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Preparation of 4,4′-(1-[$^2H_6$]methylethylidene)bis-[2,3,5,6-$^2H_4$]phenol and its application to the measurement of bisphenol A in beverages by stable isotope dilution mass spectrometry

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Abstract

The preparation of 4,4′-(1-[$^2H_6$]methylethylidene)bis-[2,3,5,6-$^2H_4$]phenol, bisphenol A-d_{14}, was achieved in excellent yield by reaction of [$^2H_6$]acetone with [$^2H_6$]phenol in the presence of deuterium chloride. The product thus obtained was shown by mass spectroscopy to be a mixture of the $^2H_{14}$, $^2H_{13}$ and $^2H_{12}$ isotopomers in the relative proportions of 82.3:16.2:1.5, respectively. An isotope dilution gas chromatography–mass spectrometric method using bisphenol A-d_{14} as an internal standard was developed to measure the level of bisphenol A in beverages. The procedure involves extracting bisphenol A into dichloromethane, and then purifying the analyte by back extraction into dilute aqueous sodium hydroxide. Conversion of bisphenol A and its internal standard, bisphenol A-d_{14}, to their corresponding *O*-bis(trifluoroacetyl) derivatives by treatment with trifluoroacetic anhydride gave compounds with good chromatographic properties and whose mass fragmentomerty is such that loss of $M-CH_3$ and $M-C^2H_3$ are the base peaks in the mass spectra of the analyte and internal standard, respectively. Quantification of bisphenol A was achieved by comparing the area of the $M-15$ ion to that of the corresponding ion of bisphenol A-d_{14}. The characteristics of our assay are: an analyte recovery of better than 95%, a root mean square signal-to-noise ratio of 79:1 for 1.7 pg on column and an inter-assay RSD of better than 4% ($n=5$).

Keywords: Stable isotope dilution; Beverages; Food analysis; Bisphenol A; Phenols

1. Introduction

For a variety of technical reasons, chemicals such as plasticisers, cross-linking agents, dyes and monomers are either present in food packaging materials or are added during their manufacture [1,2]. Consequently, these chemicals are potential sources of contaminants in that they can migrate from the packaging material into the food they protect [3,4]. In addition to being sources of taints and off-flavours [5,6], some of these chemicals have also been associated with pathological conditions in both humans and wildlife [7–12]. In particular, plasticisers, viz. the diesters of phthalic acid, and phenolic compounds such as nonylphenol have been reported to possess estrogenic activity, which can interfere with the physiological role of endogenous hormones in animals [13,14]. In this respect, some concerns have been raised regarding the safety of the starting material, bisphenol A (BPA), used in the manufacture of polymeric food packing materials such as epoxy resins and polycarbonate bottles. Thus some

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authorities have reacted to these concerns by imposing a daily intake limit for BPA [15]. A challenge arising from this regulation is to develop a sensitive and selective method to quantify the concentration of BPA in the complex matrices of food. We report here a sensitive and highly selective method, based on the technique of isotope dilution mass spectrometry, for determining the concentration of BPA in beverages.

2. Experimental

2.1. Chemicals and reagents

[$^2H_6$]Phenol (phenol-d_6), and [$^2H_6$]acetone (acetone-d_6), were purchased from Cambridge Isotope Labs. (Andover, MA, USA) and Aldrich (Milwaukee, WI, USA), respectively. The isotopic purity of these reagents were stated by the manufacturers to be 98% and 99.5% (respectively) and were used without further purification. Deuterium chloride (99.5%) was obtained as a 37% (w/w) solution in ^2H_2O from Aldrich. BPA and trifluoroacetic anhydride were purchased from Aldrich. The chemical purity of these reagents were better than 99% and were used without further purification. Dichloromethane, isooctane, methanol, diethyl ether and water were distilled directly into glass storage containers. Both 0.05 *M* sodium hydroxide and sodium hydrogen carbonate were prepared as solutions in 20% and 10% (w/w), respectively, aqueous sodium chloride. All other reagents were of analytical grade and unless stated otherwise were used without further purification.

