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HPLC with Fluorescence Detection for Determination of Bisphenol A in Canned Vegetables: Optimization, Validation and Application to Samples from Portuguese and Spanish Markets

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Abstract: Bisphenol A (BPA) is one of the chemicals used to produce both polycarbonate plastics and epoxy resin coatings. Research has shown that small amounts of BPA can migrate into the foods and beverages enclosed in these types of containers. In this research, an analytical method based on high-performance liquid chromatography with fluorescence detection (HPLC-FLD) was developed and validated for the determination of BPA in canned vegetables. The results were confirmed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was performed, to identify the coating material of each tin can. Nineteen cans of vegetables were taken as study samples (eleven samples from the Spanish market, and eight samples from the Portuguese market). Excellent linear correlation (r^2 = 0.9999) was observed over the range of 0.01 to 0.25 mg/L. Limit of detection (LOD) and limit of quantification (LOQ) values were calculated to be 0.005 mg/kg and 0.01 mg/kg, respectively. Good recoveries, between 72% and 90% were obtained at three different levels of concentration (RSD% = 4.6). BPA was not detected in the samples. The proposed HPLC-FLD was found to be suitable for the determination of BPA in canned vegetables.

Keywords: bisphenol A; epoxy resins; canned vegetables; HPLC-FLD; LC-MS/MS

1. Introduction

People are exposed to contaminants through several sources. Packaging can be a potential source of contamination through the migration of substances from the packaging material into the food [1]. This fact is especially relevant, as most of the food found at the supermarkets is packaged in order to prevent its deterioration and to extend its shelf life [1–3] In order to ensure food safety, the monitoring of migration from food contact materials has become a priority issue. For researchers, the study of the contaminant bisphenol A (BPA) and its impact on the endocrine system are of major concern, because BPA exhibits estrogenic properties arising from its structural resemblance to the human 17β-estradiol [4] and disturbs the endocrine and reproductive systems [5–7].

Bisphenol A [2,2-bis(4 hydroxyphenyl)propane] has the chemical formula $C_{15}H_{16}O_2$ and results from the condensation of 2 mol of phenol with 1 mol of acetone in the presence of an acid catalyst [4].

BPA is mainly (approximately 95%) used in the production of synthetic polymers including epoxy resins (as the internal coating of food and beverage cans) and polycarbonates (storage containers, reusable plastic bottles, plates, among others) [2,4]. BPA is also used in the production of plasticized PVC as inhibitor of the polymerization of vinyl chloride and, in some plasticizers as antioxidant [8].

With several limitations, BPA is authorized to be used as a monomer in the manufacture of plastic materials intended to come into contact with food in the European Union. According to Regulation (EU) no. 10/2011 [9] and its amendments, BPA has a specific migration limit of 0.05 mg/kg [10]. Moreover, BPA is not authorized to be used for the manufacture of polycarbonate infant feeding bottles [11] and polycarbonate drinking cups or bottles which, due to their spill proof characteristics, are intended for infants and young children (Regulation (EU) no. 2018/213) [10].

In recent years many methods for measuring the BPA in food have been developed. In 2019 F. Vilarinho et al. [4] carried out an exhaustive compilation and discussion of the analytical methodologies used for the determination of BPA as result of its migration from food contact materials. The determination of BPA in food is mainly performed by liquid chromatography (LC) [4,12,13] with different detectors: ultraviolet (LC/UV) [14], fluorescence (LC/FLD), electrochemical detection (LC/ECD), mass spectrometry (LC/MS), and tandem mass spectrometry (LC/MS-MS) [15] or by gas chromatography (GC) coupled with mass spectrometry (GC/MS) and tandem mass spectrometry (GC/MS-MS) [4,13,16]. According to Vilarinho et al. [4] the MS detector is the most usually used for both LC and GC, due to its unquestionable advantages which comprehend high selectivity, high sensitivity, and universality. It is important to notice that the choice of the detector, greatly influences the detection limit (LOD) and the quantification limit (LOQ) [4].

