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Determination of bisphenol A in milk by solid phase extraction and liquid chromatography–mass spectrometry

Niki C. Maragou [a], Eugenia N. Lampi [b], Nikolaos S. Thomaidis [a,*], Michael A. Koupparis [a]

[a] *Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimioupolis Zografou, 157 71 Athens, Greece*
[b] *Laboratory of Materials in Contact with Food, General Chemistry State Laboratory, An. Tsoha 16, 115 21 Athens, Greece*

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Abstract

A simple and reliable analytical method based on solid phase extraction (SPE) and liquid chromatography coupled with electrospray ionization mass spectrometry was developed for the determination of bisphenol A (BPA) in milk. The effects of the experimental parameters of the LC-ESI-MS system (mobile phase and additives, flow rate, temperature of the ionization source, cone voltage and capillary potential) on the obtained signal were assessed and the parameters were optimized to provide maximum sensitivity and detectability. In addition, the performance of three commercial SPE sorbents (C18, PS-DVB and hydroxylated PS-DVB) was evaluated using spiked water and milk, diluted with a mixture of water–methanol (8:1). By using C18 cartridges and BPA-d_{16} as internal standard, the mean relative recoveries at three fortification levels ranged between 97 and 104% and the corresponding inter-day precision (RSD%) was below 6% for 50 and 500 ng/g and below 20% for 5 ng/g fortification levels. It is shown that the ion suppression during ESI, the losses from the sample preparation procedure and the inter-day instability of LC-ESI-MS were overcome by the use of the deuterated internal standard. The concentration of BPA found in commercial canned milk samples ranged from <1.7 to 15.2 ng/g.

Keywords: BPA; Food analysis; LC-ESI-MS; Isotope labeled internal standard; SPE

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 and polycarbonates, and as an antioxidant in PVC plastics [1–3]. Epoxy resins are used as inner surface coating of food and beverage cans. Polycarbonates are used in the manufacture of plastic food containers, such as infant feeding bottles and tableware, frequently replacing glass. PVC is used in a variety of products that includes materials intended to come into contact with food, such as cling film used for food packaging. The migration of BPA from epoxy-coated can surfaces [4–9], polycarbonate plastics [10–12], and PVC products [13,14] into food simulants and food has been reported.

In addition, it has been reported that BPA exhibits estrogenic activity in *in vitro* assays at concentrations of 10–25 nM (2–5 ng/ml), competing with [^{3}H]-estradiol for binding to estrogen receptors from rat uterus [15]. During oral administration studies in mice, it was found that a dose of 2 ng/g of BPA affected the size of reproductive organs of male offspring of mice fed by this substance during pregnancy, and a dose of 20 ng/g of BPA significantly decreased efficiency of sperm production by 20% relative to control males [16].

Hence, the potential adverse effect of BPA in human health through beverage and food consumption has generated a great concern during the last years. The reported analytical methods for the determination of BPA in food involve mainly GC–MS [7,17–19] and HPLC with fluorescence [4,20–23] or diode array detection [24]. UV absorbance and fluorescence detection usually demand a tedious sample preparation due to their limited selectivity and sensitivity to complex matrices. Moreover, derivatization of BPA has been proposed to increase sensitivity with fluorescence detection, adding further extraction and reaction steps [23]. Furthermore, in these techniques the identification of the target compound has a high degree of uncertainty, because it is based only on the comparison of the retention time

* Corresponding author. Tel.: +30 210 7274 317; fax: +30 210 7274 750.
E-mail address: ntho@chem.uoa.gr (N.S. Thomaidis).

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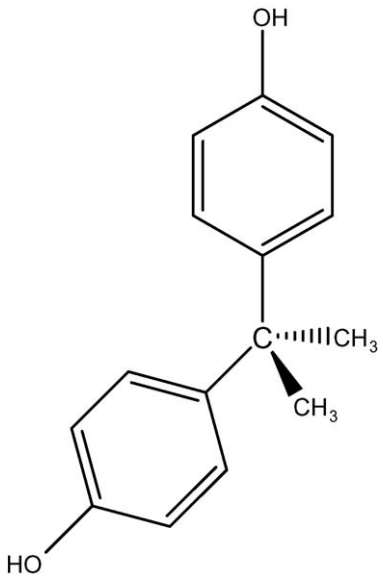

Fig. 1. Structure of BPA (MW 228).

of the unknown to that of a standard solution, except for the case that a diode array detector is used. On the contrary, GC–MS allows identification with a high degree of confidence, but a time-consuming step of derivatization is required. Linking HPLC with mass spectrometry can overcome these limitations. LC–MS does not require derivatization of the compounds of interest and offers more definitive confirmation of the presence of the analyte in an unknown sample, comparing to UV absorbance and fluorescence detection. Additionally, it allows quantitative determination of compounds that are not fully resolved chromatographically using single ion monitoring and it offers higher specificity and sensitivity.

