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Sample preparation including sol–gel immunoaffinity chromatography for determination of bisphenol A in canned beverages, fruits and vegetables

R. Braunrath, M. Cichna*

Institute of Analytical Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria

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Abstract

The paper describes the development of a very simple method to prepare samples of canned food (beverages, fruits and vegetables) for the determination of bisphenol A by isocratic HPLC with fluorescence detection. The new sample preparation method makes use of the selectivity of bisphenol A antibodies immobilized in a silica matrix by an inexpensive and simple sol–gel technique. In spite of applying highly complex food matrices, immunoaffinity columns could be used for clean-up of at least 15 real samples. Limits of detection ($S/N = 3$) ranged from 0.1 ng/ml for beverages to 4.3 ng/g for vegetables.

Keywords: Sol–gel immunoaffinity chromatography; Bisphenol A; Canned food; Sample pretreatment; Food analysis

1. Introduction

A number of both naturally occurring and anthropogenic substances are able to disrupt the endocrine system. These so-called endocrine disruptors can interfere by either mimicking, modulating or antagonizing the effects of the natural hormone, 17ß-estradiol. The estrogenic activity of bisphenol A [2,2′-bis(4-hydroxyphenyl)propane, BPA) has been reported for the first time by Krishnan et al. in 1993 [1]. During the past decade the estrogenic effects of BPA have been further investigated in both in vitro and in vivo experiments [2–7]. Some of these studies indicate that BPA can show estrogenic effects at concentrations far below those being tested in toxicological studies [2,5–7].

It is very likely that humans are exposed to low doses of BPA since it is an important monomer in the production of polycarbonates and epoxy resins and is also applied as an antioxidant in polyvinyl chloride (PVC) plastics. Epoxy resins are used as protective coatings in metal food and beverage cans. Several studies have shown that traces of non-reacted BPA can be leached from the epoxy lining and migrate into food. Low levels of BPA have already been detected in infant formula concentrates [8], canned vegetables [9–11], fruits [10,11], desserts, fish, meat products and soups [11]. Due to differences in sampling, however, data published from different research groups can hardly be compared. Brotons et al. [8] were only interested in the BPA levels of the liquids in the cans. In contrast, Goodson et al. [11] usually carried out their analysis by homogenizing the whole content of a can. In vegetable cans BPA concentrations were in the range from 9 to 48 ng/g whereas in beverages BPA levels were below the limit of detection (2 ng/g). Yoshida et al. [10] who separately analysed the aqueous and the solid portions of vegetable and fruit cans detected BPA mainly in the solid parts with a maximum level of 95 ng/g.

In several studies experiments have been carried out with food simulants in order to investigate the process of BPA leakage from can coatings [8,12–13].

In canned foodstuffs BPA is usually quantified by high-performance liquid chromatography (HPLC) in combination with either UV [8,10] or fluorescence detection [9] or by gas chromatography coupled to mass spectrometry (GC–MS) [11]. Due to the complexity of food matrices extensive

* Corresponding author. Tel.: +43 1 4277 52374; fax: +43 1 4277 9523.
E-mail address: margit.cichna@univie.ac.at (M. Cichna).

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clean-up steps have to be carried out before the sample can be injected into the chromatographic system. Up to now, liquid–liquid extraction and solid–phase extraction (SPE) are the most common sample preparation steps applied [9,10].

To our knowledge immunoaffinity chromatography, which has proved to be a highly efficient method for removing interfering matrix components contained in complex food samples [14–21], has never been included in a sample pretreatment scheme for determination of BPA in food.

There is, however, a recent paper which describes the use of immunoaffinity columns in a sample preparation scheme for the determination of BPA in serum [22]. These columns had been prepared by a rather complicated, time consuming and costly method consisting of covalently coupling polyclonal antibodies to CNBr-activated Sepharose 4B. This immobilisation method can, however, be replaced by the much simpler, less costly and more versatile method to immobilise antibodies by inclusion in the pores of a sol–gel generated silica matrix [19,23–28].

The current paper presents a simple analysis method allowing the determination of low levels of BPA in complex food matrices like canned beverages, fruits and vegetables. The method is based on eliminating interfering matrix compounds by sol–gel immunoaffinity chromatography followed by determination of BPA by HPLC with fluorescence detection.

2. Experimental

2.1. Reagents and materials

Purified polyclonal bisphenol A antibodies (5 mg/240 μl phosphate-buffered saline (PBS) buffer), bisphenol E, 2,2′-bis(4-hydroxyphenyl)1-propanol and BPA dimethacrylate were gifts from Japan EnviroChemicals (Tokyo, Japan). Human immunoglobulin G (IgG), bisphenol A (BPA), ∼98%, and bis(4-hydroxyphenyl)methane were obtained from Sigma (St. Louis, MO, USA). 2,2-Bis(4-hydroxyphenyl)butane and BPA diacetate were delivered from TCI (Zwijndrecht, Belgium). Acetonitrile (ACN) and methanol, both gradient grade for HPLC, were purchased from Fisher Scientific (Leicestershire, UK). Tetramethoxysilane (TMOS) was from Fluka (Buchs, Switzerland).

