Validation of an immunoassay for fast screening of bisphenol A in canned vegetables

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Abstract

The effects of BPA exposure on human health are an issue of concern and controversy. In the present work, the validation for the first time of a monoclonal antibody-based enzyme-linked immunoassay (ELISA) for BPA determination in canned vegetables is described, using HPLC as the reference method. From a collection of monoclonal antibodies, a high-sensitivity immunoassay was selected on the basis of its tolerance to organic solvents and the influence of matrix effects. This ELISA displayed a limit of detection of 3 µg kg^{-1} of BPA in whole product of canned vegetables and 15 µg L^{-1} of BPA in the liquid portion. For assay validation, processed vegetables were fortified with BPA at 10, 50, and 200 µg kg^{-1}. Sample treatment rendered crude and purified extracts. Purified extracts were analyzed by HPLC and ELISA, while crude extracts could be analyzed only by ELISA. Depending on the crop and the fortification level, good recoveries were obtained for both methods: 70.6-105 % for HPLC and 61.4-115 % (purified extracts) or 82-120 % (crude extracts) for ELISA. HPLC was more precise than ELISA. Finally, crude extracts of canned peas were analyzed by ELISA. Results (33−62 µg kg^{-1}) also compared well with those obtained by HPLC on purified extracts (23−44 µg kg^{-1}). In all samples, BPA concentration was significantly lower than the specific migration level of 600 µg kg^{-1} established by the European Commission. Therefore, the ELISA herein validated constitutes a sensitive, fast, and high-throughput technique for BPA screening in canned vegetables.

Introduction

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA) is a chemical monomer widely employed in the production of epoxy resins and polycarbonate plastics. These plastics are often used as packaging materials in processed food, whereas the resins are used as internal coatings in food and beverage cans to protect the product from direct contact with metals. Unfortunately, the residual non-polymerized BPA can be released from plastic containers and can linings. The amount of BPA leached depends on the processing temperature, liquid composition, and pH.[1]

From the toxicological point of view, BPA has estrogenic properties and several studies have proved a wide range of adverse effects in animal models.[2] In humans, biomonitoring studies indicate a widespread exposure to BPA through different routes such as the environment and food.[3] The extensive use of BPA and the consequent increasing BPA exposure have raised a great concern about potential harmful effects on human health, which is controversial among the scientific community.[4] International regulatory organisms clearly stated that further studies to asses BPA safety are required.[5−7] Meanwhile, a specific migration

limit (SML) of 600 µg kg^{-1} from food contact plastic materials was set by the European Commission.[8]

Surveys of BPA in canned foods have been carried out in several countries.[9,10] BPA residues in canned products differed considerably among food types. In this sense, vegetables are an important group in the food pyramid and canned vegetables account for about 10% of total vegetable intake. In the last decade, BPA has been detected in canned vegetables at concentrations between 10 and 100 µg kg^{-1}, depending on the country, vegetable, and sample (solid portion or whole content).[11−12] Yoshida and co-workers[13] analyzed separately the liquid and the solid portions of canned vegetables, reporting that BPA was mainly detected in the solid portion in the 18−95 µg kg^{-1} range. On the contrary, Brotons et al.[14] detected high BPA concentrations, between 29 and 458 µg kg^{-1}, in the liquid portion of canned vegetables. All these values are below the SML for BPA, but the low-dose exposure ongoing research demands adequate analytical techniques for a thorough and affordable BPA monitoring. Accordingly, revision and optimization of existing methods for BPA determination and the research on new ones would be very useful to ensure the compliance with current legislation and for the thorough risk assessment of human exposure to low doses of BPA.

BPA analysis has mostly been performed by gas chromatography-mass spectrometry and liquid chromatography coupled to fluorimetry or mass spectrometry.[12] These methods are highly sensitive and specific but they require sophisticated equipment and may be laborious, particularly as regards to sample treatment. Immunoassays offer an inexpensive and quick alternative or complement to traditional chromatographic methods because they are easy to perform and do not require complex instruments. In this sense, the production of polyclonal[15−20] and monoclonal[18,21−23] antibodies against BPA has been described. These antibodies have been used in different formats such as ELISA,[15−17,21−24] immunosensors[18,19] or as immunosorbents[20,25,26] for sample clean-up. These immunochemical techniques have been mainly applied to environmental or biological samples, whereas their application to BPA determination in food has been very limited, even though food is the primary route of BPA exposure. Braunrath et al.[25] reported that traces of BPA from 0.1 to 38 µg kg^{-1} were detected in canned food samples by immunoaffinity chromatography coupled to HPLC with fluorescence detection. Recently, polyclonal antibody-based immunoassays for BPA determination in canned vegetables were reported[27]. However, BPA analysis was limited to corn samples, and some of those samples were incomprehensibly spiked at levels even below the estimated limit of quantification.

