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Determination of bisphenol A in wine by sol-gel immunoaffinity chromatography, HPLC and fluorescence detection

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Determination of bisphenol A in wine by sol-gel immunoaffinity chromatography, HPLC and fluorescence detection

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Abstract:

The paper presents a highly selective analytical method for the determination of traces of bisphenol A (BPA) in wine and the results of a survey 59 wine samples sourced from vats (steel, wood and plastic), glass bottles and Tetra Briks. The procedure consists of sample clean-up by sol-gel immunoaffinity chromatography followed by determination of BPA by high performance liquid chromatography and fluorescence detection. The method has a LOD (S/N=3) of 0.1 ng/ml and a LOQ (S/N=6) of 0.2 ng/ml. In 13 of 59 wine samples the BPA concentration was below the LOQ. The mean and median for all wine samples with BPA concentrations above the LOQ were 0.58 and 0.40 ng/ml, respectively. These values – the first set of data on BPA in wine - are far lower than previously published BPA levels derived from migration experiments using wine simulants. Experiments carried out by submerging plastic stoppers in ethanol-water (11:89, v/v) up to 11 weeks indicated that detectable amounts of BPA can be leached from some stoppers.

Key words:

Bisphenol A, wine, migration, immunoaffinity chromatography, sol-gel, HPLC

Introduction

Bisphenol A (BPA) is an industrial chemical primarily used in the production of polycarbonates and epoxy resins. Many food containers - like returnable milk and water bottles or baby milk bottles - are made of polycarbonate, while epoxy resins are used as protective coatings in metal food and beverage cans or to line wine storage vats. A number of studies showed a migration of BPA traces from polycarbonate based packagings (Biles *et al.* 1997a, Brede *et al.* 2003, Nerin *et al.* 2003), can coatings (Munguia-Lopez and Soto-Valdez 2001, Goodson *et al.* 2004) or wine vat coatings (Larroque *et al.* 1989) into food or wine simulants.

BPA belongs to the so-called endocrine-disrupting chemicals (EDCs) which can interfere with the function of the hormonal system of humans and animals by binding to the natural estrogen receptor. In animal experiments BPA has been shown to influence mammary gland development (Markey *et al.* 2001) and cause urethral malformations (Timms *et al.* 2005) or meiotic aneuploidy (Hunt *et al.* 2003). Recent studies indicated that BPA can be biologically active at very low doses which had not caused detectable effects in toxicological studies and had therefore been assumed to be safe for humans (Timms *et al.* 2005, Ashby 2003, Welshons 2003).

Since the food chain is considered as the main source of BPA exposure for humans, several surveys have already been carried out to determine BPA levels in canned foodstuffs and beverages (Biles *et al.* 1997b, Yoshida *et al.* 2001, Goodson *et al.* 2002, Brotons *et al.* 1995, Thomson and Grounds 2005, Braunrath *et al.* 2005) but there is a lack of realistic data for wines. Both the "Opinion of the Scientific Committee on Food on BPA" (SCF 2002) and the "European Union risk assessment report on BPA" (EURAR 2003) were based on exposure models derived from data published by Larroque *et al.* (1989) which were, however, obtained in experiments with wine simulants. Following the "Opinion of the Scientific Committee on Food on BPA" the European Commission reduced the Specific Migration Limit for BPA from 3 mg/kg to 0.6 mg/kg (EC 2004).

A recent paper by Lambert and Larroque (1997) advanced the methodology for the determination of BPA (and other phenolic compounds) in wine which had been in contact with epoxy resins by HPLC and fluorescence detection but due to its high detection limit the method could only be validated by using fortified samples.

