congener of BPA used by the chemical industry in the
72 manufacture of phenolic resins (Merck index, 2001), can also be considered potential
73 migrant and food contaminant (Grumetto et al., 2008).

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

82 It is well known that BPA has an estrogenic activity, demonstrated by *in vivo* laboratorial
83 experiments in rats and mice with oral administration of BPA (Ashby and Odum, 2004). *In*
84 *vitro* human studies have shown that the estrogenic effect is attained by binding to the
85 nuclear estrogen receptor (Blair et al., 2000; Nagel et al., 1998) whereas the conjugated
86 metabolites are devoid of this activity (Matthews et al., 2001; Shimizu et al. 2002). To the
87 congener BPB, endocrine disruptive properties similar to those of BPA are endorsed
88 (Yoshihara et al., 2004), specifically estrogenic and anti-androgenic activities (Kitamura et

al., 2005). It is believed that BPA could be associated to 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 estrogenic 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, an European Union (EU) 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 and 2010). A similar report released by the Food and Drug Administration (FDA), also found no reason for major concern (U.S. Food and Drug Administration, 2008), although it was criticized for including mostly industry-funded studies and excluding newer data obtained in the National Toxicology Program report. More recently, in January of 2010, FDA has published recommendations for parents on minimization of children's exposure (U.S. Food and Drug Administration, 2010). Currently, the tolerable daily intake set by EU commission and the reference dose established by Environmental Protection Agency (EPA) is 0.05 mg/kg body weight/day (European Union 2010).

Several studies have reported BPA concentrations in foods, beverages, and infant formulae (reviewed in Ballesteros-Gómez et al., 2009). However, there is still need to update exposure analysis including assessing and accounting for variability in 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), pressurized liquid extraction (PLE) (Carabias-Martínez et al., 2006), solid-phase microextraction (SPME) (Chang et al., 2005, Nerín et al., 2002), 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 environmental friendly sample preparation proposal is most likely the DLLME procedure which 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 analyzed 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 multipesticide 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 (Shao et al., 2007; Maragou et al., 2006). Alternatively, some authors make use of GC-MS, a technique that provides a higher resolution, although has the inconvenience of requiring the prior derivatization 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. (Wang et al., 2009) who proposed the simultaneous extraction/derivatization 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 et al., 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 determination of BPA and BPB in food matrixes, specifically canned soft drinks, beers and powdered infant formulae. Special attention was

given on the optimization 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 formulae samples commercialized in Portugal.

Materials and Methods

Reagents and materials

Standards of bisphenol A (BPA; 99% purity) and bisphenol B (BPB; $\geq 98\%$ purity) were purchased from Sigma (West Chester, PA; USA). Internal standard (I.S.) d_{16} -Bisphenol A (BPAd₁₆, 98 atom % D) was also purchased from Sigma. Derivatization 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. Ultrahigh 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 analyzed. If BPA and/or BPB were detected the lot of 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 concentration between 0.02 and $10 \mu\text{g L}^{-1}$ (depending on the experiment) were prepared in 0.5% potassium carbonate solution (0.5% K_2CO_3). Individual working solution of BPAd₁₆ (1 mg L^{-1}) was prepared in 0.5% K_2CO_3 from a stock solution of 20 mg L^{-1} in MeOH. All the 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 analyzed was obtained by the internal standard method.

Sampling

A total of 22 samples of canned soft drinks comprising 6 brand colas, 7 energetic drinks, 6 carbonated drinks and 2 no carbonated flavor drinks were randomly purchased in local supermarkets. A total of 8 samples of canned beers were also purchased in local supermarkets. All the samples were stored at room temperature ($\pm 20^{\circ}\text{C}$) protected from light and opened only at the moment of analysis.

A total of 7 samples of canned powdered infant formulae were also purchased in local supermarkets. The powdered infant formulae 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 formulae.

Sample Preparation

Soft drinks and beers

Approximately 10 mL ($\approx 10\text{ g} \pm 0.2\text{ g}$) of sample were placed into a 25 mL screw cap plastic tube with conical bottom, spiked with 100 μL of BPA_{d16} solution (to reach 10 $\mu\text{g L}^{-1}$), and 5% K₂CO₃ 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 was 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 formulae

Approximately 10 mL ($\approx 10\text{ g} \pm 1\text{ g}$) of sample were placed into a 25 mL screw cap plastic tube, spiked with 100 μL of BPA_{d16} solution (to reach 10 $\mu\text{g L}^{-1}$) and 5 mL of TCA solution (10% in MeOH) was added. After vortexing for 1 min and centrifuged at 11 000 rpm for 5 min the upper layer was transferred to another tube and 5% K₂CO₃ solution was added until pH ≥ 10 . Then 10 mL of the extract was 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 μ L.