In the present study, the development and validation of an analytical method by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) to quantify BPA in canned vegetables are reported. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to confirm the results obtained. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was performed to identify the coating material of each of cans of samples selected for the study. Nineteen canned vegetables (eleven samples from the Spanish market, and eight samples from the Portuguese market) were evaluated in order to check their compliance with the legislation in force in the European Union.

2. Materials and Methods

2.1. Chemicals and Standard Solutions

All reagents were analytical grade. Acetonitrile LC Grade (Lichrosolv, Merck KGaA, Darmstadt, Germany). Methanol LC-MS Grade was provided from Merck (Darmstadt, Germany). Ultrapure water (type I) was obtained from an Automatic Plus purification system (Wasserlab, Navarra, Spain). Standard Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane; 4,4'-isopropylidene-diphenol), with a purity >99% was acquired from Aldrich-Chemie (Steinheim, Germany).

BPA stock solution was prepared by dissolving the BPA in acetonitrile (ACN) in order to obtain 1000 mg L^{-1}. Standard solutions of concentration between 0.005 and 0.25 mg L^{-1} were prepared by diluting the stock solution with the ACN/Water (10:90).

2.2. Samples Preparation

Nineteen commercial canned vegetables were purchased in three different local supermarkets of Lisbon (Portugal) and Santiago de Compostela (Spain). The canned samples have different food products: namely, three samples of canned fine peas, two of artichoke hearts, two of sweet corn, one of chilli peppers, one of asparagus (thick gauge), one of red peppers—all natural, one of rolled mushrooms, one of Almagro eggplant, one of whole peeled tomato, one of white cabbage, one of cooked lentils, one of vegetables of Macedonia, one of mung bean sprouts and two of green beans

(Table 1). All canned foodstuffs were sampled in duplicate, therefore the result is the mean of two independent measurements Samples were stored at room temperature until analysis.

Table 1. Samples selected for the present study (P—Portugal; E—Spain).

Origin (Country)	Samples Used for Trial
1E	Alegrias Riojanas
2E	Asparagus buds
3E	Red peppers—whole in brine
4E	Artichoke hearts
5E	Sweet corn
6E	Peas with extra-fine carrots
7E	Laminated mushrooms
8E	Eggplant Almagro
9E	Fine peas
10E	Whole peeled tomato
11E	White cabbage
1P	Very fine peas
2P	Cooked lentils
3P	Vegetables of Macedonia
4P	Sweet corn
5P	Mungo bean sprouts
6P	White cooked quinoa
7P	Green beans
8P	Artichoke Hearts

Following the preparation of foods described by Nerín et al. (2002) [17] and because the objective of the paper was also to first screen BPA in canned vegetables, the liquid phase in contact with canned foods was analyzed. To determine the amount of BPA present in each sample, the net content of each can was leaked into a container. Then 900 µL were removed from this container and 100 µL of can was added. This mixture was filtered and then injected into HPLC-FLD and LC-MS/MS.

2.3. HPLC-FLD

The high-performance liquid chromatography (HPLC) system used in this study was an Agilent Technologies 1200 Series (Waldbronn, Germany) constituted by a quaternary pump, a degassing device, an autosampler, a column thermostat system, a diode array detector, a fluorescence detector and an Agilent ChemStation for LC and ChemStation for LC 3D Systems software (version B.04.0x).

2.4. LC-MS/MS

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) system used in this study consisted of an Accela autosampler, a column thermostat system and Accela 1250 pump fitted with a degasser, coupled to a TSQ Quantum Access Max Triple Quadrupole controlled by Xcalibur 2.1.0 (Thermo Fisher Scientific, San José, CA, USA). MS data were acquired in the negative Atmospheric Pressure Chemical Ionization (APCI) mode. The operating conditions were: spray voltage 2500 V; nebulizer gas (N_2), 35 psi; vaporizer temperature 400 °C; capillary temperature 350 °C. As the collision gas was used Argon (1.5 mTorr). The selected precursor ion for BPA was m /z 227.2 and the two SRM transitions monitored were 227.2–212.1 and 227.2–133.2 m/z with a collision energy of 21 and 29 V respectively (Table 3).

