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Sensitive gas chromatographic-mass spectrometric (GC-MS) method for the determination of bisphenol A in rice-prepared dishes

A. Zafra-Gómez[a]*, J.C. Morales[b], O. Ballesteros[a] and A. Navalón[a]

[a]*Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avda. Fuentenueva s/n, E-18071 Granada, Spain;* [b]*Instituto de Investigaciones Químicas, CSIC – Universidad de Sevilla, 49 Americo Vespucio, E-41092 Seville, Spain*

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Certain chemicals possess the potential to modulate endocrine systems, and thereby interfere with reproductive and developmental processes. Bisphenol A is suspected to be one of them. The compound is widely used as a plastic additive, lacquer, resin, or plastic and can usually be found in food samples. An accurate and reproducible gas chromatographic-mass spectrometric (GC-MS) method to detect and measure trace amounts of the compound in rice-prepared dishes samples is proposed. Solid–liquid extraction with acetonitrile was carried out in order to isolate and pre-concentrate the analyte. The solvent was removed and a silylation step using *N*,*O*-*bis*(trimethylsilyl)trifluoro acetamide/pyridine (BSTFA/PYR) was carried out. The silylated compound was identified and quantified by GC-MS using a DB-5 MS column. Bisphenol F was used as a surrogate internal standard. The detection limit was 2.0 ng g^{-1} while inter- and intra-day variability was less than 6%. Due to the absence of reference materials, the method was validated using standard addition calibration and a recovery assay. Recoveries for spiked samples were between 90% and 105%.

Keywords: gas chromatography-mass spectrometry (GC/MS); clean-up; bisphenol A; food-contact materials; canned foods; rice

Introduction

Various epidemiological and laboratory studies describing in some cases severe disturbances in the endocrine system of humans and wildlife have been published in the past few years (Rivas et al. 2005; Paris et al. 2002). Today, it is well known that around 100 industrial chemicals show oestrogenic activity in addition to their desired chemical properties. Most of these endocrine disrupter chemicals (EDCs) are widely used organic compounds that are ubiquitous in the environment and in biological samples (Harris et al. 1997; Fromme et al. 2002). At the same time, EDCs accumulate in certain tissues in humans and their effects could be passed to future generations via the placenta and/or milk (Hood 2005; Maffini et al. 2006; Lopez-Espinosa et al. 2008).

Most of these compounds, commonly called endocrine disrupter chemicals (EDCs), are synthetic organic chemicals introduced into the environment by way of anthropogenic inputs. Aware of the problem, both the European Union and the US Environmental Protection Agency (USEPA) have authored a 'priority' list of substances for further evaluation of their role in endocrine disruption (Groshart and Okkerman 2000; US Environmental Protection Agency (USEPA) 2003) and indicate the need to assess the levels and effects of EDCs.

One of the representative compounds of the group of EDCs is bisphenol A (BPA). BPA is a compound with high reactivity and is the raw material for a large amount of manufactured products, such as epoxy resin, or polysulfones. It is also used as an antioxidant or stabilizer. However, one of the most important applications of the compound is the production of polycarbonate plastics, which are mainly a condensed polymer of BPA and carbonyl chloride or diphenyl carbonate. Since it is transparent, has excellent heat resistance and impact resistance, and can be used for high-temperature uses and in microwave ovens, it is used in items such as children's tableware, coffee-makers and food containers.

Some studies have reported that BPA has an oestrogen-like action of toxicity; moreover, it was discovered that the compound was released from a flask made of polycarbonate and showed binding to the oestrogenic receptor. In fact, BPA-induced

*Corresponding author. Email: azafra@ugr.es

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progesterone receptors in cultured breast cancer cells and its activity were about 1/5000 that of oestradiol (Krishnan et al. 1993). Different studies show statistically significant reproductive and developmental toxicity in rats and mice at high doses of BPA (Morrissey et al. 1987). Nevertheless, there is still controversy over whether low doses of BPA can cause reproductive and developmental effects in humans (Goodman et al. 2006).