Application of LC–MS with electrospray ionization (ESI) has been described for the determination of BPA in water [25,26] and biological fluids [27–29]. LC–MS with atmospheric pressure chemical ionization (APCI) has been applied for the determination of BPA in fish tissue [30] with a sample preparation procedure involving microwave assisted extraction, centrifugation and SPE. LC coupled with tandem mass spectrometry has recently been applied to beverages [31] and breast milk [32]. The latter method describes an on-line column-switching-LC-APCI-tandem MS method for the determination of BPA (and other seven phenols) in breast milk. However, sample preparation was not fully automated, even if on-line C18 SPE was used, because it was found that before the SPE procedure, an additional step of lipids and proteins removal, with the addition of 2-propanol and centrifugation, had to be preceded [32]. To the best of our knowledge, LC–MS has not been applied for the determination of BPA in a complex food matrix like milk, combined only with SPE for the sample preparation.

Despite the advantages of combining liquid chromatography with mass spectrometry, ESI and APCI processes are susceptible to competition/suppression matrix effects. All polar/ionic species in the solution being sprayed, whether derived from the analyte or not, are potentially capable of being ionized [33]. These effects have been reported and a number of approaches have been developed to compensate for signal suppression, e.g. mobile phase additives [34], application of isotope labeled internal standard [35–37], standard addition [38], two dimensional separation and low injection volumes [39].

This paper describes a simple analytical protocol for the determination of BPA in milk using a simple dilution step, SPE with C18 cartridges and LC-ESI-MS. The optimization of the LC–MS parameters is reported and their effect on the obtained signal is investigated. The performance of three SPE sorbents on spiked water and diluted milk is also reported and discussed. Additionally, the advantages of the application of the deuterated internal standard concerning the sample preparation losses, the matrix effects and the instability of the LC–MS instrument are emphasized. Finally, the developed method is validated and applied to commercial canned milk samples.

2. Experimental

2.1. Standards and materials

Standard BPA (97%) was purchased from Fluka (Buchs, Switzerland) and the isotope labeled internal standard BPA-d_{16} ($C_{15}D_{16}O_2$, 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 for six months.

Methanol and acetonitrile of HPLC grade were obtained from LAB SCAN (Dublin, Ireland), ammonium hydroxide (25%) was obtained from Merck (Darmstadt, Germany) and acetic acid of 99.9% purity from Carlo Erba (Rodano, Italy). 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).

ISOLUTE solid phase extraction (SPE) cartridges packed with 500 mg of silica-based bonded C18 endcapped material (3 cc), or 100 mg poly(styrene-co-divinylbenzene), (PS-DVB) (ISOLUTE 101, 3 cc), or 200 mg hydroxylated PS-DVB (ISOLUTE ENV+, 6 cc) were supplied by International Sorbent Technologies (UK).

2.2. Evaluation of SPE sorbents

The SPE procedure was initially optimized for C18 cartridge only, using 1 g of blank milk (3.5% fat) spiked with BPA and BPA-d_{16} at a level of 100 ng/g for each compound and diluted with 9 ml of water–methanol (8:1, v/v), in order to obtain extracts as clean as possible. The addition of water reduces the viscosity of the sample, thus a better flow rate is achieved during SPE. The addition of methanol aims at the destabilization of the milk's emulsion [17]. It is likely that if milk fat globule membranes are not disrupted, BPA or the internal standard may not be effectively and reproducibly extracted.

The C18 cartridge was conditioned with 5 ml of methanol and equilibrated with 5 ml of water. Then the diluted sample was loaded and mixtures of water–methanol with methanol content

ranging from 0 to 40% were tested as 'wash solvent' and mixtures of methanol–water with methanol content ranging from 80 to 100% were tested as 'elution solvent'. The most selective combination that removed the interferences, produced the clearest extracts and eluted BPA satisfactorily was 8 ml of water and 2 ml of methanol–water (40:60, v/v) successively in the 'wash step' and 3 ml of methanol–water (90:10, v/v) in the 'elution step'.

To compare the performance of three different SPE sorbents with non-polar primary interactions (C18, PS-DVB and hydroxylated PS-DVB), two protocols were applied on spiked water and spiked blank milk (3.5% fat).

By the first protocol, the cartridges were conditioned with methanol and equilibrated with water. The volumes of these solvents were selected according to the mass of the sorbent of each cartridge. Five ml of each solvent were used for the C18 cartridge, 2 ml for the PS-DVB and 4 ml for the hydroxylated PS-DVB cartridge. After that, the optimized procedure for the C18 cartridge, described above, was followed. In particular, 1 g of water or milk, spiked with BPA and BPA-d_{16} at a level of 100 ng/g for each compound and diluted with 9 ml of water–methanol (8:1, v/v), was applied to the SPE cartridges at a flow rate of about 10 ml/min with the aid of vacuum. When the extraction was completed, the interferences were washed with 8 ml of water and 2 ml of methanol–water (40:60, v/v) successively. The cartridges were dried first under high vacuum, and afterwards by a gentle nitrogen stream. The elution was accomplished with 3 ml of methanol–water (90:10, v/v).

According to the second protocol, the SPE cartridges were conditioned with methanol and equilibrated with aqueous HCl (pH 3). The volumes of the solvents were the same as for the first protocol. After the equilibration of the cartridges, 1 g of water or milk, spiked with BPA and BPA-d_{16} at a level of 100 ng/g for each compound and diluted with 9 ml of a mixture of aqueous HCl (pH 3) and methanol (8:1, v/v) was passed through the SPE cartridges. The conditions of the wash- and the elution-step remained the same as for the first protocol.

