



Determination of bisphenol A in canned food by microwave assisted extraction, molecularly imprinted polymer-solid phase extraction and liquid chromatography-mass spectrometry

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ABSTRACT

Bisphenol A (BPA), a known potential endocrine disrupting compound (EDC) is expected to be present in low quantities in canned food due to its migration from the inner surface coating of cans made of epoxy resins. A selective and confirmatory analytical method, based on microwave assisted extraction (MAE), molecularly imprinted solid phase extraction (MISPE) using a polymer prepared by a non-covalent molecular imprinting technique and liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI/MS) was developed for the determination of BPA in canned pineapple, tuna and mushrooms. First, the effect of the loading medium of hydro-organic solutions on the binding of BPA and its deuterated analogue on the MISPE sorbent was investigated. Subsequently, the effects of the experimental conditions of the microwave assisted extraction (solvent, sample mass/solvent volume, time and temperature) on the obtained recovery of BPA from canned food were assessed and the parameters were optimized to provide maximum recovery and selectivity. It was demonstrated that the combination of MAE with MISPE permits the use of a selective extraction solvent (methanol/water, 4/6, v/v), simplifying the sample preparation steps and enhancing sample clean-up of complex food matrices. The method was validated in different food matrices, using BPA-d16 as internal standard and quantitative relative recoveries were determined. The precision (RSD %) of the method ranged from 7% to 10% and the limit of detection was at low ng/g level for all food matrices. The determined concentration of BPA in commercial canned samples ranged between 7.3 and 42.3 ng/g.

1. Introduction

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) (Fig. 1), known as BPA, is a chemical substance widely used in industry as a monomer in the production of epoxy resins [1], which are used as inner surface coating of food and beverage cans. The migration of BPA from epoxy coated can surfaces and other packaging material to food has been recently reviewed [2].

The interest on the BPA migration to food and human exposure was triggered by the fact that this substance was shown to exhibit estrogenic activity in *in vitro* assays at concentrations of 10–25 nM (2.3–5.7 ng/ml) [3]. The potential adverse effect of BPA in human health through

beverage and food consumption has generated a great concern during the last years. In 2015 the European Food Safety Authority (EFSA) published a scientific opinion on the risks to public health related to the presence of BPA in foodstuffs. Based on benchmark dose values at lower confidence limit (BMDL₁₀) and taking into account possible effects in mammary gland, reproductive, neurobehavioural, immune and metabolic system, a temporary Tolerable Daily Intake (t-TDI) of 4 µg/kg bw per day was established [4]. An overview of the current status of BPA exposure and relevant health effects covering reproductive, developmental, metabolic, immuno, respiratory, hepatic and renal toxicity and carcinogenesis of BPA is described by Ma et al. [5]. In addition to this, data from epidemiological studies showed that BPA exposure itself

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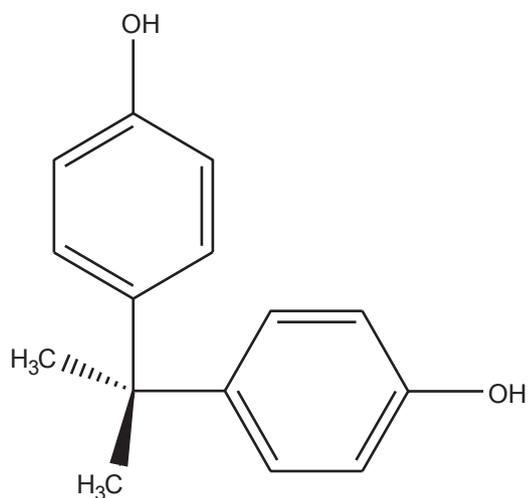


Fig. 1. Molecular structure of Bisphenol A.

increased the risk of obesity in children [6]. In order to protect human health a European regulated specific migration limit (SML) of BPA from varnishes or coatings into or onto food is set at 0.05 mg of BPA per kg of food (mg/kg), excluding the use of BPA in articles intended for infants and young children [7].

Based on the above it becomes evident that accurate and sensible analytical methods for the determination of BPA in different kinds of food are necessary in order to generate reliable data for a safe estimation of human dietary exposure to BPA. As a result, a number of analytical methods has been developed for its determination in several types of food [2,8–27]. In particular, the analytical techniques that have been applied for the determination of bisphenol A in complex canned foods consisting of solid and liquid phase, concern mainly gas chromatography - mass -spectrometry (GC-MS) [8–15], high performance liquid chromatography (HPLC) with UV detection [16–18] and fluorescence detection (FLD) [19–21], liquid chromatography coupled with mass spectrometry (LC-MS) [22] and liquid chromatography with tandem mass spectrometry (LC-MS/MS) [23–26]. Among the aforementioned analytical techniques, liquid chromatography coupled to mass spectrometry offers the advantage of high selectivity compared to the UV and FLD detectors, as well as the advantage of simplicity compared to gas chromatography, since the latter requires derivatisation of BPA.

Still, laborious sample preparation with multiple steps for the isolation of BPA and clean-up of food samples are usually required, especially when the analysis concerns solid samples. Solid phase extraction with different kinds of sorbents, such as Florisil [16], Oasis-HLB cartridges [22], PSA [23], Carb/PSA [24], and immunoaffinity columns [19,20,26] has been widely applied for the clean-up of solid food extracts. Among them, molecularly imprinted polymers (MIP) have also been used for the isolation of BPA from food samples [17,18,25,27]. Molecular imprinting is a technique that has gained wide acceptance as a means for producing specific recognition sites in synthetic polymers. The method involves complexation in the solution of a target compound (template) with appropriate functional monomers, through either covalent or non-covalent bonds. The self-assembled complex is “frozen in place” by the initiation of a polymerization reaction with an excess of a cross-linker molecule. Removal of the template molecule liberates specific recognition sites in the polymer network that are complementary to the template in terms of shape, size, and position of functional groups. The resulting MIP is expected to selectively recognise and interact with the template in preference to other molecules of different shape, size or functionalities.