Five millilitre polypropylene (PP) centrifuge tubes and 20 ml scintillation vials (PP) were from Bibby Sterilin (Staffordshire, UK). 15 ml glass centrifuge tubes, polyethylene (PE) frits (13 mm diameter) and polytetrafluorethylene (PTFE) frits (9 mm diameter) were obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation

2.2.1. Immunoaffinity columns and C_{18} cartridges

Immunoaffinity columns and C_{18} cartridges were operated using a 16-port Waters Sep-Pak SPE station (Milford, USA). Food samples were homogenized using an Ultra-Turrax mixer. Centrifugation was carried out with a Sigma centrifuge (Model 4 K 10, Vienna, Austria).

2.2.2. HPLC instrument and detectors

The modular liquid chromatograph used consisted of a high pressure gradient pump (Model L-7100, Merck), a column thermostat (Model bfo-04 dt, W.O. electronics, Langenzersdorf, Austria) and a six-port injection valve (Model 7161, Rheodyne) equipped with a 100 μl stainless steel injection loop. Two fluorescence detectors Model 1080 (Merck) were used. At the beginning of the study detection was carried out with detector 1. Due to apparatus problems it was replaced by another detector of the same type (detector 2). Since the two detectors were used at different parameter settings different absolute signal intensities were obtained. The signal-to-noise ratio, however, was the same. Fluorescence detection was carried out at 275/305 nm. Bisphenol A diacetate was detected with a UV detector (Model L-4000, Merck). Peaks were integrated using the McDacq software (Bischoff, Leonberg, Germany).

2.3. Standard solutions and buffers

A stock solution of BPA was prepared by dissolving 20.0 mg of BPA in 20.0 ml acetonitrile (ACN). Working solutions of BPA were prepared by diluting the stock solution with mobile phase. All BPA standard solutions were stored at 4 °C.

Phosphate-buffered saline (PBS), pH 7.6, was prepared by dissolving 12.46 g $Na_2HPO_4 \times 2H_2O$, 1.56 g $NaH_2PO_4{\cdot}2H_2O$ and 8.5 g NaCl in 1 l bidistilled water.

2.4. Experiments for the selection of laboratory material

A number of experiments had to be carried out to avoid systematic errors by adsorption of BPA on exposed surfaces or leaching from laboratory materials.

2.4.1. Selection of centrifuge tubes and scintillation vials

The laboratory containers used (5 ml centrifuge tubes and 20 ml scintillation vials, both consisting of polypropylene, and—for comparison—15 ml glass centrifuge tubes) were filled with either PBS buffer, ACN–water (40:60, v/v), BPA (20.0 ng/ml) in PBS or BPA (20.0 ng/ml) in ACN–water (40:60, v/v). After incubation at room temperature (experimental details are given in Table 1) the concentration of BPA in the test solution was determined by injecting an aliquot into the HPLC system.

2.4.2. Selection of column frits

2.4.2.1. Adsorption experiments. In a first test PE frits (13 mm diameter) or PTFE frits (9 mm diameter) were put into scintillation vials filled with 2 ml of either 5.0 ng BPA/ml

Table 1
Adsorption and leakage of BPA to/from plastic and glass containers

Container	Material	Test solution	Volume of test solution (ml)	Incubation time (h)	Adsorption/leakage of BPA
Adsorption experiments					
5 ml Centrifuge tube	PP	BPA in PBS	4	42	n.d.
		BPA in ACN–water (40:60, v/v)	4	21	n.d.
20 ml Scintillation vial	PP	BPA in PBS	4	42	n.d.
		BPA in ACN–water (40:60, v/v)	4	21	n.d.
15 ml Centrifuge tube	Glass	BPA in PBS	4	42	n.d.
		BPA in ACN–water (40:60, v/v)	4	21	n.d.
Leakage experiments					
5 ml Centrifuge tube	PP	PBS	4	18	n.d.
		ACN–water (40:60, v/v)	4	18	n.d.
20 ml Scintillation vial	PP	PBS	4	22	n.d.
		ACN–water (40:60, v/v)	4	22	n.d.
15 ml Centrifuge tube	Glass	PBS	4	17	n.d.
		ACN–water (40:60, v/v)	4	17	n.d.

Concentration of the BPA test solutions used in adsorption experiments: 20 ng/ml. PP: Polypropylene; n.d.: not detectable.

PBS or 5.0 ng BPA/ml ACN–water (10:90, v/v). After incubation at room temperature (see Table 2) aliquots of the test solutions were injected into the HPLC system.

PE frits which had reduced the BPA concentration in the first test were transferred into a scintillation vial filled with 2 ml of the strong eluent (ACN–water, 40:60, v/v). After 45 min an aliquot of the supernatant solution was injected into the HPLC system to determine the amount of BPA desorbed.

2.4.2.2. Leakage experiments. PTFE frits were transferred to scintillation vials filled with 7 ml of either PBS or ACN–water (40:60, v/v). After an incubation time of 16 h aliquots of the supernatant solutions were injected into HPLC.

2.5. Preparation of sol–gel columns

2.5.1. Immunoaffinity columns

A mixture of 0.2 ml of a 0.04 M aqueous hydrochloric acid, 0.75 ml bidistilled water and 3.4 ml TMOS was sonicated under ice-cooling for 30 min. In a pre-weighed beaker 48 μl of the BPA antibody solution (5 mg/240 μl PBS buffer) were diluted with PBS to 1 ml and stored on ice. A 1 ml aliquot of the pre-hydrolyzed TMOS was added to the cold antibody solution. After gelation the beaker was weighed and kept at room temperature during the aging of the gel. The aging process was stopped when the gel had lost 50% of its initial mass. Then the silicate glass (about 1 g) was manually ground in an achate mortar and—without sieving—packed into a 3 ml glass column (Merck) equipped with a PTFE frit. The resulting immunoaffinity column was washed with 15 ml of ACN–water (40:60, v/v), followed by 20 ml of PBS. The columns were stored in PBS at 4 °C.