The extracts from all the SPE procedures were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 ml of water. Re-dissolution in water was preferred because re-dissolution in the mobile phase, pure methanol or acetonitrile produces irregular peak shapes and decreased sensitivity. This could be attributed to differences in viscosity between the injected sample solution and the mobile phase [40]. Prior to LC–MS measurement, the solutions were filtered with a 0.22 μm syringe driven filter unit (Millipore, Bedford, MA, USA).

2.3. LC–MS measurements

Identification and quantification of BPA were carried out using 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). Chromatographic separation was performed using a LiChrospher 100-RP18 (250 mm × 4 mm, 5 μm) reversed phase LC column. The chromatographic parameters (composition and flow rate of mobile phase) and the operating parameters of ESI (cone voltage, temperature of the ionization source and capillary potential) were optimized. The optimum working conditions were as follows: isocratic elution with a mobile phase consisted of methanol–water (70:30, v/v) at a flow rate of 0.9 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 500 °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-d_{16}. Two standards with concentrations 10 and 100 ng/ml were measured daily in order to check the system suitability.

2.4. Method validation

After the selection of the optimum conditions for the sample preparation step and the LC–MS measurements, the method was thoroughly evaluated using aqueous BPA standard solutions and spiked blank milk (3.5% fat), packed in a plastic bottle of PET (polyethylene terephthalate) that did not contain detectable quantity of BPA.

The linearity of the response of the LC–MS system was examined with a calibration curve, obtained by measuring aqueous standard solutions of nine different concentrations (5, 10, 50, 100, 150, 200, 300, 500 and 700 ng/ml) containing 100 ng/ml of internal standard, with two replicates per concentration. Linear regression analysis was performed using the ratio *analyte peak area/internal standard peak area* against analyte concentration.

In order to examine the overall matrix effects on the determination and investigate whether standard addition is indispensable for the quantification of BPA in milk, another calibration curve was constructed by analyzing blank milk samples spiked with the internal standard at 100 ng/g and BPA at five different concentrations (5, 10, 50, 200 and 500 ng/g). This curve was compared to a calibration curve of aqueous standard solutions of BPA. The comparison was accomplished by constructing a correlation curve in which the analytical parameters (ratio *analyte peak area/internal standard peak area*) obtained from the spiked milk samples at the five fortification levels were plotted on the *y*-axis and the analytical parameters obtained from the standard solutions of BPA at the five corresponding concentrations were plotted on the *x*-axis.

The instrumental LOD and LOQ were defined as $(3.3 \times \mathrm{SD}_{n=10})/b$ and as $(10 \times \mathrm{SD}_{n=10})/b$, respectively. SD was the standard deviation of the response of ten replicate injections of 5 ng/ml BPA standard solution and b was the slope of a calibration curve ranging from 5 to 200 ng/ml. The LOD and LOQ of the method were determined using the same mathematical equations, but in this case SD stood for the standard deviation of the response of ten independent replicate analyses of milk samples fortified with BPA at a concentration of 5 ng/g.

For the assessment of the accuracy and the precision, the method was applied to blank milk samples that were spiked with 100 ng/g BPA-d_{16} and three fortification levels of BPA,

5, 50 and 500 ng/g. Analysis of six replicates during one day were conducted for the repeatability test ($n = 6$, intra-day precision), and analysis of two replicates in three different days over a period of one week, were conducted for the reproducibility test ($n = 2\,k = 3$, inter-day precision), for each fortification level. The accuracy of the method was determined as recovery relative to the internal standard. Blanks of water and milk were analyzed at every experiment.

2.5. Application of the method

The validated method was applied to eight products of canned condensed milk of different brands and to one product of canned powdered infant formula, purchased from local supermarkets in Athens in May and June 2005. When the method was applied to canned condensed milk, after the spiking of 1 g of the sample with the internal standard at 100 ng/g, this was diluted 1:1 with water in order to obtain a milk sample with fat content that did not exceed 3.5%. Afterwards 18 ml of a mixture of water–methanol (8:1, v/v) were added and solid phase extraction with C18 cartridges followed. The powdered infant formula was reconstituted with water at the ratio prescribed on the packaging (approximately 18.71 g of powdered milk in 120 ml of water) and 1 g of the product solution was treated as full fat milk.

The internal surface of the body and the lid of each can was screened with FT-IR (System 2000 FT-IR, Perkin Elmer) equipped with a specular reflectance attachment. The obtained spectra of transmittance in the region 4000–600 cm^{-1} were compared by a matching algorithm (Euclidean search) to the spectra of known epoxy resins and other polymers, in order to identify the coating type of the can surface.

3. Results and discussion

3.1. Optimization of LC-ESI-MS conditions

The response in ESI-MS detection is strongly dependent on the conditions of liquid chromatography and the interface [33,34]. These parameters were optimized in order to achieve maximum sensitivity for the determination of BPA in aqueous standard solutions and milk. The ionization in ESI source occurs in the solution state, therefore, the mobile phase and the modifiers may affect the production of the ions and consequently the signal response of the solute [31,33,34]. Different compositions of mobile phases consisted of 70% organic solvent (methanol or acetonitrile) and 30% aqueous solution (water or 0.01% NH_3 in water or 0.01% CH_3COOH in water) were examined and the results from the measurements of 1000 ng/ml standard solution of BPA are shown in Table 1. In all cases the mass spectrum of BPA standard confirmed that the most abundant ion was the *m/z* 227, which has been assigned as the deprotonated molecule $[M - H]^-$ [25–27,30,31]. The compositions of methanol–water and methanol-0.01% NH_3 in water gave mass spectra, which contained the ions with *m/z* 211 and 212 with relative abundance between 3 and 15%. The *m/z* 211 has been related to the additional loss of oxygen $[M - H–O]^-$, whereas the *m/z* 212 fragment probably results from a cleavage of one of the CH_3 groups $[M - H–CH_3]^-$ [30]. The mobile phase of acetonitrile-0.01% NH_3 in water gave a mass spectrum, which contained the ion with *m/z* 113, additionally to the already reported ions. This ion could be related to the loss of both acidic protons $[M - 2H]^{2-}$, a phenomenon that may be enhanced by the basicity of ammonia.

