DEVELOPMENT AND VALIDATION OF A LC-MS/MS METHOD FOR THE ANALYSIS OF BISPHENOL A IN POLYETHYLENE TEREPHTHALATE

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ABSTRACT

Bisphenol A (BPA) is widely recognized being an endocrine disrupter and it is employed in many food packaging applications. Although it is not intended to take part in the manufacture of polyethylene terephthalate (PET) food grade, the presence of BPA in recycled PET should not be neglected. To satisfy the increasing need to ensure "BPA-free" articles, a liquid chromatography-tandem mass spectrometry method was developed. The crucial step in the sample preparation was the total dissolution/reprecipitation of the polymer. The repeatability of the method (RSD%, n=6) was lower than 7.6%, while HorRat values ranged between 0.3 and 0.5. Limits of detection and quantitation were 1.0 and 3.3 ng g^{-1}, respectively. Recovery ranged from 89 to 107%. The method was applied to 23 samples of virgin and recycled pellets, preforms and bottles. Migration tests were also carried out. Results shown significantly higher levels of BPA in recycled PET.

Keywords: Bisphenol A; PET; Liquid Chromatography; Tandem Mass Spectrometry; Migration; Recycled PET.

1. INTRODUCTION

2,2-bis(4-hydroxyphenyl) propane, also called Bisphenol A or BPA, is an industrial chemical that is widely used as a monomer or additive for the manufacture of polycarbonate (PC) plastics and epoxy resins and other polymeric materials, certain paper products (e.g. thermal paper) and printing inks (European Food Safety Authority CEF, 2015). Many chemicals, termed Endocrine Disrupters (ED), have the capability of interfering with the endocrine system, and whether or not BPA belongs to this category of compounds is still under discussion within the scientific community. In view of this, the European Food Safety Authority (EFSA) Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) has discussed and endorsed a scientific protocol for the upcoming re-evaluation of BPA hazard, scheduled to be started in 2018 (Gundert-Remy, Barizzone, Croera, Putzu, & Castoldi, 2017). Nevertheless, BPA is known to affect hormonal homeostasis, because of its ability to mimic oestrogen binding (Kim, Yun, & Ryu, 2011; Kunz et al., 2011; Kuruto-Niwa, Nozawa, Miyakoshi, Shiozawa, & Terao, 2005). Moreover, it has been reported that BPA exhibits anti-androgenic activity that can interact with the pregnane X receptor and the thyroid and glucocorticoid receptors (Ehrlich, Calafat, Humblet, Smith, & Hauser, 2014; Welshons, Nagel, & vom Saal, 2006; Žalmanová et al., 2016).

Since a huge number of studies have demonstrated the harmful effects of BPA on living organisms, its presence in food contact materials must be controlled, since this is the major source of human exposure. In its last Scientific Opinion, EFSA established a temporary-Tolerable Daily Intake (t-TDI) of 4 μg kg^{-1} body weight per day (European Food Safety Authority CEF, 2015). Bisphenol A is included in the European Union List of the Regulation EU No 10/2011 and the recently approved

Specific Migration Limit (SML) is 0.05 mg kg^{-1} (The European Commission, 2011, The European Commission, 2018).

Although migration testing in the food prevails, migration is usually tested using "food simulants", which are test mediums imitating each food category, and are used as substitutes for food to facilitate the chemical analysis. Determination of BPA in food matrices often requires a complex sample preparation prior to the instrumental analysis. Solvent extraction (SE) and solid phase extraction (SPE) are the most widely used techniques for the isolation of BPA from solid and liquid samples, respectively, mainly because of their simplicity and wide-range applicability (Ackerman et al., 2010; Bono-Blay et al., 2012; H. Gallart-Ayala, Moyano, & Galceran, 2010; Maragou, Lampi, Thomaidis, & Koupparis, 2006). However, many novel techniques have been developed for increasing the efficiency of extraction, time and solvent saving.

The reported low dose effects of BPA and the new SML proposed by the EU Commission has given rise to the development of analytical methods with Limits of Detection (LoDs) low enough to assess the human exposure at these levels. The determination of BPA in food is mainly carried out by gas chromatography-mass spectrometry (GC-MS) (McNeal et al., 1999; Salafranca, Batlle, & Nerín, 1999), liquid chromatography-fluorescence detection (LC-FLD) (Alabi, Caballero-Casero, & Rubio, 2014; Gallo et al., 2017; Nerín, Philo, Salafranca, & Castle, 2002; Xiong et al., 2018), and liquid chromatography-mass spectrometry (LC-MS) (Cheng et al., 2017; Héctor Gallart-Ayala, Moyano, & Galceran, 2007; Salatti-Dorado, Caballero-Casero, Sicilia, Lunar, & Rubio, 2017). Gas chromatography provides higher peak resolution, while liquid chromatography offers the advantage of simplicity over GC for which a derivatization step is necessary. Other techniques like electrochemical detection (LC-ED) (D'Antuono, Campo Dall'Orto, Lo Balbo, Sobral, & Rezzano, 2001; Y. Li et al., 2016; Shi, Liang, Zhao, Liu, & Tian, 2017), and immunoassays (Feng et al., 2009; Maiolini et al., 2014), have been used to a lesser extent. Within this scope, a comprehensive overview is offered by Ballesteros-Gómez and coworkers (Ballesteros-Gómez, Rubio, & Pérez-Bendito, 2009).