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) ionization chamber, and a 7683B Series injector/autosampler.

Heart-cutting GC separation was performed with the columns set as follows: primary column, 5 m x 0.32 mm I.D. x 0.10 μ m film DB-5HT (J&W Scientific, Folsom, CA, USA), secondary column, 15 m x 0.25 mm I.D. x 0.25 μ m film thickness DB-5MS (J&W Scientific) and restrictor, 4.31 m x 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 (see **Figure 1**). The oven temperature program 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.

[Insert Figure 1 about here]

Mass spectrometric parameters were set as follows: electron impact ionization 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.

[Insert Table 1 about here]

Results and Discussion

Optimization of extraction and derivatization conditions

In order to develop and optimize a DLLME procedure for an effective and reproducible extraction of low 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 derivatizing 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 effects of its volumes

As referred above the extraction of BPA by DLLME has been 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 $BPA_{d_{16}}$ as internal standard, in high complex food matrixes such as soft drinks, beers and powdered infant formulae. Initially, the procedure was optimized in beverages (soft drinks and beers), being the best two combinations of extractive and dispersive solvents further evaluated in powdered infant formulae (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: i) higher density than water, (ii) immiscibility with water, (iii) good solubility for analytes, and (iv) good chromatographic behavior. Tetrachloroethylene, trichloroethylene, carbon disulfide, chlorobenzene, and carbon tetrachloride were the extractive solvent evaluated. The dispersive solvents evaluated were: methanol, acetonitrile and acetone. These solvents fulfill 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 $\text{pH} \geq 10$) and analyzed; 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 derivatizing). The data presented in the **Figure 2 A) B) and C)** indicates 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 formulae. The results obtained were similar to these 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.

[Insert Figure 2 about here]

The next step performed in the optimization 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 μ L, 50 μ L, 70 μ L and 90 μ L. Lower volumes were avoided due to the lower volume of sedimented phase formed, with subsequent effects on reproducibility. The enhancement of the volume of T4CE from 30 to 90 μ L resulted in an increased volume of sedimented phase from 20 to 70 μ L in the beverages, and from 15 to 65 μ L in the powdered infant formulae. The best results in terms of enrichment factor and peak area were obtained with 30 μ L 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 μ L, providing a somewhat lower although very acceptable enrichment factor, as can be seen in **Table 2**. With the optimized 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 μ L of AA and 30 μ L of extractive solvent were tested in soft drinks. The volumes of sedimented phase obtained were quite similar (20 μ L) during all experiments. However, increasing the volume of MeCN from 1 mL to 2 mL resulted in decreased extraction efficiency. From the obtained results no obvious variation on extraction efficiency can be seen when 0.5 mL or 1 mL of MeCN were used. However, taking into consideration that lower volumes of dispersive solvent given 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 derivatizing reagent, effects of its volume and reaction time

Among the derivatization methods (e.g. trimethylsilylation and acetylation) used for BPA GC-MS determination, acetylation was chosen taking into account its availability, simplicity of use and time consumed. The selected derivatization "*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 derivatization reaction yield. The influence of derivatizing 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 derivatization reaction yield was observed when volumes of 20 and 30 μL were used, thus 30 μL was chosen as a volume of derivatizing reagent. The addition of K_2CO_3 solution to the sample allowed a $pH \geq 10$, assuring the neutralization of acetic acid produced during the acetylation reaction.

Under the optimal conditions chosen, experiments were made with increasing reaction times, from 1 min 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 derivatization procedures.

Pre-treatment of powdered infant formulae

Due to the fact that powdered infant formulae 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 formulae and breast milk involve deproteinization with an organic solvent miscible with water such as methanol (Shao et al., 2007; Maragou et al., 2006) and 2-propanol (Ye e tal., 2006). Combination of methanol with trichloroacetic acid has been found to be useful for protein precipitation 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 the **Figure 3 A)**. The best results were obtained with 2.5% TCA in methanol.

However, the chromatograms achieved with this deproteinization 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 deproteinization. As can be seen in the **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. Taking into account these factor 10% TCA in methanol was selected.

