

Article

# Development and Validation of an HPLC-DAD Method for the Simultaneous Extraction and Quantification of Bisphenol-A, 4-Hydroxybenzoic Acid, 4-Hydroxyacetophenone and Hydroquinone in Bacterial Cultures of *Lactococcus lactis*

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**Abstract:** Bisphenol-A, a synthetic organic compound with estrogen mimicking properties, may enter bloodstream through either dermal contact or ingestion. Probiotic bacterial uptake of bisphenol can play a major protective role against its adverse health effects. In this paper, a method for the quantification of BPA in bacterial cells of *L. lactis* and of BPA and its potential metabolites 4-hydroxybenzoic Acid, 4-hydroxyacetophenone and hydroquinone in the culture medium is described. Extraction of BPA from the cells was performed using methanol–H<sub>2</sub>O/TFA (0.08%) (5:1 *v/v*) followed by SPE. Culture medium was centrifuged and filtered through a 0.45 µm syringe filter. Analysis was conducted in a Nucleosil column, using a gradient of A (95:5 *v/v* H<sub>2</sub>O: ACN) and B (5:95 *v/v* H<sub>2</sub>O: ACN, containing TFA, pH 2), with a flow rate of 0.5 mL/min. Calibration curves (0.5–600 µg/mL) were constructed using 4-*n*-Octylphenol as internal standard (1 > R<sup>2</sup> > 0.994). Limit of Detection (LOD) and Limit of Quantification (LOQ) values ranged between 0.23 to 4.99 µg/mL and 0.69 to 15.1 µg/mL respectively. A 24 h administration experiment revealed a decline in BPA concentration in the culture media up to 90.27% while the BPA photodegradation levels were low. Our results demonstrate that uptake and possible metabolism of BPA in *L. lactis* cells facilitates its removal.

**Keywords:** HPLC-DAD; bisphenol A; 4-Hydroxybenzoic Acid; 4-Hydroxyacetophenone hydroquinone; *Lactococcus lactis*

## 1. Introduction

Bisphenol-A is the organic synthetic compound 4, 4'-Isopropylidenediphenol, which is widely used in thermal paper industry and as a component of synthetic plastic (vinyl-chloride) due to its mechanical and extreme-temperature resistance [1]. BPA, was found to exhibit estrogenic properties, arising from its structural resemblance to the human 17β-estradiol [2], thus it is also referred to as xenoestrogen or endocrine disruptor. The main routes of human exposure to BPA are the consumption of food in BPA containing packages [3] and through dermal contact with BPA rich materials [4]. Following exposure, BPA is mainly metabolized in humans to BPA-glucuronide through the hepatic glucuronide transferase and excreted from the body [5,6]. However, a percentage of BPA, referred to as “free” or “active” BPA, remains in the blood circulation for up to a week and can interfere with endogenous biological processes [4]. Due to its estrogenic and genotoxic effects and its wide

occurrence, BPA presents a risk to humans and animals and hence it is the object of great environmental concern [7–9].

Although part of bisphenol is degraded via photo-oxidation [10–12], monitoring of its levels is deemed necessary in various environmental sources [13]. To this end, biodegradation has been proposed as an advanced technique for BPA elimination [13–15], since a variety of microorganisms have been identified to decompose BPA [15,16]. Probiotics are bacteria beneficial to the host, which have been recognized as safe for human consumption and are used in the production of food products [17,18]. Recently they have been reported to be ideal against heavy metal toxicity since they bind and sequester metals [19]. Moreover, the probiotic bacteria *Bifidobacterium breve* and *Lactobacillus casei* when administered to rats showed a prophylactic effect against the detrimental effects of BPA, by reducing its intestinal absorption and facilitating its excretion, [18]. Likewise, the ability of *Bacillus* strains [20–22], certain *Lactococcus* strains [23] and *Shingomonas paucimobilis* [24] to degrade bisphenol has been documented. Recently a simplified method for the extraction of bisphenol from bacterial culture suspensions has been reported [25]. Moreover, the compounds 4-hydroxy acetophenone (HAP), 4-hydroxy benzoic acid (HBA) and Hydroquinone (HQ) have been proposed as major bacterial metabolites of BPA bacterial degradation pathways [14,26,27]. However, all these studies employed the determination of bisphenol in the cell free culture supernatant and not in the bacterial cells even though they examine the kinetics of bisphenol.

Analytical methods for the determination of bisphenol in various food samples [3], as well as in biological fluids and environmental samples [28], have been extensively reviewed. The determination of BPA has also been investigated in more complex matrices such as rat tissues [29], human breast milk [30] resin-based dental restorative materials [31] and saliva [32]. However, to the best of our knowledge, no method has been reported for the determination of bisphenol in bacterial cells.