However, reported molecularly imprinted solid phase extraction (MISPE) applications in fish [17] and fruits [18] apply HPLC-UV for the

detection of BPA. Acetonitrile was used for the initial extraction of BPA from fish and toluene was used for the purification of the extract before MISPE [17]. Fruits were extracted with acetone and further adjusted to about pH 7.0 by adding NaOH [18]. There is also one reported application which combines MISPE with LC-MS/MS detection [25]. In that application extraction of BPA from canned fish, corn and pork was carried out with acetonitrile (ACN) and sonication, followed by liquid-liquid extraction with hexane, solvent change to water and solid phase extraction with MIP. Similar protocols with multiple sample preparation steps, use of several organic solvents and necessity of solvent change are usually followed when solid phase extraction is applied prior to liquid chromatography analysis [16,19,20,22–24,26].

On the other hand, microwave assisted extraction (MAE) is a well-established automated green extraction technique, offering many advantages such as the combined reduction of the extraction time, solvent and energy consumptions, as well as the possibility of performing simultaneous multiple extractions, thus increasing the number of samples processed daily [28]. However, this efficient technique has been applied in limited number of studies that deal with the determination of BPA in environmental and biological samples [29–31].

The aim of the present project was to combine the efficiency of MAE to quantitatively extract BPA from complex solid samples (pineapple, tuna and mushrooms) and the specificity of the MIP to isolate BPA from the co-extracted matrix, with the minimum possible use of organic solvents. In order to achieve this combination a proper solvent should be chosen which would serve as a microwave assisted extraction solvent and afterwards as a MISPE loading medium, without a need of solvent change. A thorough study on the impact of the loading medium on the retention of BPA was accomplished by testing hydro-organic solutions with acetonitrile (ACN) and methanol (MeOH) as loading media using standard solutions. Based on the generated results two kinds of solvents, H₂O and MeOH/H₂O (4/6, v/v) were tested and compared as MAE solvents using real samples. The optimization of MAE and MISPE resulted in a straightforward and selective analytical method for the determination of BPA in canned pineapple, tuna and mushrooms with the use of water and methanol only. The method was validated in the different food matrices, using BPA-d16 as internal standard.

2. Materials and methods

2.1. Standards and materials

Standard BPA (97%) was purchased from Fluka (Buchs, Switzerland) and the isotope labeled internal standard BPA-d16 (C₁₅D₁₆O₂, 98% atom D) from Sigma Aldrich (WI, USA). Stock solutions (100 mg/L) of each of the two compounds were prepared in methanol and used for further dilutions. These solutions were stored at –15 °C.

Methanol (MeOH) and acetonitrile (ACN) of HPLC grade were obtained from LAB SCAN (Dublin, Ireland). Water used as HPLC solvent and for the preparation of BPA standard working solutions was purified with a Milli-Q water system (Millipore, Bedford, MA, USA).

MIP-SPE cartridges: Six 3-mL polypropylene SPE cartridges packed with 200 mg polymer synthesized by a non-covalent technique reported in previous work [32] were used for the solid phase extraction of BPA from canned mushrooms, tuna and pineapple. In brief, the MIP preparation included BPA as template molecule, a mixture of methacrylic acid and 2-vinylpyridine as monomers, ethylene glycol dimethacrylate as cross-linker and ACN as the polymerization porogen. The polymer was Soxhlet extracted with a mixture of MeOH/acetic acid (9/1 v/v) for 18 h in order to remove BPA and release the binding sites. The polymer was finally sized between 20 and 71 μm particles through a series of grinding and sieving steps.

2.2. Samples of canned food

Three kinds of canned food, (a) tuna in water and salt, (b)

mushrooms in water and salt and (c) pineapple slices in syrup (syrup density 18%), were purchased from local supermarkets in Athens. The whole content of each can was homogenised with a laboratory homogeniser and stored at $-15\text{ }^{\circ}\text{C}$. Aliquots of the homogenised samples were used for the optimisation of MAE parameters and the method validation. Six different samples in total were measured for the determination of BPA content.

2.3. Method optimization

As already mentioned, the aim of the project was to select a proper solvent that could serve as a microwave extraction solvent and as a MISPE loading medium. For this reason, MISPE optimization was performed in first place, in order to define the loading medium, as regards the kind of organic solvent and its percentage, which could be applied. Based on the findings of the MISPE optimization, the effect of other parameters of microwave assisted extraction of BPA from samples of tuna, mushrooms and pineapple was investigated, such as the time, the temperature and the ratio of sample mass to solvent volume.

2.3.1. MISPE optimization

It has been reported that different physicochemical properties of different solvents, may affect the formation of supramolecular complexes, thus influencing shape and distance parameters that are “locked” into the forming polymer. In order to recreate and maintain these shape and distance parameters, it is expected that optimum re-binding conditions would occur in conditions that are the same, or are very similar to the conditions used for polymerization [33]. In addition, there are references which give the perspective of use of solvents with high water content for selective binding of the analyte [34]. Based on this hypothesis and taking into consideration that acetonitrile (ACN) was used as porogen during polymerization of BPA-molecularly imprinted polymer used in the present study, (described in detail in previous work [32]), this solvent was initially selected to conduct the loading experiments.