[Insert Figure 3 about here]

Optimization of chromatographic conditions

To increase sample throughput during GC analysis, which would consequently reduce the laboratory operating costs, a multi-dimensional GC system with Deans switch device, was used. This system allows 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 (see **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 x 0.32 mm x 0.10 μm) was combined with a second narrower chromatographic DB-5 capillary column (15 m x 0.25 mm x 0.25 μm). This set of columns requires a 4.31 m x 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 optimized conditions used 169.2 kPa in the front inlet until 172 kPa at the end of the run and 166.4 kPa 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**).

[Insert Figure 4 about here]

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In terms of data acquisition both factors (i) number of ions in the given time window, and (ii) dwell time (time spent monitoring a single ion) were optimized in SIM mode to achieve the best selectivity and to maximize 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 of 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 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 optimization of SIM conditions was achieved with matrix-matched standards, being the ion 270 used for quantification of the derivatized BPA due it is high selectivity. For quantification of acetyl BPB and acetyl BPAd₁₆ 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 ionizable 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 formulae (**Table 2**). Soft drinks and beers showed similar behavior 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/I.S. ratio obtained against the concentration of each analyte. The results obtained demonstrated a good linearity within the tested interval, with determination coefficients (R²) always higher than 0.998 in the two distinct matrixes studied.

Recovery

The optimized DLLME procedure includes a simultaneous derivatization which makes the comparison between preextraction and postextraction spiked 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 formulae, 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 formulae (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 3:1 was reached, whereas the LOQs were determined 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 formulae 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 formulae 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., 2005), powdered infant formulae 1700.0 ng L⁻¹ (Maragou et al., 2006) and breast milk 280.0 ng L⁻¹ (Ye et al., 2006). Similar behavior was observed in canned beverages, with levels of LODs herein reported better than those described by other authors e.g. 45.0 µg 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).

[Insert Table 2 about here]

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 formulae samples from the retail market in Portugal.

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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 $\mu\text{g L}^{-1}$ with levels found being extremely variable, ranging from 0.03 to 4.02 $\mu\text{g L}^{-1}$. These levels are in agreement with those reported by Geens et al. (2010), who found an average concentration of 1.0 $\mu\text{g L}^{-1}$ (ranging from 0.06 to 8.10 $\mu\text{g L}^{-1}$) in 45 canned beverages from Belgium, by Cao et al. (2009), who found an average concentration of 0.57 $\mu\text{g L}^{-1}$ (ranging from 0.13 to 4.5 $\mu\text{g L}^{-1}$) in 72 samples from Canada, and by Braunrath et al. (2005), which reported an average level of 1.0 $\mu\text{g L}^{-1}$ (ranging from 0.1 to 3.4 $\mu\text{g L}^{-1}$) in 6 soft drinks analyzed from Austria. In contrast, Goodson et al. (2002) found no positive sample from 7 soft drinks analyzed, probably due to the high LOD of the method (2 $\mu\text{g L}^{-1}$).

In beers, BPA was detected in 6 of 8 samples, with an average level higher than that obtained in soft drinks, 1.94 $\mu\text{g L}^{-1}$ (ranging from 0.29 to 4.70 $\mu\text{g L}^{-1}$), which is in agreement with the amount reported by Braunrath et al. (2005), 1.5 $\mu\text{g L}^{-1}$, for one sample.

BPB was found in 12 of 22 soft drinks samples analyzed, with levels ranging from 0.07 to 0.16 $\mu\text{g L}^{-1}$. The highest levels were found in the brand colas samples, all of which were positive. In beers BPB was found in 3 of 8 samples, with levels ranging from 0.06 to 0.08 $\mu\text{g L}^{-1}$. 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.

[Insert Table 3 about here]

The results found for BPA and BPB in powdered infant formulae samples are summarized 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 $\mu\text{g L}^{-1}$. The concentrations here detected are similar to those reported by Shao *et al.* (2007), which found one positive sample from ten powdered infant formulae samples analyzed, with a level of 0.49 $\mu\text{g kg}^{-1}$. However, other authors reported distinct levels, i.e. not detected (Goodson et al., 2002), from 1.60 to 2.60 $\mu\text{g kg}^{-1}$ (Liu et al., 2008), lower than 1.70 $\mu\text{g kg}^{-1}$ (Maragou et al., 2006), or from 2.30 to 10.23 $\mu\text{g kg}^{-1}$ (Cao et al., 2008). This discrepancy may be partly attributed to the origin and number of the samples analyzed, or more likely to the type of method used in the determination.