2.2. Preparation of 4,4′-(1-[$^2H_6$methylethylidenebis-[2,3,5,6-$^2H_4$]phenol

A mixture of acetone-d_6 (0.5 ml, 0.42 g, 6.6 mmol), phenol-d_6 (2.7 g, 27 mmol) and 37% (w/w) deuterium chloride in deuterium oxide (0.4 ml, 4 mmol) was allowed to stand at room temperature. After 12 days, the resulting orange solid reaction mixture was dissolved in diethyl ether (50 ml), and the ethereal solution was then poured into saturated aqueous sodium hydrogen carbonate (50 ml). The organic layer was separated and the aqueous phase was extracted with diethyl ether (2×25 ml). The organic layer and extract were combined, washed with saturated brine (50 ml) and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure gave an oil, which solidified upon addition of chloroform (ca. 2 ml). Recrystallization of an aliquot of the crude product (1.0 g) from chloroform–isooctane gave 4,4′-(1-[$^2H_6$]methylethylidene)bis-[2,3,5,6-$^2H_6$]phenol, (BPA-d_{14}), as colourless crystals (880 mg). The isotopic composition of this material was determined by a mass spectrometric assay to be such that it contains 82.3% BPA-d_{14}, 16.2% BPA-d_{13} and 1.5% BPA-d_{12}.

2.3. Stock solutions

Stock solutions of BPA (2100, 210.0, 21.00 and 2.10 μg ml^{-1}) and BPA-d_{14} (2090, 209.0 and 20.90 μg ml^{-1}) were prepared in methanol. Mass spectrometric measurements of the deuterated internal standard revealed its isotopic purity is not affected by storage in methanol. All stock solutions were kept at 4°C until required.

2.4. Sample preparation

A solution of BPA-d_{14} in methanol (20.90 μg ml^{-1}, 50 μl, 1.05 μg) and sodium chloride (2 g) were added to a beverage (10 ml)[1]. The resulting sample was extracted with dichloromethane (40 ml) and the extract was washed with aqueous sodium hydroxide (0.05 *M*, 10 ml). The organic layer was discarded and the pH of the aqueous phase was adjusted to ca. 2 with concentrated hydrochloric acid. The resulting acidified aqueous solution was extracted with dichloromethane (20 ml) and the extract was washed with aqueous sodium hydrogen carbonate (0.05 *M*, 5 ml) and dried (Na_2SO_4). Evaporation of the solvent at atmospheric pressure gave a residue, which was treated with trifluoroacetic anhydride (200 μl). After standing at room temperature for 30 min, the excess reagent was removed by heating the reaction vial in a hot block at 60°C for approximately

[1]In the case of carbonated beverages these were degassed prior to the addition of the internal standard by either allowing the beverages to stand at room temperature for 24 h or pouring the beverage from one beaker into another several times.

5 min. The resulting residue was dissolved in isooctane (200 μl), and an aliquot of the solution was analysed by gas chromatography–mass spectrometry (GC–MS).

2.5. Gas chromatography–mass spectrometry

Gas chromatography was performed on a Hewlett-Packard (HP) HP-5 Trace Analysis fused-silica capillary column (25 m×0.2 mm; 0.33 μm) housed in a 5890 series II plus gas chromatograph fitted with an electronic pressure control unit. Helium was used as the carrier gas at a constant flow of 0.5 ml min^{-1} after an initial head pressure of 2.1 kg cm^{-2} for 0.8 min. The oven temperature was initially set at 100°C and, after 1 min, was ramped at 25°C min^{-1} to 150°C and then raised to 210°C at 5°C min^{-1} before being ramped at 25°C min^{-1} to 280°C. The final oven temperature of 280°C was maintained for 2.5 min. Sample injections were performed in the splitless mode using an HP 7673 autoinjector and a purge activation time of 0.75 min. The injector and detector temperatures were set at 250 and 280°C, respectively.