In the present study, the development and validation of an analytical method for the quantification of BPA in bacterial cells is reported for the first time using the widespread probiotic bacterium *Lactococcus lactis* (ATC 11454). The method is also validated in bacterial culture supernatants for the simultaneous determination of the bacterial metabolites reported to be produced during the bacterial major metabolic pathway [14], namely 4-hydroxy-acetophenone (HAP), 4-hydroxy benzoic acid (HBA) and Hydroquinone (HQ). The application of this method demonstrates the uptake of bisphenol by *Lactococcus lactis* cells and allows its quantification.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Acetonitrile was purchased from VWR Chemicals (Paris, France). Methanol and Absolute ethanol were purchased from Fisher Scientific (Loughborough, UK). Trifluoroacetic acid (TFA),  $\text{KH}_2\text{PO}_4$  and  $\text{FeCl}_3$  were purchased from AppliChem GmbH (Darmstadt, Germany), while NaCl,  $\text{CaCl}_2$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{MgSO}_4$  were purchased from Merck KGaA (Darmstadt, Germany). All the chemicals used were HPLC Grade, unless specified otherwise.

Bisphenol-A (BPA) and the internal standard 4-*n*-Octylphenol (OP) were purchased from Alfa Aesar (Karlsruhe, Germany). 4-Hydroxyacetophenone (HAP), 4-Hydroxybenzoic Acid (HBA) and Hydroquinone (HQ) were purchased from Acros Organics (Ceel, Belgium).

The bacterial strain from which the liquid culture supernatant and the pellet originated and used for the spike-recovery studies, was *Lactococcus lactis* (ATCC 11454). The liquid culture medium that was used for the growth of *L. lactis* was Brain Heart Infusion broth (BHI broth) and was purchased from AppliChem GmbH (Darmstadt, Germany). An artificial bacterial culture medium was prepared, namely Basal Salts Medium (BSM), composed of  $\text{KH}_2\text{PO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$  1 g/L each,  $\text{MgSO}_4$  0.1 g/L, NaCl and  $\text{CaCl}_2$  0.05 g/L each and  $\text{FeCl}_3$  0.01 g/L. BPA was used as a sole carbon source in BSM in varying concentrations for the feeding experiments. For the retrieval of the bacterial culture supernatant and pellet 100  $\mu\text{L}$  of an *L. lactis* glycerol stock solution were inoculated in a 250 mL

flash with BHI broth. Following a 24-h incubation at 28 °C under constant stirring, the culture was centrifuged at 5000 rpm and the precipitate was transferred to a 250 mL flask containing BSM. At the end of the experiment, the culture was centrifuged, the precipitate was placed on a filtering paper and its weight was recorded following removal of excess water. The recorded weight is referred as 'wet cell weight'. The culture supernatant and the wet cell weight were used for the spike-recovery assays.

Solid Phase Extraction columns, frits and C<sub>18</sub> sorbent material, 35–75 U were purchased from Grace Davison Discovery Sciences (Bannockburn, IL, USA). Membrane filters with 0.45 µm diameter pores were purchased from Merck KGaA (Darmstadt, Germany).

## 2.2. Standard Preparation

### 2.2.1. Stock Solution Preparation

Stock solutions 500 µg/mL of BPA, HAP, HBA and HQ were prepared by dissolving 25 mg of the analyte in 50 mL methanol. The stock solution of BPA (50 mg/mL) was prepared by dissolving 2.5 g of BPA in 50 mL ethanol. The internal standard stock solution of 500 µg/mL was prepared by dissolving 25 mg of OP in 50 mL methanol.

### 2.2.2. Working Standard Solution Preparation

Seven-point and six-point working standard solutions were prepared for analytes at concentrations of 0.5, 10, 50, 100, 200, 400, and 600 µg/mL for BPA, at 0.5, 2, 4, 6, 8 and 10 µg/mL for HAP and HQ and at 1, 2, 4, 6, 8 and 10 µg/mL for HBA and the internal standard 4-*n*-octylphenol (75 µg/mL).