In particular, 1-mL standard solutions containing 200 ng/mL BPA and 200 ng/mL BPA-d16 were prepared, in duplicate, in the following ACN/H₂O media: 0/10, 1/9, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1, 9.5/0.5 and 10/0, v/v and were loaded on MIP-SPE cartridges at a flow rate of 0.2 mL/min. The corresponding standard solutions were prepared in MeOH/H₂O media. After loading, 9 mL MeOH were applied for the elution. The fractions of the loading and the fractions of elution were collected separately, evaporated to dryness, reconstituted with 1.0 mL of water and measured by LC-MS. Exactly the same experiments were conducted with standard solutions prepared in the corresponding MeOH/H₂O media. Apart from the composition of the loading medium it is important to evaluate how much volume of the selected solvent can be loaded without having a breakthrough. For this reason, experiments were performed using higher volumes of 10 mL of the two critical loading solvents, MeOH/H₂O (7/3) and ACN/H₂O (5/5) (Section 3.1). At every experiment the cartridges were first washed with 3 mL of methanol, in order to remove possible residues from previous experiment and then conditioned with 3 mL of the loading solvent. The monitored parameter was the absolute recovery of BPA and that of the internal standard.

2.3.2. Microwave assisted extraction optimization

A MARS XPress microwave appliance (CEM Corporation, USA) was used for the extraction of BPA from canned food. The main objective was the selection of such an extraction solvent which would achieve the highest possible extraction of BPA, the minimum co-extraction of matrix interferences and at the same time it would allow the direct loading of the MAE extract to the MIP-SPE cartridges without the necessity of solvent change. Taking into consideration that only materials or solvents with permanent dipoles can get heated under microwaves [28], making theoretically MAE more efficient in the presence of water, and

based on the results from the evaluation of MIP (Section 3.1), where it was found that water and MeOH/H₂O (4/6) can be used at high volumes of 500 mL and 100 mL, respectively, without loss in recovery, these two solvents were tested for microwave assisted extraction. After the selection of the solvent, the effect of other MAE parameters on BPA recovery was investigated and optimized for the most complex matrix of tuna. These parameters were the ratio of sample mass to solvent volume (m/V), the time and the temperature of the extraction.

Extraction Solvent: Pure water (H₂O) and MeOH/H₂O (4/6, v/v) were tested as microwave assisted extraction solvents. The impact of these two solvents on the overall recovery, the matrix effect and on the extraction yield of BPA, for the three matrices (mushrooms, tuna and pineapple) was studied. For every matrix two samples of 1 g were weighed. One of the samples was used as control while the other was spiked with 200 ng of BPA and BPA-d16 from the very beginning of the analytical procedure. This spiked sample was used for the estimation of the overall recovery. The samples were transferred in the microwave vessels and 10 mL of extraction solvent was added. Microwave assisted extraction took place for 2 min at 100 °C. After the extraction, the vessels were left to cool down to approximately 40 °C and they were centrifuged at 4000 rpm for 5 min twice, then they were filtered with a 0.45 μm syringe driven filter. At this point, from every extract an aliquot of 100 μL was used for immediate measurement with LC-MS in order to compare the LC-MS chromatograms of MAE extracts to the corresponding ones that had been further subjected to MISPE. The rest of the extracts were used for solid phase extraction with MIP cartridges under the conditions that are described in Section 3.3. The final eluents were evaporated to dryness at 45 °C under a gentle nitrogen stream. At this point the control samples were spiked with 200 ng of BPA-d16 for the matrix effect study. All the dried samples were reconstituted with 1 mL of water, filtered with a 0.22 μm syringe driven filter unit (Millipore, Bedford, MA, USA) and then measured by LC-MS. The quantification of the overall recovery (%R_{overall}), the matrix effect (% Signal suppression) and the % extraction yield was performed using Equations (1), 2 and 3, respectively. The peak area of BPA-d16 was selected for the recovery and the matrix effect calculations for more accurate results, since all samples contained BPA. The concentration of the standard solution used in the calculations corresponds to the theoretical concentration of BPA-d16 in the measured solutions of the spiked samples.

$$\% R_{\text{overall}} = \frac{\text{Peak area (spiked sample)}}{\text{Peak area (standard solution)}} \times 100 \quad (1)$$

$$\% \text{ Signal suppression} = \left(1 - \frac{\text{Peak area (sample spiked after MISPE)}}{\text{Peak area (standard solution)}} \right) \times 100 \quad (2)$$

$$\% \text{ Extraction yield} = \% R_{\text{overall}} + \% \text{ Signal suppression} \quad (3)$$

Temperature – Time – m/V ratio: The tested temperature levels were 40, 60, 80 and 100 °C, with 1 g of tuna sample, 10 mL MeOH/H₂O (4/6) extraction solvent and extraction time of 5 min. The tested extraction time was 2, 5, 15 and 30 min at 100 °C with 1 g of tuna sample and 10 mL MeOH/H₂O (4/6) extraction solvent. For the m/V ratio the following pairs were tested (2 g, 10 mL), (1 g, 10 mL), (1 g, 20 mL), (1 g, 30 mL), (1 g, 40 mL) and (1 g, 50 mL) corresponding to m/V values equal to 0.200, 0.100, 0.050, 0.033, 0.025 and 0.020 g/mL. For each evaluated factor three samples (n = 3) spiked with 200 ng BPA-d16 were tested. The overall recovery of BPA-d16 was calculated according to Eq. (1). Statistical analysis (t-tests) at confidence level of 95% was performed with Statgraphics Centurion for every two levels of each factor under evaluation in order to examine whether mean recoveries obtained for the different levels differ significantly between them. It is noted that results for m/V ratio 0.020 which corresponds to pair (1 g 50 mL) are not included in calculations as this volume was not manageable.

2.4. LC-MS measurements

Identification and quantification of BPA were carried out according to a method described previously [35]. A Thermo Finnigan LC-MS system (San Jose, USA) consisted of a Spectra System P 4000 pump, a Spectra System AS 3000 autosampler with the volume injection set to 10 μL and a Surveyor MSQ quadrupole mass spectrometer equipped with an electrospray ionization LC-MS interface (ESI) was used for the measurements of samples for the optimization of MISPE and the optimization of MAE solvent. Chromatographic separation was performed using a Discovery C18 (150 mm \times 2.1 mm, 3 μm) reversed phase LC column. The working conditions were as follows: isocratic elution with a mobile phase consisted of methanol-water (70/30 v/v) at a flow rate of 0.2 mL/min. ESI was applied in the negative ionization mode and the capillary was held at a potential of 3.5 kV. The cone voltage was 70 V and the ionization source was set at a temperature of 350 $^{\circ}\text{C}$.