The present paper describes a method for the determination of BPA in wine which includes a slightly modified sample clean-up procedure previously

developed for the determination of BPA in canned beverages, fruits and vegetables (Braunrath and Cichna 2005). The method offers a detection limit low enough to finally allow the analysis of real wine samples. The application of the method described in this paper provides the first set of data on BPA in wine samples

Experimental

Reagents and Chemicals

Purified polyclonal bisphenol A (BPA) rabbit antibodies (5 mg/240 µl PBS buffer) were a gift from Japan EnviroChemicals, Ltd. (Tokyo, Japan). BPA was bought from Sigma (St. Louis, MO, USA), tetramethoxysilane (TMOS) from Fluka (Buchs, Switzerland). Acetonitrile (ACN), gradient grade for HPLC, was delivered from Fisher Scientific (Leicestershire, UK). The BPA stock solution was prepared by dissolving 20.0 mg of BPA in 20.0 ml ACN. Lower concentrated BPA solutions were obtained by diluting the stock solution with mobile phase. All BPA solutions were stored at 4 °C. Phosphate-buffered saline (PBS), pH 7.6, was prepared by dissolving 12.46 g $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$, 1.56 g $\text{NaH}_2\text{PO}_4 \times 2 \text{H}_2\text{O}$ and 8.5 g NaCl in 1 litre of bidistilled water.

Preparation of Sol-Gel Columns

Sol-gel immunoaffinity columns were prepared according to a previously described protocol (Braunrath and Cichna 2005). In brief, TMOS was hydrolysed under slightly acidic conditions in an ultrasonic bath for 30 min. After mixing 1 ml hydrolysate with 1 ml of PBS containing 1 mg of BPA antibody a gel was formed within a few min. When the resulting sol-gel glass had lost 50% of its initial mass it was manually ground in an achate mortar and packed into a 3 ml glass column (Merck, Darmstadt, Germany) equipped with a polytetrafluorethylene frit (Merck).

After flushing the immunoaffinity column in sequence with 20 ml of PBS, 15 ml of ACN-water (40:60, v/v) and 20 ml of PBS, it was stored in PBS at 4 °C.

Sol-gel filter columns were prepared following the same procedure but by mixing PBS (without BPA antibodies) with pre-hydrolysed TMOS.

Wine samples

59 wine samples were analysed in the present study. 31 samples were obtained from four Austrian vintagers from the Wachau, a famous Austrian wine-growing region. Vintagers kindly provided information about wine storing conditions, e.g. the tank material and storage time. Ten samples were taken directly from vats, 21 had already been filled into glass bottles. Twenty-eight wine samples (17 filled into glass bottles and 11 Tetra Brik wines) were bought in local markets. Since white wine production is more important in Austria (especially in the Wachau) than red wine production the study comprised more white wine (46) than red wine (13) samples.

Sample Preparation

The sample pretreatment procedure is a slight modification of the procedure developed for the analysis of canned beverages, fruits and vegetables (Braunrath and Cichna 2005) which was now adapted to the analysis of wine. It is very simple and consists of just a few steps.

Wine samples were degassed in an ultrasonic bath for 5 min before taking an aliquot of 25 ml which was mixed with 25 ml of PBS. The pH values of these samples were in the range from 3.0 to 3.9. In order to enable interactions of BPA and the antibodies immobilised in the immunoaffinity column the pH value of the sample was adjusted to pH 7.0 by adding diluted sodium hydroxide. To avoid clogging of the immunoaffinity column the samples were filtered through a

borosilicate frit. The sample was then applied to a sol-gel pre-column which was on-line coupled with an immunoaffinity column using a flow-rate of about 1 ml/min. After sample application the immunoaffinity column was decoupled from the pre-column and washed with 5 ml of ACN-water (10:90, v/v) before eluting BPA with 4 ml of ACN-water (40:60, v/v). The eluate was collected in a 5 ml measuring flask and filled up to the ring mark with bidistilled water. An aliquot of 100 µl was injected into the HPLC system. The immunoaffinity column was regenerated with 20 ml of PBS.

HPLC separation and detection

In the present work two HPLC systems were used operated with different phase systems and detectors.

HPLC system 1, used to determine BPA concentrations in wine samples, consisted of a high pressure gradient pump (Model L-6200, Merck), a column thermostat (Column oven 655 A-52, Merck), a six-port injection valve (Model 7161, Rheodyne) equipped with a 100 µl injection loop and a fluorescence detector (Model 1080, Merck) set at 275/305 nm. Wine samples were analysed using a LiChrospher 60 RP-Select B column, 5 µm, 250 x 4.0 mm I.D. (Merck). The mobile phase consisted of water-ACN (70:30, v/v). The flow rate was 1.0 ml/min. Peaks were integrated using the Stratos V 3.0 software (Polymer Laboratories, Darmstadt, Germany). At the end of each HPLC analysis the analytical column was flushed with water/ACN (40:60, v/v) for 10 min.