2.5. Chromatographic Conditions

The chromatographic conditions for the HPLC-FLD and the LC-MS/MS analyses are similar. Table 2 shows the chromatographic conditions for BPA separation/quantification and Table 3 indicates the gradient elution conditions [18]. For LC-MS/MS analysis the organic solvent used was methanol instead of acetonitrile.

Table 2. Chromatographic conditions for the determination of bisphenol A (BPA) as results of its migration to canned vegetables.

Chromatographic conditions of HPLC-FLD and LC-MS/MS methods	
Analytical column	Luna® C18 (2) 100 Å (150 mm × 3 mm, 5 μm particle size)
Pre-column	SecurityGuard™ cartridges for C18 HPLC columns with 2.0 to 3.0 mm internal diameters (ID)
Temperature	25 °C
Mobile phases	HPLC-FLD: Milli-Q water (type I) (A) and Acetonitrile (B) LC-MS/MS: Milli-Q water (type I) (A) and Methanol (B)
Flow rate	0.5 mL/min
Injection volume	20 μl
FLD detector	λem 305 nm; λex 225 nm
MS spectrometer conditions	
Acquired in	Negative atmospheric pressure chemical ionization (APCI) mode
Spray voltage	2500 V
Nebulizer gas	(N_2), 35 psi
Vaporizer temperature	400 °C
Capillary temperature	350 °C
Collision gas	Argon (1.5 mTorr)
Selected precursor ion for BPA	m/z 227.2
SRM transitions monitored	227.2 > 212.1, collision energy: 21V
SRM transitions monitored	227.2. > 133.2, collision energy: 29V

Table 3. Gradient elution conditions of HPLC-FLD and LC-MS/MS methods.

Time (min)	**%A**	**%B***
0.0	70.0	30.0
2.00	70.0	30.0
23.00	0.0	100.0
30.00	0.0	100.0
32.00	70.0	30.0
35.00	70.0	30.0

*B corresponds to acetonitrile in HPLC-FLD or methanol in LC-MS/MS.

2.6. ATR-FTIR

Infrared spectra were acquired using an ATR-FTIR spectrometer (ATR-PRO ONE FT-IR4700, Jasco, Japan) equipped with a Diamond optical element (in the range from 4000–650 cm^{-1}). ATR-FTIR spectrometer was controlled by the software Spectra Manager™ Suite (version 2). The identification was carried out by using the IR Spectral libraries of Polymers & Related Compounds (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the software KnowItAll® (version 17.4.135.B).

2.7. Recovery

To determine recovery of BPA, a spiking experiment using a canned vegetable BPA free (blank) was performed. The spiking levels chosen were 0.01, 0.05 and 0.25 mg kg^{-1} of BPA, respectively. HPLC results were plotted into the BPA calibration curve. Spiking assays were performed by different operators, in three different days and the recovery was calculated by comparing the theoretical spiking and practical BPA value obtained from HPLC.

2.8. Precision

Repeatability and precision were determined by running spiking experiments on a representative sample at 3 different spiking levels (0.01, 0.05 and 0.25 mg kg^{-1}). Repeatability was carried out in a single day and precision (internal reproducibility) was performed during three

different days and with different operators. Data are expressed in terms of % of relative standard deviation (%RSD).

3. Results and Discussion

3.1. Method Validation

Validation parameters including linearity, precision (internal reproducibility), limit of detection (LOD) and limit of quantification (LOQ) were determined according to the International Conference on Harmonisation (ICH) guidelines Q2 (R1) [19,20].

