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A Sensitive Gas Chromatographic-Mass Spectrometric (GC–MS) Method for the Determination of Bisphenol A in Rice-Prepared Dishes

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Abstract

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: Bisphenol A; Migration; Gas chromatography–mass spectrometry (GC–MS); Rice-Prepared Dishes.

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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., 1997; Paris, et al., 2002). Today, it is well known that around 100 industrial chemicals show estrogenic activity in addition to their desired chemical properties. Most of these endocrine disrupter chemicals (EDC's) are widely used organic compounds, which are ubiquitous in the environment and in biological samples (Fromme et al., 2002; Harris et al., 1997). At the same time, ECD's accumulate in certain tissues in humans and their effects could be passed to future generations via placenta and/or milk (Maffini et al., 2006; Hood, 2005; 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 United States (EPA) have authored a "priority" list of substances for further evaluation of their role in endocrine disruption (Groshart and Okkerman, 2000; Multi-Year Plan [FY2000-2012] for Endocrine Disruptor) and indicate the need to assess the levels and effects of EDCs.

One of the representative compounds of the group of EDC's 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 estrogen-like action of toxicity; moreover, it was discovered that the compound was released from a flask made of polycarbonate and showed binding to the estrogenic receptor. In fact, BPA induced progesterone receptors in cultured breast cancer cells and its activity was about 1/5,000 that of estradiol (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 still controversy over whether low doses of BPA may cause reproductive and developmental effects in humans (Goodman et al. 2006).

Significant published data is available on 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 (Munguia-Lopez and Soto-Valdez, 2001, Sajiki et al., 2005; Le et al, 2008; Poças and Hogg, 2007; Mungia-Lopez et al., 2002).

Sterilization conditions, normally applied by industry for the scenarios used in our 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 of these factors.

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

Gas chromatography-mass spectrometry has traditionally been used as the main analytical methodology for analyzing BPA together with other EDCs (nonylphenol, phthlates or bisphenol A diglycidyl ether) in water, milk or beverages (Mol et al., 2000; Rodríguez et al., 2003; Helaleh et al., 2001; Ding and Chiang, 2003; 2000; Schoene et al., 1994; Nakamura et al., 2001; Fine 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 prior to consumption using GC-MS as their analytical technique.

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Alternatively, liquid chromatography (Katayama et al., 2001; Shao et al., 2005 and 2007; Petrovic and Barceló, 2001; Maragou N.C. 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 analyzed 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 high-purity. Water was purified with a Milli-Q plus system (Millipore, Bedford, USA).

Acetonitrile (HPLC-gradient. PAI-ACS), hexane (UV-IR-HPLC, PAI), absolute ethanol (HPLC-gradiente, PAI), ethyl acetate (UV-IR-HPLC, PAI-ACS), 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%, sylilation 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 µg/ml) were prepared in ethanol. BPF was selected as the surrogate due to the structure similarity to BPA and to the fact that 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 three months.

Sample preparation

Rice-prepared dishes are a pre-cooked meal which main component is rice (50-65 %) and 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.

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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 analyzed.

As well, rice-prepared samples were collected previous to 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 ºC until treatment. The analysis was performed with the minimum possible delay and as described below in the *Extraction and derivatization* section in order to avoid sample degradation.

Extraction and derivatization

Prior to extraction, sample was carefully homogenized with a mixer and sample (20 g) were placed in 50 mL falcon tube. After that, te sample was spiked with bisphenol F as a surrogate (100 $ng \cdot g^{-1}$). 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 r.p.m. at room temperature. Supernatant was transferred into a 25 mL separation funnel, and the extraction was repeated twice. NaCl (5 g) were 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, could be 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 re-dissolved 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 to carry out the derivatization. BPA and BPF are suitable for derivatization due to their chemical structure. Once the derivatization process was completed (1 minute), 1 µL of the reaction mixture was injected into the GC–MS system.

Apparatus and software

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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 capillary column DB-5MS fused-silica, 30 m × 0.25 mm I.D.; 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). The GC–MS operation control and the data process were carried out by ChemStation software.