Significant published data are available on bisphenol A diglycidyl ether (BADGE) or BPA migration from can coatings into foods and food simulants (Biles et al. 1997; Kawamura et al. 1999; Yoshida et al. 2001; Goodson et al. 2002; Kang and Kondo 2003; Cabado et al. 2008), but little is known about how the migration level would be influenced by damage to the can or storage conditions. However, some workers have investigated the effects of the food-processing conditions on the migration of BPA from food or from simulating liquids (Munguía-López and Soto-Valdez 2001; Munguía-López et al. 2005; Sajiki et al. 2005; Poças and Hogg 2007; Le et al. 2008).

Sterilization conditions, normally applied by industry for the scenarios used in this study, are relatively extreme (high temperature and sterilization time). In view of the potentially long shelf-life of most canned foods and the current interest surrounding the exposure to BPA from food packaging, it was considered important to obtain information on all these factors.

The Scientific Committee for Food (SCF) reviewed the tolerable daily intake (TDI) for BPA and the TDI reduced its value to a temporary value of $0.01\,mg\,kg^{-1}$ body weight day^{-1}. Therefore, it is of crucial importance to devise analytical methodology for detecting and quantifying these compounds in food.

Gas chromatography-mass spectrometry (GC-MS) has traditionally been used as the main analytical methodology for analysing BPA together with other EDCs (nonylphenol, phthlates or BADGE) in water, milk or beverages (Schoene et al. 1994; Ding and Chiang 2003; Mol et al. 2000; Helaleh et al. 2001; Nakamura et al. 2001; Fine et al. 2003; Rodríguez et al. 2003; Casajuana and Lacorte 2004). In contrast, there are very few examples where GC-MS has been applied to powdered (Kuo and Ding 2004) or solid foods (Thomson and Grounds 2005). Goodson et al. (2004) investigated the potential effects, on the migration of BPA from can coatings, of cooking or heating foods in the can before consumption using GC-MS as their analytical technique. Alternatively, liquid chromatography (Katayama et al. 2001; Petrovic and Barceló 2001; Shao et al. 2005, 2007; Maragou et al. 2008) and capillary electrophoresis (Tsukagoshi et al. 2002) have also been used.

In this paper, an accurate, simple, and reproducible method to detect and quantify trace amounts of BPA in rice-prepared dishes is reported. The main purpose of the study was the application of the method as routine methodology in a food company for BPA contamination control. Samples taken from different supermarkets were also analysed as a preliminary step and the method was checked by recovery assays in spiked samples.

Materials and methods

Reagents and standards

Unless noted otherwise, all chemicals and solvents purchased were of high purity. Water was purified with a Milli-Q plus system (Millipore, Bedford, MA, USA).

Acetonitrile (HPLC-gradient, PAI-ACS), hexane (UV-IR-HPLC, PAI), absolute ethanol (HPLC-gradient, PAI), ethyl acetate (UV-IR-HPLC, PAI-ACS), and sodium chloride (PA-ACS-ISO) were supplied from Panreac (Barcelona, Spain). All solvents and reagents were checked to ensure they were free of contamination from bisphenol A. Bisphenol F (BPF) purum, ≥98.0% (Fluka), bisphenol A (BPA) purum, ≥99%, silylation agent *N*,*O*-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA) puriss. p.a., for GC, ≥99.0% and pyridine anhydrous, 99.8% (Sigma-Aldrich) were supplied by Sigma-Aldrich-Fluka (Madrid, Spain).

Stock solutions of analyte BPA and surrogate BPF ($100\,\mu g\,ml^{-1}$) were prepared in ethanol. BPF was selected as the surrogate due to its structural similarity to BPA and because it has not been detected in a previous study in canned food (Goodson et al. 2002). Mixtures of the analytes for working standard preparation and sample fortification were also prepared in ethanol. All stock solutions and mixtures were stored at −10°C in the dark until use, remaining stable for at least 3 months.