HPLC system 2 was used in order to confirm the identity of BPA when migration experiments with plastic stoppers were carried out. It consisted of a high pressure gradient pump (Model L-6200, Merck), an autosampler (Model AS-2000 A) and a coulometric electrode array detector (ESA, Chelmsford, MA, USA) equipped with two cell blocks consisting of eight working electrodes. The analytical column was a HP-Hypersil PDS column, 5 µm, 250 x 4 mm I.D. Mobile

phase A was 0.01 M sodium acetate buffer (pH 4.8, adjusted with acetic acid), mobile phase B ACN. The gradient program was as follows: 0 min: 77% A / 23% B, 10 min: 76.1% A / 23.9% B, 20 min: 73.6% A / 26.4% B, 30 min: 66.8% A / 33.2% B, 40 min: 60% A / 40% B, 62 min: 60% A / 40% B. The flow-rate was 0.6 ml/min, the injection volume 100 µl. Potentials were set at +300, +400, +500, +550, +600, +650 and +700 mV against palladium reference electrodes, the eighth electrode did not work. Peaks were integrated using the CoulArray Win software.

External calibration

HPLC system 1 was calibrated by injecting nine BPA standard solutions in the concentration range from 0.3 to 100.0 ng/ml in mobile phase. Analysis functions were obtained by linear regression of peak areas on standard concentrations.

Standard addition method

In order to determine BPA recoveries the standard addition method was carried out with one white wine (sample 33) and one red wine (sample 55). The non-spiked and three spiked samples (+ 100, 200 and 300 µl of a 100.0 ng/ml BPA standard / 25 ml wine sample) were analysed as described above. Peak areas were plotted against the amount of analyte added. Recoveries were determined by dividing the slope of the linear regression line for the standard addition by the slope of the linear regression line of BPA standard solutions.

Solid phase extraction

Solid phase extraction experiments were carried out using 3 ml C₁₈ cartridges packed with 500 mg Isolute (International Sorbent Technology, Mid Glamorgan, UK). The C₁₈ column was flushed with 6 ml of ACN, followed by 6 ml of ACN-water (10:90, v/v). Wine samples were applied at a flow-rate of about 1 ml/min.

After washing the column with 10 ml of ACN-water (20:80, v/v) elution was carried out with 4 ml of ACN, collecting the eluate in a 5 ml measuring flask. The eluate was evaporated under a gentle nitrogen stream to about 2 ml before filling up the flask with bidistilled water to the ring mark.

Migration of BPA from plastic stoppers

Each of four plastic stoppers from different manufacturers was stored submerged in a 50 ml centrifuge tube filled with 10 ml of ethanol-water (11:89, v/v). Since in most wine samples the alcohol concentration was in the range from 11 to 12%, migration experiments were carried out with 11% ethanol instead of 10% ethanol conventionally used as alcoholic simulant. At the end of the storage period a 100 µl aliquot of the ethanolic solution was directly injected into HPLC system 1.

Results and discussion

Sample clean-up

Fig. 1 shows typical chromatograms obtained by injecting (A) a BPA standard solution, (B) a white wine and (C) a red wine sample, both purified by immunoaffinity chromatography. In order to illustrate the increased efficiency in removing matrix components obtained by the immunoaffinity column the same wine samples were alternatively purified by solid-phase extraction using phase systems and operating procedures (as described in the experimental section) which had been optimized for maximum BPA recovery (Braunrath and Cichna 2005). The chromatograms obtained are documented in Fig. 2.

[Insert Figure 1 and Figure 2 about here]

Comparing Fig. 1 (B) and Fig. 1 (C) with Fig. 2 clearly demonstrates the significantly lower efficiency of the SPE cartridges in removing matrix components (Please note the different scaling of the y-axis in Fig. 1 and Fig. 2).

Method performance characteristics

HPLC system 1 was calibrated by injecting 100 µl of BPA standard solutions from 0.3 to 100.0 ng/ml, obtaining a good linearity ($r > 0.9998$, $n=9$) between BPA concentration and peak area. The limit of detection (LOD), calculated for a signal-to-noise ratio of 3, was 0.5 ng/ml.