We previously reported the production and partial characterization of a collection of monoclonal antibodies (MAbs) against BPA from several haptens, and a preliminary application to BPA detection in spiked canned vegetables[28]. In the present work, using those MAbs as primary immunoreagents and HPLC as the reference method, a comprehensive validation study of an ELISA for the determination of BPA in canned vegetables was performed. To this aim, the best monoclonal antibody in terms of tolerance to organic solvents and minimum matrix effects was selected. Fortified and real canned vegetable samples were analyzed by ELISA and HPLC, and the results were compared in terms of accuracy and precision. Finally, the suitability of the ELISA as an affordable, high throughput screening technique of BPA concentrations in canned vegetables is discussed.

Materials and methods

Chemicals and immunoreagents

Analytical standard of bisphenol A was from Sigma-Aldrich (Madrid, Spain). Water for HPLC analysis was obtained from a Milli-Q system (Millipore, Bedford, MA). HPLC-grade methanol, acetone, and acetonitrile were from Scharlau (Barcelona, Spain). Analytical reagent-grade hydrochloric acid was from Merck (Darmstadt, Germany). GC residue analysis-grade anhydrous sodium sulphate was from Scharlau.

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Ovalbumin (OVA) and *o*-phenylenediamine (OPD) were obtained from Sigma-Aldrich. Bovine serum albumin fraction V (BSA) was purchased from Roche Diagnostics (Barcelona, Spain). Peroxidase-labelled rabbit anti-mouse immunoglobulins were obtained from Dako (Glostrup, Denmark).

Hapten synthesis, preparation of hapten-protein conjugates, and production of MAbs to BPA were previously described.[28] The haptens used in the selected ELISA are depicted in Figure 1.

Bisphenol A

Immunizing hapten
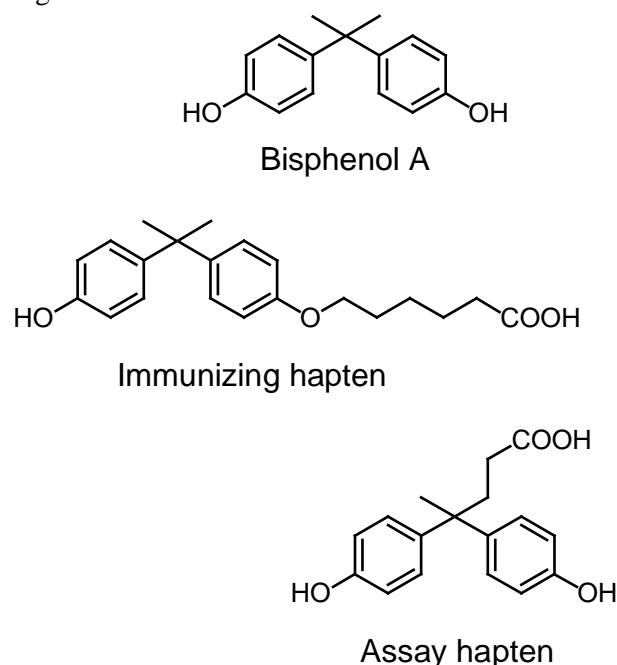
Assay hapten

Fig. 1 Chemical structures of bisphenol A and of the haptens used to develop the immunoassay.

ELISA instrumentation

Ninety-six-well ELISA polystyrene high binding plates were from Costar (Cambridge, MA). ELISA plates were washed with a 96 PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria). Absorbances in ELISA wells were read in dual-wavelength mode (490-650 nm) with a SpectraMax 190 microplate reader from Molecular Devices (Sunnyvale, CA). Data processing and analysis were performed using the SOFTmax PRO software from Molecular Devices.

HPLC instrumentation

All the equipment for HPLC analysis was supplied by Waters (Milford, MA). HPLC was performed on a Waters 2695 separation module, including a pump and an injection system, and equipped with a Waters 2475 fluorescence detector. Data acquisition and processing were performed using the Waters Empower 2 software.

Samples and fortification

Cans of asparagus, corn, pea, and tomato; glass jars of asparagus, corn, and pea; and crushed tomato in a plastic container were purchased from local supermarkets and were analyzed for incurred residues of BPA by HPLC. BPA residues were detected in all canned vegetables while they were not detected in asparagus, corn, and pea packed in glass jars. Crushed tomato

packed in a plastic container contained BPA at 5−7 µg kg^{-1}. This processed tomato and vegetables in glass jars were used as blank samples for fortification. Three levels of fortification were prepared (10, 50, and 200 µg kg^{-1}) from stock standard solutions of BPA at 500 ng mL^{-1} and 50 µg mL^{-1} in acetonitrile.