A Shimadzu LC-MS 2010EV with Prominence LC20AB pump with autosampler and an electrospray ionization interface was used for the measurements of samples for the optimization of the rest of MAE parameters and the validation of the method to the three matrices. The method was adapted and chromatographic separation was performed on a Thermo C18 column (150 mm \times 4.6 mm, 3 μm) with an isocratic elution of a mobile phase consisted of methanol-water (70/30 v/v) at a flow rate of 0.5 mL/min. The volume injection set to 40 μL . The optimum conditions of the interface included ESI (-) capillary potential of 3.5 kV, cone voltage at -1 V and the ionization source temperature at 300 $^{\circ}\text{C}$.

For each analysis the full scan spectrum was acquired (m/z 100–300) for identification purposes and quantitative analysis was performed using single ion monitoring (SIM) with m/z 227 for BPA and m/z 241 for BPA-d16.

2.5. Method validation

The optimized method (Section 3.3) was evaluated using aqueous BPA standard solutions and spiked samples of tuna, mushrooms and pineapple.

Standard calibration curves were constructed by measuring aqueous standard solutions of 5, 10, 20, 35, 50 and 100 ng/mL BPA concentrations containing 20 ng/mL of internal standard. In addition, matrix calibration curves were obtained by analyzing BPA-unsiked samples and spiked with BPA at 5, 10, 20, 35, 50 and 100 ng/g (concentrations in the measured solutions 5–100 ng/mL). In all samples the spiked concentration of the internal standard was 20 ng/g. Linear regression analysis was performed using the ratio *analyte peak area/internal standard peak area* against analyte concentration.

The method LOD and LOQ were defined as $\frac{3 \times SD}{b}$ and $\frac{10 \times SD}{b}$, respectively, where SD stands for the standard deviation of the response of eight independent replicate analyses of BPA-unsiked food samples and b is the slope of the matrix matched curve generated with samples fortified with low concentrations up to 35 ng/g.

For the assessment of the accuracy and the precision, the method was applied to food samples that were spiked with BPA at a fortification level of 200 ng/g for tuna and at 20 ng/g for mushrooms and pineapple. These two spiking levels, one low (20 ng/g) and one high (200 ng/g) were selected in order to cover the range of anticipated BPA levels based on the literature for these three kinds of food matrices, as reported in Section “3.5. Concentration levels of BPA in canned food”. Analysis of four replicates during one day were conducted for the repeatability test ($n = 4$, intra-day precision) for all matrices, and analysis of four replicates in two different days over a period of one week, were conducted for the reproducibility test ($n = 4$, $k = 2$, inter-day precision) for tuna samples. The accuracy of the method was determined as recovery relative to the internal standard. The recovery (% R, Eq. (4)) was calculated by subtracting the concentration measured in

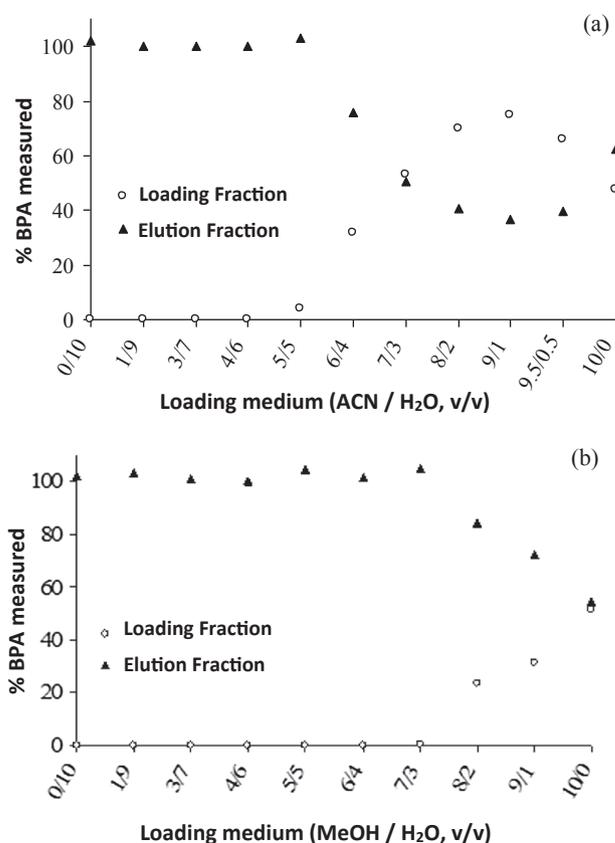


Fig. 2. Effect of the loading medium (a) ACN/H₂O, (b) MeOH/H₂O. Loading 1 mL. Elution with 10 mL MeOH.

the non-spiked sample from that measured in the spiked sample and then dividing with the spiked concentration (C_{ADDED}). Similarly, the absolute recovery of BPA from the three matrices was calculated using as analytical parameter the BPA peak area.

$$\% R = \frac{C_{\text{SPIKED SAMPLE}} - C_{\text{NONSPIKED SAMPLE}}}{C_{\text{ADDED}}} \times 100 \quad (4)$$

3. Results and discussion

3.1. Evaluation of MIP-SPE sorbents with standard solutions

Fig. 2(a) and (b) illustrate the % percentage of BPA measured in the fractions of loading and elution against the composition of the loading media of ACN and MeOH, respectively. Identical plots were obtained for BPA-d16 as well, proving the similarity of the two analogues as regards their binding on the polymer. It is shown that the two organic solvents have a different impact on the retention of BPA.