Even though a few publications have been devoted to the investigation of BPA in bottled water (Xu, Ying, Su, Yang, & Wang, 2010), there is no scientific literature concerning the determination of BPA content in polyethylene terephthalate. A reasonable explanation is the fact that BPA is not thought to take part in the composition of the polymer itself as it is not used in the manufacture of PET. However, we should consider the use of recycled-PET (R-PET) as a possible source of BPA coming from printing inks or other materials (Vinković, Rožić, & Galić, 2017). In addition, we could advance the hypothesis of cross-contamination not only during the recycling process, but also during the manufacture of virgin PET. This might be due to environmental contamination of BPA which can also take place inside the factory during PET production process. Briefly, PET synthesis involves a trans-esterification reaction at high temperature (150 – 220 °C), where a polymerisation catalyst is normally added (e.g. antimony trioxide) forming bis(hydroxyethyl) terephthalate (BHET). Follows the polymerisation and polycondensation reaction of BHET. Solid state polymerisation (SSP) might be required in which a high MW PET is produced (Awaja & Pavel, 2005; Welle, 2011). In these steps, the machinery involved and the raw materials used can lead to a low, but still probable degree of BPA contamination.

Whilst, taking into consideration a migration study from PET packaging, other sources of BPA contamination could be bottle closures, environmental contamination and the food itself (Bach, Dauchy, Chagnon, & Etienne, 2012), which might have been polluted prior to packaging.

In order to satisfy an increasing request from the polymer industry, mainly from manufacturers of "BPA-free" items and infant feeding bottles, the aim of the current work concerned the development of an analytical method for the determination of Bisphenol A in polyethylene terephthalate and performing a migration study on PET-bottles, in order to check the compliance of the materials to European legislation. A second purpose was to establish whether a certain level of BPA in the polymer can be ascribed to the employment of recycled PET resins in the manufacture of polyethylene terephthalate bottles and items. Care should be taken when analysing BPA because of its rather ubiquitous character, thus it is very important to correct the results by an appropriate blank test to

ensure the reliability of the results. This ubiquitous occurrence, also reported by other authors (Deceuninck et al., 2014; Salgueiro-González et al., 2012), is the reason why it was not possible to establish a method LoD and LoQ, since a PET certified reference standard material with known level of BPA is not available.

2. MATERIALS AND METHODS

2.1. Samples

A total of 23 PET samples were used for the determination of BPA. The samples came from five different companies within the EU. One company supplied only virgin PET, three companies supplied only R-PET, and one company supplied a mixture of virgin PET and R-PET. In addition, samples included different PET forms such as pellets, preforms and bottles. Respecting a confidentiality agreement, the manufacturers of origin are not mentioned.

2.2. Reagents and materials

Standards of Bisphenol A (CAS 80-05-7 purity ≥ 99%) and the isotope labelled internal standard Bisphenol A-d_{16} (CAS 96210-87-6, 98% atoms D) were purchased from Sigma Aldrich Quimica (Madrid, Spain). Purified water was obtained with a Milli-Q Plus system (Millipore, Bedford, MA, USA), and methanol HPLC grade, ethanol HPLC grade, acetic acid and chloroform (for both purity >98%) were purchased from Scharlau Chemie S.A (Sentmenat, Spain). 1,1,1,3,3,3-hexafluoro-2-propanol (99% purity) was purchased from Fluorochem Ltd (Hadfield, UK). All reagents were used without further purification. Filters were PTFE Acrodisc® Syringe Filter 13 mm, 0.2 µm pore size GHP from Waters (USA), nitrogen evaporator was a TECHNE sample concentrator (Cole-Parmer Ltd., UK). The ultrasounds generator was a Branson 3510 (frequency applied 40 Hz). Stock solutions of BPA and BPA-d_{16} were prepared in methanol and stored at 4 °C in the dark, with an expiry date of one week.

2.3. Instrumentation

The analyses of standard solutions and samples were carried out with an ACQUITY UPLC® H-Class System with autosampler and quaternary solvent manager (QSM), coupled with an AQUITY Xevo® TQ tandem quadrupole mass spectrometer equipped with Electrospray Ionization source in negative mode (ESI^-), both from Waters (Milford, MA, USA). The chromatographic separation was performed on ACQUITY UPLC® BEH C_{18} column (100 x 2.1 mm internal diameter, 1.7 µm particle size, 130 Å pore size); preceded by a BEH C_{18} VanGuard™ pre-column (5 x 2.1 mm i.d., 1.7 µm particle size), from Waters (Ireland). The column temperature was held at 35 °C. The injection volume was 10 µL. The mobile phase consisted of methanol and water. The flow rate was 0.3 mL min^{-1} and the gradient elution program was as follows: 1 min isocratic 10:90 (V/V) MeOH/H_2O, 1-5 min linear gradient to 100% (V/V) MeOH, 5-8 min held at 100% (V/V) MeOH. The fragmentation pathway study and the optimization of the mass spectrometry conditions were achieved by direct infusion of a 10 µg g^{-1} methanolic standard solutions of each compound (BPA as the analyte and BPA-d_{16} as the IS) into the ion source with a flow rate of 20 µL min^{-1}. ESI final conditions were: capillary voltage 3.00 kV, sampling cone voltage 43 V, extractor cone voltage 2 V, RF lens voltage 0.10 V, source temperature 150 °C, desolvation temperature 450 °C, cone and desolvation gas (N_2) flows 50 and 500 L h^{-1}, respectively. The acquisition MS method was Multiple Reaction Monitoring (MRM), performed by collision-induced dissociation (CID) of the precursor ions in the collision cell. Two transitions between the precursor ion and the most abundant product ions were monitored for the identification of BPA and BPA-d_{16}, and the ion transition with relatively higher intensity was selected for quantitation. The collision gas (Ar) flow rate was 0.20 mL min^{-1} and Table 1 summarizes MRM parameters for both compounds. Data were acquired and processed by *MassLynx* (ver. 4.1).