Sample treatment

Homogenized samples (5 g) were thoroughly mixed for 3 min with 50 ml of acetone, 25 µL of 0.1 M HCl (150 µL for asparagus samples), and 30 g of anhydrous sodium sulphate, using a blender jar. Next, the mixture was filtered through a microfiber GF/A filter. Ten ml of the mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 10 mL of 10% methanol in water. This solution was considered as the crude extract.

Sample clean-up

Solid−phase extraction cartridges (Oasis HLB 6 cc, Waters) were conditioned with 10 mL of methanol followed by 10 mL of 10% methanol in water. Next, 10 mL of crude extract was passed trough the cartridge at a flow rate of 1 mL min^{-1}. After washing with 10 mL of 10% methanol in water, the cartridge was allowed to dry by passing air through it. BPA retained was carefully eluted with 5 mL of methanol at a flow rate of 0.2 mL min^{-1}. Finally, the solvent was evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 1 mL of acetonitrile. This solution was the purified extract and it was ten times more concentrated than the crude extract.

ELISA determinations

General conditions. ELISA plates were coated overnight with OVA−hapten conjugates in 50 mM carbonate buffer, pH 9.6. A volume of 100 µL per well was used throughout all assay steps, and all incubations were carried out at room temperature. After each incubation, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Assay conjugate and antibody concentrations were optimized by checkerboard titrations. To obtain reproducible results, glass tubes were always used and exactly the same pipetting and dispensing procedures were applied to standards and samples.

Preparation of BPA standards. From a 2.28 mg mL^{-1} (10 mM) stock solution of BPA in DMF, eight serial dilutions from 142500 to 0.072 ng mL^{-1} were prepared in the same solvent. From these intermediate solutions in DMF, standards from 712.5 to 0.00036 ng mL^{-1} were obtained by diluting 1/200 each of the eight concentrations in the adequate solution (2% methanol or 2% acetonitrile in water for crude and purified extracts, respectively). For sample dilution, it should be kept in mind that standards contained 0.5% DMF. As the assay procedure involved the addition of the same volume of the appropriate antibody concentration, BPA standards in the final assay ranged from 356.25 to 0.00018 ng mL^{-1}.

Dilution of samples. For accurate and precise determinations, samples were adequately diluted to achieve the same solvent content as standards. Thus, crude extracts were first diluted with 10% methanol in water to enter the working range. Then, all crude extract samples were diluted 1/5 with water to render samples ready to be assayed in 2% methanol. Likewise, purified extracts in acetonitrile were first diluted in this solvent to enter the working range. Then, all purified extract samples were diluted 1/50 with water to render samples in 2% acetonitrile, the same solvent content as standards.

ELISA procedure. A conjugate-coated format with indirect detection of the specific antibody was followed. First, plates were coated with the corresponding OVA-hapten conjugate. Then, 50 µL of BPA standards or samples were added to triplicate wells, followed by 50 µL of the appropriate antibody concentration in PBS 2X (PBS, 10 mM phosphate, 0.15 M NaCl, pH 7.5), containing 0.002% Tween 20, and plates were incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labelled rabbit anti-mouse immunoglobulins diluted 1/2000

in PBST (PBS containing 0.05% Tween 20). Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulphuric acid, and the absorbance was read at 490 nm with a reference wavelength of 650 nm. Standard curves were obtained by plotting absorbance values against the logarithm of BPA concentration. Sigmoidal curves were fitted to a four-parameter logistic equation.[29] BPA concentrations in samples were determined by interpolation of the mean absorbance values on the standard curve run in the same plate.

HPLC determinations

HPLC analysis of BPA was performed on a Waters Atlantis T3 4.6x250 mm (5 µm particle size) column. A mixture of water/acetonitrile (60:40) was used as mobile phase at a flow rate of 1.1 mL min^{-1}. Sample injection volume was 30 µl and the retention time of BPA was 12.7 min. BPA fluorescent detection was carried out using 275 and 300 nm as excitation and emission wavelengths, respectively. Calibration standards of BPA at 5, 10, 50, 200, and 600 ng mL^{-1} were prepared in acetonitrile from a stock solution of 50 µg mL^{-1}. Standard curves were obtained by linear regression of mean values of peak areas. BPA concentration on samples was calculated by the following formula:

$$[BPA] \ (\mu g \ kg^{-1}) = c \times v \ / \ v' \times w \times r$$

were c is the BPA concentration (ng mL^{-1}) calculated from the standard curve, v is the volume of acetonitrile used to dissolve the dried eluate (1 mL), v' is the sample volume loaded onto the Oasis cartridge (10 mL), w is the sample weight (5 g), and r is the recovery obtained during method validation. In each analytical batch, a standard with known concentration was injected to verify the variability of retention time and the validity of calibration curves. BPA concentration in real samples was corrected with the recovery factor obtained during the validation.