The use of a series of standards solutions of known concentration allowed to test the linearity of the method. The calibrations curves were constructed using six concentration levels (0.005, 0.010, 0.025, 0.050, 0.100 and 0.250 mg L^{-1}) and they were fitted to a linear equation. The linearity was tested in acetonitrile/water (10:90, v/v) as solvent. Three calibration curves were performed in three different days. All of them have shown good linearity in the studied concentration range with coefficients of determination (r^2) equal to 0.9999, indicating suitability for BPA quantification in the selected calibration range. Table 4 shows the parameters of linearity.

The limits of detection and quantification, calculated according to ACS guidelines [21] (defined as three and ten times the height signal of the noise level, respectively) were presented in Table 5. The method presents enough sensitivity to detect the BPA at the regulatory level, once the experimental LOD (0.005 mg kg^{-1}) is considerably lower than the specific migration limit (SML) of 0.05 mg kg^{-1}established by the Regulation (EU) no. 10/2011 and its amendments, which are in force [4,10].

Table 4. Recovery, repeatability and intermediate precision of the HPLC-FLD method.

Compound	Recovery (%) (n = 6)			Repeatability (RSD%) (n = 6)			Intermediate Precision (RSD%) (n = 18)		
	0.01 [a] mg kg^{-1}	0.05 [a] mg kg^{-1}	0.25 [a] mg kg^{-1}	0.01 [a] mg kg^{-1}	0.05 [a] mg kg^{-1}	0.25 [a] mg kg^{-1}	0.01 [a] mg kg^{-1}	0.05 [a] mg kg^{-1}	0.25 [a] mg kg^{-1}
BPA	90	77	72	9.94	3.48	3.26	4.53	4.70	0.57

[a] Fortification level.

Table 5. Linearity, limit of detection and quantification of BPA according to the International Conference on Harmonisation (ICH) guidelines Q2 (R1), and using high-performance liquid chromatography (HPLC) with fluorescence detection (FLD).

Day	Equation	r^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Range (mg kg^{-1})
1	$y = 224.71x - 0.1121$	0.9999	0.005	0.01	0.01–0.25
2	$y = 223.92x - 0.2539$				
3	$y = 219.54x - 0.113$				

Figure 1 shows the chromatogram obtained by HPLC-FLD with 0.005 mg kg^{-1}standard solution, that is the limit of detection of the method.

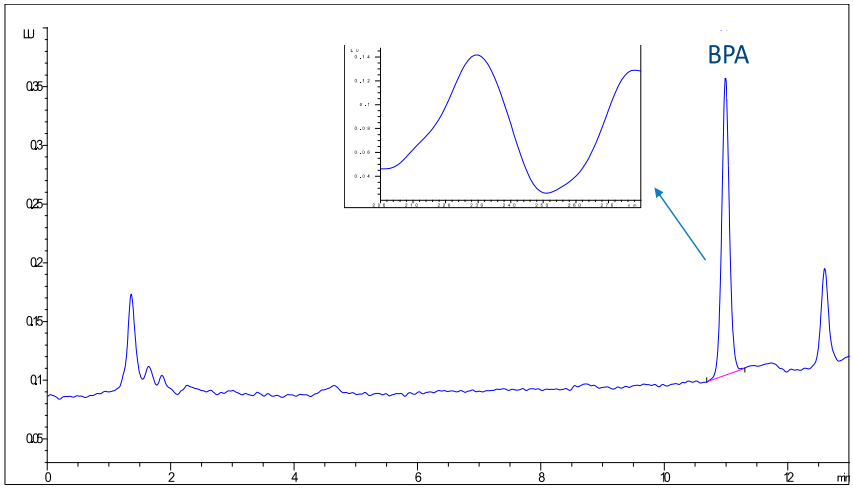


Figure 1. Chromatograms obtained by HPLC-FLD with a 0.005 mg·kg^{-1}standard solution: luminescence units (LU) vs. retention time (min).