2.4. Sample preparation

The first step of the sample preparation could be considered based on the chemolysis principle. Many solvents and combinations thereof were studied. The optimized procedure was as follows:

149 approximately 100 g of PET pellets were cryogenically ground using a steel blender cooled with
150 liquid nitrogen. Exactly about 0.4 g of powdered PET were weighed in a 20 mL glass vial with screw
151 cap and 0.07 g of 3.0 µg g^{-1} Bisphenol A-d_{16} methanol solution were added, as internal standard. To
152 ensure the complete dissolution of the polymer, 7.5 g (about 4.7 mL) of hexafluoro isopropanol
153 (HFIP) were added and the vial was kept in an ultrasonic bath for 3 h, holding the temperature
154 between 35 and 45 °C to minimize a possible degradation of the analyte. After cooling down at room
155 temperature for 5-10 minutes, 6.5 g (about 8 mL) of high purity methanol (reprecipitation solvent)
156 were added and the vial was shaken for 1 minute, in order to guarantee close contact between solvent
157 and polymer. Thus, the vial was kept at 4 ºC for 1 hour to support the polymer precipitation. The
158 liquid phase was transferred to a centrifuge tube. Subsequently, residual precipitated polymer was
159 washed twice with 1.0 mL of pure methanol and the liquid phases were combined into the centrifuge
160 tube. The extract was centrifuged at 247 *g* for 10 minutes. Supernatant was collected and evaporated
161 to 2 mL under a gentle nitrogen flow at room temperature. The concentrated extract was filtered into
162 a 2 mL vial using a PTFE filter, 0.2 µm pore size.
163 In the case of PET bottles, pieces of about 5 mm^2 were cut prior to insertion into the glass vial, whilst
164 PET preforms were shattered into little pieces and then cryogenically ground likewise for PET pellets.
165 Blank and samples were prepared and analysed in triplicate.

166 **2.5. Calibration**

167 2.5.1. Calibration with internal standard

168 The calibration curve was made by preparing eight standard solutions with known concentration of
169 BPA and BPA-d_{16} in methanol. The concentration of BPA increased from 3.0 to 1700.0 ng g^{-1},
170 whereas the concentration of BPA-d_{16} was fixed at 200.0 ng g^{-1}. Each standard solution was analysed
171 three times and the average peak areas ratio of BPA and IS were plotted against the corresponding
172 concentration ratio using a linear regression model. The calibration curve obtained was used to
173 calculate the relative response factor (RRF) of the analyte to the internal standard, as the slope of the

curve. Samples that showed on initial injection, a higher content of BPA than the calibration range were diluted to bring their response within the range of the calibration and reinjected. Their absolute BPA concentration was then calculated taking the dilution factor into consideration. For routine analysis, the relative response factor of BPA with respect to BPA-d_{16} was calculated daily by analysing three independent replicate standard solutions of a mixture of 400.0 ng g^{-1} BPA and BPA-d_{16} and three independent replicate standard solutions of a mixture of 1500.0 ng g^{-1} BPA and BPA-d_{16} in methanol. The average value of the three replicate acquisitions was used for each concentration.

2.5.2. Calibration with external standard

The calibration curve was made in the same way as the IS method (section 2.5.1). Each standard solution was analysed three times and the average peak areas were plotted against the concentrations of BPA using linear regression model. Taking into consideration the whole sample preparation procedure, a formula for the quantification of BPA in PET samples was created (equation (1)).

$$C_{BPA} = C_{sol} * F * w_{conc} * \frac{w_{HFIP}+w_{MeOH}}{w_{cent}} * \frac{1}{w_P} \quad (1)$$

Where C_{BPA} [ng BPA per g of PET] is the concentration of BPA in the polymer, C_{sol} [ng BPA per g of concentrated extract] is the concentration of BPA in the solution injected, calculated by using the calibration curve formula, F is the dilution factor, w_{conc} [g] is the weight of the concentrated extract, w_{HFIP} and w_{MeOH} [g] are the weights of hexafluoro isopropanol used for the dissolution and the sum of methanol used for reprecipitation and wash, respectively, w_{cent} [g] is the weight of the extract after centrifugation, and w_P [g] is the weight of the polymer.

2.6. Migration tests

Migration experiments were performed on bottle samples, according to Reg. (EU) No 10/2011, by

197 immersion of a previously cut 1.5 x 4.0 cm^2 plastic piece into a 20 mL glass vial filled with 20.0 g of
198 food simulant. Each vial was hermetically closed with a PTFE cap. The dimensions have been chosen
199 based on the convention that 1 kg of food is packaged with 6 dm^2 of food contact material, and both
200 faces of the bottle were considered (The European Commission, 2011). Thus, the equivalent condition
201 of 3 dm^2 per kg of food simulant was used. Considering that bottles investigated are thought to contain
202 foodstuffs with a shelf-life longer than 30 days at room temperature, an accelerated test was
203 performed at 60 °C for 10 days. Bottles S8B, S9B, S10B, S11B, S12B, S13B and S14B were
204 submitted to the migration test with distilled water (which represented food simulant A), 3% (w/V)
205 acetic acid solution in water (food simulant B) and 20% (V/V) ethanol solution in water (food
206 simulant C). For quantitation, 16.0 g of food simulant were collected in a different 20 mL glass vial
207 and an aliquot of 0.07 g of 92.0 μg g^{-1} BPA-d_{16} in methanol was added as IS, in order to reach 400.7
208 ng g^{-1} of BPA-d_{16}. All food simulants were directly injected into the LC-MS system without any
209 additional sample treatment. Since bottle S14B is also designed to contain edible oils, a migration
210 test with sunflower oil (food simulant D2) was performed respecting the same experimental
211 conditions employed for other simulants. The oil was also spiked with BPA-d_{16} after the migration
212 time, obtaining 400.0 ng of IS per g of oil. The crucial point was the extraction of BPA from the oil.
213 To overcome this hurdle, a method of total lipid extraction designed by Bligh and Dyer (Bligh &
214 Dyer, 1959) was opportunely modified and optimized for this analysis, as follows: first, 7.5 mL of a
215 solution 2:1 (V/V) MeOH:$CHCl_3$ were added to 2.0 mL of oil in a glass vial and vortexed for 1
216 minute. Afterwards, 2.5 mL of $CHCl_3$ were added, followed by further shaking with vortex for 1
217 minute. Finally, 2.5 mL of distilled water were added and mixed again as before. A centrifugation at
218 224 *g* was used to allow two phases to separate. The aqueous (top) phase was recovered from the
219 organic (bottom) phase by transferring it to a clean glass vial with a glass Pasteur pipette. The
220 extracted aqueous solution was concentrated to 2 mL under gentle nitrogen flow at room temperature
221 and filtered before the instrumental analysis. All migration tests were carried out in triplicate and the
222 results were corrected by subtracting the obtained concentration of BPA for its concentration in blank

223 samples, which were also analysed three times.