Results and discussion

In our previous work, fifteen hybridomas secreting MAbs against BPA were cloned and stabilized.[28] Four of these MAbs were selected for the present study on the basis of their highest affinity (lowest limit of detection) to BPA using homologous and heterologous haptens in a conjugate-coated ELISA format. Firstly, as sample extracts contained organic solvents, the influence of these solvents on MAb activity was studied.

Influence of organic solvents on MAb activity

Sample treatment prior to the chromatographic analysis of BPA included a solvent extraction step followed by a SPE cleanup step. During this procedure two extracts were generated: 1) crude extracts in 10% methanol in water, which were used for SPE cleanup, and 2) purified extracts in acetonitrile, which were ready for HPLC analysis. These two extracts were used to carry out the comparison between ELISA and HPLC analysis of BPA in canned vegetables. Therefore, the influence of these organic solvents on MAb activity had to be studied prior to ELISA determinations. As methanol and acetonitrile are water miscible, extract dilution is appropriate to minimize their effect, but dilution should not be as high as to produce an excessive loss of sensitivity. Applying the extraction procedure described in the Materials and Methods section, a sample containing 10 µg kg^{-1} BPA − a concentration close to the limit of detection (LOD) of the immunoassays − would theoretically render a crude extract at 1 ng mL^{-1} and a purified extract at 10 ng mL^{-1} BPA. On the other hand, the lower limit of the working range of the immunoassays developed with the selected MAbs is approximately 0.1 ng mL^{-1} BPA. Therefore, the crude and purified extracts of this sample should respectively be diluted not more than 1/10 and 1/100 in assay buffer to be properly analyzed. Accordingly, the final composition of diluted extracts was 1% methanol or 1% acetonitrile in assay buffer

for crude and purified extracts, respectively. Then, competitive standard curves were obtained in assay buffers containing 1% methanol or 1% acetonitrile (2% organic solvent for standard preparation), using water as control. The study was carried out for the immunoassays developed with the four MAbs selected, searching for differences in organic solvent tolerance. The results are shown in Table 1. As it can be observed, in most cases the assay parameters, A_{max} (maximum absorbance) and I_{50} (concentration giving 50% inhibition of the maximum absorbance), obtained in the presence of solvents were higher than those obtained in water. Consequently, all MAbs were affected by the presence of methanol or acetonitrile in the assay buffer at the proportions assayed. Therefore, for the analysis of BPA by ELISA in crude or purified extracts of canned vegetables, standards must be prepared in 2% methanol or 2% acetonitrile in water, respectively, to avoid solvent effects. As the immunoassay developed with MAb BPAH-15 was the most affected, this monoclonal antibody was discarded for further studies.

Table 1 Influence of organic solvents on immunoassay parameters[a]

Standards prepared in[b]	MAb BPAB-11		MAb BPAB-31		MAb BPAH-15		MAb BPAH-34	
	A_{max}	I_{50} (nM)	A_{max}	I_{50} (nM)	A_{max}	I_{50} (nM)	A_{max}	I_{50} (nM)
Water	0.95	0.7	1.21	0.9	1.14	1.5	1.04	1.1
2% Acetonitrile	1.14	1.1	1.45	1.2	1.43	2.5	1.34	1.4
2% Methanol	1.05	0.9	1.43	1.1	0.71	3.9	1.23	1.2

[a] Data are the average of triplicate determinations. [b] Concentrations in the final assay were 1% organic solvent.

Study of matrix effects

When immunoassays for a given analyte are applied to a particular matrix, they can undergo different matrix interferences depending on the robustness of the MAb used. A thorough evaluation of matrix effects on the immunoassays developed from the available MAbs is therefore essential. Consequently, the three remaining MAbs to BPA were evaluated to find out the one providing minimum matrix effects. On the other hand, blank samples were needed to carry out this study. All canned vegetables –peas, corn, asparagus, and tomatoes– purchased from local supermarkets contained BPA above 10 µg kg^{-1} in the whole content as determined by HPLC. In contrast, BPA was not detected in vegetables –peas, corn, and asparagus– packed in glass jars, while it was detected at very low concentrations (5–7 µg kg^{-1}) in crushed tomatoes packed in plastic containers. Therefore, these blank samples were processed to obtain the corresponding crude and purified extracts. These extracts and the liquid portions of vegetables packed in glass jars were used to study the influence of matrix components on the three MAbs/immunoassays evaluated.