Observing the elution fraction in Fig. 2(a) it is apparent that for a loading medium of 100% ACN (ACN/H₂O, 10/0) the retention of BPA is approximately 60%. Addition of a low content of water to this medium (ACN/H₂O, 9.5/0.5) seems to weaken the retention of BPA and further addition of water (ACN/H₂O, 9/1) leads to the minimum observed retention. However, from that point and on, higher content of water in the ACN loading medium seems to enhance BPA retention gradually, reaching complete retention (100%) at (ACN/H₂O, 5/5) and further, until the use of pure water as loading medium (ACN/H₂O, 0/10).

Although it is anticipated that MISPE will have a strong molecular recognition when using as loading medium the porogen applied during polymerization, incomplete retention of BPA (60%) was observed in the present study for 100% ACN as loading medium. The weak retention of BPA could be attributed to the strong eluting power of ACN, according

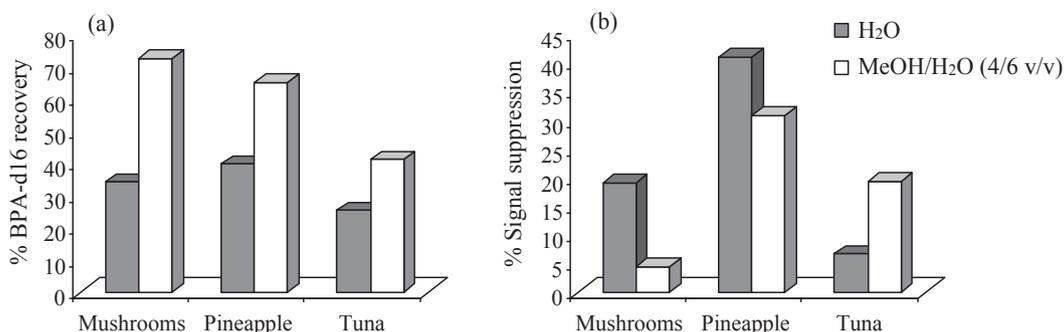


Fig. 3. Effect of the extraction solvent on (a) the recovery, (b) the matrix effect (1 g sample, 10 mL solvent, 2 min, 100 °C).

RT: 0.00 - 10.50 SM: 156

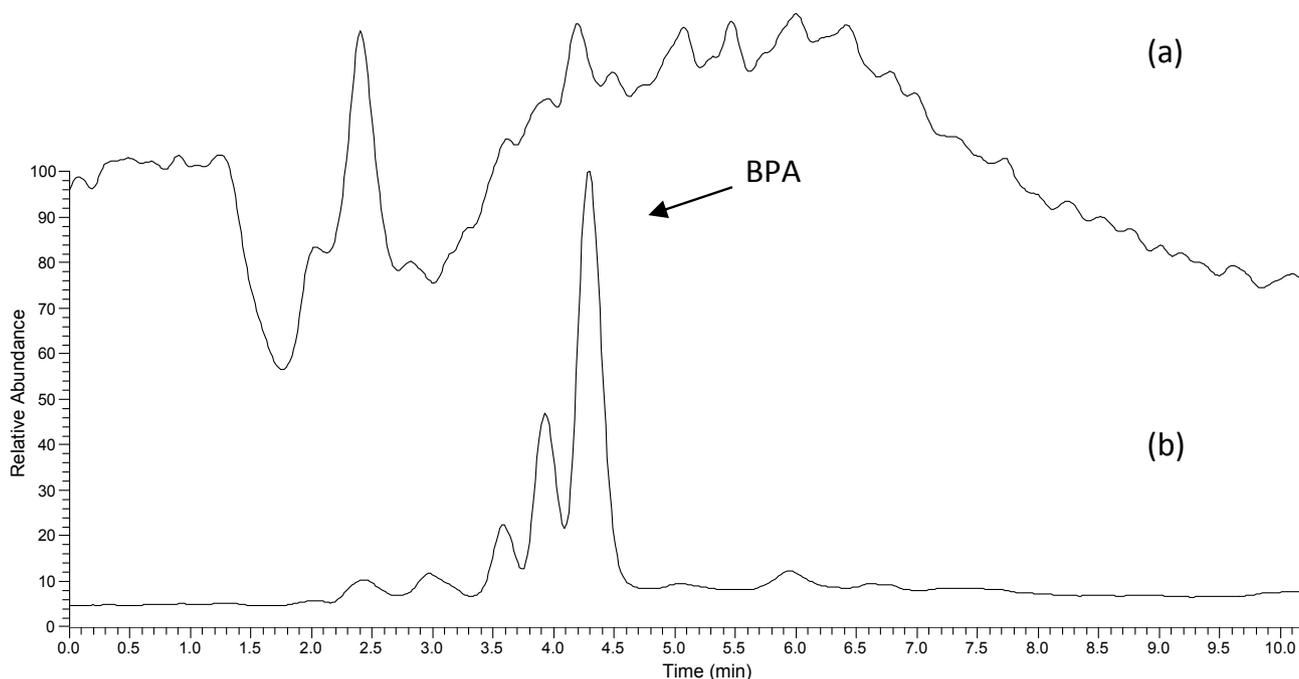


Fig. 4. SIM chromatograms (m/z 227) of a tuna extract containing 100 ng BPA/ml (a) before MISPE and (b) after MISPE.