## 2.3. Sample Preparation

For the bacterial pellet method development, 0.1 g of wet cell weight *L. lactis* was added in a test tube with 8 mL methanol, 2 mL of double distilled water containing 0.08% (*v/v*) TFA and 150 µL internal standard from the 500 µg/mL stock. The concentration of the internal standard in the final 1 mL volume was 75 µg/mL. The test tube was transferred to an ice-filled container and was placed under a homogenizer for 5 min. The homogenate was separated in ten Eppendorf centrifuge tubes and was centrifuged for 10 min in 8000 rpm. The supernatant was collected and mixed with an equal volume of ddH<sub>2</sub>O containing 0.08% TFA. BPA extraction was performed using Solid Phase Extraction (SPE). An SPE cartridge was prepared using 0.5 g of C<sub>18</sub> sorbent in an SPE column. The SPE cartridge was conditioned with 10 mL methanol, 5 mL ddH<sub>2</sub>O and 5 mL ddH<sub>2</sub>O containing 0.08% TFA. The sample was loaded on the cartridge and a manual flow of 1 mL/min was employed. The cartridge was, then, washed with 5 mL ddH<sub>2</sub>O and elution was performed using 4 mL methanol. The eluate was collected and placed under a rotary evaporator in a 37 °C water bath, to dryness. Finally the sample was reconstituted with 1 mL acetonitrile and stored in an aluminum foil-covered Eppendorf tube at –20 °C. All experiments were conducted in dark or covered tubes to avoid photodegradation of BPA.

For the bacterial supernatant, an 8.5 mL sample of the culture was added in a test tube along with 1.5 mL internal standard from the stock solution. The sample was placed in 10 Eppendorf centrifuge tubes (1 mL per tube), and was centrifuged for 10 min in 8000 rpm. The supernatant was collected and filtered through a 0.45 µm diameter pore filter. The filtrate was stored in an aluminum foil-covered Eppendorf tube at –20 °C.

## 2.4. BPA Administration (Feeding) Assay

For the feeding assays 6 falcon tubes of 50 mL volume were filled with BHI broth and were inoculated with 100 µL of a *L. lactis* glycerol stock solution. The cultures were incubated for 24 h at 28 °C under constant stirring. The cultures were, then, centrifuged and the medium was discarded. A total of 50 mL of fresh BSM was added to each culture along with varying quantities from the BPA stock solution (50 mg/mL) so that the final BPA concentration in each culture would be 50, 100, 150, 200, 400 και 500 µg/mL respectively. The cultures were incubated for another 24 h at 28 °C

under constant stirring. The remaining BPA in the cultures was quantified using the method and the calibration curves that were described.

### 2.5. Photodegradation Assay

To investigate potential photodegradation of BPA, a falcon tube of 50 mL containing BSM and BPA at a concentration of 50 µg/mL was prepared and was incubated under the same conditions as the feeding assays. BPA was quantified at the beginning and the end of the 24 h incubation period. The presence of photodegradation products was examined in the resulting chromatograms.

### 2.6. HPLC-DAD Conditions and Methods

The instrument used consisted of an LC20<sub>AD</sub> pump and an SPD-20A photodiode array detector (DAD) purchased from Shimadzu (Kyoto, Japan). Separation of BPA was achieved on a Nucleosil 100 C<sub>18</sub> column (250 × 4.6 mm, 5 µm) purchased by Macherey-Nagel GmbH & Co. (Duren, Germany) with a binary mobile phase system at room temperature. The injection was performed using a 100 µL Hamilton syringe, with an injection volume of 80 µL. Mobile Phase A consisted of a 0.1% TFA in ddH<sub>2</sub>O solution and Acetonitrile at a ratio of 95:5, *v/v*. Mobile Phase B consisted of a 0.08% TFA in ddH<sub>2</sub>O solution and Acetonitrile at a ratio of 5:95, *v/v*. The gradient started at 100% Mobile Phase A which was brought down to 80% over 1 min, 70% over 4 min, 55% at 15 min, 40% at 17 min, 20% at 20 min and finally to 10% over 2 min and then was kept at this level until the end on the analysis. The flow rate was constant at 0.5 mL/min. BPA was detected at 220 nm with a band width of 2.0 nm. HAP, HBA and HQ were detected at 280 nm with a band width of 2.0 nm. Identification was conducted by matching the peak retention times (*Rt*) with those of the pure standards. Internal standard calibration was used by plotting the peak area ratio of the analyte to the peak area of the internal standard against the concentration of the analyte.

## 3. Results and Discussion

### 3.1. Optimization of Sample Preparation

The homogenization of the sample was performed to lyse the cells and release the adsorbed BPA for the feeding assay. Initially, ethyl acetate was used instead of methanol and a manual homogenization was attempted with a glass homogenizer, but cell lysis was deemed inadequate. Hence methanol was selected as the extraction solvent. The addition of an equal volume ddH<sub>2</sub>O containing 0.08% (*v/v*) TFA after centrifugation aimed to increase the solubility of BPA due to further dilution.