2.5.2. Sol–gel pre-columns

0.5 g amounts of pure sol–gel glass (prepared as described above, but without adding antibodies) were packed into 8 ml glass columns (Merck) equipped with glass microfibre filters GF/F from Whatman (Kent, UK). Columns were flushed with about 20 ml of PBS and stored in PBS at 4 °C. Before using a pre-column it was conditioned with 10 ml of ACN–bidistilled water (10:90, v/v). After a single use the pre-columns were discarded.

Table 2
Adsorption and leakage of BPA to/from frits

Frit material	Diameter (mm)	Incubation time (min)	Test solution	Adsorption/leakage of BPA
Adsorption experiments				
PE	13	45	BPA in PBS	Approx. 30%
		45	BPA in ACN–water (10:90, v/v)	n.d.
PTFE	9	30	BPA in PBS	n.d.
		30	BPA in ACN–water (10:90, v/v)	n.d.
Leakage experiments				
PTFE	9	16	PBS	n.d.
		16	ACN–water (40:60, v/v)	n.d.

Adsorption experiments: Concentration of the BPA test solutions used in adsorption experiments: 5 ng/ml. n.d.: not detectable.

2.6. Solid–phase extraction

Solid phase extraction experiments were carried out using 3 ml C_{18} columns packed with 500 mg Isolute (International Sorbent Technology, Mid Glamorgan, UK). C_{18} columns were pre-conditioned with 6 ml of ACN, followed by 6 ml of ACN–water (10:90, v/v). Samples were applied at a flow-rate of approximately 1 ml/min. After washing the cartridge 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. Since injection of BPA solutions with ACN concentrations > 40% into the HPLC system did not yield peaks with Gaussian peak shape the eluate had to be diluted with water. In order to obtain a definite volume the eluate was evaporated under a gentle nitrogen stream to about 1 ml before filling up the flask with bidistilled water to the mark. A100 μl aliquot was injected into the HPLC system.

2.7. HPLC separation and detection

2.7.1. Phase system

The analytical column was a Spherisorb S ODS1, 250 mm × 4.6 mm i.d., 5 μm (Knauer, Berlin, Germany) operated with a mobile phase consisting of 50 mM sodium acetate buffer (adjusted to pH of 4.8 with acetic acid) – ACN (60:40, v/v). All separations were carried out at 25 °C applying a flow-rate of 1 ml/min.

2.7.2. External calibration

The HPLC system was calibrated by injecting six standard solutions in the concentration range from 0.5 to 100 ng/ml in mobile phase. The analysis function was obtained by linear regression of peak areas on standard concentrations.

2.8. Analysis methods for real samples

Canned beverages [soft drinks (orange and lemon), energy drinks and beer], fruits (pineapple, peaches and lychees) and vegetables (peas, corn and haricot beans) were bought in local supermarkets. Sealed samples were stored at room temperature.

2.8.1. Sample preparation of beverages

Beverages were transferred into a beaker and degassed in an ultrasonic bath. An aliquot of 15.0 ml was diluted 1:1 with PBS buffer. If necessary, 1 M NaOH was added to adjust the pH to 7.0. Then the sample was applied to the immunoaffinity column. After washing the column with 5 ml of ACN–water (10:90, v/v) elution was carried out 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 ACN–water (40:60, v/v). The column was regenerated with 20 ml of PBS.

2.8.2. Sample preparation of fruits and vegetables

For the analysis of fruits the whole content of the can was homogenised. In the case of vegetables the liquid part was removed by sieving and only the solid portion was homogenized.

One gram of the homogenised sample was filled in a centrifuge tube and after spiking with a known amount of BPA (see Section 2.8.3) shaken vigorously on a vortex mixer for 30 s. 1 ml of ACN was added and the mixture was shaken for 1 min. After centrifugation at 2800 × *g* for 5 min the supernatant was removed with a Pasteur pipet. The solid residue was extracted for a second time. The combined extracts were filtrated through a glass microfibre filter GF/F (Whatman). The filtrate was diluted 1:10 with water. A PTFE suction tube was used to apply the sample to a sol–gel pre-column which was on-line coupled with the immunoaffinity column. Sol–gel pre-columns were used as filter columns. They prevented small particles originating in the sample extracts from clogging the immunoaffinity columns. After the sample solution left the pre-column the column was discarded. After removing sample components adsorbed in the immunoaffinity column by washing with 5 ml of ACN–water (10:90, v/v) the analyte was eluted 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 ACN–water (40:60, v/v). The analytical column was regenerated with 20 ml of PBS.

2.8.3. Standard addition

Samples were spiked in the range from 10 to 30 ng of BPA. One unspiked and three spiked samples were analysed as described above. Peak areas were plotted against the amount of analyte added. The recovery was determined by dividing the slope of the linear regression line for the standard addition by the slope of the linear regression line of standard solutions.

3. Results and discussion

3.1. Selection of laboratory materials for the analysis of BPA

In our laboratory many sample preparation steps are carried out using plastic containers which had to be tested with regard to their adsorption and BPA leakage properties under the experimental conditions. PBS and ACN–water (40:60, v/v) were chosen as test media since both play an important role in the operation of our immunoaffinity columns. ACN–water (40:60, v/v) is used for elution of the analyte from the immunoafftnity column, PBS for regeneration of the columns. For comparison 15 ml glass centrifuge tubes were also subjected to the same test. At the end of the incubation time (17–42 h) aliquots of the test media were injected into the HPLC system.