The analytical performance of the whole analysis method was assessed by applying the standard addition method. Correlation coefficients, mean recoveries, standard deviations of the recovery and the LODs ($S/N=3$) and LOQs ($S/N=6$) are given in Table 1.

[Insert Table 1 about here]

Since the sample preparation procedure results in an analyte enrichment factor of five the whole analysis method offers a LOD and LOQ of 0.1 and 0.2 ng/ml, respectively.

BPA levels in wine samples

In order to get a first impression in the BPA concentrations found in commercially available wine, 59 wine samples were collected, analysed and the results listed together with information on factors which might influence the BPA concentrations. The data obtained are shown in Table 2 and Table 3.

[Insert Table 2 and 3 about here]

In two of ten samples taken directly from wine vats BPA concentrations were below the LOQ (see Table 2a). In seven samples BPA levels ranged from 0.2 to 0.5 ng/ml; only in one sample a significantly higher BPA level of 2.1 ng/ml was found. Mean and median were 0.50 ng/ml and 0.20 ng/ml, respectively. In six of

21 wine samples which were obtained from local vintagers but had already been filled into bottles the BPA concentrations were below the LOQ. In 15 wine samples BPA concentrations ranged from 0.2 ng/ml to 1.5 ng/ml, with a mean of 0.61 ng/ml and a median of 0.50 ng/ml.

In five of the 28 wine samples purchased at local markets the BPA concentration was below the LOQ. In 23 samples BPA levels were in the range from 0.2 to 1.6 ng/ml. The mean was 0.58 ng/ml, the median 0.40 ng/ml.

In general, BPA concentrations were very low. The mean and median for all wine samples with BPA concentrations above the LOQ were 0.58 and 0.40 ng/ml, respectively. In all samples BPA concentrations were far below the Specific Migration Level of 0.6 mg/kg established by the EU Commission in 2004.

The data obtained indicate that neither the material of the vat nor the storage time of the wine has an influence on the BPA concentration in the wine sample. Furthermore, no significant difference has been found between the BPA levels in white wine or red wine samples. In addition there was no relation between the alcohol concentration and the BPA level of the sample. Neither the packaging material (glass bottle or Tetra bri) nor the top of the glass bottle (plastic, metal or cork) had a measurable effect on the BPA level.

Migration of BPA from plastic stoppers.

In order to investigate if BPA can migrate from a plastic stopper four different plastic stoppers were stored in 50 ml centrifuge tubes submerged in 10 ml of ethanol-water (11:89, v/v). Table 4 gives the BPA amounts determined in the ethanolic solutions after storage periods from five to 11 weeks by injecting aliquots of the incubation solutions into HPLC system 1. Figure 3 shows the chromatogram of the ethanolic solution after incubation of stopper 4 for 11 weeks.

[Insert Figure 3 and Table 4 about here]

From two stoppers detectable amounts of BPA were released into the ethanolic solution. In order to confirm the identity of BPA one incubation solution was also injected into HPLC system 2 which was equipped with a coulometric electrode array detector. Figure 4 shows the chromatograms of (A) a BPA standard solution and (B) the incubation solution. Both chromatograms contained a peak with a retention time of 48 min, indicating the presence of BPA in the ethanolic solution. Figure 5 demonstrates the similarity of the current-voltage curves of the 48 min peaks, adding a further evidence for the identity of BPA peaks in the chromatogram of the ethanolic solution and thus migration of BPA from the plastic stopper.

[Insert Figure 4 and Figure 5 about here]

However, migration of BPA from plastic stoppers will only lead to insignificant contributions to the BPA concentrations. (The migration of 30 ng BPA from a plastic stopper into a wine sample contained in a 750 ml glass bottle would result in a concentration of about 40 pg BPA / ml wine which is far below the Specific Migration Level of 0.6 mg/kg established by the EU Commission).