An elaborate and efficient vortex assisted liquid-liquid microextraction with octanol was used for the trace analysis of bisphenol in water and wastewater samples [33]. However, an attempt to use liquid-liquid extraction with ethyl acetate in the samples of the present study resulted to the formation of an emulsion zone between the two phases, a fact that led to greatly varying percentages of BPA recovery due to the loss of the analyte during the procedure. Thus, solid phase extraction was implemented, since SPE is a common step in the extraction procedures of bisphenol from complex matrices, such as rat tissues [29] or human serum [5] and its benefits in the biomonitoring of bisphenol have been demonstrated [28,33]. In the present study a flow rate of 1 mL/min was selected throughout the SPE procedure, as effective and timely favorable, while higher flow rates did not provide full retention of BPA by the C<sub>18</sub> material, especially at high BPA concentrations employed and resulted in lower recovery values. A 37 °C water bath was selected to facilitate the evaporation of methanol after SPE. The slightly higher evaporation temperatures of 45 °C that have been reported for the analysis of bisphenol and 4-nonylphenol [29] resulted in heat-induced decomposition of the internal standard when used in the present study. Our results for BPA extraction are in accordance with a recently published report on a methanol extraction of bisphenols from bacterial cultures and solid matrices [25] in which, however, SPE was not employed and lower concentrations of BPA were used [25], resulting in recoveries up to 95%. The SPE step used in the present study greatly improved recovery which

amounted to 99.38–100.18% in culture supernatants and to 90.8–102.46% in bacterial cells, in the wide range of concentrations employed (Table 1).

**Table 1.** Analyses of the calibration curves for analytes in the cell culture supernatant and the cell culture precipitate.

Analyte in Media/Std. Curve Equation/R <sup>2</sup>	Nominal Conc. (µg/mL)	Mean Calculated Concentration (µg/mL) (Mean ± SD)	Relative Bias (%)	Precision		Recovery Rate (Mean ± SD)
				Intra-Day (RSD %)	Inter-Day (RSD %)	
BPA in BSM Supernatant y = 0.0295x + 0.0077 1	0.5	0.48 ± 0.05	−3.6	8.7	10.4	96 ± 13
	10	9.94 ± 0.08	−0.6	0.1	0.8	99.4 ± 0.7
	50	50.2 ± 0.7	0.4	0.8	1.4	100 ± 1
	100	100 ± 2	0.05	0.8	1.7	100 ± 1
	200	200 ± 2	−0.2	0.7	1.2	100 ± 1
	400	401 ± 3	0.2	0.2	0.8	100.2 ± 0.6
BPA in <i>L. lactis</i> precipitate y = 0.0232x + 0.0092 0.9999	0.5	0.41 ± 0.03	−18.5	3.2	7.4	82 ± 5
	10	9.1 ± 0.4	−9.2	2.3	4.7	91 ± 4
	50	48 ± 1	−3.7	2.7	2.7	96 ± 2
	100	98 ± 3	−2.0	2.0	2.9	98 ± 2
	200	205 ± 3	2.5	0.4	1.4	103 ± 1
	400	400.6 ± 0.9	0.2	0.2	0.2	100 ± 0.2
HAP in BSM y = 0.202x + 0.0134 0.9994	0.5	0.52 ± 0.02	3.4	2.8	3.8	103 ± 3
	2	2.06 ± 0.05	2.9	0.2	2.4	103 ± 2
	4	3.84 ± 0.04	−4.1	0.7	1.0	95.9 ± 0.8
	6	6.1 ± 0.1	1.1	1.0	2.3	101 ± 2
	8	8.0 ± 0.2	0.6	2.1	2.2	101 ± 2
	10	10.0 ± 0.1	−0.3	0.3	1.0	99.7 ± 0.8
HBA in BSM y = 0.0407x + 0.0073 0.994	1	0.91 ± 0.03	−9.5	2.7	3.3	90 ± 2
	2	1.86 ± 0.04	−6.9	0.3	2.2	93 ± 3
	4	4.1 ± 0.2	2.3	3.2	4.2	102 ± 2
	6	6.2 ± 0.1	3.0	2.1	2.3	103 ± 2
	8	8.3 ± 0.3	4.2	3.0	3.1	104 ± 3
	10	9.62 ± 0.04	−3.8	0.3	0.4	96.2 ± 0.3
HQ in BSM y = 0.0351x − 0.0082 0.999	0.5	0.48 ± 0.01	−3.8	1.4	2.1	96 ± 2
	2	2.2 ± 0.1	7.5	3.4	6.5	108 ± 5
	4	3.91 ± 0.03	−2.2	0.03	0.8	97.8 ± 0.6
	6	5.91 ± 0.09	−1.5	1.6	1.5	98 ± 1
	8	7.9 ± 0.2	−1.4	0.5	2.0	99 ± 2
	10	10.1 ± 0.2	1.3	1.4	2.2	101 ± 2

In an effort to perform the analysis of BPA in the BHI medium, which is recommended for growth of *L. lactis*, the nutrients in the broth resulted in the presence of peaks in the chromatogram that appeared to interfere with the tested bacterial metabolites peaks. The artificial bacterial medium (BSM) was prepared to tackle the problems that arose in the analysis using the reference BHI medium. Moreover, in this manner BPA was the sole carbon source for the bacterium, a fact that contributes in its biotransformation.