The results (see Table 1) indicated that both the PP centrifuge tubes and the PP scintillation vials could be used for sample pre-treatment without causing systematic errors in the determination of BPA.

3.2. Preparation and characterisation of the immunoaffinity column

3.2.1. Selection of frits

In our previous studies [19,23–24] sol–gel immunoaffinity columns and pre-columns had been equipped with thin glass microfibre frits and operated by a low-pressure pump. This was changed for the present paper. In order to increase the sample throughput the pre-column was on-line coupled to the immunoaffinity column and the column combination operated by a SPE station.

Preliminary experiments had, however, indicated that the thin glass microfibre frits were whirled up by the liquid phase stream. It was therefore investigated if glass frits could be replaced by other frits being immediately available in our laboratory, e.g. frits consisting of PE (13 mm diameter) for the pre-columns and PTFE frits (9 mm diameter) for the immunoaffinity columns without causing systematic errors by BPA adsorption or leakage from the frits.

The results obtained for different incubation periods and solutions are summarised in Table 2. Incubation of a PE frit in the purely aqueous standard solution (BPA in PBS) resulted in a decrease of the BPA concentration to 72% of the original concentration. The amount missing had been adsorbed to the frit since it could quantitatively be recovered by desorption with ACN–water (40:60, v/v). However, since the pre-columns served as single-use columns whirling up of the microfibre filters could be kept to a minimum and thus did not pose a big problem. We therefore continued to equip the pre-columns with glass microfibre frits.

On the other hand, PTFE frits could replace glass fibre frits in immunoaffinity columns, since neither BPA adsorption nor leakage of BPA from PTFE frits were observed.

3.2.2. Retention mechanism

In order to elucidate the mechanism of BPA retention breakthrough curves obtained with three different sol–gel columns were compared. The columns were identically synthesized but one column contained 1 mg of BPA antibodies, the second 1 mg of “non-specific” human immunoglobulins and the last one consisted only of pure sol–gel matrix. A BPA standard solution (20 ng/ml) in PBS was applied to each column while collecting the eluate in 1 ml fractions. The breakthrough curves obtained with the columns not containing BPA antibodies were almost identical. In both cases BPA was already detected in the second eluate fraction. In contrast, BPA was strongly retained in the column having entrapped BPA antibodies. Breakthrough of BPA was not observed before having applied 200 ng of BPA. These data indicate that retention of BPA in the immunoaffinity columns occurs due to specific interactions with the BPA antibodies.

3.2.3. Loading medium

Since sample preparation of solid samples usually includes extraction steps with organic solvents, e.g. methanol or acetonitrile, it was investigated if small amounts of organic solvents in the sample feed solution would already lower the recovery of BPA. 5 ml of a BPA standard solution (2 ng/ml) in either PBS buffer or ACN–water (10:90, v/v) were applied to a column at a flow-rate of 1.0 ml/min. After washing the column with 5 ml of either PBS buffer or ACN–water (10:90, v/v) BPA was eluted with 4 ml of ACN–water (40:60, v/v). The experiments showed that using a loading medium consisting of ACN–water (10:90, v/v) did not effect the recovery of BPA.

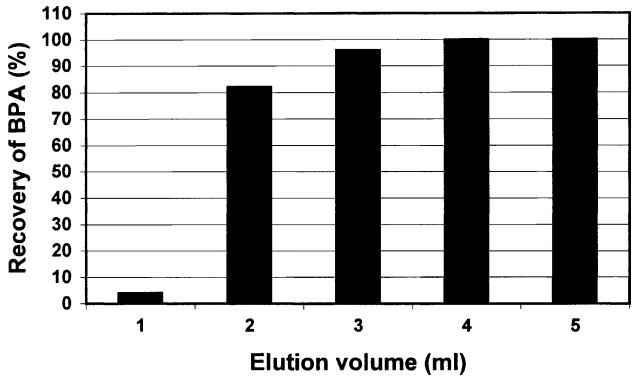

Fig. 1. Influence of elution volume on recovery of BPA. Feeding solution: 12 ml of BPA (1 ng/ml) in ACN–water (10:90, v/v). Washing solution: 5 ml of ACN–water (10:90, v/v). Elution medium: ACN–water (40:60, v/v).

3.2.4. Conditioning of new immunoaffinity columns

Before new immunoaffinity columns could be used for retention of BPA they had to be flushed with 15 ml of the eluent ACN–water (40:60, v/v) (see Section 2.5.1) to remove a substance interfering with the chromatographic determination of BPA.

3.2.5. Elution conditions

Previous investigations showed that an ACN–water (40:60, v/v) mixture allows the quantitative elution of different analytes from sol–gel immunoaffinity columns without causing irreversible denaturation of the antibodies [19,23–24]. Quantitative recovery of the analytes could be achieved by flushing the columns with volumes from 3 to 8 ml of ACN–water (40:60, v/v), depending on the affinity constants of the antibodies entrapped in the sol–gel matrix.