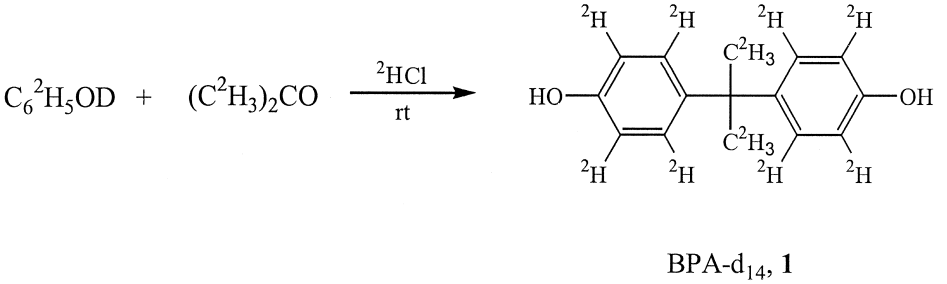
The capillary column was directly interfaced to an HP 5972 mass-selective detector, which was operated in the electron impact mode. The energy of the electron beam was 70 eV. The ion source temperature was 180°C. The abundance of the ions selected for monitoring the analyte and its internal standard were optimised by using the most abundant ion from a cluster of ions separated by 0.05 in the range of ±0.5 of the ion chosen in the full scan mode. In the case of the *O*-bis(trifluoroacetyl) derivative of the analyte the ions were 420.25, 406.25 and 405.25 and in the case of the internal standard the ions are 432.35, 417.35 and 416.35. The dwell time for each ion was set to 40 ms to achieve a scan rate of 3 Hz so as to reduce imprecision of area measurement.

2.6. Calibration curve

A calibration curve was constructed by plotting the peak area ratios of the analyte target ion, m/z 405.25, to that of the internal standard target ion, m/z 416.35, against the corresponding molar ratios of the analyte to the internal standard over the range 1:40 to 40:1. The plot was linear over this range ($y=0.821x+0.177$, $r^2=0.999$).

3. Results and discussion

Reaction of acetone-d_6 and phenol-d_6 in the presence of deuterium chloride (Fig. 1) gave BPA-d_{14} [16]. The isotopic purity of this material was determined by a mass spectrometric assay to be such that it contained 16.2% BPA-d_{13} and 1.5% BPA-d_{12}. The formation of these less isotopically enriched isomers of BPA is, undoubtedly, a consequence of the moisture present in both phenol and acetone because of their hydroscopic nature. It seems feasible to assume that under the acidic reaction conditions the deuterium atoms of acetone partially exchange with the protons of water prior to reaction with phenol. The assignment of the *para–para* stereochemistry to the reaction product was made on the basis of the mass spectrum of its *O*-bis(trifluoroacetyl) derivative (Fig. 2). The ratio of the intensity of the base peak, $M-C^2H^3$, to that of the molecular ion was identical with that of an authentic sample of the *para–para* isomer of BPA. This ratio



BPA-d_{14}, **1**

Fig. 1. Summary of reaction conditions used for the preparation of BPA-d_{14}.

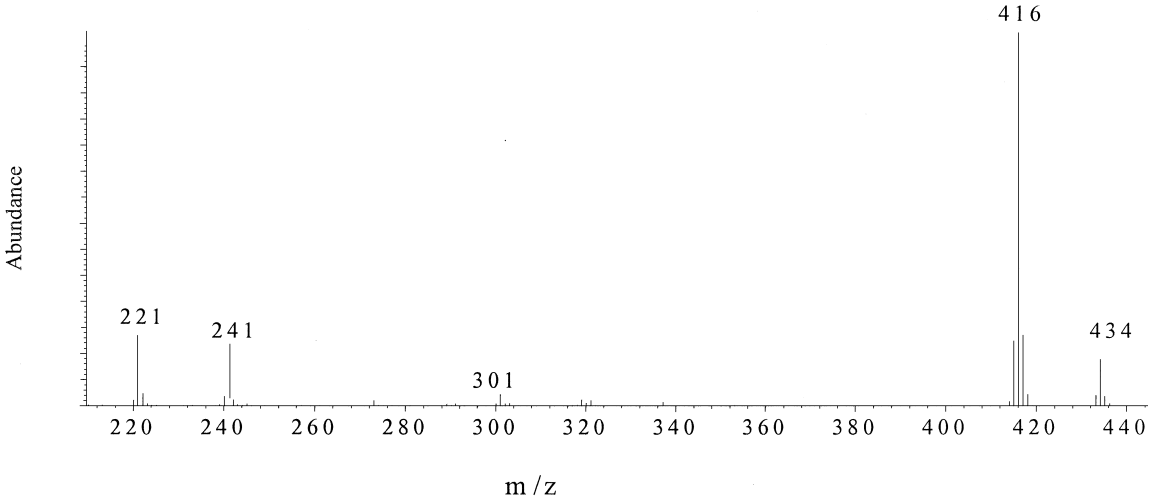


Fig. 2. Electron impact mass spectrum in the region m/z 200–450 of the *O*-bis(trifluoroacetyl) derivative of BPA-d_{14}.

has been reported [17] to be unique for the *ortho–ortho*, *ortho–para* and *para–para* aromatic substitution patterns of BPA.