Purified extracts. Purified blank extracts of peas, corn, asparagus, and tomatoes in acetonitrile were diluted 1/2 and 1/4 in the same solvent. Acetonitrile was used as control. All extracts were diluted 1/50 in water and subsequently fortified with BPA at 0.5 ng mL^{-1} by adding 10 µl of BPA at 100 ng mL^{-1} in DMF to 2 ml of each diluted extract. Thus, all fortified blank samples contained BPA at 0.5 ng mL^{-1} and 2% acetonitrile but distinct proportions of initial extracts. This BPA fortification is equivalent to 25 ng mL^{-1} in purified extracts and to 25 µg kg^{-1} in packed products. These samples were analyzed by the three immunoassays. The final BPA concentration in all the assays was 0.25 ng mL^{-1}, a concentration close to the I_{50} value

(around 1 nM) for all competitive curves. The results expressed as recoveries are shown in Table 2. Overall, acceptable recoveries (65–135%) were obtained with the three immunoassays and for the four vegetable matrices studied. Purified extracts could be analyzed by ELISA simply after diluting them 1/50 in water. The only exception was the analysis of tomato extracts by the immunoassay developed with MAb BPAB-31, which would require at least a 1/4 dilution of purified extracts to minimize matrix effects.

Table 2 Study of matrix effects of purified extracts

Immunoassay developed with MAb	Sample[a]	Recovery (%)[b]			
		Peas	Corn	Asparagus	Tomato[c]
BPAB-11	Acetonitrile (control)	95	90	90	90
	Purified extract	123	129	120	133
	Purified extract 1/2	104	108	129	99
	Purified extract 1/4	103	106	118	105
BPAB-31	Acetonitrile (control)	89	96	95	95
	Purified extract	122	133	91	197
	Purified extract 1/2	103	120	100	154
	Purified extract 1/4	97	112	67	135
BPAH-34	Acetonitrile (control)	101	115	113	113
	Purified extract	91	108	91	84
	Purified extract 1/2	95	99	92	93
	Purified extract 1/4	89	108	73	102

[a] Purified extracts in acetonitrile were diluted 1/50 in water and 1/2 in assay buffer. The final assay buffer contained 1% acetonitrile. [b] Data obtained by analyzing diluted purified extracts fortified at 0.5 ng mL^{-1} BPA. Mean values from three independent determinations. [c] Data corrected with BPA detected before fortification.

Crude extracts. A similar procedure to that applied for purified extracts was carried out. In this case, crude extracts of vegetables were prepared in 10% methanol in water, a solution also used for the initial dilution of crude extracts (1/2 and 1/4) and as control. According to the ELISA protocol, all blank samples, that is, the control one and crude extracts without diluting and diluted 1/2 and 1/4, were further diluted 1/5 in water and fortified with BPA at 0.5 ng mL^{-1} as described previously. Likewise, all fortified blank samples contained BPA at 0.5 ng mL^{-1} and 2% methanol but distinct proportions of initial crude extract. This BPA fortification is equivalent to 2.5 ng mL^{-1} in crude extracts and 25 µg kg^{-1} in packed products. These samples were analyzed by the three immunoassays. The results shown in Table 3 indicate that the immunoassays developed with MAbs BPAB-11 and BPAB-31 were strongly affected (recovery values higher than 140%) by three of the four vegetable extracts tested. In these cases, crude extracts should be diluted at least 1/4 to minimize matrix effects. In contrast, recoveries obtained with MAb BPAH-34 were fairly good (75–125%) for the four vegetables even without diluting crude extracts. Therefore, among the MAbs evaluated, the immunoassay developed with MAb BPAH-34 was the most robust for reliable BPA determination in canned vegetable extracts.

Liquid portions. The whole product of peas, corn, and asparagus packed in glass jars was filtered through a fine nylon screen. The liquid portions were collected and serially diluted

with water from 2 to 500 times. Each serial dilution was compared with water as control in terms of maximum absorbance obtained with the three immunoassays evaluated (MAbs BPAB-11, BPAB-31, and BPAH-34). Similar results were obtained for the three vegetables tested (data not shown). Among the immunoassays, the one developed with MAb BPAH-34 was the least affected by the liquid matrix components. Figure 2 shows the influence of the liquid portion of peas packed in glass jars on this immunoassay. As it can be observed, the liquid portion should be diluted 250 times (500 times in the final assay) to minimize matrix effects.