A series of experiments was carried out in order to investigate the elution conditions for BPA. After loading the immunoaffinity columns with 12 ml of BPA standard solutions (1 ng/ml) in ACN–water (10:90, v/v) the columns were washed with 5 ml of ACN–water (10:90, v/v) and eluted with 1 to 5 ml of ACN–water (40:60, v/v). Fig. 1 shows that eluting the columns with 1, 2, and 3 ml of 40% ACN resulted in recoveries of 4, 82 and 96%, respectively. Quantitative recovery of BPA was achieved with $\geq$4 ml of ACN–water (40:60, v/v).

3.2.6. Binding capacity of the affinity columns

The binding capacity was determined by overloading the immunoaffinity columns with 10 ml of a BPA standard

solution (100 ng/ml) in ACN–water (10:90, v/v) using a flow-rate of 1 ml/min. After washing the columns with 5 ml of ACN–water (10:90, v/v), BPA was eluted with 4 ml of ACN–water (40:60, v/v). Columns having entrapped 1 mg of antibodies showed a binding capacity of 280 ng BPA.

3.2.7. Recovery of BPA

In order to determine the recovery of BPA a newly prepared immunoaffinity column was repeatedly loaded with 15.0 ml of a BPA standard solution (5.3 ng BPA/ml) in ACN–water (10:90, v/v). After washing the column with 5 ml of ACN–water (10:90, v/v), elution was carried out with 4 ml of ACN–water (40:60, v/v). Recovery of BPA was 98% with a standard deviation of 3% ($n = 5$). In order to investigate if BPA recovery is influenced by either the volume or the concentration of the BPA standard solution applied, the same column was repeatedly loaded with a total BPA amount of 30.0 ng by varying feeding volume and feeding concentration. Recovery was maintained at almost 100% when either 5, 10, 15 or 30 ml of BPA standard solutions containing 6, 3, 2 and 1 ng BPA/ml, respectively, were applied to the column. An influence of feeding volume or feeding concentration on the recovery was not observed. Since quantitative elution of BPA from the immunoaffinity column could be achieved with an elution volume of 5 ml an enrichment factor of 6 could be achieved by applying 30.0 ml of a BPA containing solution.

In order to determine batch to batch reproducibility immunoaffinity columns packed with sol–gel glasses from different batches were subjected to recovery tests. Standard deviation of recovery obtained with columns from different batches was as low as observed for repeatedly loading one and the same column. These results indicate that both preparation and operation of sol–gel immunoaffinity columns can be carried out highly reproducibly.

3.2.8. Cross-reactivity

The selectivity of immunoaffinity columns is limited by the retention of substances showing cross-reactivity with the antibodies immobilized. In Table 3 cross-reactivity data, determined in a competitive enzyme-linked immunosorbent assay (ELISA) by the supplier of the antibodies, are summarized. Cross-reactivities for substances not listed in the table [1,2-bis(4-hydroxyphenyl)-2-propanol, 4,4′-bis(4-hydroxyphenyl)pentanoic acid, 4,4′-dihydroxydiphenyl ether, 4,4′-dihydroxybenzophenone, bisphenol S, bis(4-(2-hydroxyethoxy)phenyl)sulfone, bisphenol A diglycidyl ether and BPX-33] were below 0.4%. In contrast to the competitive ELISAs where the amount of antibodies is limited, however, immunoaffinity columns offer an excess of antibodies. For this reason, ELISA cross-reactivity data cannot be used for quantitative prediction of cross-reactivities in immunoaffinity columns.

Substances showing cross-reactivity >1% in the ELISA were therefore subjected to retention tests in immunoaffinity columns. 500 ng of the test substance (5 ml of a 100 ng/ml solution in PBS) were loaded to the column. After washing the column with 5 ml of ACN–water (10:90, v/v) the test compound was eluted with 5 ml of ACN–water (40:60, v/v). Its concentration in the eluate was determined by injecting an aliquot into the HPLC system. With the exception of 2,2′-bis(4-hydroxyphenyl)-1-propanol cross-reactivities in the immunoaffinity column were significantly higher than cross-reactivities determined in ELISA experiments (see Table 3). Since BPA diacetate and BPA dimethacrylate do not show fluorescence, a UV detector was coupled to the HPLC system to determine their concentration in the eluates. The sensitivity of the UV detector was, however, not sufficient to enable detection of such low concentrations. For these two compounds therefore only cross-reactivity data obtained by the ELISA are given.

Table 3
Cross-reactivity and HPLC retention data of substances structurally similar to BPA

Substance	Cross-reactivity (%)		t_R (min)
	ELISA	Immunoaffinity column	
BPA	100	100	10.8
2,2′-Bis(4-hydroxyphenyl)butane	22.5	81.7	14.9
BPA diacetate	11.1	n.d.[a]	>40
2,2′-Bis(4-hydroxyphenyl)-1-propanol	9.3	8.6	7.1
Bisphenol E	6.6	28.8	>30
BPA dimethacrylate	2.7	n.d.[a]	>40
Bis(4-hydroxyphenyl)methane	1.0	5.0	7.4

Mobile phase: 50 mM sodium acetate buffer, pH 4.8–ACN (40:60, v/v). Flow-rate 1 ml/min.

[a] Since BPA diacetate und BPA dimethacrylate did not show fluorescence their HPLC retention times were determined by using an UV detector. Its sensitivity was, however, not sufficient to enable assessing their cross-reactivities.