The injector port of the GC was set at 250 ºC. The silylated samples were automatically injected using the splitless-injection mode. The transfer line of the GC to the MS was set at 280 ºC, and the electron ionisation (EI) ion source of the MS set at 250 ºC. The GC oven temperature program applied was as follows: the initial oven temperature was set at 150 ºC, held for 0.5 min, then the temperature was increased to 280 ºC via ramp of 20 ºC min^{-1} and held for 5.0 min, finally the temperature was raised to 300ºC at 40 ºC 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 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/ion) was used. The retention times for BPF and BPA trimethylsilyl derivatives were 8.9 min 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

Statgraphics Centurion XV, vs 15.1.02 software package (1982-2006 Statpoint Inc) was used for the statistical analysis of 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, *e.g.*, 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 3:1, 1:1 and 1:3) were tested for 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 that 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 separating funnel. Although carbon tetrachloride and chloroform showed good results (close to 90% of de-fating 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 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.

The optimisation 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, temperature of process and reaction time were tested on the analytical response. The three factors were simultaneously optimised by

application of a 2^3 central composite design plus face centred (with three centred points). The BSTFA/Pyridine concentration was studied from 0 to 100%, temperature from 25 (room temperature) to 95 ºC and the reaction time was varied between 0 and 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 show a characteristic chromatogram obtained in SCAN mode (A) and SIM mode (B) for a silylated sample containing BPA and BPF as surrogate.

Figure 1

Another feature of the application of derivatization reactions is that trimethylsilyl derivatives produce ions with higher *m/z* in the GC–MS system in contrast to those obtained from underivatizated 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 analyzed, 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.

Figure 2

The figure displays the EI mass spectra and tentative fragmentation of the bis-*O*-TMS derivative of bisphenol A 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.

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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 surrogate.

The standard addition calibration was carried out by fortification of homogenized rice samples aliquots. 20 g of raw samples free of BPA were placed in a 50 mL falcon tubes collection. Then, samples were 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 in the "*Extraction and derivatization"* section. Linearity of the calibration using standard addition graphs was tested according to the Analytical Methods Committee (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 2

Validation was performed according to the U.S. Food and Drug Administration (FDA) guideline for bioanalytical assay validation (U.S. Department of Health and Human Services, 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.X to 100 ng g^{-1}) was selected for method application.

The response of 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, we should test the linearity of 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.

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Precision. The precision expressed as relative standard deviation (RSD), at three concentration levels, was obtained from thirty replicates of spiked samples obtained from 10 aliquots of a homogenate of 10 different rice dishes and analyzed during the same day (repeatability) and in three different days (reproducibility). A total of 30 analyses were carried out in order to calculate precision. The relative standard deviation is lower than 10% in all cases as is shown in 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 20g of rice samples with the analyte, at three concentration levels were analyzed. The recoveries for BPA were between 90% and 105 % in all cases as is shown in Table 3.

Table 3

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 which 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 has been used that includes the decision limit, CC_α, and the detection capability, CC_β (COMMISSION DECISION, 2002). Decision limit (CCα) 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. Detection capability (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 commission decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. 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 appear 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 (3 fragments were used).

Application of the method

The proposed method has been 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 which could include polycarbonate plastics. 250 samples of rice-prepared dishes, picked up from different points were analyzed. 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 BPA can be detected and quantified reliably in rice-prepared dishes combining GC-MS with a solid-liquid extraction procedure. The assay involves a preconcentration and the removal of 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 had a low limit of detection for BPA, and has been successfully applied to spiked and non-spiked rice samples. Proper sample collection in conjunction with sound storage practice prior to 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.

Our 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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FIGURE CAPTIONS

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).