Table 3 Study of matrix effects of crude extracts

Immunoassay developed with MAb	Sample[a]	Recovery (%)[b]			
		Peas	Corn	Asparagus	Tomato[c]
BPAB-11	10% Methanol in water	105	105	87	87
	Crude extract	286	153	132	178
	Crude extract 1/2	217	140	117	134
	Crude extract 1/4	173	133	112	97
BPAB-31	10% Methanol in water	97	97	80	80
	Crude extract	231	152	183	116
	Crude extract 1/2	172	116	126	98
	Crude extract 1/4	148	122	100	94
BPAH-34	10% Methanol in water	94	94	85	85
	Crude extract	77	90	112	123
	Crude extract 1/2	88	116	94	104
	Crude extract 1/4	95	114	95	110

[a] Crude extracts in 10% methanol were diluted 1/5 in water and 1/2 in assay buffer. The final assay buffer contained 1% methanol. [b] Data obtained by analyzing diluted crude extracts fortified at 0.5 ng mL^{-1} BPA. Mean values from three independent determinations. [c] Data corrected with BPA detected before fortification.
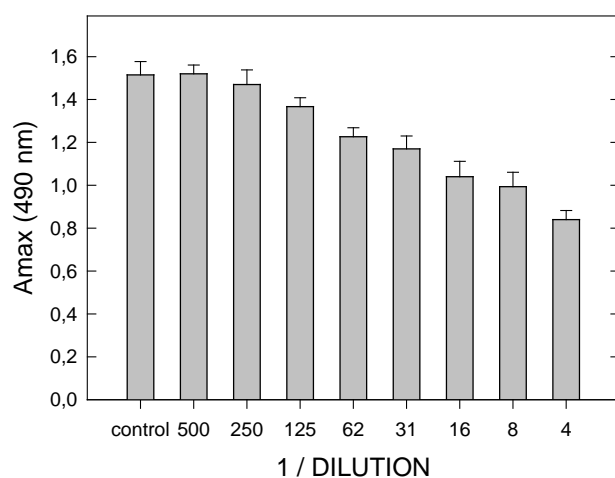

Fig. 2 Influence of the liquid portion dilution of peas packed in glass jars on the BPA immunoassay (MAb BPAH-34). Values are the mean of eight replicates.

Analytical parameters of the BPA immunoassay

A representative standard curve obtained with the selected MAb BPAH-34 in the conjugate-coated competitive ELISA format is presented in Figure 3. BPA standards were prepared in water. The BPA I_{50} value was 0.24 ng mL^{-1} (1.05 nM) and the limit of detection, estimated as the BPA concentration giving 10% inhibition of the maximum absorbance, was 0.03 ng mL^{-1}. The working range, defined as the concentration giving 20−80% inhibition, was 0.06−0.84 ng mL^{-1} BPA. Similar analytical parameters were estimated from standard curves prepared in 2% methanol or 2% acetonitrile in water, which were used for BPA determinations in crude or purified extracts, respectively. As the final dilution of crude extracts in the ELISA wells was 1/100, the actual BPA LOD in whole product was 3 µg kg^{-1}, and the working range was 6−84 µg kg^{-1}. For the liquid portions, which were diluted 1/500, the LOD and working range was 15 and 30−420 µg L^{-1}, respectively.

With regard to specificity, MAb BPAH-34 is quite specific for bisphenol A. It only showed little cross-reactivity (CR) to the closely related compound bisphenol E (4,4-ethylidenebisphenol, CR < 10%) and weak CR to bisphenol F (bis-(4-hydroxyphenyl)-methane, CR < 0.5%). Other compounds such as phenol, *p*-cresol, bisphenol A diglycidyl ether, dibutyl phthalate, 4-nonylpehnol or equol were not recognized (CR < 0.01%).
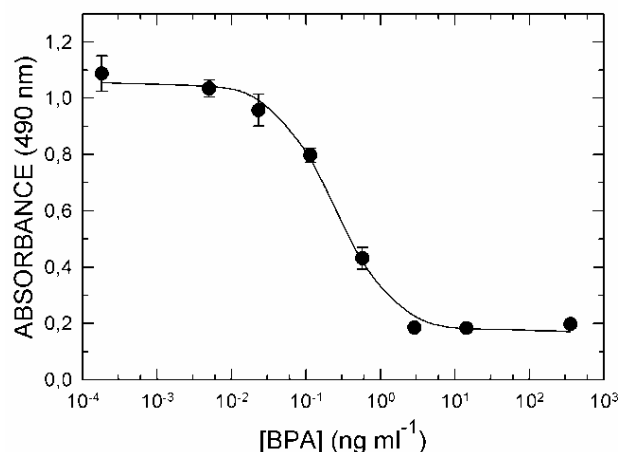
Fig. 3   Representative standard curve for the BPA ELISA (conjugate: OVA-BPVA at 0.8 µg ml^{-1}; MAb BPAH-34 at 30 ng ml^{-1}) used to analyze BPA in crude extracts of canned vegetables. Standards were prepared in water.