As an important step in the validation process, is to demonstrate that the proposed methodology is fit to its purpose. This was evaluated through recovery assays, spiking a BPA free-sample. A working session with six replicates for each spiking level was performed in three different days. According to the results report in Table 4, it is confirmed that the method internal reproducibility is appropiate even at low concentrations, with average recoveries (n = 18) ranging from 72 % to 90%. Repeatability was always lower than 3.48%, except at 0.01 mg kg^{-1}, which was 9.94%, and intermediate precision was always lower than 4.70%, indicating good precision. Poustka et al. (2007) [22] in their research, reported the results of bisphenols monitoring in various categories of canned food obtained from the Czech retail market through the validation of analytical procedure HPLC coupled with FLD. They obtained recoveries, not below 75%, and LOD and LOQ of 0.002 and 0.01 mg kg^{-1}, respectively. In its turn, Errico et al. (2014) [23] in its work performed the determination of bisphenol A (BPA) in canned tomatoes by a method based on solid-phase extraction followed by liquid chromatography coupled to fluorescence spectrophotometry obtaining a LOQ of 0.26 µg BPA kg^{-1} tomato.

Figures 2 and 3 show the chromatogram obtained by HPLC-FLD with a fortification level of 0.01 mg kg^{-1}and a chromatogram obtained by HPLC-FLD of a sample of "sweet corn" where BPA was not detected, respectively.

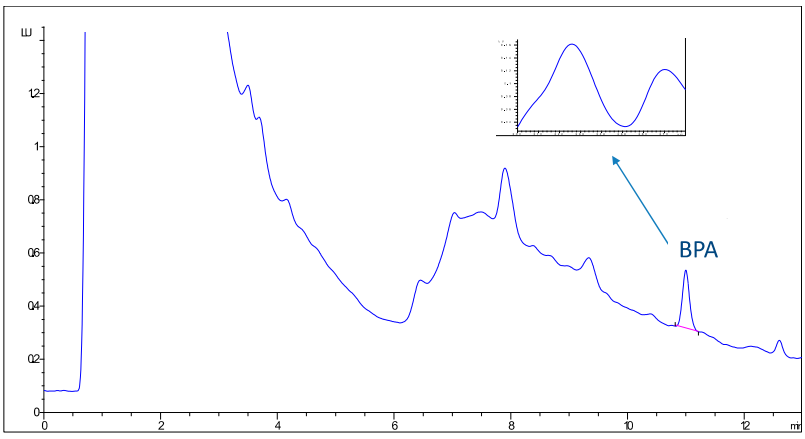


Figure 2. HPLC-FLD chromatogram of a blank sample ("sweet corn") with a fortification level of 0.01 mg kg^{-1}. Luminescence unit (LU) vs Retention Time (min).

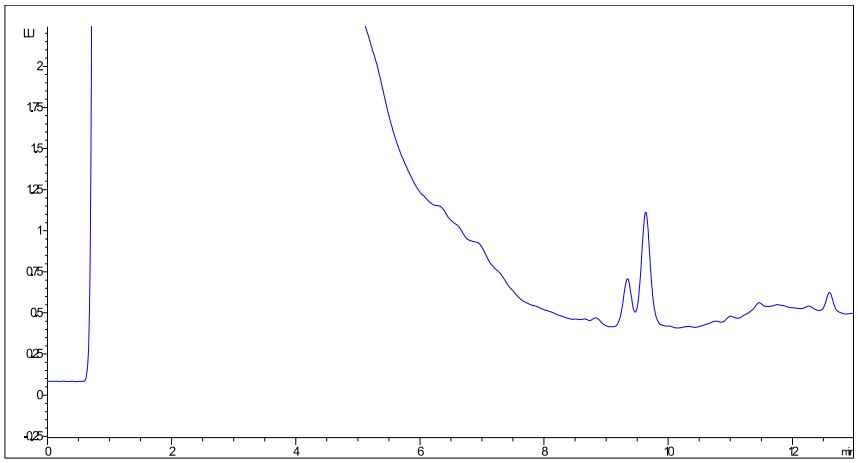


Figure 3. Chromatogram obtained by HPLC-FLD with a blank sample of "sweet corn": luminescence unit (LU) vs. retention time (min).