### 3.2. Method Validation

The results of the linear regression analysis performed using the “Linear Regression” package of Microsoft Excel 2013 software are presented in Table 1, while detection limits and Retention time variance are presented in Table 2. The coefficient of determination values (R<sup>2</sup>) for the standard curves in BSM supernatant and *L. lactis* cells were 1 and 0.999 respectively, while for HAP, HBA and HQ in BSM supernatant the values exceeded 0.9994, 0.994 and 0.999 respectively, indicating excellent linearity. The Relative Bias was calculated by computing the percentage of the difference of the mean calculated concentration to the nominal concentration divided by the nominal concentration of the analyte. All results are within the accepted range of ±10%. Intra-day precision was calculated by performing a total of three injections of the nominal concentration on the same day, while inter-day

precision was calculated by performing six injections of the nominal concentration over three days. Both were computed as % R.S.D. = (S.D./mean) × 100. None of the values exceeded 6.0% for all analytes, with the exception of the values obtained for the lowest nominal value of 0.5 µg/mL of BPA in bacterial cells and in the medium, which might be due to the fact that this nominal value is below LOD (Table 2). Since an elaborate extraction protocol was required for the bacterial cells, the use of an internal standard was deemed necessary, to compensate for sample losses during sample preparation. The internal standard 4-*n*-octylphenol was selected, due to the fact that it is well resolved and it has a structure similar to bisphenol. Moreover, it is not expected to be present in samples, since only 4-*t*-octylphenol has been reported to be found at very low concentrations in environmental samples and human milk [34,35]. Our results, as presented in Table 1, indicate that the method is highly precise and repeatable. Recovery rates were well within range of 10% error, reaching close to 100% recovery, thus further supporting the precision of this method. The recovery results presented in Table 1 are in good correlation with those reported for rat serum (95%) and higher than those reported for rat testis (78.6%) and liver (84.0%) [29].

**Table 2.** Analyses LOD, LOQ and *Rt* values for all analytes.

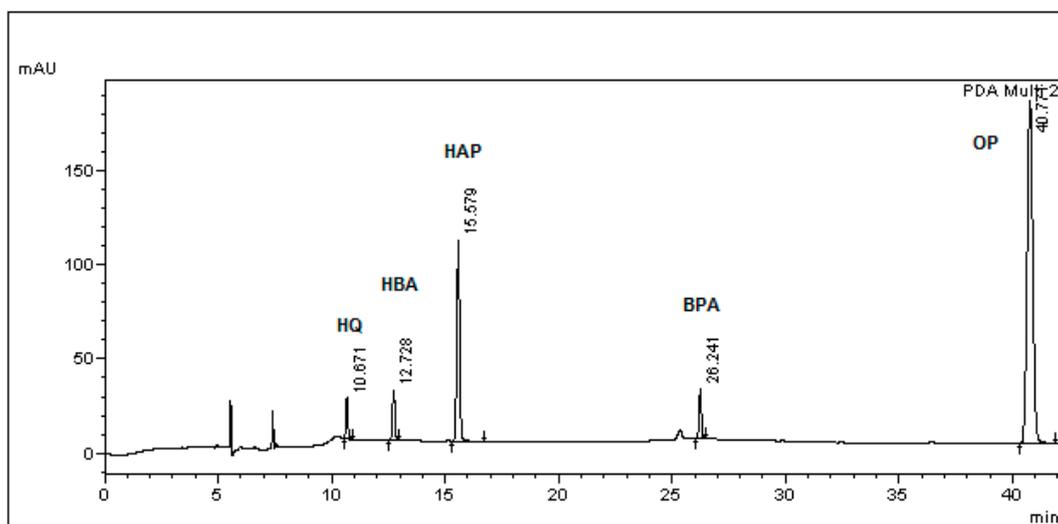
Std. Curve	Retention Time (Min) Mean ± SD (RSD %)	LOD (µg/mL)	LOQ (µg/mL)
BPA in <i>L. lactis</i>	26.68 ± 0.13 (0.41)	4.99	15.01
BPA in BSM	26.71 ± 0.09 (0.38)	0.64	1.93
HAP in BSM	15.66 ± 0.05 (0.29)	0.24	0.73
HBA in BSM	12.77 ± 0.12 (0.23)	0.73	2.21
HQ in BSM	10.80 ± 0.08 (0.90)	0.32	0.98