[Insert Table 4 about here]

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 formulae. Good results were obtained using a mixture of tetrachloroethylene as extractive solvent, acetonitrile as dispersive solvent and acetic anhydride as derivatizing 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 internal standard increased the reliability of the results found in complex food matrixes. The optimized method allowed an appreciable decrease in the LODs and LOQs when compared to previous methods reported in 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 formulae commercialized in Portugal.

Acknowledgments

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Figure Captions

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

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

Figure 3 – Comparison of the average of peak area response using: A) different deproteinization solutions (normalized to the standard peak area- Std =100%) and B) different concentration of TCA solution in methanol (normalized to the standard peak area- Std =100%) (*n* = 3).

Figure 4 - Total ion chromatogram of a soft drink sample with 2 µg L⁻¹, 0.15 µg L⁻¹ and 0.10 µg L⁻¹ of BPA_{d16}, BPA and BPB, respectively, using A) conventional mode GC-MS system B) Deans switch GC–MS system; and ion chromatogram in SIM mode for BPA_{d16} (ion 224), BPA (ion 270) and BPB (ion 213).

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

Analyte [M] ⁺	t _R (min)	Time windows (min)	Data acquisition rate (scans/s)	SIM ions <i>m/z</i> (% abundance)
BPA d ¹⁶ (I.S.) [MW+86] ⁺	6.65	3.5-6.95	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)

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

Analyte	Beverages									Powdered infant formulae								
	Concentration level						Linearity range	LOQ	LOD	Concentration level						Linearity range	LOQ	LOD
	0.05 µg L ⁻¹			0.2 µg L ⁻¹						0.5 µg L ⁻¹			2 µg L ⁻¹					
	% Recovery	% RSD	EF	% Recovery	% RSD	EF	(µg L ⁻¹)	(ng L ⁻¹)	(ng L ⁻¹)	% Recovery	% RSD	EF	% Recovery	% RSD	EF	(µg L ⁻¹)	(ng L ⁻¹)	(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