3.2. LC-MS/MS

In order to optimize and obtain the appropriate signal response in LC-MS/MS, acetonitrile and methanol were introduced as mobile phases. The best signal was achieved with methanol-water as a mobile phase.

MS data were acquired in selected reaction monitoring (SRM) mode once the optimization of the MS/MS parameters was performed using the built-in perfusion system. The ion m/z 227.2 has been assigned as the deprotonated molecule $[M–H]^-$, the transition m/z 227.2 > 212.1 was related with the additional loss of oxygen $[M–H–O]^-$ and the transition m/z 227.2 > 133.2 with the loss of phenol group [4].

LC-MS/MS is valuable to confirm the results of HPLC-FLD. However, this technique has a higher LOD (0.01 mg kg^{-1}) than HPLC-FLD (0.005 mg kg^{-1}). The Figure 4 shows the chromatogram obtained by LC-MS/MS for the 0.01 mg kg^{-1}standard solution of the BPA.

In our study, any of the samples presented detectable BPA according to HPLC-FLD, therefore it was not necessary to analyse them by LC-MS/MS. However, we decided to analyse the samples by LC-MS/MS and also none of them showed detectable amounts of BPA by this technique.

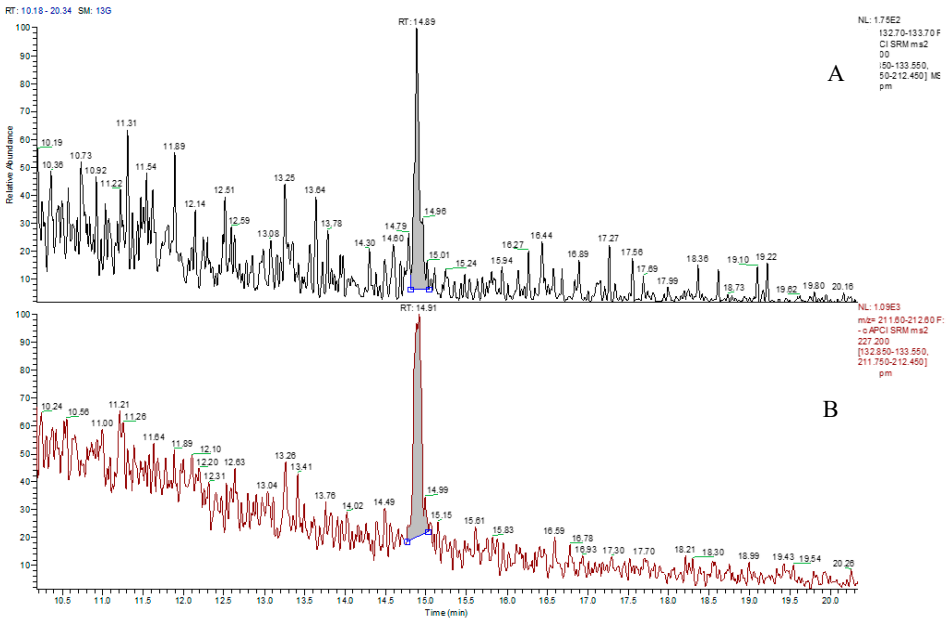


Figure 4. Chromatograms obtained by LC-MS/MS for the 0.01 mg kg^{-1}standard solution of the BPA: (**A**) the transition 227.2 > 133.1 m/z; (**B**) the transition 227.2 > 212.1 m/z.

3.3. ATR-FTIR

For the analysis of the cans by ATR-FTIR, small pieces of each sample (can) were cut with an area suitable to be used in the spectrophotometer. Pieces of the cover, bottom and side of each tin can were analyzed. Subsequently, each piece of the sample was analyzed by ATR-FTIR regarding their inner part. The identification was carried out by comparing obtained spectra with those of the IR Spectral libraries of Polymers and Related Compounds (Bio-Rad Laboratories, Inc.) database.