Recovery studies of BPA in fortified samples

Peas, corn, and asparagus packed in glace jars and crushed tomatoes packed in a plastic container were used as blank samples. BPA was not detected in vegetables packed in glace jars by HPLC while BPA was detected at 5−7 µg Kg^{-1} in crushed tomatoes packed in a plastic container. Each matrix was fortified with BPA at 10, 50, and 200 µg Kg^{-1}. Samples were processed according to the procedure described in Materials & Methods to render the corresponding crude and purified extracts.

Purified extracts were analyzed by ELISA and HPLC. For ELISA analysis, purified extracts in acetonitrile were diluted 1/50 in 0.5% DMF in water to minimize matrix effects and, at the same time, to enter directly into the standard curve working range. Purified extracts from the 200 µg Kg^{-1} fortification were previously diluted 1/8 in acetonitrile. Reproducibility and recovery data obtained for each vegetable by the two analytical methods are shown in Table 4. As it can be observed, HPLC reproducibility was better than that of ELISA since coefficient of variations (CV) ranged from 1.3 to 9.6% (mean CV of 5.9) and from 6.7 to 30.5% (mean CV of 18.5), respectively. On the other hand, both methods yielded

acceptable recoveries although ELISA range was wider (61.4−115%) than that of HPLC (70.6−105%). Irrespective of the crop, both methods rendered mean recoveries below 90% (84.1% for ELISA and 86.6% for HPLC), which may be ascribed to BPA losses during clean-up and additional evaporation/dissolution steps.

Table 4 Recovery studies by ELISA and HPLC of purified and crude extracts of processed vegetables fortified with bisphenol A (n = 5 replicates)

| Matrix | Fortification level (μg kg^{-1}) | Purified extracts | | | | | | Crude extracts | | |
| | | HPLC | | | ELISA | | | ELISA | | |
		Mean (μg kg^{-1})	CV (%)	Recovery (%)	Mean (μg kg^{-1})	CV (%)	Recovery (%)	Mean (μg kg^{-1})	CV (%)	Recovery (%)
Peas	10	10.5	4.5	105.0	11.4	20.9	114.0	12.0	26.2	120.0
	50	39.9	5.3	79.8	34.5	14.6	69.0	49.4	14.0	99.0
	200	169.7	5.9	84.9	143.3	10.2	71.7	236.5	21.4	118.0
Corn	10	10.2	3.2	102.0	9.9	30.5	99.0	8.2	22.9	82.0
	50	40.7	6.0	81.4	30.7	19.8	61.4	39.7	21.4	89.4
	200	165.1	1.3	82.6	183.2	25.5	91.6	204.3	16.1	102.0
Aspara	10	9.3	5.5	93.0	8.8	15.1	88.0	10.6	30.3	106.0
	50	45.4	9.4	90.8	39.1	11.8	78.2	43.8	18.2	87.6
	200	141.3	3.1	70.6	149.8	6.7	75.0	177.7	21.3	88.9
Tomat	10	9.4	9.6	94.0	11.5	29.0	115.0	8.7	34.8	87.0
	50	39.1	7.6	78.2	36.5	24.0	73.0	41.9	27.4	83.8
	200	152.6	8.8	76.3	149.9	13.0	75.0	177.5	12.2	88.8
Mean			5.9	86.6		18.5	84.1		22.2	96.8

One of the main advantages generally attributed to immunoassays is the possibility of reducing sample purification steps in food analysis. Accordingly, BPA was analyzed in crude extracts by ELISA to check the performance and robustness of this technique. After diluting the 200 μg Kg^{-1} fortifications 1/8 in 2% methanol in water, all samples were diluted 1/5 in 0.625% DMF in water to enter the working range and to minimize matrix effects. Crude extract results are also shown in Table 4. A direct comparison with purified extract results can be established since crude and purified extracts came from the same fortified samples. Recovery values for ELISA analysis of crude extracts were also acceptable, ranging from 82 to 112%, with a mean value (96.8%) closer to 100% than those obtained with purified samples. Moreover, ELISA reproducibility for crude extracts (mean CV of 22.2%) was in a similar range to that of the ELISA of purified extracts but higher than that of HPLC. These results demonstrated that the clean-up step can be omitted for ELISA determinations without significantly affecting their analytical parameters. When compared with the reference method, ELISA afforded an acceptable precision and accuracy. Therefore, BPA can be properly analyzed by ELISA at levels as low as 10 μg Kg^{-1} in crude extracts of canned vegetables.