The nominal concentrations of 0.5 µg/mL BPA in BSM and cell precipitate were below the calculated LOD values (Table 2) and were thus excluded from the estimation of recovery results described above. The values for the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from the calibration curves. The LOD was calculated to 3.3 times the ratio of the Standard Error (intercept) to the Coefficients *x*, while the LOQ was 10 times this ratio. The retention time means, standard deviations and RSDs were calculated using the data from one chromatogram of each of the nominal concentrations used. The value of an LOD of 4.99 µg/mL estimated in the present study for BPA in bacterial cells at the range of 10–600 µg/mL, is higher than the value of 2.8 ng/mL reported for rat tissues, but over a narrower range of concentrations of 0.15–150 µg BPA/mL [29]. A lower value of LOD was found for BPA in the less complex sample of BSM, amounting to 0.64 µg/mL (Table 2), although the same range of concentrations was employed (Table 1), possibly due to lower matrix complexity. Similar low LOD values were estimated for all possible metabolites tested, as shown in Table 2. Although these compounds have been proposed as possible bisphenol bacterial metabolites, they were identified using MS and NMR analysis of the HPLC isolated fractions [27]. In fact, in the studies on the determination of bisphenol degradation using HPLC-DAD [20,23], no validation was included. The method presented in this work allows the simultaneous quantification of bisphenol A and three of its major bacterial metabolites.

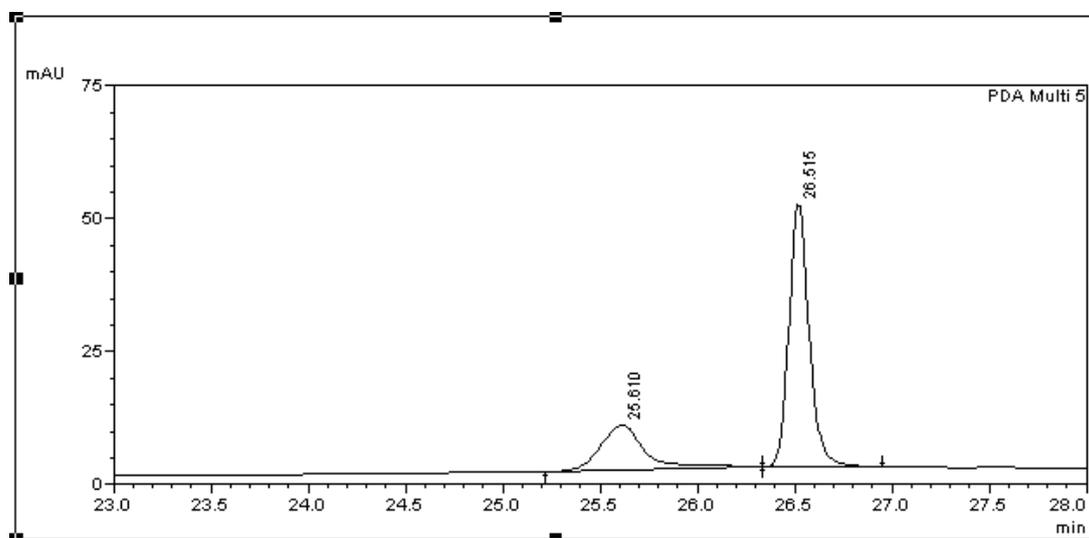
### 3.3. Feeding Results

A typical chromatogram of HQ, HBA and HAP and BPA is presented in Figure 1. Analyte peaks of HQ, HBA, HAP, BPA and internal standard OP are shown with a retention time (*Rt*) of 10.671, 12.728, 15.579, 26.241 and 40.771 min respectively. The peak that appears at 25.6 (Figure 1) might be attributed to a BPA photodegradation product, since it was also identified during the photodegradation experiment and is presented in Figure 2. Photodegradation of BPA via photooxidation has been confirmed [10,12], and the products of this process presented higher toxicity than the parent compound [11]. Since the feeding experiments were conducted under normal light, it was deemed

appropriate to test if any BPA loss was due to photodegradation. In these experiments the initial BPA concentration was calculated at 48.65  $\mu\text{g}/\text{mL}$  and the concentration following the 24 h incubation amounted to 44.18  $\mu\text{g}/\text{mL}$ . Hence, the BPA loss due to photodegradation was 9.19%. These values are in accordance with those reported for bisphenol A degradation by UV irradiation in the absence of hydrogen peroxide [36].