With the internal standard in hand, we turned our attention to the analytical method development phase of the project. In this respect, a review of the literature reveals that the analysis of BPA by GC–MS has been performed with or without derivatization of the analyte [2,18–23]. Whilst acceptable chromatographic behaviour of BPA was observed under the conditions used to elute the compound from a fused-silica capillary column, this was not the case for the internal standard, BPA-d_{14}. The mass spectrum of the internal standard (Fig. 3) after chromatography on a fused-silica capillary column revealed its isotopic purity had deteriorated because of deuteron–proton (^{2}H–H) exchange in the aromatic portion of the molecule. This phenomenon had been previously reported [24] to occur with various monodeuterated phenols that had been chromatographed on fused-silica columns and was attributed to an aromatic electrophilic exchange of deuterium

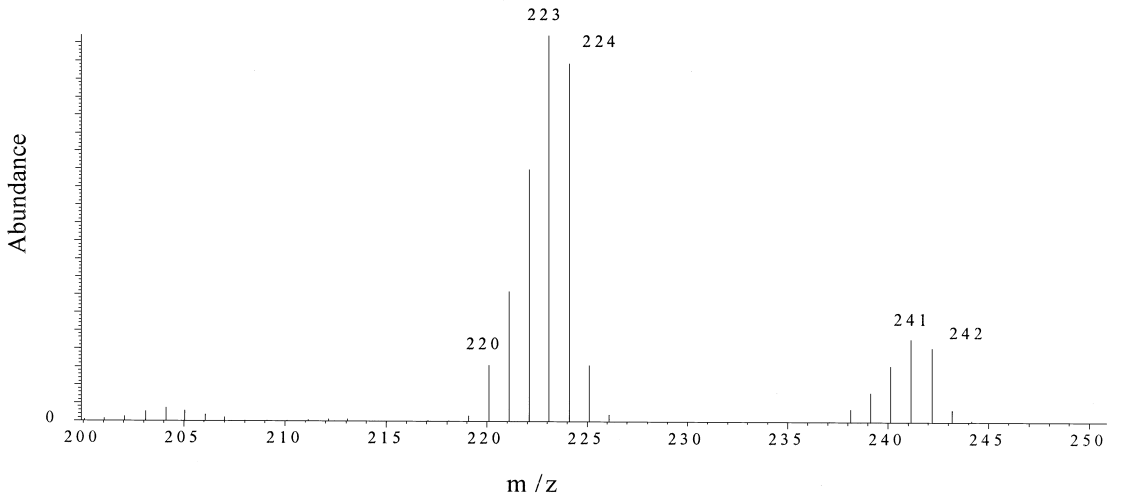


Fig. 3. Electron impact mass spectrum in the range of m/z 200–250 of BPA-d_{14} after chromatography on a fused-silica column.

atoms with active hydrogen atoms located on the internal surface of the columns.