Sample preparation

Rice-prepared dishes are a pre-cooked meal whose main component is rice (50–65%) and they also contain a large variety of other minor components (vegetables such as carrots, green pepper, onion, peas, meat such as chicken, pig or beef, and other ingredients such as oil, salt, garlic and spices). The rice-prepared dishes are presented in plastic containers.

Samples were randomly selected from different supermarkets and also from a rice-prepared dishes production plant situated in Seville, Spain. A total of 250 different rice-prepared dishes were acquired and analysed.

Also, rice-prepared samples were collected before the packing procedure directly in the production plant. These samples were homogenized and tested as negative controls for BPA and, at the same time, were used for fortification and/or recovery studies.

All samples were carefully mixed and homogenized using a food mixer. The homogenized samples were stored in glass bottles, previously cleaned with nitric acid (1 : 1; v/v), in the dark and at 4°C until treatment. Analysis was performed with the minimum possible delay and as described below in order to avoid sample degradation.

Extraction and derivatization

Before extraction, the sample was carefully homogenized with a mixer and a sample (20 g) was placed in 50 ml falcon tube. After that, the sample was spiked with bisphenol F as a surrogate (100 ng g^{-1}). Extraction was carried by adding 5 ml of acetonitrile and shaking vigorously in a vortex mixer for 1 min. The mixture was centrifuged 2 min at 6000 rpm at room temperature. Supernatant was transferred into a 25 ml separation funnel, and the extraction was repeated twice. NaCl (5 g) was added to the combined organic phase in the separation funnel and three phases – entailing a solid sodium chloride phase, saturated NaCl aqueous solution phase and an organic phase – were observed. After removing the solid and the aqueous layer, the acetonitrile phase was washed with *n*-hexane (2 ml) to remove fat traces and decanted into a 10 ml assay tube in order to evaporate to dryness in a speed-vac system. The extract was redissolved using 1.0 ml of ethyl acetate and transferred into a chromatographic vial.

After evaporation to dryness under nitrogen, 50 µl of a mixture of ethyl acetate/BSTFA/pyridine (2 : 1 : 1; v/v/v) were added into the tube to resuspend the residue and carry out the derivatization. BPA and BPF are suitable for derivatization due to their chemical structure. Once the derivatization process was completed (1 min), 1 µl of the reaction mixture was injected into the GC-MS system.

Apparatus and software

GC analysis was performed using an Agilent 6890 Series GS System GC fitted with a splitless injector for a low background; a split/splitless deactivated glass liner (78.5 mm × 6.3 mm × 4.0 mm, wool packed) for capillary injection port was used. A J&W Scientific, Inc. (Folson, CA, USA) capillary column DB-5MS fused-silica, 30 m × 0.25 mm internal diameter, 0.25 µm film thickness, 5% phenyl–95% dimethyl arylene siloxane was used. Detection was carried out with a 5973 mass-selective single quadrupole detector (Agilent Technologies, Santa Clara, CA, USA). The GC-MS operation control and the data process were carried out with ChemStation software.

The injector port of the GC was set at 250°C. The silylated samples were automatically injected using the splitless-injection mode. The transfer line of the GC to the MS was set at 280°C, and the electron ionization (EI) ion source of the MS set at 250°C. The GC oven temperature programme was applied as follows: the initial oven temperature was set at 150°C, held for 0.5 min, then the temperature was increased to 280°C via ramp of 20°C min^{-1} and held for 5.0 min. Finally, the temperature was raised to 300°C at 40°C min^{-1} and maintained for 2.5 min. The total run time was 15.0 min. The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0 ml min^{-1}. Delay time was 3 min in order protect the ion multiplier of the MS instrument from saturation and the sample volume in the direct injection mode was 1 µl.

For qualitative analysis, the Mass Spectrometric Detector (MSD) was operated in full-scan mode. The conditions for electron impact ionization (EI) were ion energy of 70 eV, multiplier 1800 and the mass range scanned was 50–400 m/z. The MS was tuned everyday to m/z 69, 219 and 502 with perfluorotributylamine (PFTBA) as a calibration standard.