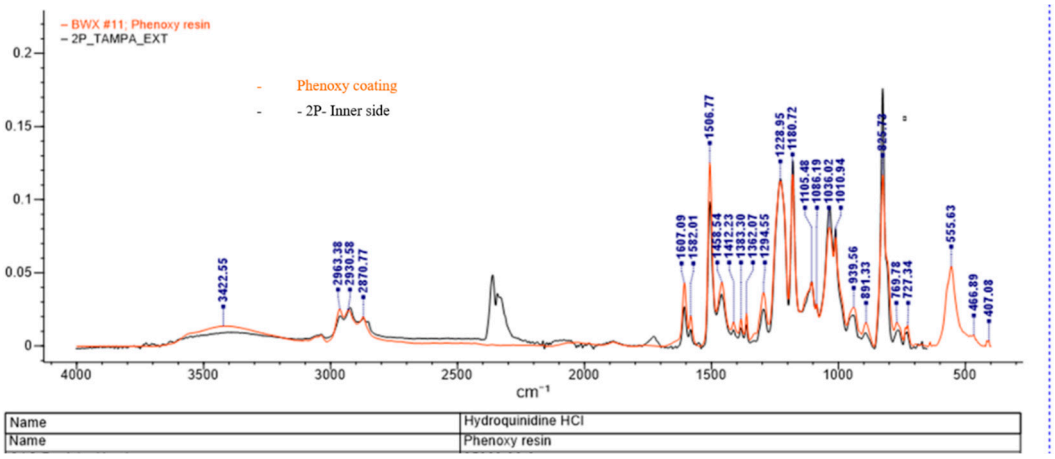
Analyzing the results listed in Table 6, it can be seen that polyester, phenoxy and epoxy coatings are those that are used in the samples. However, the polyester is the most used, as 78% of the samples have a polyester coating. Figure 5 shows, as an example, two spectra obtained for the samples 2P (phenoxy coating) and 3S (polyester coating), respectively.

Table 6. Main compound identified for each of the samples individually.

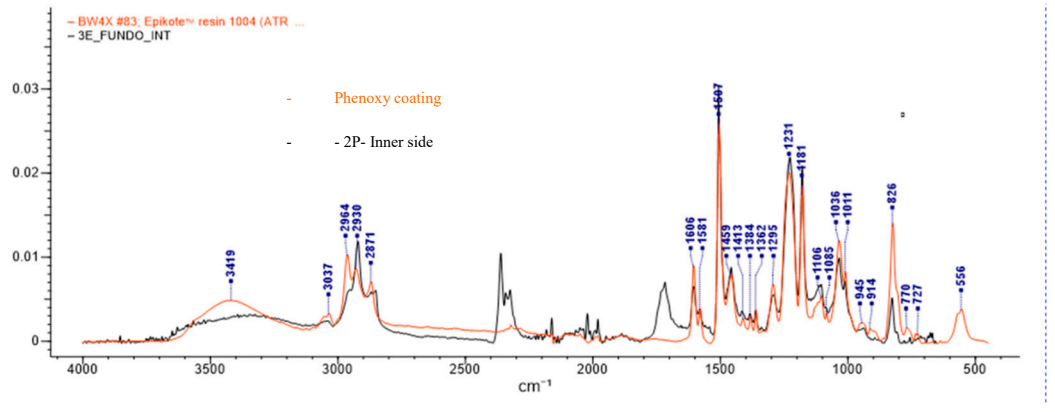
Samples/ Country	Part of the Tin Can Evaluated	Main Compound Identified
1S	Inner Body	Epoxy coating
	Inner Cover	Polyester
1P	Inner Bottom	Polyester
	Inner Side	Phenoxy coating
	Inner Cover	Polyester
2S	Inner Bottom	Modified phenolic coating
	Inner Side	Epoxy coating
	Inner Cover	Epoxy coating
2P	Inner Bottom	Polyester
	Inner Side	Phenoxy coating
	Inner Cover	Polyester
3S	Inner Bottom	Epoxy coating
	Inner Side	Polyester
	Inner Cover	Polyester
3P	Inner Bottom	Polyester
	Inner Side	Polyester
	Inner Cover	Polyester
4S	Inner Bottom	Polyester
	Inner Side	Polyester
	Inner Cover	Polyester
4P	Inner Bottom	Polyester
	Inner Side	Phenoxy coating
	Inner Cover	Phenoxy coating
5S	Inner Bottom	Polyester
	Inner Side	Polyester
	Inner Cover	Polyester
5P	Inner Bottom	Polyester
	Inner Side	Polyester
	Inner Cover	Polyester
6S	Inner Bottom	Phenoxy coating
	Inner Side	Polyester
	Inner Cover	Polyester
6P	Inner Bottom	Phenoxy coating
	Inner Side	Polyester
	Inner Cover	Polyester
7S	Inner Bottom	Phenoxy coating
	Inner Side	Phenoxy coating
	Inner Cover	Polyester
7P	Inner Bottom	Polyester
	Inner Side	Polyester
	Inner Cover	Phenoxy coating