The extent to which this phenomenon occurred with the monodeuterated phenols was noted [24] to be directly dependent on the residence time of the analyte on the column. This was also found to be the case for BPA-d_{14}. Although decreasing the residence time of BPA-d_{14} on the capillary column was exploited to reduce the loss of isotopic purity it did not overcome the more serious limitation of inter-variation of isotopic purity between runs. This was particularly problematic when BPA-d_{14} was chromatographed after the column had been used for other analyses. A potentially more successful solution to the problem at hand was the conversion of BPA-d_{14} to more volatile compounds. In this respect, conversion of BPA to its corresponding *O*-bis-(trimethylsilyl) ether is the only reported derivative used for the purpose of GC–MS [2,19]. Our initial work with this derivative of BPA-d_{14} demonstrated the viability of this approach towards reducing the loss of isotopic purity. Indeed, the volatility of the *O*-bis(trimethylsilyl) ether derivative of BPA-d_{14} is such that no loss of isotopic purity occurred under the chromatographic conditions used to effect its elution from a 25 m fused-silica column. Mindful of the fact that electron withdrawing groups deactivate aromatic systems towards electrophilic substitution [25], i.e. the mechanism by which ^{2}H–H exchange proceeds in phenols, it occurred to us that the acylation of the hydroxy groups of BPA-d_{14} should, in principle, give a volatile derivative that is more deactivated towards ^{2}H–H exchange than the *O*-bis(trimethylsilyl) derivative. Thus reaction of BPA-d_{14} with trifluoroacetic anhydride gave the corresponding *O*-bis(trifluoroacetyl) compound, which, in addition to being significantly more volatile than the corresponding *O*-bis(trimethylsilyl) derivative, was found not to undergo ^{2}H–H exchange when subjected to excessively long residence times on a fused-silica capillary column.

Further advantages of using the *O*-bis(trifluoroacetyl) derivatives of both BPA and BPA-d_{14} are that these compounds have higher molecular masses than those of the corresponding *O*-bis(trimethylsilyl) compounds (Fig. 4) and that fluorine, unlike silicon, is monoisotopic. In the context of selected ion monitoring, the higher masses of the trifluoroacetyl derivatives make these compounds more desirable than the trimethylsilyl derivatives because of the favourable signal-to-noise ratio improvement at the higher end of the mass range of a mass spectrometer.

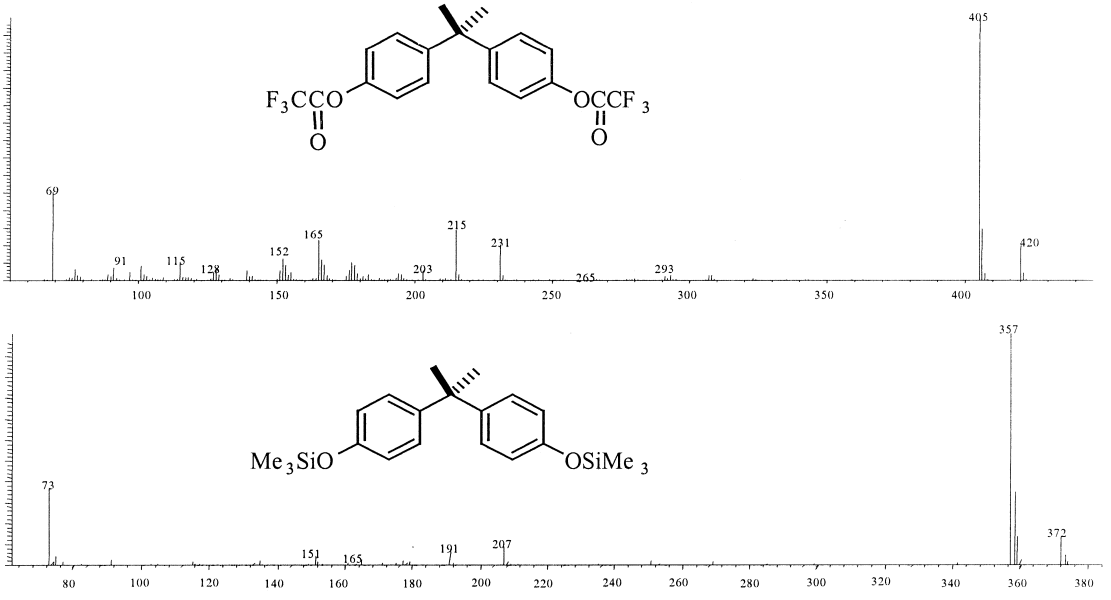


Fig. 4. Electron impact mass spectra of the *O*-bis(trifluoroacetyl) (top) and *O*-bis(trimethylsilyl) (bottom) derivatives of bisphenol A.