Cross-reacting substances, however, do not pose a problem when they can be separated from the analyte by the chromatographic conditions applied. The isocratic elution conditions outlined in Section 2.7.1 made it possible to separate BPA from all cross-reacting substances tested (see Table 3). By both injecting higher concentrations and using the UV detector the retention times of BPA diacetate and BPA dimethacrylate could be determined too. (Standard solutions prepared by dissolving these substances in PBS were highly unstable. Even when stored at 4 °C for only one day BPA diacetate and BPA dimethacrylate where already completely degraded to BPA).

3.3. Solid–phase extraction

Solid phase extraction is a frequently applied clean-up step in BPA analysis [10,29–32]. To compare the efficiency of removing interfering matrix compounds by either immunoaffinity columns or C_{18} cartridges a number of food samples were analysed twice by applying solid phase extraction instead of immunoaffinity chromatography.

In preliminary experiments the optimum washing conditions for C_{18} cartridges had to be found. Four pre-conditioned

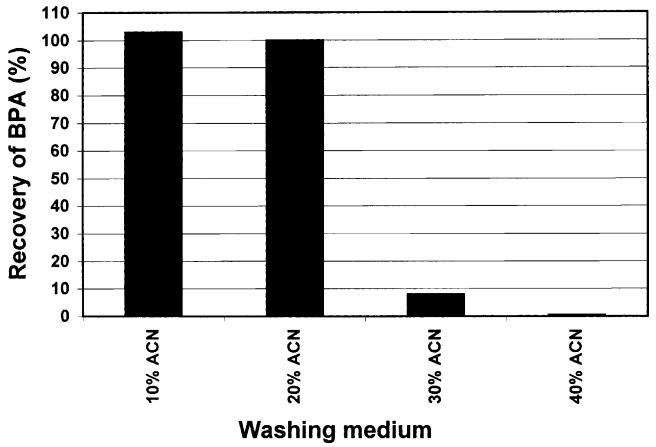

Fig. 2. Influence of washing medium on recovery of BPA from C_{18} cartridges. Feeding solution: 5 ml of BPA (5 ng/ml) in ACN–water (10:90, v/v). Volume of washing solution: 10 ml. Elution medium: 4 ml of ACN.

C_{18} cartridges were loaded with 5 ml of a BPA solution (5 ng/ml) in ACN–water (10:90, v/v). The loaded columns were washed with 10 ml of either 10, 20, 30 or 40% ACN in water. After eluting BPA and diluting the eluate with water as described in Section 2.6. recoveries of BPA were determined by HPLC. The results indicated that both washing with 10 ml of ACN–water (10:90, v/v) and ACN–water (20:80) yielded quantitative recovery of BPA (see Fig. 2). Washing the C_{18} cartridge with ACN–water (30:70, v/v), however, resulted in a recovery of only 8%. Thus, after sample application C_{18} cartridges were washed with 10 ml of ACN–water (20:80, v/v).

3.4. Determination of BPA in real samples

In order to investigate the potential of sol–gel immunoaffinity columns for removing interfering matrix components an analysis method including immunoaffinity chromatography was developed for the determination of BPA in canned beverages, fruits and vegetables.

3.4.1. Sample preparation

3.4.1.1. Beverages. Sample preparation procedure developed for determination of BPA in beverages needed just a few steps. In order to enable binding of BPA to the antibodies beverages were diluted with PBS buffer. If necessary pH value was adjusted to pH 7.0. After these simple steps the samples were applied to the immunoaffinity columns (see Section 2.8.1). Aliquots of the eluates were injected into the HPLC system.

The efficiency of the sample preparation method developed is illustrated by the chromatograms in Fig. 3. Fig. 3a shows a typical chromatogram obtained by directly injecting a BPA standard solution. Direct injection of an orange soft drink sample diluted 1:1 with PBS led to the chromatogram shown in Fig. 3b. Fig. 3c and d were obtained by injecting