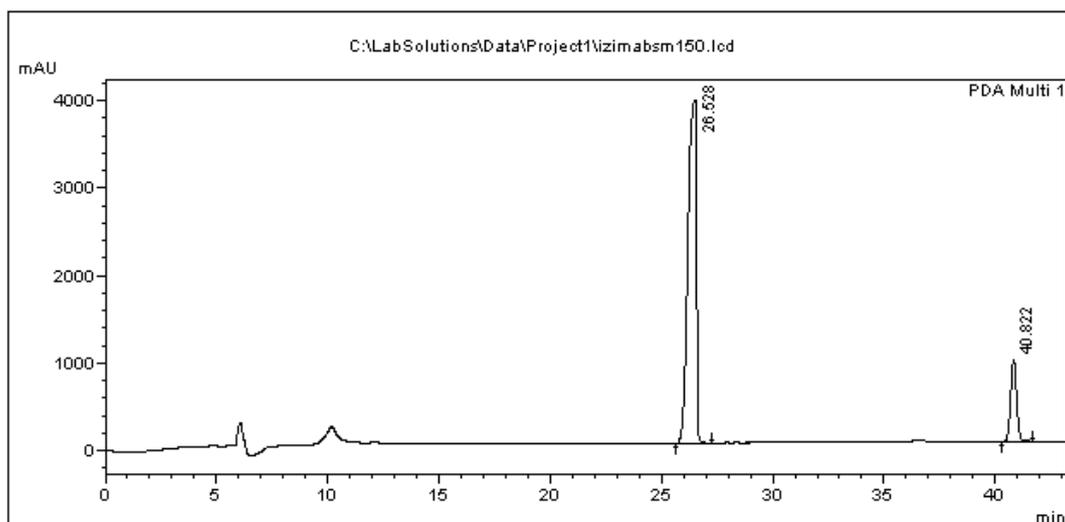


**Figure 1.** A chromatogram containing all analytes at a concentration of 10  $\mu\text{g}/\text{mL}$  and internal standard at 75  $\mu\text{g}/\text{mL}$  in fresh BSM without *L. lactis* inoculation. The first two peaks with  $R_t$  of 5.7 min and 7.5 min are background noise from acetonitrile solvent and TFA.

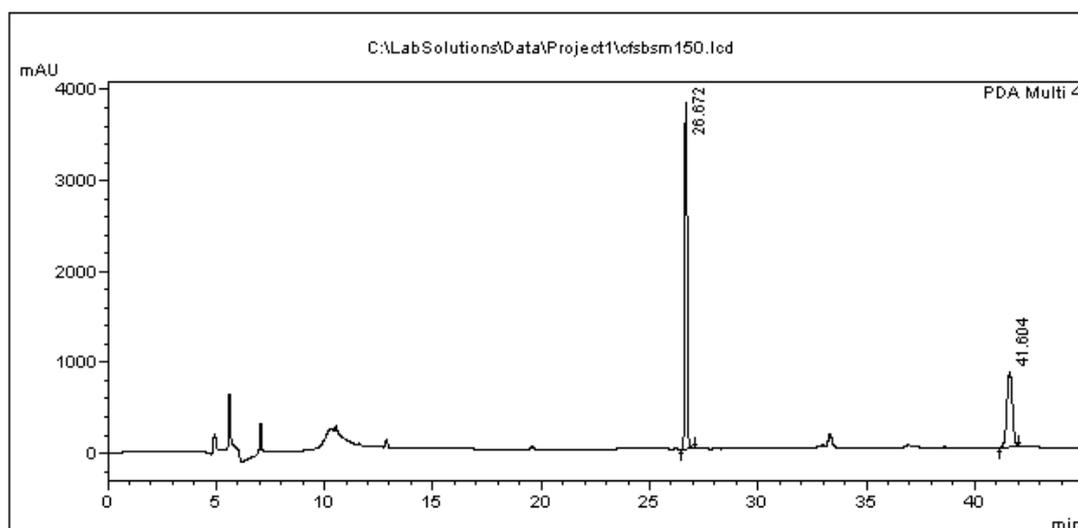


**Figure 2.** Chromatogram displaying BPA and its identified photodegradation product with a  $R_t$  of 26.515 min and 25.610 min respectively. The compounds were detected at a wavelength of 300 nm.