Furthermore, the monoisotopic nature of fluorine does not reduce the intensity of an ion by distributing its current to isotopomeric ions. Consequently, the *O*-bis(trifluoroacetyl) derivative of BPA should, in principle, be a more sensitive derivative than the *O*-bis(trimethylsilyl) derivative when the corresponding target and qualifier ions, *viz M*, $(M+1)-15$ and $M-15$, are monitored in both sets of derivatives. This proved to be the case in practice. The root mean squared, signal-to-noise ratios for 6 pg of both the *O*-bis(trifluoroacetyl) and the *O*-bis(trimethylsilyl) derivatives of BPA were 221:1 and 157:1, respectively. It is also noteworthy that a rms signal-to-noise ratio of 79:1 was recorded for 1.7 pg on-column of the *O*-bis(trifluoroacetyl) derivative of BPA.

The base peak in the electron impact mass spectra of the two derivatives, and indeed BPA, corresponds to loss of a methyl group from the molecular ion. This can be rationalised in terms of resonance stabilisation of the resulting *tert.*-benzylic carbocation **3** (Fig. 4) formed by loss of a methyl radical from the molecular ion. An alternative minor fragmentation pathway involves loss of one of the aryl groups from the molecular ion to give the fragment **4** –a *tert.*-benzylic carbocation, which, in the case of R=H, has been reported [26] to be an intermediate in the preparation of BPA by a Friedel–Crafts reaction of acetone with phenol. Interestingly, loss of the aryl group is the major fragmentation pathway in the positive ion chemical (methane) ionisation mass spectra of BPA and its two derivatives. Subsequent loss of methane from the benzylic carbocation **4** gives a fragment ion, the proposed structure of which is that shown as the allene **5**.

The next task in the method development was the isolation of the analyte and its internal standard from beverages. In this respect, our strategy was influenced by the weakly acidic nature of phenols [27]. It was envisaged that BPA, and its internal standard, could be extracted from a beverage into an organic solvent and then purified by back extraction into dilute aqueous sodium hydroxide. It also occurred to us that further sample purification could be effected by back extraction of compounds more acidic than BPA into aqueous sodium hydrogen carbonate. Thus, our sample preparation protocol would initially involve extracting BPA and its internal standard, BPA-d_{14}, into an organic solvent and then back extracting the acidic components of the extract into dilute aqueous sodium hydroxide. Further purification of the analyte would then be achieved by back extraction of compounds more acidic than BPA into aqueous sodium hydrogen carbonate after acidification and extraction of the alkaline aqueous phase with an organic solvent. In light of the fact that the electron donating groups on the phenyl rings of the internal standard activate the molecule towards an electrophilic aromatic substitution reaction [25] there was concern that the deuterium atoms of BPA-d_{14} could be labile under the acidic and basic conditions employed to effect its isolation from a beverage and subsequent purification. This concern was addressed by subjecting BPA-d_{14} to the conditions encountered in the sample preparation procedure. The mass spectrum of the *O*-bis(trifluoroacetyl) derivative of BPA-d_{14} thus obtained revealed its deuterium atoms are stable under the conditions of the sample preparation procedure. However, ^{2}H–H exchange was found to occur when BPA-d_{14} was allowed to stand in aqueous sodium hydroxide at room temperature overnight.

Application of our sample preparation protocol to distilled water that had been fortified with a known amount of BPA revealed that the recovery of the analyte was less than 30%. A systematic investigation of the various stages within the protocol revealed that losses were occurring at the two liquid–liquid extraction stages. It was also evident from this investigation that the volatility of BPA and its *O*-bis(trifluoroacetyl) derivative are such that significant losses occur when the sample is allowed to stand under a stream of nitrogen after the solvent (or excess trifluoroacetic anhydride) has evaporated (Fig. 5). Although these evaporative losses could be reduced by minimising the time the sample spent under a stream of nitrogen, a more practical approach was to concentrate the solutions to approximately 0.2 ml under a stream of nitrogen and then evaporate the remaining solvent (or derivatising reagent) to dryness by placing the samples in a hot block at 60°C. With respect to the losses at the two liquid–liquid extraction stages, the problem was considered to be a consequence of the hydroxy groups of BPA producing a dipole moment that results in an unfavourable partitioning of BPA be-