Table 1
Mean value of retention time (t_R), peak area and S/N ratio ($n = 2$) of 1000 ng/ml standard solution of BPA for different mobile phases at a flow rate of 0.7 ml/min

Mobile phase, organic solvent–aqueous solution (70:30, v/v)	t_R (min)	Peak area (*m/z* 227)	S/N
MeOH–water	7.94	4.2×10^6	2137
MeOH–0.01% NH_3 in water	6.64	2.8×10^6	502
MeOH–0.01% CH_3COOH in water	7.45	1.0×10^6	353
ACN–water	4.44	0.4×10^6	715
ACN–0.01% NH_3 in water	4.10	1.7×10^6	1124
ACN–0.01% CH_3COOH in water	4.46	1.3×10^6	1229

MS conditions: temperature of ionization source, 350 °C, cone voltage, 80 V and capillary potential, 3.5 kV.

Comparing methanol–water with acetonitrile–water as mobile phases, the former gave significantly higher response of the standard solution, which is in agreement with previous studies [31,35]. Generally, the mobile-phase parameters which affect the operation of the ESI interface (thus, ionization) include its boiling point, surface tension and conductivity [33]. Taking into consideration that acetonitrile has higher boiling point than methanol, it could be assumed that this parameter is responsible for the lower signal, since desolvation of the droplets formed is probably less favoured in the presence of acetonitrile. It has been reported that the addition of ammonia in mobile phase of acetonitrile–water (0.5% NH_3 in water) [26] and methanol–water (0.1% NH_3 in water) [31] in the negative ESI mode increased the response of BPA. However, in our study this phenomenon was observed only for acetonitrile–water mobile phase. The presence of ammonia in methanol–water mixture decreased the signal of BPA standard solution 1.5-fold, and the signal of spiked milk samples 3-fold (data not shown). The addition of acetic acid in acetonitrile–water, which has been suggested by other researchers [27], increased the response of BPA standard. However it decreased the signal when it was added to methanol–water. Eventually, methanol–water was selected as the best mobile phase since it gave the optimum sensitivity.

The effect of the flow rate of the mobile phase and the cone voltage on the signal response was investigated simultaneously (Fig. 2). The optimum response was obtained for a flow rate of 0.9 ml/min and cone voltage of 70 V. It can be assumed that a voltage lower than 70 V was not effective enough to ionize BPA quantitatively. Additionally, the increase of cone voltage above 70 V decreased the signal intensity gradually. This can be explained by the gradual fragmentation of BPA, which was observed in the mass spectrum: gradual decrease of the intensity of *m/z* 227 $[BPA–H]^-$, accompanied by gradual increase of *m/z* 211 $[BPA–H–O]^-$ and 212 $[BPA–H–CH_3]^-$.

The effect of the source temperature and the capillary potential on the signal intensity, S/N ratio and fragmentation were also studied. The ionization source was set at temperatures between

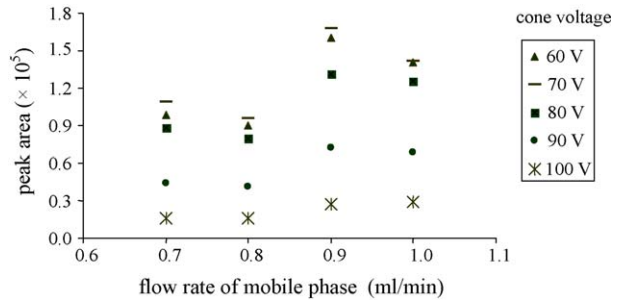

Fig. 2. Influence of the flow rate of the mobile phase on signal intensity of 100 ng/ml standard solution of BPA at different cone voltages. Mobile phase: methanol–water (70:30, v/v). Temperature of ionization source: 350 °C. Capillary potential: 3.5 kV.

350 and 500 °C: the higher the temperature was set, the higher the signal and the S/N ratio became. This can be attributed to the relatively high flow rate of the mobile phase that was used (0.9 ml/min). As the temperature increase, the solvents of the mobile phase may evaporate more effectively and the formation of the ions is better accomplished. No fragmentation was observed in the mass spectrum. The capillary potential did not show any effect on the response of standard BPA when two different values were examined (2.5 and 3.5 kV).