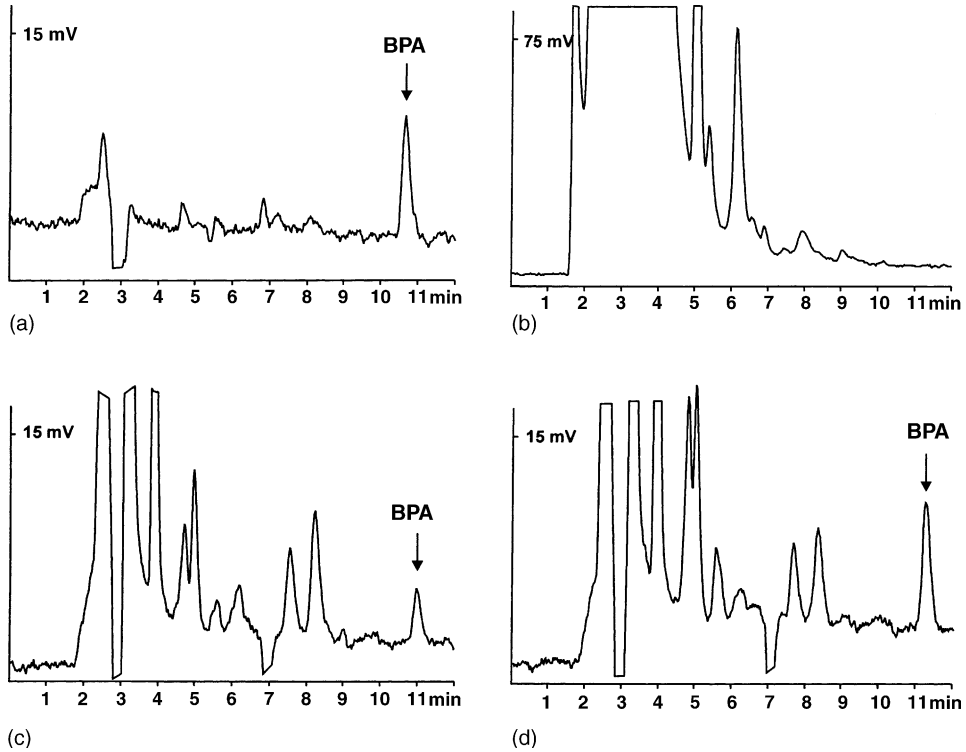

Fig. 3. Chromatograms obtained by (a) directly injecting a BPA standard solution (2 ng/ml in ACN–water (10:90, v/v), (b) directly injecting an orange soft drink diluted with PBS 1:1, (c) injecting the unspiked orange soft drink purified by immunoaffinity chromatography and (d) the orange soft drink, spiked with 10 ng of BPA, purified by immunoaffinity chromatography. HPLC injection volume in all cases: 100 μl. Note that purification by immunoaffinity chromatography resulted in the enrichment of BPA by a factor of 3 (volume of the soft drink used: 15 ml, volume of the eluates: 5 ml).

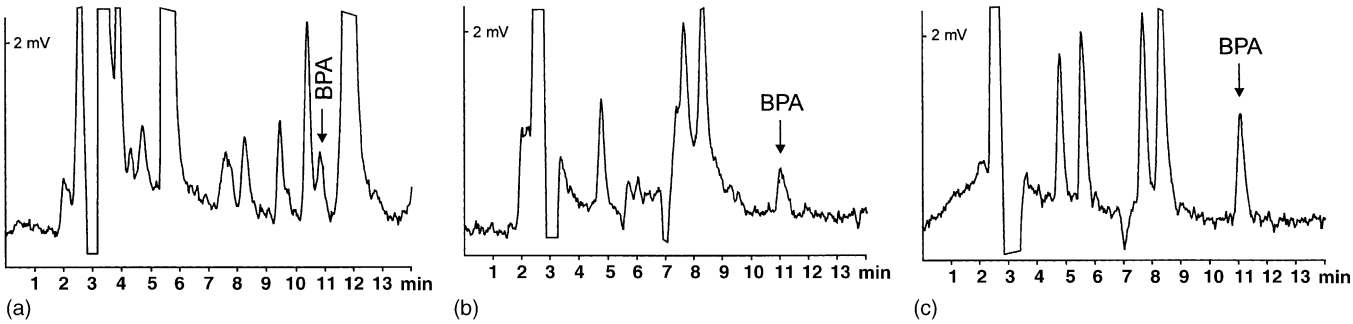

Fig. 4. Chromatograms obtained by (a) injecting an unspiked peach extract purified by SPE, (b) injecting an unspiked peach extract purified by immunoaffinity chromatography and (c) injecting the peach extract, spiked with 10 ng of BPA, purified by immunoaffinity chromatography. HPLC injection volume in all cases: 100 μl. Note that purification resulted in the dilution of BPA by a factor of 5 (amount of sample used: 1 g, volume of the eluates: 5 ml).

an orange soft drink purified by immunoaffinity chromatography. Fig. 3c is the chromatogram of the unspiked sample, Fig. 3d that of the soft drink spiked with 10 ng of BPA.

3.4.1.2. Fruits and vegetables. Fruits and vegetables were extracted twice with ACN. After filtration and dilution 1:10 with water the extract was loaded to the immunoaffinity column. Aliquots of the eluates were injected into the HPLC system.

Fig. 4 shows the chromatograms of peach extracts. The chromatogram in Fig. 4a was obtained by applying the sample preparation method but replacing immunoaffinity chromatography by SPE. Fig. 4b and c are chromatograms from samples prepared by using immunoaffinity columns. Fig. 4b is the chromatogram of an unspiked peach sample and Fig. 4c the chromatogram of the same sample spiked with 10 ng of BPA. Fig. 4a shows that samples prepared by SPE instead of immunoaffinity columns yield chromatograms where the BPA peak is overlapped by peaks of matrix components while the inclusion of immunoaffinity columns results in a baseline separation of the analyte peak from other peaks.

Fig. 5 presents chromatograms of pea extracts obtained by direct injection (Fig. 5a), after SPE (Fig. 5b) and after immunoaffinity chromatography (Fig. 5c and d). The chromatograms clearly illustrate the increase in selectivity resulting from including the immunoaffinity column in the clean-up procedure.