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

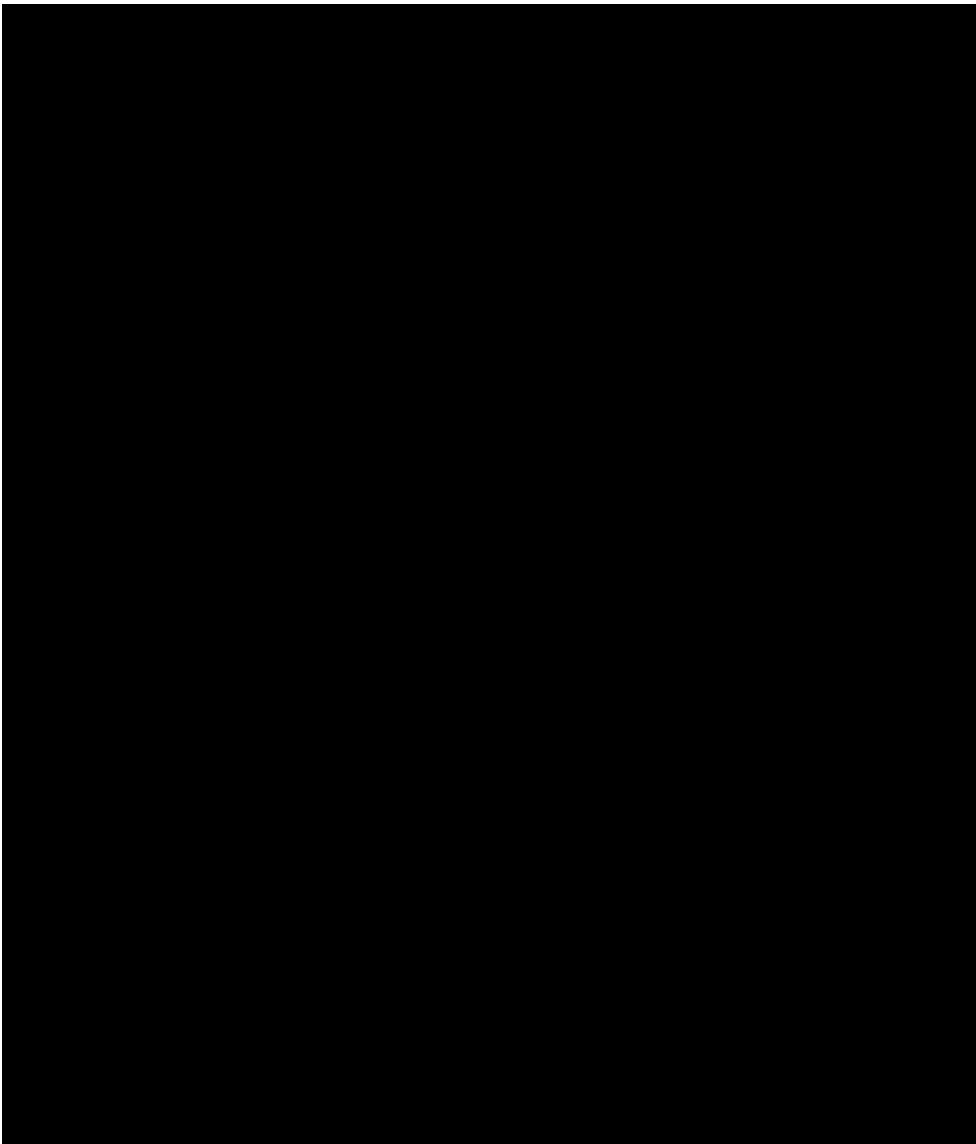


Table 4 - BPA and BPB levels found in canned powdered infant formulae (average and relative standard deviation %RSD, $n=2$).