The SIM chromatograms (*m/z* 227 for BPA and *m/z* 241 for BPA-d_{16}) of a standard solution, containing 500 ng/ml of BPA and 100 ng/ml of BPA-d_{16}, as well as the mass spectra at the corresponding retention times, under the optimum selected conditions, are given in Fig. 3. The molecular structure of BPA-d_{16} (MW 244) is identical to that of BPA (Fig. 1), with the exception that all the protons are replaced with deuteriums. The mass spectrum of BPA-d_{16} indicated that the most abundant ion was

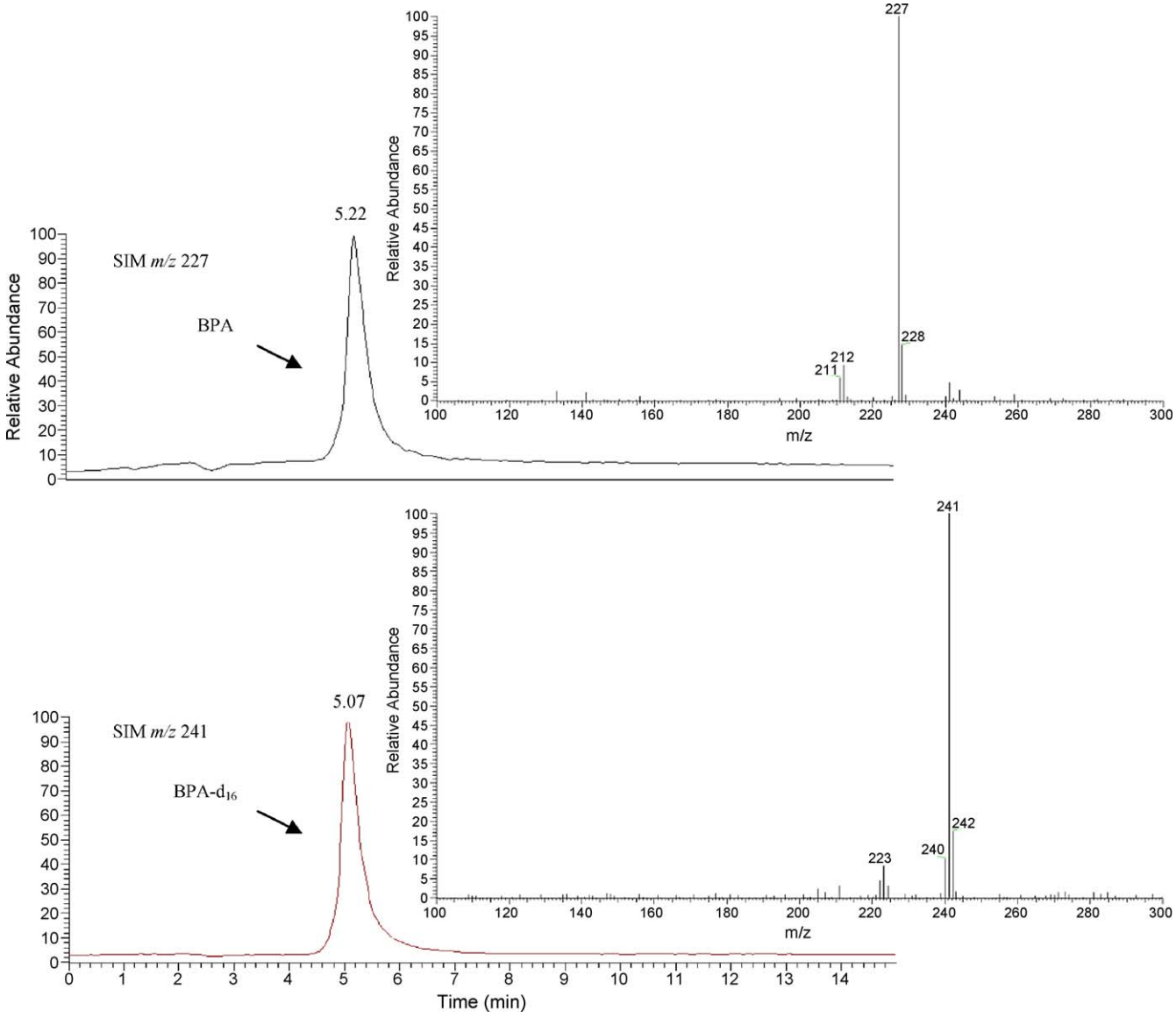

Fig. 3. SIM chromatograms of a standard solution containing 500 ng/ml of BPA (*m/z* 227) and 100 ng/ml of BPA-d_{16} (*m/z* 241) and full scan spectrum at the retention time of each compound.

Table 2
Significant improvement of the overall method recovery by the application of the deuterated internal standard (C represents the concentration of the measured solution (ng/ml). Concentration range: 5–500 ng/ml. Calibration points: 5)

	y: analyte peak area $\times 10^{-3}$	y: (*analyte peak area/I.S. peak area*) $\times 10^{3}$
Spiked milk samples	$y = (-29 \pm 51) + (7.52 \pm 0.22)$ C $r = 0.999$	$y = (4.3 \pm 9.3) + (7.54 \pm 0.039)$ C $r = 0.99996$
Standard solutions	$y = (2.4 \pm 21) + (14.6 \pm 0.090)$ C $r = 0.99994$	$y = (-19 \pm 17) + (7.47 \pm 0.071)$ C $r = 0.9999$
Slope ratio × 100	Absolute recovery: 52%	Relative recovery: 101%

m/z 241. BPA-d_{16} was transformed into BPA-d_{14} (MW 242) in water, as the two acidic deuterium atoms of BPA-d_{16} are immediately exchanged against protons when dissolved in a protic medium [29]. This is the reason that *m/z* 241 was assigned as the deprotonated molecule [BPA-d_{14}–H]$^-$.