Analysis of BPA in canned peas by ELISA and HPLC

Five cans of peas of three different brands were purchased from local supermarkets. These samples were processed to obtain crude and purified extracts, which were analyzed by ELISA and HPLC, respectively. As shown in Table 5, BPA was detected in all samples at concentrations in the 23−44 μg kg^{-1} range by HPLC, and the analysis of crude extracts by ELISA resulted in detection of BPA in the 33−62 μg kg^{-1} range. It is remarkable to notice that different cans from the same brand contained very similar BPA levels as determined by HPLC, while for the ELISA method they differed significantly. This fact reaffirms that the ELISA method was clearly more imprecise than the HPLC method. On the other hand, it seems that ELISA analysis tended to overestimate (mean 140%) the values obtained by HPLC. The origin of this effect is questionable but a possible explanation may be found in the different content of peas packed in glass jars (where the study of matrix effects was carried out) and in cans. However, it can not be ruled out that ELISA of crude extracts afforded more realistic results, because a loss of analyte recovery may be associated with the additional clean-up step required by HPLC analysis. In any case, these concentrations were far below the current specific migration limit (600 μg kg^{-1}) fixed by the European Commission for BPA, and similar to those found by different groups worldwide.[11,12]

As far as is referred to BPA in the liquid portions of cans, Brotons et al.[14] found concentrations of BPA as high as 460 μg L^{-1} in the liquid content of canned peas, levels that are worrying indeed. In this study, BPA concentrations in the liquid portions of canned peas were analyzed by ELISA and all were below the limit of quantification of the technique (30 μg L^{-1}).

Consequently, ELISA determinations of BPA in canned vegetables can be very useful as a screening technique, since less sample treatment is required and a lot of samples can simultaneously be analyzed in an ELISA plate.

Table 5 Analysis of BPA in canned peas by HPLC (purified extracts) and by ELISA (crude extracts) (n = 2 replicates)

Sample	HPLC[a]		ELISA	
	Mean (μg kg^{-1})	CV (%)	Mean (μg kg^{-1})	CV (%)
CON-1	42.3	12.2	52.6	28.1
CON-2	40.0	0.4	53.0	26.1
CON-3	43.4	4.0	43.6	10.9
CON-4	43.6	5.0	61.2	7.2
CON-5	43.4	4.0	38.8	16.0
HAC-1	23.4	4.8	33.1	11.6
HAC-2	25.5	5.3	37.4	23.2
HAC-3	25.8	4.4	42.1	35.2
HAC-4	26.6	0.5	40.1	33.3
HAC-5	24.7	2.0	38.8	14.9
DAU-1	26.5	5.8	46.2	12.1
DAU-2	27.0	5.5	36.5	38.3
DAU-3	29.1	0.2	47.3	13.2
DAU-4	26.8	8.7	35.3	22.8
DAU-5	28.7	4.9	43.3	5.9

[a] Data were corrected considering that method recovery is 100% (actual method recovery was estimated as 90%).

Conclusions

To our knowledge, this is the first time that a comprehensive validation study of an immunoanalytical technique for BPA determination in canned vegetables is reported. The usefulness of a collection of MAbs to develop a high-sensitivity, robust enzyme immunoassay for the determination of BPA in several canned vegetables has been proved. MAb selection was based on studies of organic solvent tolerance and influence of matrix effects. The MAb selected allows the high sensitivity determination of BPA in crude extracts of the whole canned product (LOD around 3 µg kg^{-1}) and in the liquid portions of cans (LOD around 15 µg L^{-1}) with the simplicity and rapidity inherent to immunoassays.

Fortified and real samples of canned vegetables were analyzed by HPLC and ELISA. Overall, ELISA was less precise and accurate than HPLC. However, the application of this ELISA as a screening technique previous to HPLC can suppose a significant methodological improvement for the cost-effective and high sample throughput determination of BPA in canned vegetables at levels of regulatory relevance.

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