224

225 **3. RESULTS AND DISCUSSION**

226 **3.1. Method performance**

227 3.1.1. Limit of Detection (LoD), Limit of Quantitation (LoQ), working range and linearity

228 LoD and LoQ were 1.0 and 3.3 ng g^{-1}, respectively. The calculation of LoD and LoQ was carried out
229 following the *Eurachem* guidelines (Magnusson & Örnemark, 2014), by multiplying the "adjusted"
230 standard deviation of ten distinct reagent blanks by a factor of 3 and 10, respectively. In view of the
231 fact that a sample blank was not available because all samples analysed presented an appreciable level
232 of BPA, LoD and LoQ shall be considered as instrument LoD/LoQ. The instrumental working range
233 was assessed as described in the earlier section (2.5.1.). To verify the adequateness of the linear
234 regression model, the control diagnostic was performed. The Pearson linear correlation coefficient
235 (ρ) and the determination coefficients (R^2) were both 0.9999, the residuals were lower than 4.8% and
236 the relative standard deviation of the slope was 4.7%. The working range was 3.3 – 1700.0 ng g^{-1} and
237 the RRF was 0.9828. The JRC guidelines on validation procedures for analytical methods in control
238 of Food Contact Materials (Bratinova, Raffael, & Simoneau, 2009) states that the maximum relative
239 standard deviation of the slope should not exceed 8%, while the residuals calculated for the lowest
240 level (LoQ) should be less than 20%, and less than 15% for all other levels. Therefore, the linearity
241 was successfully verified over the working range employed.

242 3.1.2. Selectivity and repeatability

243 One factor that contributes to the overall selectivity of the method is the employment of a separation
244 technique such as UHPLC. In addition, the use of two MRM transitions per compound supported the
245 correct identification of the analyte, and the selectivity afforded by the MRM methodology meant
246 that less sample clean-up was required compared with other detection methods hence saving time and

reducing the costs of solvents. The repeatability was evaluated by analysing six independent replicates of three different samples over the working range of the method. The repeatability relative standard deviation (RSD_r) ranged from 6.1 to 7.8%, while Horwitz ratio ($HorRat_{(r)}$) values were between 0.3 and 0.5 (see Appendix A for calculates and results). RSD_r and $HorRat_{(r)}$ values are in accordance with the JRC guidelines (Bratinova, Raffael, & Simoneau, 2009), for which accepted $HorRat_{(r)}$ values are between 0.3 and 1.3.

3.1.3. Trueness and recovery

Trueness was stated quantitatively in terms of "bias" by comparing the response of the method to a reference material with a known value taking part in the proficiency test *EURL FCM 01/2017 BPA*, organized by the Joint Research Centre in support of EC No 882/2004 on official controls. The certified reference material consisted of two aqueous solutions of BPA (SOL 1 and SOL 2). Each solution was analysed in replicates of six by direct injection into the instrument after spiking with an aliquot of IS methanolic solution. Since this reference material can be considered equal to a standard solution, the calculated bias acts as trueness index of the instrumental method only. The relative percentage bias (b %) was calculated with equation (2) and was 2% for SOL 1 and 0% for SOL 2.

$$b\ \% = \frac{x_{mean} - x_{ref}}{x_{ref}} * 100 \qquad (2)$$

Where x_{mean} [ng g^{-1}] is the mean of the results of the current method and x_{ref} [ng g^{-1}] is the "true value" (reference value provided by the collaborative study). A t-test was also performed, and the calculated concentrations were not statistically different from the "true value", with a 95% of confidence interval (results shown in appendix A).

The recovery tests were carried out at three levels, spiking 0.4 g of powder sample S8F with 100.0, 200.0, and 400.0 ng of BPA and 200.0 ng of BPA-d_{16}, corresponding to 250.0, 500.0, and 1000.0 ng of BPA per g of PET, and 500.0 ng of BPA-d_{16} per g of PET, respectively. The internal standard method leads to recoveries ranging from 89 to 107%, while with the external standard method

271 recoveries were between 73 and 81% (see Appendix A for recovery calculations). The calculated
272 recoveries are present in Table 2. To make the present method fully applicable to food contact
273 materials testing, the availability of reference standard PET materials with known concentration of
274 BPA would be required for verifying method trueness on real samples.
275
276
277 3.1.4. Matrix effect
278 In contrast to food matrices, PET-extracted solutions are not complex samples in terms of amount of
279 various constituents. However, the presence of substances like residual monomers or oligomers,
280 colorants, plasticizers and other additives, can affect the ionization of the target analyte, resulting in
281 ion suppression or enhancement during LC-MS analysis, which is also called matrix effect. Matrix
282 effect has been widely studied and recognized as a source of error in quantitative LC-MS/MS analysis
283 of food samples (Niessen, Manini, & Andreoli, 2006). In this work, matrix effect was investigated by
284 comparing the slopes of calibration curves prepared in matrix extract and in solvent. For creating the
285 solvent calibration curve, solutions of BPA 49.9, 100.0, 295.9, 494.1, 799.9 and 1487.6 ng g^{-1}, and
286 BPA-d_{16} 62.5, 125.3, 370.8, 619.1, 1002.3 and 1864.9 ng g^{-1} in methanol were prepared. Matrix-
287 matched calibration solutions were obtained by spiking extracts coming from 2.4 g of PET solution
288 after the entire sample treatment procedure with different aliquots of a 5000.0 ng g^{-1} BPA and BPA-
289 d_{16} stock solution in methanol in order to achieve concentrations of 58.4, 110.6, 272.8, 457.3, 748.5
290 and 1150.7 ng g^{-1} of BPA, and 73.1, 138.6, 341.9, 573.0, 937.9 and 1441.9 ng g^{-1} of BPA-d_{16}. For
291 reaching the same dilution factor, the total weight was brought to 2.7 g with pure methanol. The
292 average area (n = 3), subtracted from blank, of BPA and BPA-d_{16} chromatographic peaks were plotted
293 against their concentrations and the matrix effect (ME %) was obtained by equation (3).