For quantitative analysis, single-ion monitoring (SIM) acquisition mode (dwell time = 100 ms per ion) was used. The retention times for BPF and BPA trimethylsilyl derivatives were 8.9 and 10.7 min, respectively. The mass spectrum of silylated BPF showed a base peak at 344 m/z, corresponding to the molecular ion, and the mass spectrum of silylated BPA showed the molecular ion peak at 372 m/z, whereas the base peak appears at 357 m/z, corresponding to the loss of a methyl group.

A Statgraphics Centurion XV, v.15.1.02 software package (1982–2006; Statpoint Inc. Technologies, Warrenton, VA, USA) was used for statistical analysis of the data.

Results and discussion

Isolation and pre-concentration procedure

Rice-prepared dishes contain a complex mixture of a large number of components (rice, meat, peas, oil, fat, etc.). Determinations at the trace level require isolation and a pre-concentration step of the samples to reach nanogram levels of concentration. An extraction procedure was selected as appropriate to obtain the analyte from the homogenized rice dish samples. The extraction was optimized by adjusting parameters that influence in analyte extraction, for example, the nature of extraction solvent, extraction time or cleaning solvent.

A large number of mixtures of diethyl ether, methanol and acetonitrile were tested for extraction of analytes from samples. Pure solvents and different mixtures of diethyl ether, methanol and acetonitrile (ratios of 3 : 1, 1 : 1 and 1 : 3) were tested for the

extraction of analytes from samples. Extraction yield for analyte isolation (at 100 ng g^{-1} spiking samples) was in all cases between 60% and 90%, except for pure acetonitrile which showed the highest yield (96%).

In addition, *n*-pentane, *n*-hexane and chlorinated solvents (dichloromethane, chloroform or carbon tetrachloride) were tested to remove fat traces from acetonitrile extracts in a separating funnel. Although carbon tetrachloride and chloroform showed good results (close to 90% of the defating ratio, and less than 15% of analyte losses), they were not used due to their high toxicity for humans and the environment. *n*-Pentane showed a relative defating ratio very close to *n*-hexane, but the analyte loss was 40% for pentane and only 12% for *n*-hexane. Therefore, acetonitrile was selected for BPA extraction and *n*-hexane was used for extract cleaning.

Derivatization method

Trimethylsilyl derivatives of BPA and BPF were obtained using a BSTFA/pyridine mixture as the silylation reagent. This reagent was selected because of its fast reactivity with compounds containing hydroxyl groups, its high volatility resulting in no co-elution of early eluting peaks, and low thermal degradation and good solubility of the derivatized compounds in common organic solvents. Derivatized samples had a better separation of the analyte under GC-MS analysis because of their higher volatility and lower interaction with the stationary phase.

Optimization of the derivatization procedure was carried out by applying the experimental design methodology. The effect of varying the percentage of silylation agent (BSTFA/pyridine 1 : 1, v/v) in ethyl acetate, the temperature of the process and the reaction time were tested on the analytical response. The three factors were simultaneously optimized by application of a 2^3 central composite design plus face centred (with three centred points). The BSTFA/pyridine concentration was studied from zero to 100%, from a temperature of 25°C (room temperature) to 95°C, and the reaction time was varied from zero to 60 min. A 50% of silylation agent in ethyl acetate (v/v), 1 min and room temperature were the procedural conditions selected.

Gas chromatographic-mass spectrometric analysis

An increase in the signal-to-noise ratio for BPA was clearly observed in the derivatized sample. Figure 1 shows a characteristic chromatogram obtained in SCAN mode (a) and SIM mode (b) for a silylated sample containing BPA and BPF as surrogate.