to a similar study where the same phenomenon was observed to MISPE of caffeine [36]. It is noteworthy that this U shape curve that shows a critical minimum at 10% of water in acetonitrile for the retention of target analyte has been reported again for molecularly imprinted polymers of BPA and β -estradiol prepared with 4-VPY and EDMA during evaluation of MIP as HPLC stationary phase [37], as well as for molecularly imprinted polymers of terbutylazine prepared with MAA and EDMA during evaluation of MIP as solid phase extraction sorbent [34]. In addition, in the non-covalent imprinting technique the complex of template and functional monomer is formed *in situ* by non-covalent interactions, such as hydrogen bonding, electrostatic forces, Van der Waals forces or hydrophobic interactions, and the rebinding of template molecules with MIPs is also carried out by the same non-covalent interactions [38]. Therefore, it could be expected that in the presence of pure ACN, in the absence of water, hydrogen bonding interactions between BPA and MIP become dominant [37]. On the other hand, the complete retention of BPA for loading media with high water content, above 50%, could be attributed mainly to specific and non-specific hydrophobic interactions between BPA and MIP. Specific hydrophobic interactions could possibly concern π - π interactions between the aromatic phenyls of BPA with the aromatic rings of pyridine (monomer unit of the MIP) [32]. In addition to this, another specific hydrophobic interaction between the propyl bridge of BPA with the polymer could

potentially take place, as suggested in the relevant study of terbutylazine [34]. In the intermediate region of loading media with composition between (ACN/H₂O, 9.5/0.5) and (ACN/H₂O, 6/4) it could be suggested that mainly non-specific hydrophobic interactions retain BPA on MIP.

In Fig. 2(b) it is shown that the binding of BPA on the MIP presents a different pattern for loading media with methanol. It is demonstrated that BPA is totally retained on the MIP cartridges for loading media of pure water (MeOH/H₂O, 0/10) up to (MeOH/H₂O, 7/3). For higher methanolic content, the retention during loading gradually decreases, as BPA begins to be detected in the loading fractions and the amount of the compound measured in the elution fractions decreases.

The critical compositions of the hydro-organic solutions, (MeOH/H₂O, 7/3) and (ACN/H₂O, 5/5), were selected and tested for analyte breakthrough at a higher volume of 10 mL, where it was shown that most of the loaded amount was obtained in the loading fractions indicating incomplete retention, and only 39% and 21% of the compound was obtained during the normal elution step, respectively. On the contrary, as we have previously reported no BPA loss was observed when 100 mL (MeOH/H₂O, 4/6 v/v) or 500 mL of H₂O were loaded [32]. Based on these findings a loading medium of (MeOH/H₂O, 4/6 v/v) was selected for MISPE isolation of BPA.

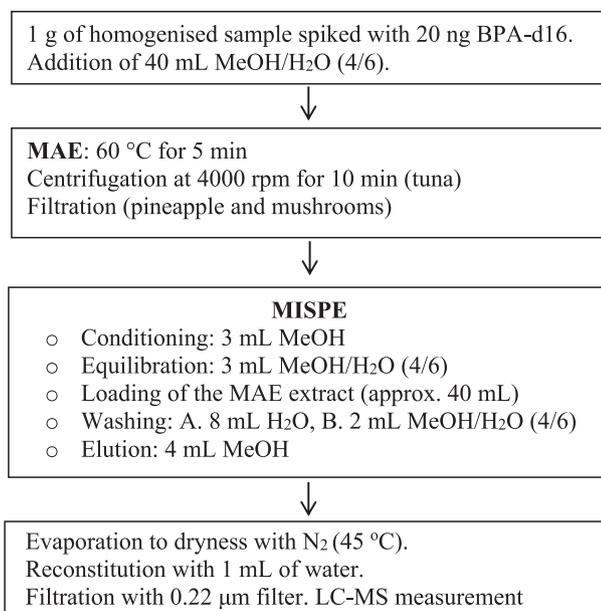


Fig. 5. Final method protocol for the determination of BPA in canned samples by MAE, MISPE and LC-MS.

3.2. Optimisation of MAE – MISPE method

3.2.1. Effect of microwave assisted extraction solvent

The study of the effect of the MAE solvent type on the overall recovery of BPA and BPA-d16 from canned food showed that the system MeOH/H₂O (4/6) is more appropriate compared to pure water, providing the highest overall recoveries for all three matrices as illustrated in Fig. 3(a). The more in-depth investigation regarding the stages of the analytical procedure, where losses take place, showed that the matrix effect, expressed as % signal suppression and presented in Fig. 3(b), depends on the matrix under evaluation. In particular for pineapple and mushrooms the use of pure water generated more intense signal suppression in comparison to MeOH/H₂O, probably because of the co-extraction of polar, water-soluble components from these high-water matrices, which could affect significantly the ionization of BPA and BPA-d16 in the electrospray source. On the contrary, for tuna, stronger signal suppression was observed with the use of MeOH/H₂O, probably because of the presence of fatty, less polar components of this matrix that are more efficiently extracted with the presence of MeOH, and disturb analyte electrospray ionization. The quantification of the extraction yield of BPA during MAE showed that MeOH/H₂O was the most efficient solvent for all three matrices.

3.2.2. Effect of temperature, time and m/V ratio

The statistical analysis showed that temperature and time do not affect significantly the recovery of BPA-d16, whereas the m/V ratio

Table 1

Calibration curves, LOD and LOQ for the different matrices. C: ng/ml for standard solutions and ng/g for the food samples. Concentration range: 5–100 ng/g. y: (analyte peak area/I.S. peak area).

Matrix	Calibration Curve	LOD (ng/g)	LOQ (ng/g)
Standard	$y = (-12.4 \pm 4.8) \times 10^{-2} + (55.4 \pm 0.9) \times 10^{-3} C$ $r = 0.9995$	0.3 ^a	0.9 ^a
Tuna	$y = (-3.2 \pm 9.0) \times 10^{-2} + (54.7 \pm 1.8) \times 10^{-3} C$ $r = 0.996$	0.9	2.9
Pineapple	$y = (-6.4 \pm 6.3) \times 10^{-2} + (59.2 \pm 1.9) \times 10^{-3} C$ $r = 0.999$	1.2	4.0
Mushrooms	$y = (7.9 \pm 12.8) \times 10^{-2} + (57.7 \pm 4.4) \times 10^{-3} C$ $r = 0.992$	1.4	4.6

^a Instrumental LOD and LOQ: ng/ml.