294 $$ME\ \% = \frac{b_m}{b_s} * 100 \qquad (3)$$

295 Where b_m is the slope of the matrix-matched calibration curve, and b_s is the slope of solvent

calibration curve. For both BPA and BPA-d_{16} the matrix effect was 113.5%, therefore the percentage signal enhancement was 13.5%. This result indicates that there is a signal enhancement for both compounds; nevertheless, the matrix effect for BPA is balanced by the matrix effect for the internal standard when performing a quantitative analysis. This is reasonably explicable due to the fact that both analyte and internal standard elute at the same retention time and present the same core structure.

3.2. Comparison of the method with external standard calibration

In order to compare the current method with external standard method for quantitation, the determination of BPA was carried out on five samples using both methods for calibration. The relative percentage difference (Δ%) was calculated following equation (4).

$$\Delta\% = \frac{C_{BPA\ E.S.} - C_{BPA\ I.S.}}{C_{BPA\ I.S.}} * 100 \qquad (4)$$

Where $C_{BPA\ E.S.}$ [ng BPA per g of PET] is the concentration of BPA in PET calculated by the ES method, and $C_{BPA\ I.S.}$ [ng BPA per g of PET] is the concentration of BPA in PET calculated by the IS method. Results showed a Δ% ranging between -21 and -30%, which means that the external standard method significantly underestimates the concentration of BPA in the polymer.

3.3. Application of the method

The established method has been applied to the determination of BPA in 23 PET samples, where 10 of them were virgin, nine were 100% recycled, and four contained different percentages of recycled PET. The results of the analysis are presented in Table 3.

The concentration of BPA ranged between 25 and 432 ng g^{-1} in virgin PET, while in totally recycled PET it was found at levels between 394 and 10120 ng g^{-1}. It should be noted that only one virgin PET was found to have more than 400 ng g^{-1} of BPA, whereas all the others presented a BPA concentration lower than 121 ng g^{-1}. The relative standard deviation ranged between 1 and 19%, but in the majority of cases was below 10%. Seven out of nine R-PET samples contained more than 1.0 μg g^{-1} of BPA.

In all virgin PET samples, the concentration of BPA was higher in bottles than in preforms and pellets. However, except for sample S11, the difference is not statistically significant; therefore, it is not possible to assume that during the manufacture of virgin PET bottles a consistent contamination occurs. These data are shown graphically in the box-plot and bar-plot in Figure 1. While Figure 2 shows the chromatogram traces of sample S8B, by way of example.

3.4. Migration study

Migration experiments in water led to no detectable values of BPA for all samples, except S14B which presented a concentration of BPA between the LoD and the LoQ. In the case of simulant B, BPA was found at levels between the LoD and the LoQ, or lower than the LoD in most of the samples. Only S14B contained 3.5 ng g^{-1} of BPA migrated from the polymer. When employing food simulant C, the concentration of BPA ranged between LoD and LoQ, or lower than LoD. Only samples S13B and S14B presented values of 3.4 and 4.2 ng g^{-1}, respectively. Concerning migration test with simulant D2, no detectable levels of BPA were found to migrate from bottle S14B. The migration results are reported in Table 4. Therefore, ethanol 20% (V/V) (food stimulant C) was the food simulant where the highest concentration values of BPA were found. The migration results obtained are in accordance with previous works (Bach, Dauchy, Chagnon, & Etienne, 2012), where no detectable levels of BPA were found in PET-bottled water under normal storage conditions; and concentrations up to 4 ng L^{-1} were detected in PET-bottle water after sunlight exposure. Toyo'oka and Oshige (Toyo'oka, & Oshige, 2000) reported levels of BPA ranging between 3 and 10 ng L^{-1} in PET-bottled water, whilst other migration studies (Guart, Bono-Blay, Borrell, & Lacorte, 2011) led to no detectable levels of BPA, by performing both the UNE-EN 13130 procedure (UNE-EN 13130, 2005), and a strong ultrasonic extraction from PET samples.

4. CONCLUSIONS

Overall, a method for the analysis of BPA in PET was successfully created; which in terms of repeatability, working range, limits of detection and recovery, shall be considered reliable and suitable for its purpose. The employment of an internal standard is strongly recommended for quantitation, as it mitigates the risk of encountering systematic errors.

Importantly, we also discovered that even though BPA was not expected to be found in PET samples since it is not used for the manufacturing of PET, results showed that BPA can be present and therefore its evaluation is necessary in order to ensure "BPA-free" products. According to the Bisphenol A levels found in tested samples, a BPA concentration in the polymer above 500 ng g^{-1} PET could be considered a possible marker for the presence of recycled material in the bottle/article. Nevertheless, this assumption should be confirmed by analysing a higher number of samples, which would enable a more rigorous statistical analysis to be carried out.