3.2. Evaluation of SPE sorbents

In this study three commercially available SPE cartridges, including C18, PS-DVB and hydroxylated PS-DVB, were evaluated, subjected to two different protocols. The first protocol includes dilution of 1 g of milk with 9 ml of water–methanol (8:1, v/v) prior to solid phase extraction, while the second protocol includes dilution of 1 g of milk with 9 ml of a mixture of aqueous HCl (pH 3) and methanol (8:1, v/v). The application of the first protocol gave the following absolute recoveries of BPA from spiked water and spiked milk, respectively: 85 and 57% (C18), 83 and 51% (PS-DVB), 46 and 40% (hydr. PS-DVB). The results implied that C18 cartridge was the most suitable sorbent for the isolation of BPA from water and milk, under the specific conditions, achieving the highest recoveries for the analyte and the internal standard (I.S.). Recoveries of the I.S. were similar to those of BPA. Thus, the relative recovery of BPA obtained from C18 cartridge was 100% for water and 101% for milk, overcoming the observed loss in recovery due either to sample preparation procedure or to matrix effect. Silica-based endcapped C18 and PS-DVB sorbents possess mainly hydrophobic properties, whereas hydroxylated PS-DVB may also exert polar secondary interactions, like hydrogen bonding, which could be responsible for stronger retention and consequently for the lower recoveries.

The application of the second protocol, which includes initial acidification of the sample with HCl, resulted in the following absolute recoveries of BPA in spiked water and milk, respectively: 69 and 50% (C18), 64 and 34% (PS-DVB), 81 and 60% (hydr. PS-DVB). It was noteworthy that the initial acidification of the sample significantly increased the recovery of both BPA and BPA-d_{16} from water and milk with the use of the hydroxylated PS-DVB cartridge, which gave the highest recoveries for this procedure. Probably, the presence of the acid in the sample, loaded on this cartridge, reduced or prevented the development of strong polar interactions between the compounds and the sorbent, which render the elution more difficult. Acidification of the sample with HCl prior to SPE has been described for the determination of BPA and other phenolic xenoestrogens in water with C18, PS-DVB and sulfonated PS-DVB cartridge [41], where the last and more polar sorbent gave the highest recoveries in this case as well.

Eventually, the application of the first protocol to the C18 cartridge was selected as the final sample preparation procedure for the determination of BPA in milk. The C18 cartridge was preferred to hydroxylated PS-DVB as a more common material.

3.3. Overcoming sample preparation losses and matrix effect with deuterated internal standard

Table 2 shows the equations of the curves prepared by spiked milk samples and standards using as analytical parameter the analyte peak area and the ratio *analyte peak area/internal standard peak area*. The standard deviation of the slope and the intercept, and the correlation coefficient of every equation are also given. A general estimation of the absolute recovery of the method was accomplished by dividing the slope of the curve obtained from the spiked samples with the slope of the external calibration curve, using the peak area as the dependent variable. The mean absolute recovery of the method was found to be 52%. Performing spiking to milk sample extracts after SPE at a level of 100 ng/ml and then measuring with LC–MS, 20% signal suppression for both BPA and BPA-d_{16} was observed, relatively to the signal of a 100 ng/ml standard solution. Subtracting the 20% of the losses due to the matrix effect during ESI, from the total losses of the overall procedure (48%), the remaining losses (28%) could be attributed to the sample preparation procedure. The relative recovery of the method calculated dividing the slopes of the two corresponding curves that contained the ratio *analyte peak area/internal standard peak area* as the dependent

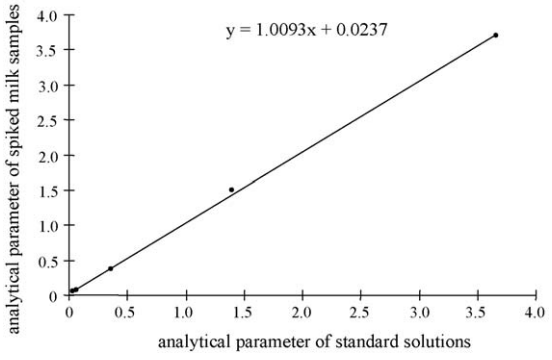

Fig. 4. Correlation curve of analytical parameters of spiked milk samples against analytical parameters of standard solutions for concentrations of 5, 10, 50, 200 and 500 ng/ml.

variable, was 101%. These results indicate that the use of the deuterated internal standard can overcome the matrix effect and the sample preparation losses, it can provide correction of the recovery and consequently improve the trueness of the method.