Table 2

Relative recovery %, absolute recovery % and RSD % at 200 ng/g for tuna and at 20 ng/g for pineapple and mushrooms (N = 4).

Matrix	Relative recovery % (RSD %)	Absolute recovery % (RSD %)
Tuna	99 (7)	57 (13)
Pineapple	92 (8)	57 (12)
Mushrooms	111 (10)	51 (11)

Table 3

BPA concentrations (ng/g) in canned food samples of different trade names. \bar{C} : mean concentration, R: range of 2 values.

Sample	$\bar{C} \pm R/2$ (ng/g)
Pineapple A	12.1 ± 0.9
Pineapple B	7.3 ± 1.1
Mushrooms A	25.8 ± 1.3
Mushrooms B	34.4 ± 1.4
Tuna A	32.7 ± 0.9
Tuna B	42.3 ± 1.5

does so. The recovery increases as the m/V ratio decreases. Apart from the obvious reason that the lower the m/V ratio, the more efficient the microwave extraction, these findings were also attributed to the fact that MAE solvent consists also the loading medium for MISPE. Therefore, the higher the volume of solvent and the lower the amount of sample, a higher percentage of matrix is removed during the loading step, contributing to more efficient purification of the sample.

Fig. 4 presents the SIM chromatograms (*m/z* 227) of a MAE tuna extract containing 200 ng BPA/mL (a) before MISPE and (b) after MISPE. The effect of MISPE on the purification of the MAE extract and consequently on the elimination of the matrix effect during LC-MS measurement is remarkable under the particular experimental conditions.

3.3. Optimum conditions

Fig. 5 describes the flow chart of the analytical procedure with the optimum conditions. Aliquots of 1 g of homogenized samples were spiked with 20 ng of internal standard, BPA-d16 (20 µL of 1 µg/mL standard solution). The samples were then transferred in the microwave vessels and 40 mL of MeOH/H₂O (4/6) were added. Afterwards, microwave assisted extraction took place for 5 min at 60 °C. The power was set at 100 W per vessel and the stirrer was set to the low velocity. After the extraction, the vessels were left to cool down to approximately 40 °C. The extracts from the tuna samples were centrifuged at 4000 rpm for 10 min, while the extracts from mushrooms and pineapple were filtered with Whatman paper, as unsatisfactory separation of solid-liquid phase was obtained with centrifugation for these samples. Prior to MISPE loading all the samples were further filtered with a 0.45 µm