Another feature of the application of derivatization reactions is that trimethylsilyl derivatives produce ions

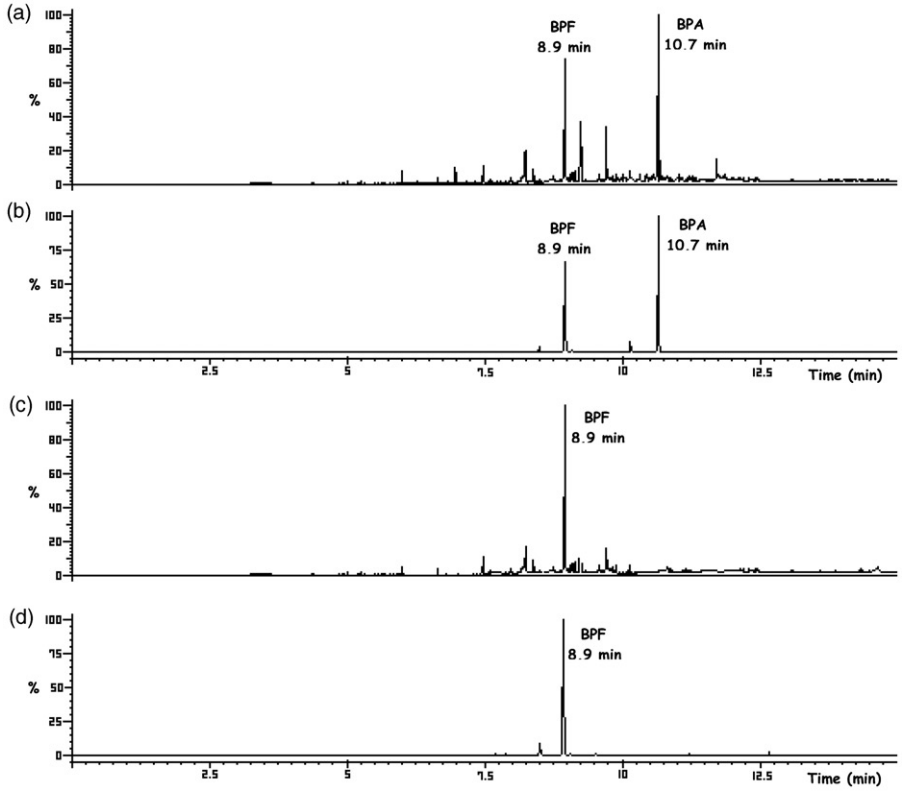

Figure 1. Chromatograms of a spiked sample in SCAN (a), SIM (b) mode (100 ng g^{-1}) and a non-spiked sample in SCAN mode (c) and SIM mode (d).

with higher a m/z in the GC-MS system in contrast to those obtained from un-derivatizated compounds. The selection of high mass fragments as quantification ions is of great interest, particularly when complex matrices, as rice-prepared dishes, are to be analysed, due to the decreased likelihood of interferences. In our research a high increase in sensitivity and selectivity was reached. The mass spectra obtained in scan mode are shown in Figure 2, which displays the EI mass spectra and tentative fragmentation of the *bis-O*-Trimethylsilyl derivative of BPA and surrogate. Either the molecular ions $[M]^+$ or the $[M\text{-}CH_3]^+$ ions were the base peaks in the derivative. The molecular ion peak of silylated BPA appears at 372 m/z, whereas the peak corresponding to loss of the methyl group is at 357 m/z. The mass spectra obtained for silylated BPF show the base peak at 344 m/z corresponding to the molecular ion. Therefore, these ions were selected to be used as the quantitation ions to obtain maximum detection sensitivity and specificity in the SIM mode. All derivatives displayed an ion at m/z 73 $[(CH_3)_3Si]^+$ which was characteristic of the TMS group and commonly observed in all TMS derivatives. The selected conditions for SIM mode are shown in Table 1.

Analytical performance: validation of the methodology

Calibration graphs for samples treated according to the analytical procedure described above were made using SIM mode. BPF was used as a surrogate.