The correlation curve of the analytical parameters obtained from the aqueous standards and the spiked milk samples is presented in Fig. 4. The statistical parameters from the application of linear regression analysis are listed in Table 3. The confidence interval of the slope contains the value 1 and the confidence interval of the intercept contains the value 0, therefore the correlation curve can be described by the equation $y = x$. Consequently, the two curves do not differ statistically at a confidence level 95%. The similarity of the two curves (constructed by spiked milk samples and standard solutions) is due to the fact that the isotopically labeled compound has similar physical and chemical properties to the unlabeled analogue and for this reason analyte and internal standard experience similar losses during SPE and similar ionization efficiency and ion suppression during electrospray ionization [33,35,37]. Therefore the ratio *analyte peak area/internal standard peak area* remains constant and for

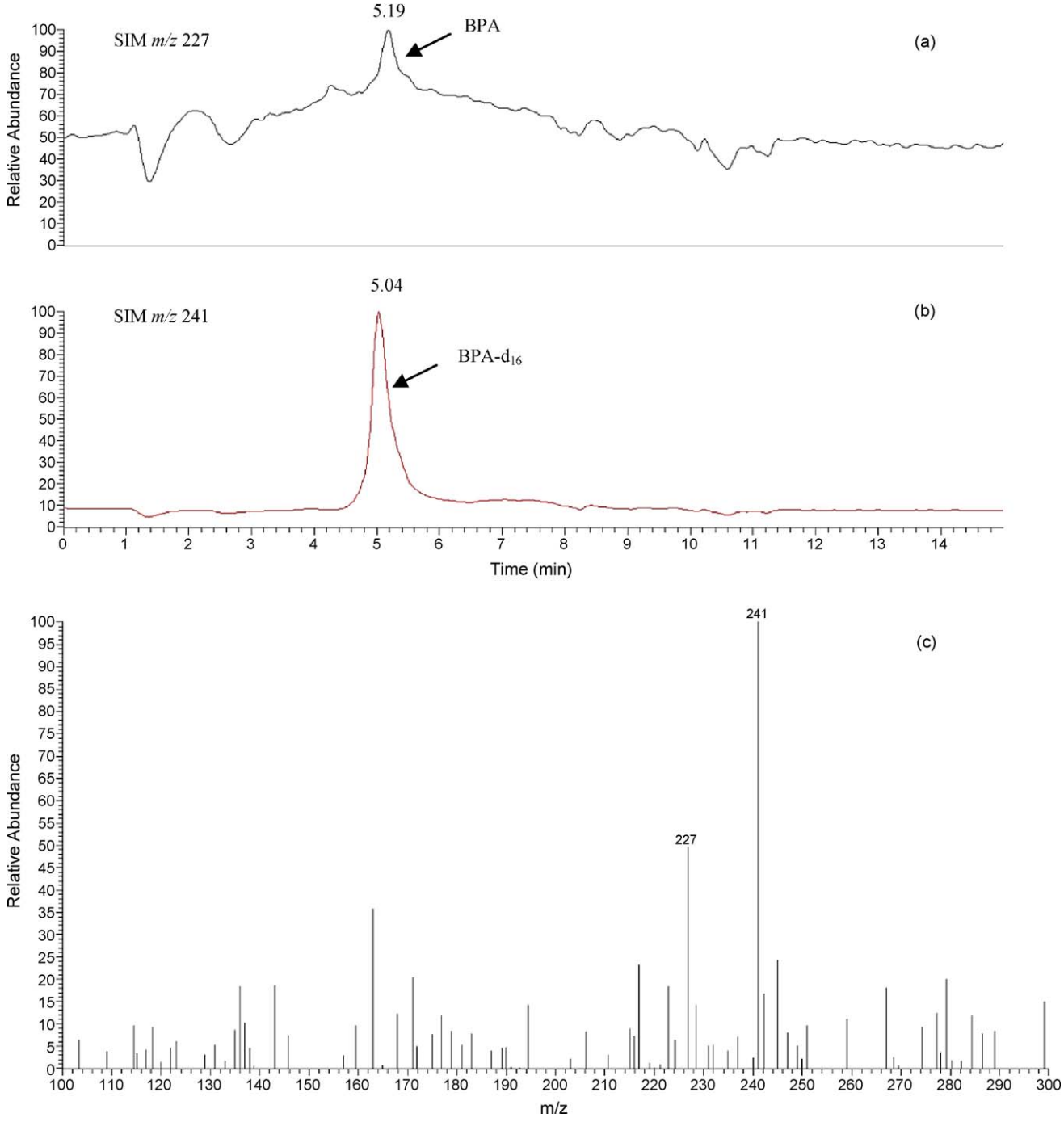

Fig. 5. SIM chromatograms of a milk sample containing approximately 5.0 ng/g of BPA (a) and 100 ng/g of BPA-d_{16} (b), along with the full scan spectra at a retention time of 5.17 min (c).

Table 3
Statistical parameters of the correlation curve of the analytical parameters of the spiked milk samples against the analytical parameters of the standard solutions

Slope (b)	1.0093
Standard deviation of slope (S_b)	0.0145
Intercept (a)	0.0237
Standard deviation of intercept (S_a)	0.0256
Correlation coefficient (r)	0.9997
Number of experimental pairs (n)	5
t_{theor} (confidence level: 95%, degrees of freedom: 3)	3.182
Confidence interval 95% b: $b \pm t_{theor} \times S_b$	Between 0.9632 and 1.0554
Confidence interval 95% a: $a \pm t_{theor} \times S_a$	Between −0.0576 and 0.1052

this reason the calibration curve, generated by aqueous standard solutions, can be used for the quantification of BPA in milk.