BPA exposure due to wine consumption

Due to the lack of literature data on BPA concentrations in wine the results obtained in the present study cannot be compared with previously published ones. The data can, however, be used to reconsider the risk assessment obtained with the help of exposure models based on the results of experiments with wine simulants by Larroque et al. (1989). In their study three series of migration experiments were carried out by placing into contact wine simulants with inert materials coated with resins. From the highest BPA migration level obtained (100 mg BPA/kg resin in series 1) the worst-case BPA concentration in

wine was calculated to be 650 ng g^{-1} , assuming that the wine has been stored in a 1500 L vat lined with 10 kg resin. The BPA concentrations obtained in the present paper were far lower than this worst-case estimate. BPA risk assessment by the Scientific Committee on Food on BPA was also based on the migration study of Larroque et al. (1989). However, the SCF calculated a significantly lower BPA concentration of 9 ng g^{-1} by using a lower BPA migration level ($10 \text{ mg BPA/kg resin}$) and assuming that the 1500 L wine vat is just lined with 1.4 kg resin.

According to the results obtained in the present study even this lower migration level is an overestimation of the actual BPA concentrations in wine samples. Based on the highest BPA level found in the present study (2.1 ng/ml), BPA exposure due to wine consumption can be estimated to be lower than $26 \text{ ng/kg body weight/day}$, assuming a person with a body weight of 60 kg and a wine consumption of 0.75 L per day. This value is about 400 times lower than the Tolerable Daily Intake (TDI) for BPA of $0.01 \text{ mg/kg body weight/day}$ proposed by the SCF.

Conclusion

The highly selective analysis method presented allows the determination of traces of BPA in wine samples without the interference of disturbing matrix compounds. Its application to 59 wine samples revealed only very low BPA concentrations. In 13 of the 59 wine samples the BPA concentration was lower than the LOQ (0.2 ng/ml). The mean and median for all wine samples with BPA concentrations above the LOQ were 0.58 and 0.40 ng/ml , respectively. These values are far lower than the estimated BPA levels derived from migration experiments using wine simulants.

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We wish to thank Heidi Schwartz for carrying out the confirmation studies with the HPLC system equipped with the electrochemical detector We are also grateful to Georg Weingart for supplying wine samples from local vintagers together with data on their storage conditions. The present study would have been impossible without the support of Japan EnviroChemicals, Tokyo, Japan, who provided BPA antibodies.

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Figures:

Fig. 1: Chromatograms of a BPA standard solution (A) and two wine samples (B,C) obtained after sample preparation by affinity chromatography: (A) BPA standard solution (10 ng/ml in ACN-water, 10:90, v/v); (B) white wine (sample 33, Table 3); C) red wine (sample 55, Table 3). HPLC system 1, injection volume 100 μ l.

Fig. 2: Chromatograms obtained after sample preparation by solid phase extraction: (A) white wine (sample 33, Table 2), (B) red wine (sample 55, Table 2).

Injection volume 100 μ l, HPLC system 1

Fig. 3: Migration of BPA from a plastic stopper. Chromatogram obtained by injecting 100 μ l aliquots of the ethanolic incubation solution into HPLC system 1.

Fig. 4: Migration of BPA from a plastic stopper. Chromatograms obtained by injecting into HPLC system 2 100 μ l aliquots of (A) a BPA standard solution (50 ng/ml in mobile phase) and (B) the ethanolic incubation solution.

Fig. 5: Current-voltage curves of the BPA standard solution (triangle) and the ethanolic incubation solution (square).

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Table 1: Analytical data

Wine	Sample volume (ml)	Standard addition correlation coefficient r	Mean recovery $\pm$ SD (%) (n=3)	LOD (S/N=3)	LOQ (S/N=6)
White	25.0	0.9919	74 ± 11	0.1 ng/ml	0.2 ng/ml
Red	25.0	0.9995	81 ± 8	0.1 ng/ml	0.2 ng/ml

Table 2a: BPA concentrations in wine samples taken directly from the vat. n.d.: below the LOQ (0.2 ng/ml)

Wine sample	Wine	Storage time (months)			BPA concentration (ng/ml)
		Steel vat	Wood vat	Plastic vat	
1	White	10	-	-	0.2
2	White	10	-	-	0.2
3	White	6	2	3	0.4
4	White	8	2	-	0.5
5	White	9.5	-	-	0.2
6	White	10.5	-	-	2.1
7	White	-	5	5	0.2
8	White	9.5	-	-	n.d.
9	Red	-	10	-	n.d.
10	Red	-	8	0.25	0.2

Table 2b: BPA concentrations in wine samples already filled into glass bottles. n.k.: storage time not known; n.d.: below the LOQ (0.2 ng/ml).