The work presented here suggests that, for foodstuffs (water, juices, nectars and other soft drinks containing fruit pulp, alcoholic beverages with an alcohol content up to 20% (V/V), and edible oils) represented by the food stimulants A (water), B (3% (v/v) aqueous acetic acid solution), C (20% (V/V) ethanol) and D1 (sunflower oil), the BPA content of virgin PET bottles does not pose a risk to consumers health, as migration tests led to concentrations of BPA lower than the new Specific Migration Limit (0.05 mg kg^{-1}), which is coming into force with the Commission Regulation EU No 213/2018. To understand the implications on a large scale, more migration experiments are needed, especially on recycled PET bottles.

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Potential conflicts of interest do not exist.

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502

503

Figure captions

Figure 1. (**a**) Box-plot showing the concentration of BPA in the 23 PET samples, sorted by the amount of recycled PET; (**b**) Bar-plot showing the concentration of BPA in the 23 PET samples (error bars representing ±standard deviation).

Figure 2. Chromatogram traces and proposed fragmentation reactions of sample S8B: (**a**) BPA quantitation reaction *m/z* 227.2→133.0; (**b**) BPA confirmation reaction *m/z* 227.2→212.2; (**c**) BPA-d_{16} quantitation reaction *m/z* 241.2→142.1; (**d**) BPA-d_{16} confirmation reaction *m/z* 241.2→222.3.

Table 1 Tandem mass spectrometry transitions for acquisition in MRM mode.

Analyte	Precursor ion [*m/z*]	Product ion [*m/z*]	Cone voltage [V]	Collision energy [eV]	Dwell time [s]
BPA (quantitation)	227.2	133.0	43	32	0.078
BPA (confirmation)	227.2	212.2	43	30	0.078
BPA-d_{16} (quantitation)	241.2	142.1	43	35	0.078
BPA-d_{16} (confirmation)	241.2	222.3	43	40	0.078

Table 2 Percentage recoveries for the three spike levels obtained by the internal standard (IS) and external standard (ES) methods (mean ± standard deviation; RSD, relative standard deviation). C_{add} is the BPA concentration added to the sample, expressed as ng of BPA per g of PET.

	IS		**ES**	
C_{add} **[ng g^{-1}]**	**[%]**	**RSD [%]**	**[%]**	**RSD [%]**
250	89 ± 15	17	73 ± 7	9
500	107 ± 16	15	74 ± 10	13
1000	100 ± 13	13	81 ± 7	9

n = 3.

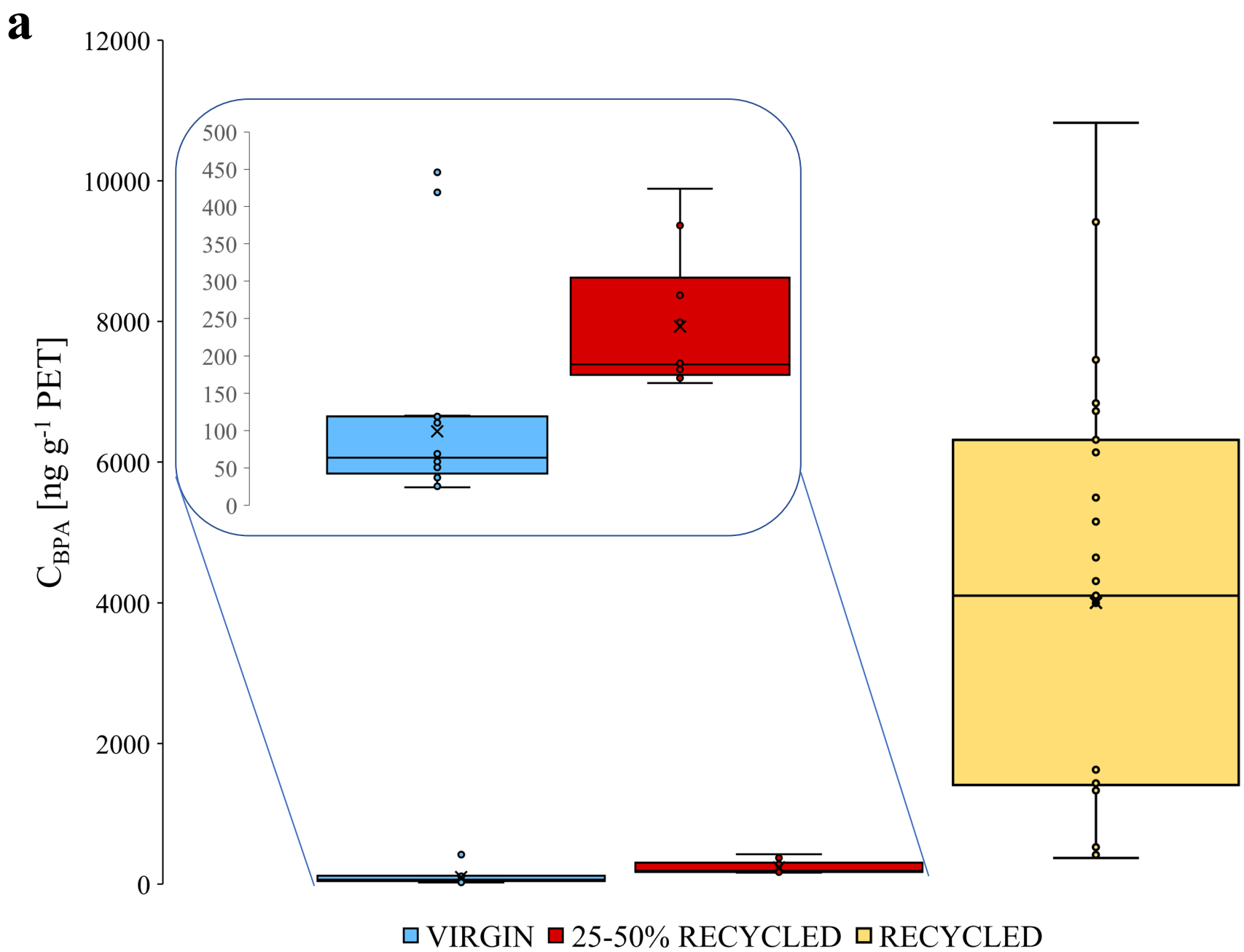
Table 3 Concentration of BPA in PET samples expressed as mean ± standard deviation (n =3) in ng of BPA per g of PET, and the corresponding percentage relative standard deviation. F, pellet; P, preform; B, bottle; V, virgin; R, recycled.