3.4. Method performance

The LC–MS system presented linearity for concentration range extended up to 700 ng/ml and the correlation coefficient always exceeded 0.999. Fig. 5 presents the SIM chromatograms (m/z 227 for BPA and m/z 241 for BPA-d_{16}) of a real milk sample containing approximately 5 ng/g of BPA and 100 ng/g of BPA-d_{16}, as well as the full mass spectra at a retention time of 5.17 min. It can be observed that the most abundant ions in the full scan mass spectrum at the corresponding retention time are the 227 (confirming the presence of BPA) and 241 (BPA-d_{16}), as it was observed for the standard solution of BPA in Fig. 3. It is noteworthy that even if the concentration of BPA in the sample is near the limit of quantification, its presence can be confirmed with high certainty.

The instrumental LOD and LOQ were found to be 0.7 and 2.2 ng/ml, respectively, whereas the LOD and LOQ of the method were determined as 1.7 and 5.1 ng/g, respectively. These values are adequate for safety control of food products since they are much lower than the specific migration legislative limit (600 ng/g) [1]. In addition, the LOD of the method is at the concentration level at which it has been reported that BPA can exhibit estrogenic activity [15,16]. The intra- and inter-day accuracy and precision data at three fortification levels are given in Table 4, showing the adequate performance of the method. Even though the 5 ng/g level correspond to the LOQ of the method, the precision and the accuracy are quite satisfactory (<20%).

Table 4
Intra- and inter-day accuracy and precision for milk samples spiked at three different levels ($n = 6$)

BPA (ng/g)	Intra-day assay		Inter-day assay	
	Mean relative recovery (%)	RSD (%)	Mean relative recovery (%)	RSD (%)
5	83[a]	12.5[a]	97	17.6
50	101	5.0	97	5.8
500	106	2.1	104	5.2

[a] $n = 10$.

Table 5
Stability of the LC–MS response and the retention times of a 100 ng/ml standard solution containing BPA and BPA-d_{16} during a two-month period ($n = 44$)

Parameters	%RSD
Analyte peak area	41.5
I.S. peak area	40.9
Analyte peak area/I.S. peak area	4.8
Analyte t_R	3.3
I.S. t_R	3.2
Analyte t_R/I.S. t_R	0.2

The stability of the LC–MS system was evaluated from two months measurements of BPA standard solutions with a concentration of 100 ng/ml. Table 5 summarizes the RSD (%) of the following parameters: peak area of BPA, peak area of internal standard, the ratio *analyte peak area/internal standard peak area*, the retention time of the two compounds and the relative retention time. Comparing the values of the RSD of the peak areas to the RSD of their ratio, a significant improvement is observed in the reproducibility of the analytical parameter, which is also reflected at the reproducibility of the calculated concentration and its overall uncertainty. Moreover, the excellent reproducibility of the relative retention time allows the use of this parameter as an additional tool for the confirmation of the presence of BPA in unknown samples, apart from the mass spectrum. This is very useful for samples with very low concentrations of BPA, as their mass spectra contain a lot of background ions with high relative intensities that render confirmation more uncertain.

Table 6
Coating type of the internal surface of the cans and the concentration of BPA found in the milk content

	Lid lacquer	Body lacquer	Concentration of BPA (ng/g)
Whole evaporated milk (Brand A)	Epoxy resin	Epoxy resin	7.11
Whole evaporated milk (Brand B)	Epoxy resin	Epoxy resin	<5.1[a]
Whole evaporated milk (Brand C)	Epoxy resin	Epoxy resin	15.2
Whole evaporated milk (Brand D)	Epoxy resin	Epoxy resin	<5.1
Whole evaporated milk (Brand E)	Epoxy resin	Epoxy resin	<1.7[a]
Whole evaporated milk (Brand F)	Epoxy resin	Epoxy resin	<5.1
Whole evaporated milk- flexible packaging (Brand G)	PVC	PVC	<5.1
Partly skimmed evaporated milk (Brand H)	Epoxy resin	Epoxy resin	<5.1
Powdered infant formula (Brand I)	Polyethylene	Polyester	<1.7

[a] LOD: 1.7 ng/g and LOQ: 5.1 ng/g.

3.5. Application of the method

Table 6 presents the coating type of food contact surface of the can samples, which were tested, as well as the concentration of BPA found in the milk content of them. The majority of the lacquers were identified as epoxy resins. BPA was detected in most of the samples. BPA was not detected in the infant formula sample, as it was expected, since the material of the coating of this can is not among the materials that contain BPA. The concentrations found ranged between <1.7 and 15.2 ng/g. The results are in agreement with previous studies that report BPA concentration in canned infant formula, powdered milk and whole milk between 0.1 and 13.2 ng/g [4] and 0.28 and 2.93 ng/g [17]. The results suggest that the levels of BPA are unlikely to be of concern, according to the recently established specific migration limit is 0.6 μg/g. However, considering that the estrogenic activity of BPA at low levels is under discussion [3] and that milk is the main nourishment of babies, determination of BPA in milk samples is essential.

4. Conclusions

The present study shows that BPA can be detected and quantified reliably in milk samples combining a very simple and time saving SPE sample preparation procedure with LC-ESI-MS, comparing it to previously reported methods [4,17,18,23,32]. A simple dilution with a mixture of water–methanol (8:1, v/v) and SPE with C18 cartridges produce clear extracts that could be analyzed with a selective and confirmatory method at very low levels, even below the LOQ. It was demonstrated that the application of the deuterated internal standard compensated for sample preparation losses, matrix effects and instrumental instability, improving significantly the accuracy and the precision of the proposed method.

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