Wine sample	Wine	Volume (L)	Alcohol (%)	Top of the glass bottle	Storage time (months)				BPA concentration (ng/ml)
					Steel vat	Wood vat	Plastic vat	Glass bottle	
11	White	0.75	12.5	Plastic	5	-	-	11	n.d.
12	White	0.75	12.5	Cork	5	-	-	11	n.d.
13	White	0.75	12.5	Cork	8	-	-	5	n.d.
14	White	0.75	12.5	Plastic	8	-	-	5	n.d.
15	White	0.75	13.0	Cork	7	-	-	8	1.0
16	White	1.00	11.5	Metal	8	-	-	5	0.2
17	White	0.75	12.5	Cork	5	-	-	8	1.2
18	White	0.75	13.5	Cork	6	-	-	6	0.5
19	White	0.75	13.5	Plastic	6	-	-	6	0.4
20	White	0.75	12.5	Plastic	5	-	-	10	n.d.
21	White	1.00	12.0	Metal	3	2	-	9	0.6
22	White	1.00	11.5	Cork	-	3.5	3.5	7	0.6
23	White	1.00	11.5	Cork	n.k.	-	-	n.k.	0.5
24	White	0.75	12.0	Cork	n.k.	-	-	n.k.	1.5
25	White	0.75	13.5	Plastic	n.k.	-	-	n.k.	0.3
26	White	0.5	12.5	Plastic	n.k.	-	-	n.k.	0.2
27	White	1.00	12.5	Cork	-	-	n.k.	n.k.	0.3
28	Red	0.75	12.5	Cork	-	11	-	2	n.d.
29	Red	0.75	13.0	Cork	-	n.k.	0.25	n.k.	0.7
30	Red	1.00	11.5	Metal	9	-	-	6	0.8
31	Red	0.75	13.0	Plastic	-	n.k.	-	n.k.	0.4

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Table 3: BPA concentrations in wine samples bought in local markets. n.d.: below the LOQ (0.2 ng/ml).

Wine sample	Wine	Container	Volume (L)	Alcohol (%)	Top	BPA concentration (ng/ml)
32	White	Glass	1.00	12.5	Plastic	1.6
33	White	Glass	0.75	12.0	Plastic	0.2
34	White	Glass	0.25	11.5	Metal	0.3
35	White	Glass	0.25	11.5	Metal	0.3
36	White	Glass	0.38	12.5	Metal	0.3
37	White	Glass	0.75	11.5	Plastic	0.4
38	White	Glass	0.75	12.5	Metal	n.d.
39	White	Glass	0.75	12.0	Cork	0.3
40	White	Glass	0.75	11.5	Plastic	0.8
41	White	Glass	1.00	12.5	Cork	1.2
42	White	Tetra Brik	1.00	10.0	-	0.2
43	White	Tetra Brik	1.50	11.0	-	0.2
44	White	Tetra Brik	1.00	12.0	-	0.8
45	White	Tetra Brik	1.00	10.0	-	0.6
46	White	Tetra Brik	1.50	11.5	-	0.4
47	White	Tetra Brik	1.00	11.5	-	0.4
48	White	Tetra Brik	1.00	11.0	-	1.0
49	White	Tetra Brik	1.00	11.5	-	0.6
50	White	Tetra Brik	1.00	11.0	-	0.3
51	White	Tetra Brik	1.00	11.5	-	n.d.
52	White	Tetra Brik	1.00	11.5	-	0.4
53	Red	Glass	0.75	12.5	Cork	n.d.
54	Red	Glass	0.75	12.0	Plastic	0.5
55	Red	Glass	1.00	12.0	Metal	0.6
56	Red	Glass	0.75	13.0	Cork	n.d.
57	Red	Glass	0.75	13.5	Cork	1.2
58	Red	Glass	0.75	12.5	Cork	0.7
59	Red	Glass	0.75	12.0	Metal	n.d.

Table 4: Migration of BPA from plastic stoppers. n.d.: below the LOD (0.5 ng/ml).

Stopper	Incubation period (weeks)	Mass of BPA migrated from the stopper (ng)
1	6	32
2	5	n.d.
3	11	n.d.
4	11	31

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