Fig. 1

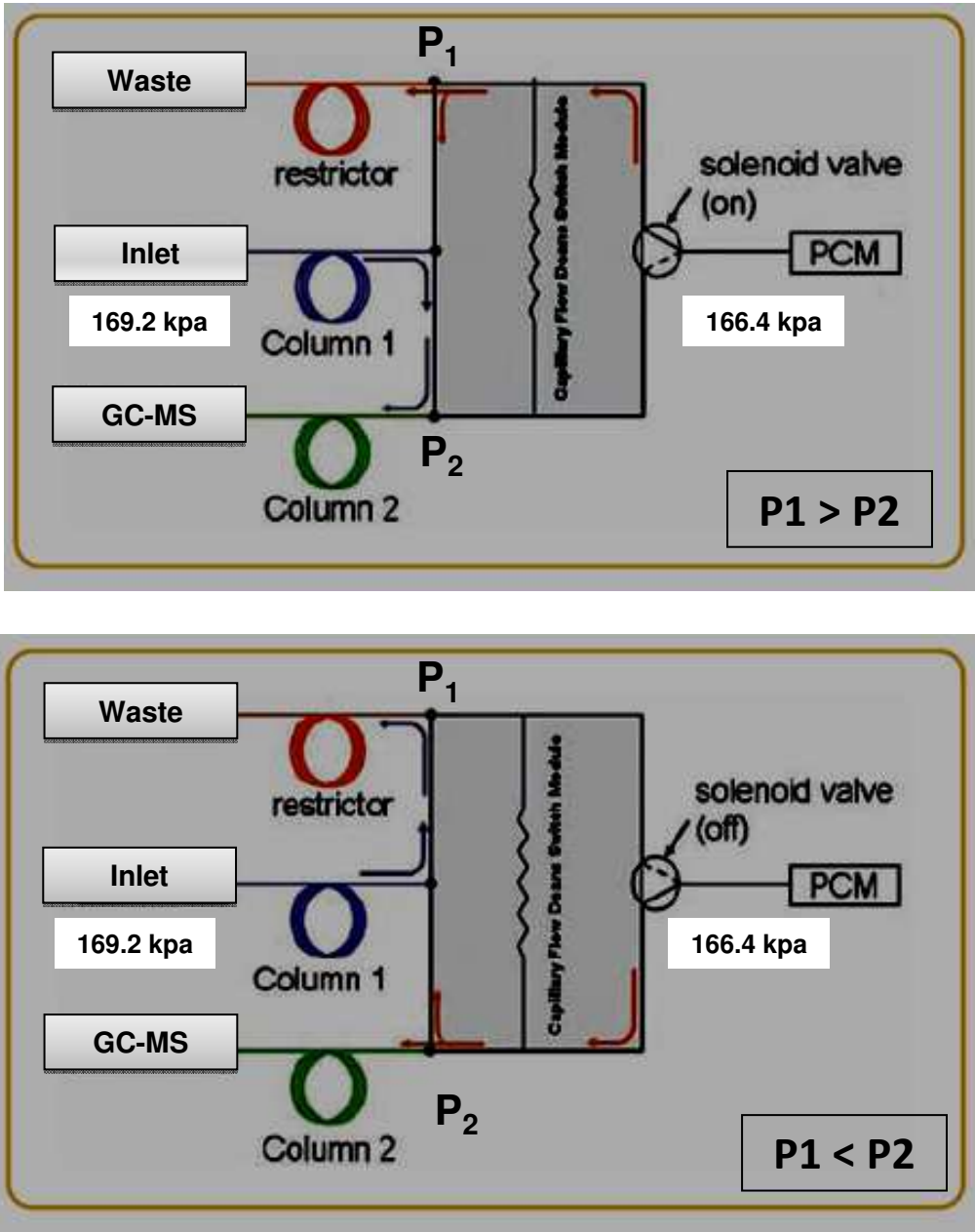


Fig. 2

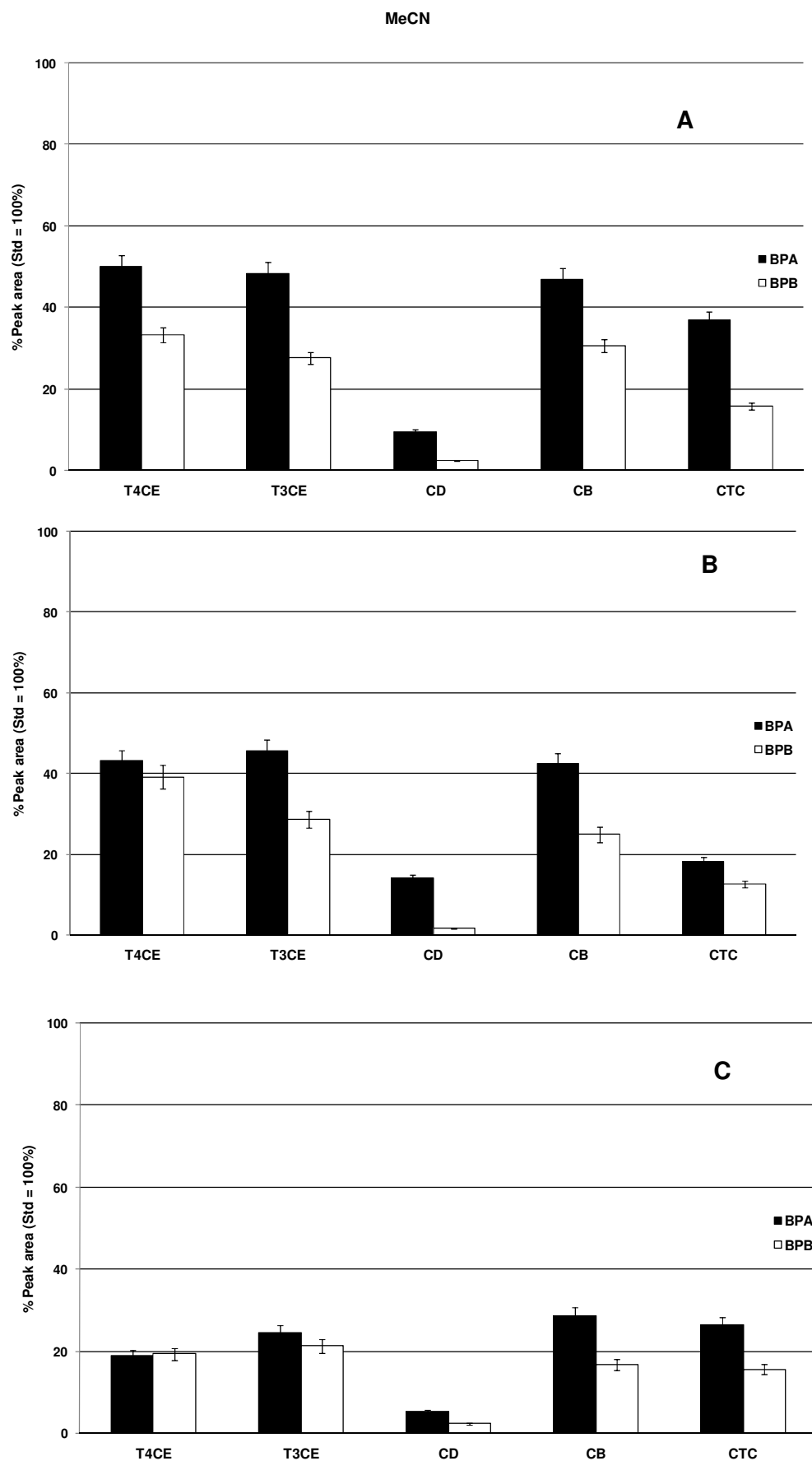


Fig. 3