8S	Inner Bottom	Epoxy coating
	Inner Side	Phenoxy coating
	Inner Cover	Polyester
8P	Inner Bottom	Epoxy coating
	Inner Side	Epoxy coating
	Inner Cover	Phenoxy coating
9S	Inner Bottom	Polyester
	Inner Side	Phenoxy coating
	Inner Cover	Phenoxy coating
10S	Inner Bottom	Epoxy coating
	Inner Side	Phenoxy coating
	Inner Cover	Phenoxy coating
11S	Inner Bottom	Epoxy coating
	Inner Side	Epoxy coating
	Inner Cover	Phenoxy coating

S: Spain; P: Portugal.



(**a**)



(**b**)

Figure 5. ATR-FTIR spectra of the: (**a**) phenoxy coating in the inner side of the sample 2P; (**b**) polyester coating in the inner bottom of the sample 3S.

Phenoxy resin has high molecular weight, a thermoplastic polymer having pendant hydroxyl groups and no oxirane ring [24]. Therefore, a broad peak of the hydroxyl group was observed at 3450.0 cm^{-1} in the FTIR spectrum, shown in Figure 5a [24]. In Figure 5b the band at 3454 cm^{-1} is related to stretching vibrations of OH groups. The bands in the range between 2800–3000 cm^{-1} correspond to stretching vibrations of CH groups such as CH_2 and CH_3 [25]. Weak bands at 1410 and 1462 cm^{-1} observed in the spectrum of polyester can be assigned to aromatic ring. The strong band at 1269 cm^{-1} that

appears in the spectrum of polyester is due to the twisting vibration of CH_2 groups [25]. The similarity between the two spectra is because Phenoxy coating is a thermoplastic polymer derived from BPA ($(CH_3)_2C(C_6H_4OH)_2$) and epoxy-epichlorohydrin ($Cl–CH_2–(C_2H_3O)$) [26].

4. Conclusions

An analytical method was validated, with success, for the accurate quantitation of Bisphenol A in canned vegetables by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Good recoveries were obtained showing that method is valid to determine the migration of BPA in these foods. The optimized method was also shown to be precise, and it presents a very low limit of detection (0.005 mg kg^{-1}) and limit of quantification (0.01 mg kg^{-1}). All the samples were analyzed by LC-MS/MS to confirm that BPA was not detected in any of them. The proposed HPLC-FLD method is an excellent tool to monitor the levels of BPA in canned vegetables due to being suitable to ensure compliance with the limit established by actual European Union legislation (SML 0.05 mg·kg^{-1}). Its application was demonstrated in real samples. We are aware of the fact that the main limitation of this study is the reduced number of samples. Therefore, in the near future, we are planning to design a study with a wider number of samples. If possible, we will try also to include samples from other countries besides Portugal and Spain, in order to compare the results and be able to reach conclusions on the influence of different the legislation systems in the number of positive samples.

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