The standard addition calibration was carried out by fortification of homogenized rice samples aliquots. A total of 20 g of raw samples free of BPA were placed in 50-ml falcon tubes for collection. Samples were then spiked with BPF as a surrogate (100 ng g^{-1}) and with BPA as analyte at growing concentrations. The standard samples were strongly shaken in order to mix surrogate, analyte, and sample and treated as described above. Linearity of the calibration using standard addition graphs was tested according to the Analytical Methods Committee (1994). The lack-of-fit test was applied to the residuals of a calibration curve. Two experimental replicates and three injections of each standard were carried out. The results for the intercept (a), slope (b), correlation coefficient (R^2), and probability level of the lack-of-fit test, P_{lof} (%), are summarized in Table 2.

Table 1. SIM mode characterization and structural assignments of the fragments.

	t_r (min)	Dwell time (ms)	Range (min)	Fragments (m/z)
Bisphenol F	8.9	100	7.5–10.0	344 $[M]^+$, 179, 157
Bisphenol A	10.7	100	10.0–15.0	357 $[M–Met]^+$, 207, 179

Table 2. Analytical and statistical parameters of the proposed method.

Parameter	Value
n	6
a	3.0×10^{-3}
s_a	2.8×10^{-3}
b (g ng^{-1})	1.20×10^{-2}
s_b (g ng^{-1})	1.25×10^{-3}
R^2 (%)	99.7
LDR (ng g^{-1})	0.8–100.0
$S_{y/x}$	2.45×10^{-3}
CC_α (ng g^{-1})	0.5
CC_β (ng g^{-1})	0.8
P_{lof} (%)	53.1

Note: n, calibration levels; a, intercept; s_a, intercept standard deviation; b, slope; s_b, slope standard deviation; R^2, determination coefficient; LDR, linear dynamic range; $S_{y/x}$, regression standard deviation; $CC_{\alpha,0.05}$, decision limit; $CC_{\beta,0.05}$, detection capability; P_{lof}, p-value for the lack-of-fit test.

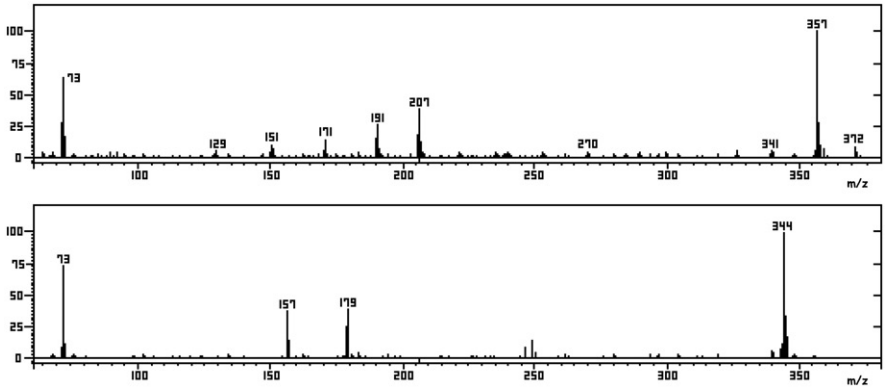

Figure 2. Mass spectra of bisphenol A and surrogate (bisphenol F) in a spiked sample.

Validation was performed according to the US Food and Drug Administration (USFDA) guideline for bioanalytical assay validation (US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER): Center for Veterinary Medicine (CVM) 2001). The analytical performance parameters assessed for the overall assay were linearity, precision, accuracy, sensitivity, and selectivity.

Linearity

A concentration range from the minimal amount detectable by this methodology up to two orders of magnitude higher (0.8–100 ng g^{-1}) was selected for method application.

The response of the compound was checked in the range of application of the analytical method by linear regression analysis by the least-squares method of peak area ratio of analyte/surrogate against different analyte concentrations. The response was linear in the range of concentrations evaluated.

In the specific case of higher BPA concentration than the selected range, one should test the linearity of the method beyond 100 ng g^{-1} or simply dilute the corresponding samples if necessary. In our application, no levels higher than 100 ng g^{-1} were found.