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

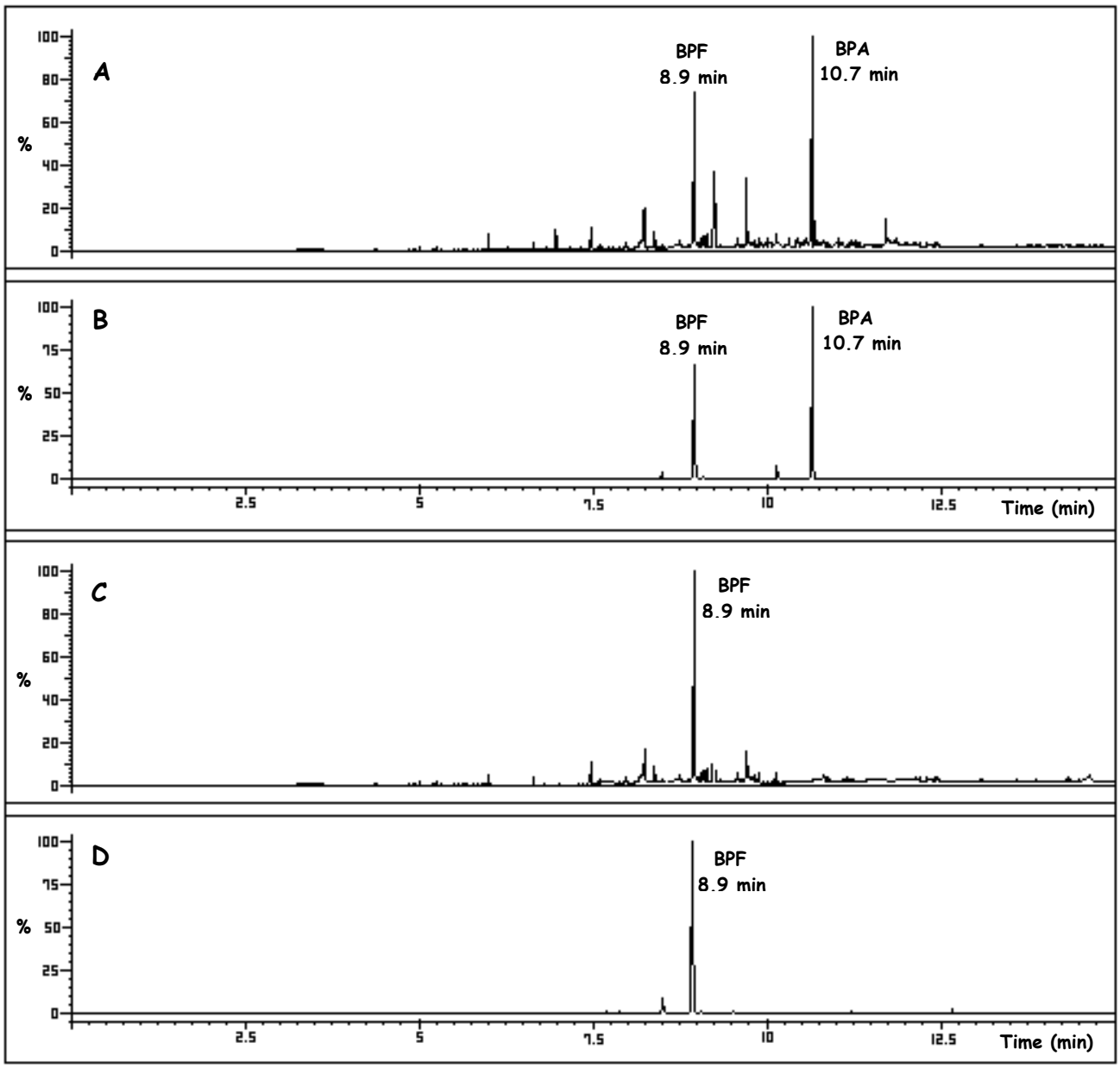
A
BPF
8.9 min
BPA
10.7 min
%
B
BPF
8.9 min
BPA
10.7 min
%
2.5
5
7.5
10
12.5
Time (min)
C
BPF
8.9 min
%
D
BPF
8.9 min
%
2.5
5
7.5
10
12.5
Time (min)