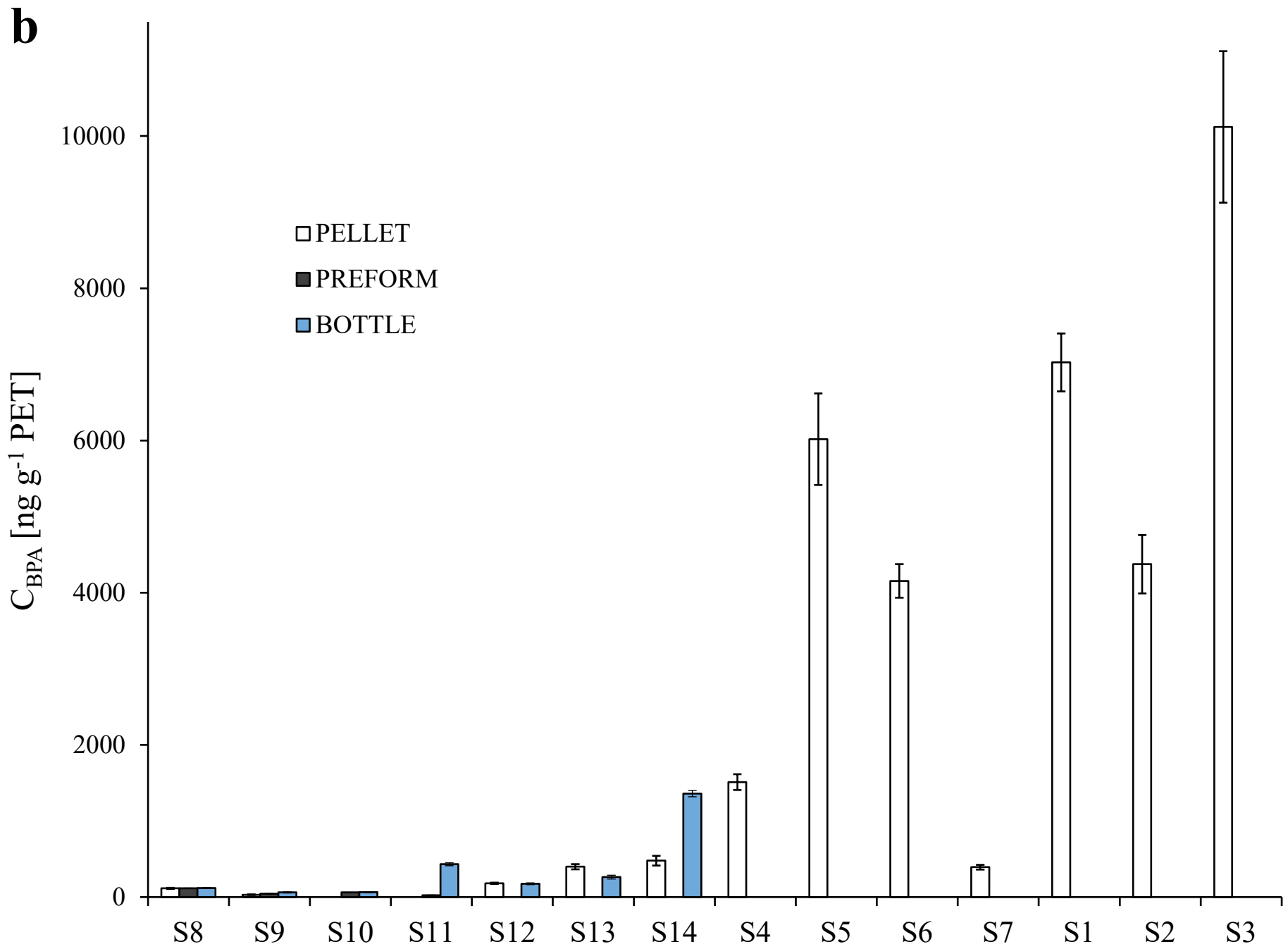
Sample (V/R)	**Mean ± s_0 [ng g^{-1}]**	**RSD %**	**Sample (V/R)**	**Mean ± s_0 [ng g^{-1}]**	**RSD %**
S1F (R)	7026 ± 381	5	S9B (V)	62 ± 8	13
S2F (R)	4375 ± 383	9	S10P (V)	62 ± 1	2
S3F (R)	10120 ± 996	10	S10B* (V)	64 ± 5	8
S4F* (R)	1511 ± 104	7	S11P (V)	25 ± 1	3
S5F (R)	6017 ± 602	10	S11B (V)	432 ± 19	4
S6F (R)	4154 ± 221	5	S12F (25% R)	181 ± 10	6
S7F (R)	394 ± 31	8	S13F (50% R)	399 ± 35	9
S8F* (V)	115 ± 7	6	S14F (R)	480 ± 63	13
S8P (V)	116 ± 4	4	S12B (25% R)	176 ± 12	7
S8B (V)	120 ± 1	1	S13B (50% R)	263 ± 26	10
S9F (V)	31 ± 6	19	S14B (R)	1360 ± 40	3
S9P (V)	44 ± 6	14			