Precision

Precision expressed as relative standard deviation (RSD) at three concentration levels was obtained from 30 replicates of spiked samples obtained from ten aliquots of a homogenate of ten different rice dishes and analysed during the same day (repeatability) and in three different days (reproducibility). A total of 30 analyses were carried out in order to calculate precision. RSD was lower than 10% in all cases (Table 3). Data indicate that the analytical method is precise (repeatable and reproducible). It is important to note that the precision varied with concentration. Important factors such as sample weight, standard preparation, instrument response or calibration uncertainty can limit precision.

Accuracy

A recovery assay was performed by comparing the analytical results for extracted samples, free of BPA, spiked at three concentration levels. The concentration of the compound was determined by interpolation in the standard addition calibration curve within the linear dynamic range and compared with the added amount. Ten replicates, by spiking 20 g of rice samples with the analyte, at three concentration levels were analysed. The recoveries for BPA were between 90% and 105% in all cases (Table 3).

Table 3. Recovery assay, precision (repeatability and reproducibility), and accuracy of BPA ($n = 30$).

	Spiked (ng g^{-1})	Found[a] ± SD (%, RSD)	Recovery (%)
Bisphenol A	10.0	9.3 ± 0.7 (6.4)	93.0
	50.0	52.8 ± 2.5 (4.7)	105.6
	100.0	93.1 ± 4.1 (4.4)	93.1

Notes: [a]Mean of thirty determinations (ng g^{-1}); SD, standard deviation; RSD, relative standard deviation.

The recoveries were quite good considering the amount of sample, and the low concentration of the analyte and complexity of the sample. The values indicate that compounds are quantitatively extracted.

Sensitivity

A fundamental aspect that needs to be examined in the validation of any analytical method is its limit of detection in order to determine if an analyte is present in the sample. In this work, a criterion for method performance was used that includes the decision limit, CC_α, and the detection capability, CC_β (European Commission 2002). CC_α is defined as the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. CC_β is defined as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. They were calculated according to the calibration curve procedure described in European Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (European Commission 2002). Decision limit and detection capacity, which are better adjusted to a statistical evaluation, are implemented. Thus, CC_α ($\alpha = 5\%$) and CC_β ($\beta = 5\%$) are also summarized in Table 2.

Selectivity

Compound was quantified using selected-ion recording mode (SIM). Analyte appears to be well resolved and free from interference peaks (Figure 1). The identity of the chromatographic peak was confirmed not only by its retention time, but also by its mass spectrum (three fragments were used).

Application of the method

The proposed method was applied as a routine method for the determination of BPA in prepared rice dishes packed in plastic containers from different brands commercialized in Spain. These containers are manufactured using different plastic materials that could

include polycarbonate plastics. A total of 250 samples of rice-prepared dishes, picked up from different points, were analysed. An example of the chromatogram obtained in SCAN mode and in SIM mode for a natural sample is included in Figure 1 (C and D). BPA does not seem to be present above the reported lower detection limits proposed in this work.

Conclusions

The present study shows that bisphenol A (BPA) can be detected and quantified reliably in rice-prepared dishes combining gas chromatography-mass spectrometry (GC-MS) with a solid–liquid extraction procedure. The assay involves a preconcentration and the removal of an interferences step in conjunction with a silyl-derivatization procedure previous to CG-MS analysis. The GC-MS analytical method developed in this study has been shown to be reliable and has a low limit of detection for BPA. It has also been applied successfully to spiked and non-spiked rice samples. Proper sample collection in conjunction with sound storage practice before analysis allows for good recovery values in all cases as demonstrated by the validation procedure employed. The method has been used routinely for monitoring the presence of BPA in the production of rice-prepared dishes and no contamination has been found.

The results indicate that the levels of BPA identified in rice-prepared dishes are unlikely to be of concern to adult health, and there is no reason for consumers to change their consumption patterns as a result of these findings.

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