Figure 1

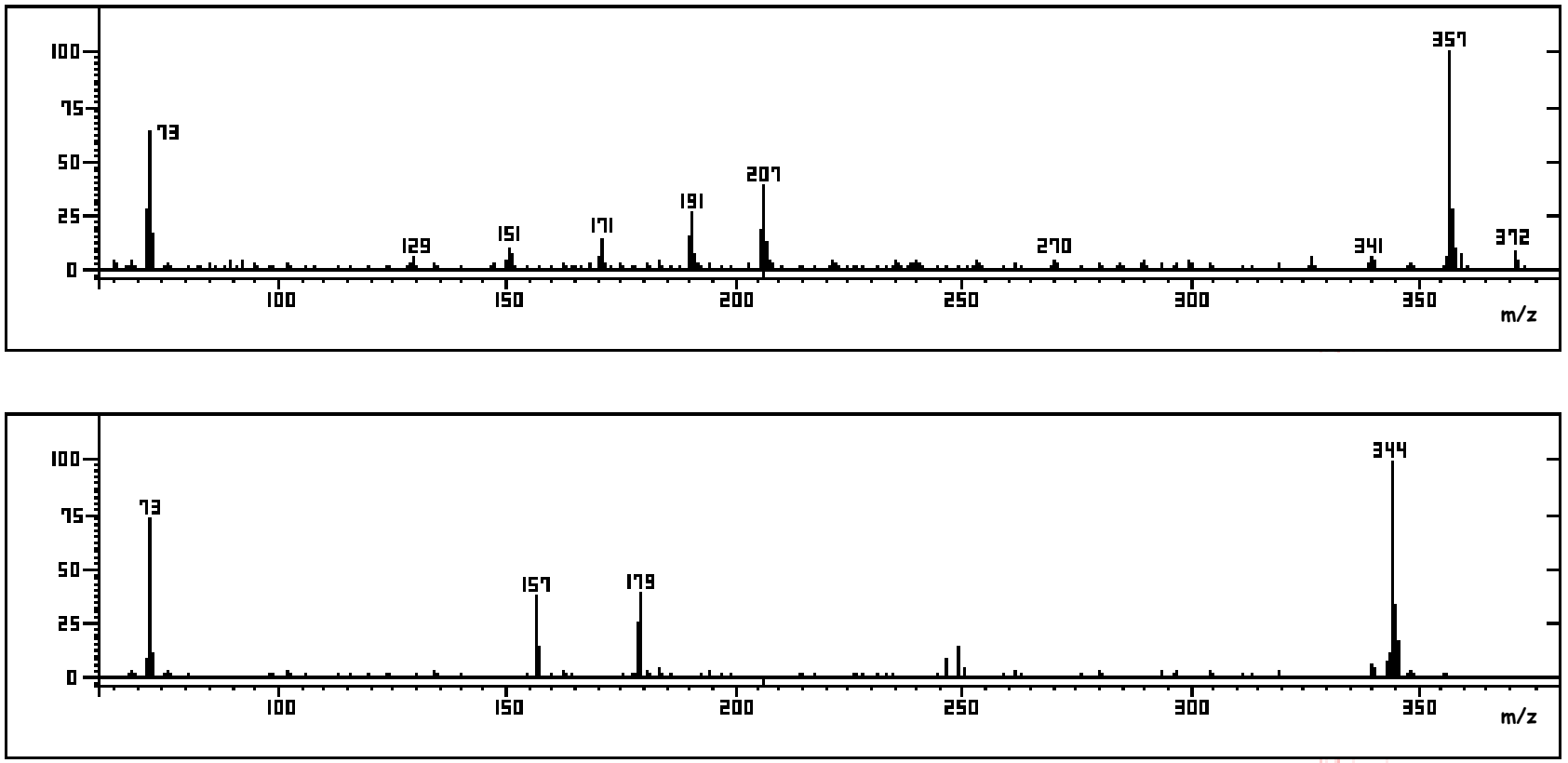

Figure 2

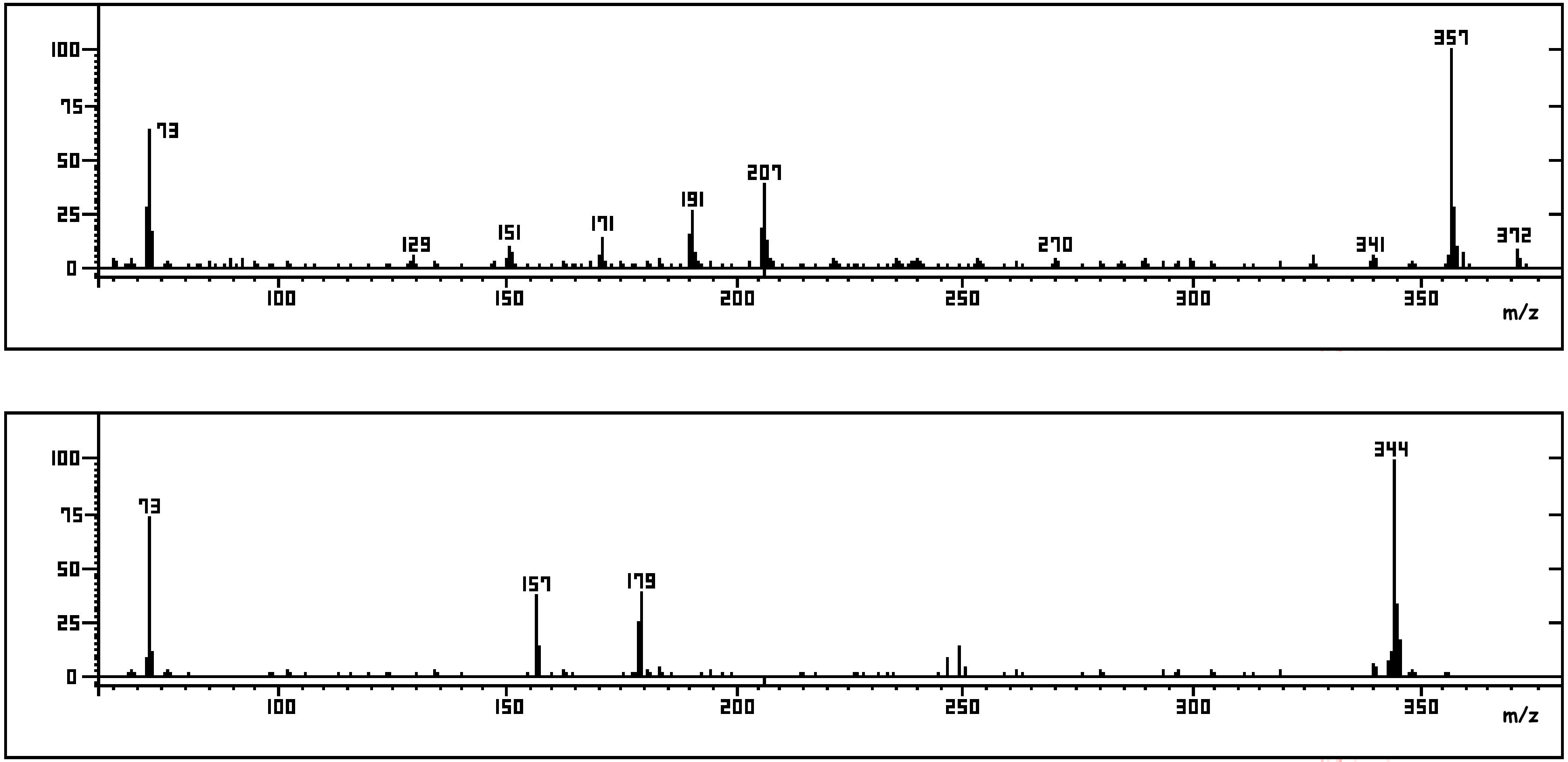

Figure 2

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

	t_r (min)	Dwell time (ms)	Range (min)	Fragments (*m/z*)
BPF	8.9	100	7.5 - 10.0	344 $[M]^+$, 179, 157
BPA	10.7	100	10.0 - 15.0	357 $[M - Met]^+$, 207, 179

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

Parameter	Value
n	6
a	$3.0 \cdot 10^{-3}$
$\mathbf{s_a}$	$2.8 \cdot 10^{-3}$
b (g ng^{-1})	$1.20 \cdot 10^{-2}$
$\mathbf{s_b}$ **(g·ng^{-1})**	$1.25 \cdot 10^{-3}$
$\mathbf{R^2}$ **(%)**	99.7
LDR (ng g^{-1})	0.8 - 100.0
$\mathbf{S_{y/x}}$	$2.45 \cdot 10^{-3}$
$\mathbf{CC_\alpha}$ **(ng g^{-1})**	0.5
$\mathbf{CC_\beta}$ **(ng g^{-1})**	0.8
$\mathbf{P_{lof}}$ **(%)**	53.1

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

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

	Spiked (ng g^{-1})	Found* ± 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

* Mean of thirty determinations (ng·g^{-1}); **SD**: standard deviation; **RSD (%)**: relative standard deviation.

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