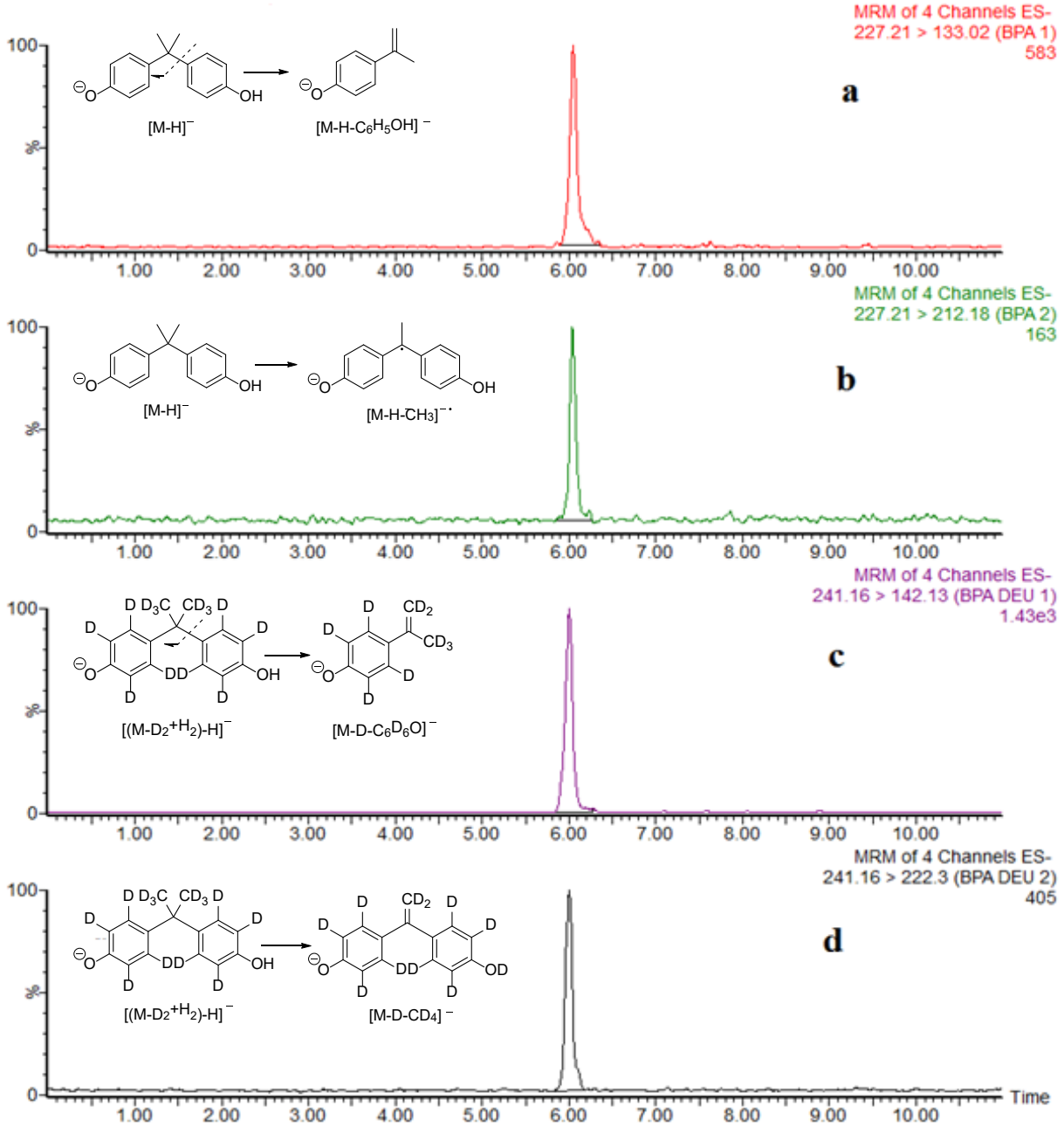
(*) n = 6.

Table 4 Results of migration tests on the four food simulants employed, expressed as mean ± standard deviation (n = 3) in ng of BPA per g of food simulant.

Sample	**Water** [ng g^{-1}]	**3% acetic acid (w/V)** [ng g^{-1}]	**20% ethanol (V/V)** [ng g^{-1}]	**Sunflower oil** [ng g^{-1}]
S9B	< 1.0	1.0 - 3.3	1.0 - 3.3	-
S11B	< 1.0	< 1.0	< 1.0	-
S10B	< 1.0	1.0 - 3.3	< 1.0	-
S8B	< 1.0	1.0 - 3.3	1.0 - 3.3	-
S12B	< 1.0	< 1.0	1.0 - 3.3	-
S13B	< 1.0	1.0 - 3.3	3.4 ± 0.7	-
S14B	1.0 - 3.3	3.5 ± 0.5	4.2 ± 0.6	< 1.0


a
12000
10000
8000
6000
4000
2000
0
C_{BPA} [ng g^{-1} PET]
500
450
400
350
300
250
200
150
100
50
0
VIRGIN
25-50% RECYCLED
RECYCLED


b
10000
8000
6000
4000
2000
0
C_{BPA} [ng g^{-1} PET]
PELLET
PREFORM
BOTTLE
S8
S9
S10
S11
S12
S13
S14
S4
S5
S6
S7
S1
S2
S3


MRM of 4 Channels ES-
227.21 > 133.02 (BPA 1)
583
a
[M-H]⁻
[M-H-C6H5OH] ⁻
MRM of 4 Channels ES-
227.21 > 212.18 (BPA 2)
163
b
[M-H]⁻
[M-H-CH3]⁻•
MRM of 4 Channels ES-
241.16 > 142.13 (BPA DEU 1)
1.43e3
c
[(M-D2+H2)-H]⁻
[M-D-C6D6O] ⁻
MRM of 4 Channels ES-
241.16 > 222.3 (BPA DEU 2)
405
d
[(M-D2+H2)-H]⁻
[M-D-CD4] ⁻
%
100
0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
Time