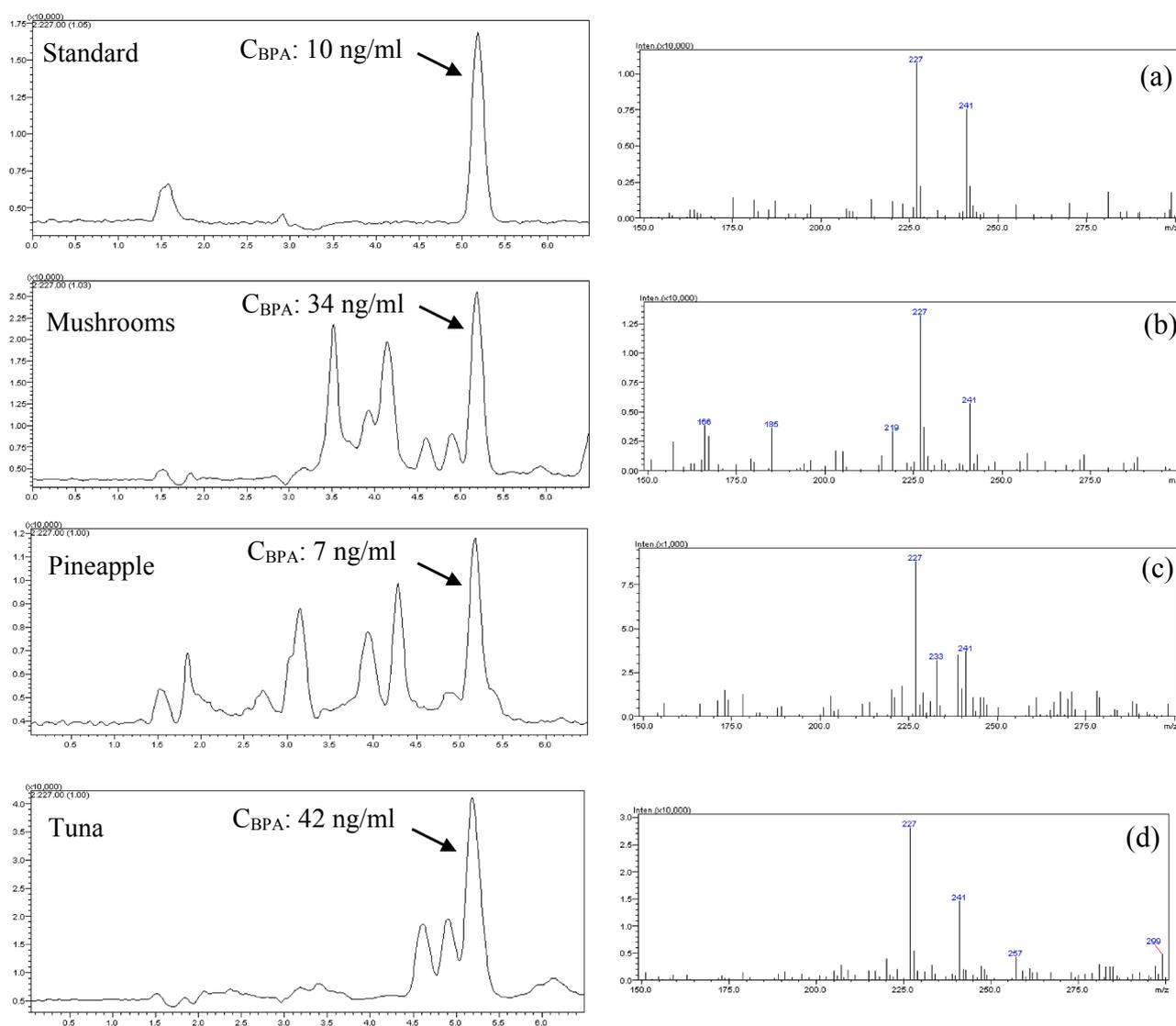


Fig. 6. SIM chromatograms (m/z 227) and the corresponding Full Scan spectra (m/z 150–300) at the retention time of BPA (5.18 min) of (a) an aqueous standard solution 10 ng/ml, and unspiked samples of (b) mushrooms, (c) pineapple and (d) tuna, with the measured concentrations of BPA.

Nylon syringe driven filter, in order to prevent blockage from small particles and to preserve the lifetime of the cartridges, and collected to a 50-mL beaker.

The final MISPE protocol that was applied to the extracts obtained from the microwave extraction of canned food included washing of the sorbents with 3 mL of MeOH and equilibration with 3 mL of MeOH/H₂O (4/6 v/v). The loading of the MAE extracts was conducted at a flow rate of 0.2–0.3 mL/min. For the washing step 8 mL water and 2 mL of MeOH/H₂O (4/6 v/v) were applied sequentially. Afterwards the sorbents were dried under high vacuum for 15 min and 4 mL of MeOH were applied at a flow rate of approximately 0.2 mL/min for the final elution. The eluants were evaporated to dryness at 45 °C under a gentle nitrogen stream, reconstituted with 1 mL of water, filtered with a PVDF 0.22 µm syringe driven filter unit (Millipore, Bedford, MA, USA) and then measured by LC–MS.

The MISPE cartridges were rinsed with 6 mL of ACN after application of food samples and they were periodically checked by blanks and standard solutions.

3.4. Method performance

Table 1 shows the equations of the calibration curves prepared by

standards and spiked food samples using as analytical parameter ratio *analyte peak area/internal standard peak area*. The standard deviation of the slope and the intercept, and the correlation coefficient of each equation are also given. The equations of the food samples calibration curves resulted with a subtraction of the BPA concentration measured in the unspiked samples from the rest of the spiked levels, as all the samples contained measurable concentrations of BPA. It is demonstrated that the overall method, and therefore the molecularly imprinted polymer, presented linearity for the tested concentration range with correlation coefficients always exceeding 0.992. The LODs and LOQs of the method are also presented in Table 1. These values are adequate for safety control of food products since they are much lower than the specific migration legislative limit of BPA (0.05 mg/kg) [7].

Comparison of the slopes of the matrix calibration curves with the standard calibration curve of Table 1, shows that the sensitivity is similar for all the food matrices and the standard solutions. This is also reflected at the quantitative relative recoveries, presented in Table 2, for the low spiking level 20 ng/g (pineapple and mushroom) and for the high spiking level 200 ng/g (tuna). Table 2 summarizes the results from the accuracy and precision experiments under intra-day conditions showing adequate performance of the method. The mean % relative recovery and the corresponding %RSD from the inter-day experiments

on tuna samples were 97.5% and 8%, respectively. The results of Table 2 indicate that the MIP interacts with deuterated BPA similarly to the template (BPA) allowing the use of the isotope analogue as surrogate and internal standard. It is shown that the use of the deuterated internal standard overcomes the losses in recovery due to matrix effect and/or to sample preparation, improving consequently the trueness of the method.

3.5. Concentration levels of BPA in canned food

Table 3 presents the mean BPA concentration that was determined in commercial samples of canned pineapple, mushrooms and tuna, of two different trade names (A and B). Fig. 6 presents the SIM chromatograms of BPA (m/z 227) of (a) 10 ng/mL BPA standard solution, and of extracts of (b) mushrooms, (c) pineapple and (d) tuna, containing approximately 34, 7 and 42 ng/g BPA, respectively. The same Figure illustrates the scan spectrum at the retention time of BPA and BPA-d16, where the ion with m/z 227 in the highest abundance corresponds to the deprotonated molecule of BPA and the ion with m/z 241 corresponds to the deprotonated molecule of the deuterated standard.

The BPA levels in canned pineapple in syrup were determined between 7.3 and 12.1 ng/g, in canned mushrooms in water and salt between 25.8 and 34.4 ng/g and in canned tuna in water and salt between 32.7 and 42.3 ng/g. These findings are within the range of the other reported surveys on BPA levels in the corresponding canned foods. In particular, the reported concentrations of BPA in canned pineapple are 5 ± 1.2 ng/g [20], between undetectable level (with LOD 0.3 ng/g) – 9.8 ng/g [10] and 13 ± 1 ng/g [21]. BPA has been determined in canned mushrooms at concentrations of 116.3 ng/g [8], 32.5–265.6 ng/g [10] and 153 ± 6 ng/g [21]. Finally, canned tuna samples in oil and water have been found to contain BPA from undetectable level (with LOD 0.2 ng/g) up to 169.3 ng/g [8,14,15,19,20,25].

4. Conclusions

The present work reports the successful coupling of microwave assisted extraction with molecularly imprinted solid phase extraction for the determination of BPA in canned food, using an appropriate solvent (methanol/water, 4/6, v/v) that simplifies sample preparation of complex food matrices and reduces the use of organic solvents, which include only methanol. BPA was detected in all the tested samples of canned food collected from the Greek market but it was in all cases below the regulated specific migration limit 0.05 mg/kg.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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