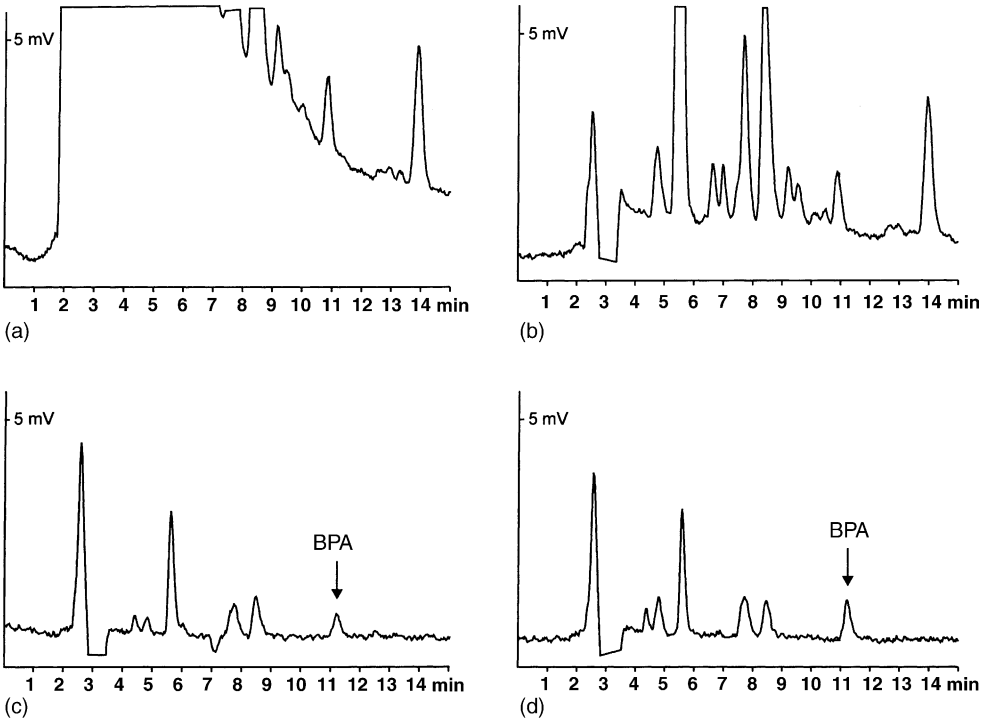

Fig. 5. Chromatograms obtained by (a) directly injecting an unspiked pea extract, (b) injecting the unspiked pea extract purified by SPE, (c) injecting the unspiked pea extract purified by immunoaffinity chromatography and (d) injecting the pea extract, spiked with 10 ng of BPA, purified by immunoaffinity chromatography. HPLC injection volumes: 100 μl. Note that BPA extraction and purification resulted in the dilution of BPA by a factor of 5 (amount of sample used: 1 g, volume of the extract: 5 ml (a), volume of the eluates: 5 ml).

Table 4
Characteristics of the analysis method

	Sample amount	Concentration of BPA ± SD	Correlation coefficient r	Mean recovery ± SD (%)[a]	Limit of detection ($S/N = 3$)
Orange soft drink	15.0 ml	0.4 ± 0.1 ng/ml	0.9959	63 ± 4.3	0.1 ng/ml
Peaches	1.0 g	6.4 ± 0.7 ng/g	0.9991	75 ± 8.2	1.1 ng/g
Pea, solid portion	1.0 g	8.5 ± 2.0 ng/g	0.9889	53 ± 6.9	1.6 ng/g

[a] From three recovery experiments at differing spiking levels.

3.4.2. Quantitative determination of BPA

The HPLC system was calibrated by injecting six standard solutions in the concentration range from 0.5 to 100 ng/ml in mobile phase. A linear relationship was obtained between BPA concentration and peak area over the whole concentration range. The correlation coefficient was 0.9999 ($n = 6$). The detection limit (signal-to-noise ratio = 3) was 0.17 ng BPA/ml.

The analytical performance of the whole analysis method was assessed by applying the standard addition method. The analytical data are summarized in Table 4 listing one sample of each group of food matrices (beverages, fruits and vegetables).

Traces of BPA were detected in all cans tested. Very low BPA concentrations were found in canned beverages, significantly higher concentrations in fruits and vegetables. Mean recoveries from 53% (pea) to 75% (peach) were obtained. Limits of detection (LOD), given in Table 4, were calculated based on the LOD ($S/N = 3$) determined with a BPA standard solution taking into account the enrichment/dilution by the sample clean-up and the BPA recovery as assessed by the standard addition method.

3.5. Stability of the immunoaffinity columns in the analysis of food samples

Immunoaffinity columns proved to be very stable. In spite of applying highly complex extracts sol–gel immunoaffinity columns could be used for clean-up of at least 15 real samples.

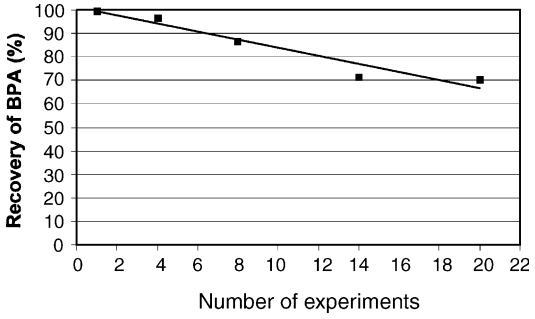

Fig. 6. Influence of the number of vegetable sample analysis cycles performed with immunoaffinity columns on the recovery of BPA. After having purified a certain number of vegetable extracts the recovery of BPA from the immunoaffinity column was determined by the use of a BPA standard solution. Feeding solution: 5 ml of BPA (5 ng/ml) in ACN–water (10:90, v/v). Washing solution: 5 ml of ACN–water (10:90, v/v). Elution medium: 4 ml of ACN–water (40:60, v/v).

However, the repeatedly use for clean-up of food extracts resulted in a slight decrease in BPA recovery. In Fig. 6 the influence of the number of applied samples on the recovery of BPA is shown.

4. Conclusion

The simple and rapid analysis method developed solves the problems posed by the complexity of food matrices by combining the selectivity of antigen-antibody interactions, chromatographic separation and fluorimetric detection. The applicability of the method was demonstrated giving chromatograms and analysis data of samples being representative for different groups of food matrices. Analysis data of all samples will be published in another paper in more detail.

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