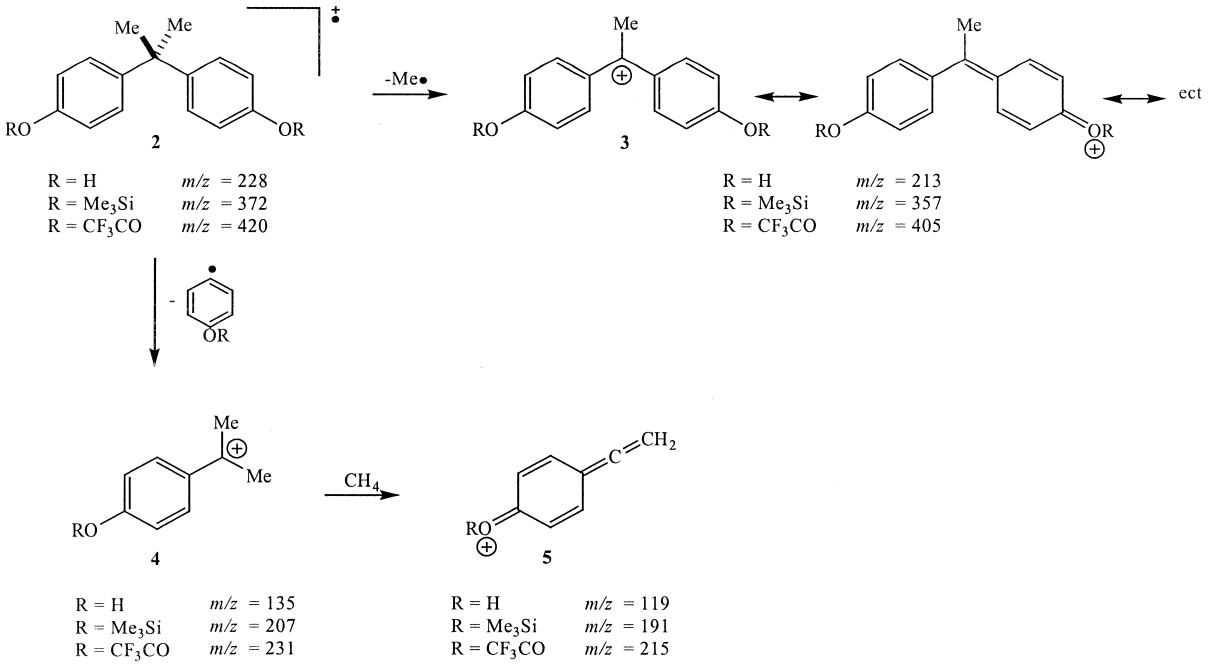


Fig. 5. Proposed electron impact fragmentation pathways of bisphenol A and its *O*-bis(trimethylsilyl) and *O*-bis(trifluoroacetyl) derivatives.

tween water and dichloromethane. Thus, decreasing the solubility of the analyte in water through a salting-out effect would, if our reasoning was correct, increase the recovery of BPA. The report by del Olmo et al. [18] that the liquid–liquid extraction efficiency of BPA from water increased as the ionic strength of the solution was increased with sodium chloride was considered to be consistent with our reasoning. In the event, addition of sodium chloride to the water fortified with BPA dramatically increased the recovery of the analyte. To avoid subsequent losses of BPA, the ionic strength of both the sodium hydroxide and sodium hydrogen carbonate aqueous solutions used at the sample clean-up stages of the protocol was adjusted with sodium chloride. When these modifications were made to our sample preparation protocol the overall recovery of BPA was determined to be better than 95%.

The accuracy and inter-assay precision of the method were determined by analysing unspiked and spiked beverages. The results are summarised in Table 1. The intra-assay precision was better than 1.0%. In summary, a both highly sensitive and selective assay for BPA in beverages has been

Table 1
Accuracy of GC–MS assay of BPA in beverages ($n=5$)[a]

Sample	Expected concentration (ng ml^{-1})	Measured concentration (mean±SD) (ng ml^{-1})	RSD (%)	Recovery (%)
Unspiked beverage	–	0.52±0.02	3.3	–
Beverage spiked with 2.18 μg of BPA	9.24	9.77±0.13	1.3	105.7
Beverage spiked with 6.33 μg of BPA	25.83	25.69±0..80	2.9	99.5

[a] The pipettes used in this work were corrected for volume using deionised water. All standards were prepared in all-glass class A 250 ml volumetric flasks.

described. The application of this methodology to a survey of the levels of BPA in Australian beverages is currently underway and will be reported in due course.

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