Typical chromatograms of the feeding assays are presented in Figure 3 (bacterial cells) and Figure 4 (bacterial culture medium). The relative blank chromatograms resulting from bacterial cells and from their culture medium any additions are shown in the Supplemental section. The peaks appearing at 10.2 min in both chromatograms does not represent one of the metabolites described in this paper, as confirmed by comparing the absorbance spectra of the peak to those of the pure metabolites analyzed.



**Figure 3.** Chromatogram of cell precipitate originating from the administration of 150  $\mu\text{g}/\text{mL}$  BPA in the bacterial cells. The peaks at 26.528 min and 40.822 min correspond to BPA and the internal standard.



**Figure 4.** Chromatogram of culture supernatant originating from the administration of 150  $\mu\text{g}/\text{mL}$  BPA in the bacterial cells. The peaks at 26.672 min and 41.604 min correspond to BPA and the internal standard.

Two major pathways have been proposed for the metabolism of bisphenol A by bacteria, the first leading to mineralization, with HBA and HAP as intermediates and the second to hydroxylation [6,16]. However, it has been pointed out that the metabolic pathways might be strain specific [14], since certain metabolites produced by one strain are not detected when another bacterial strain is used. In a similar manner, the analytes HQ, HBA and HAP were not detected in the chromatograms of the feeding assays. Absence of bacterial metabolites has also been reported for *Bacillus pumilus* [20] and certain *Lactococcus* strains [23]. In all these reports, including the present work, the presence of metabolites was examined at 24 h of culture. However, when various *Bacilli* and *Sphingomonas* strains were tested for bisphenol degrading capacity, metabolites were detected for three of them and only from 8–12 h, in a declining manner [37]. These results indicate that the time period of 24 h used in the present paper might be too long, since the metabolites under investigation might have formed and then further transformed. On the other hand, the bacterial metabolites examined in the present paper were

reported to be produced by *Bacillus* sp., yet only after 3 days of incubation in the presence of 20 mM BPA [21]. Moreover, the photodegradation of BPA metabolites has been reported for hydroxybenzoic acid as well as for hydroquinone [38,39]. It is evident that more experiments are needed in the course of bacterial incubation, since metabolism is an ongoing process and each strain presents particular metabolic characteristics.

A depletion of up to 58% in total BPA ( $\mu\text{g}$ ) was observed in our experiments, in all bacterial cultures as shown in Table 3. Complete degradation of BPA has been reported following a 48 h incubation period [37] or after 9 days of incubation [21]. In our experiments BPA was reduced by 90.27%, 64.16%, 58.60%, 67.74%, 79.83% and 85.05% in the cultures corresponding to initial BPA concentration of 50, 100, 150, 200, 400 and 500  $\mu\text{g}/\text{mL}$ . Our results suggest that the initial BPA concentration added has a profound effect on its degradation. Many bacterial strains that can degrade BPA have been reported, and this degrading ability varies among the species [14]. It should also be noted that different initial concentrations of BPA are used in the reports employing bacteria, ranging from 0.1 to 60  $\text{mg}/\text{L}$  [22,37,40] and for varying incubation periods. Bacteria can clearly employ a variety of metabolic pathways to exploit pollutants as energy sources, under the prerequisite that the pollutants are bioavailable and at levels that could trigger such mechanisms [16].

**Table 3.** Comparative data of BPA administered ( $\mu\text{g}$ ) and found in feeding assays.

BPA Administered in 50 mL Culture ( $\mu\text{g}$ )	BPA Found in Precipitate	BPA Found in 50 mL Supernatant ( $\mu\text{g}$ )	Total BPA Found	Concentration Decline in Cultures (%)
2500 (50 $\mu\text{g}/\text{mL}$ )	46.77	196.46	243.23	90.27
5000 (100 $\mu\text{g}/\text{mL}$ )	110.48	1681.74	1792.22	64.16
7500 (150 $\mu\text{g}/\text{mL}$ )	255.75	2849.48	3105.23	58.60
10,000 (200 $\mu\text{g}/\text{mL}$ )	262.20	2964.01	3226.22	67.74
20,000 (400 $\mu\text{g}/\text{mL}$ )	437.24	3595.83	4033.07	79.83
25,000 (500 $\mu\text{g}/\text{mL}$ )	421.16	3316.41	3737.57	85.05

#### 4. Conclusions

The developed analytical method provides a reliable, robust and sensitive approach for the simultaneous detection and quantification of bisphenol A and its potential metabolites in the complex matrix sample of bacterial cells. Good chromatography separation was achieved on a Nucleosil 100  $\text{C}_{18}$  column with a longer run time, which however allows the detection and separation of metabolites. LC-DAD combined with solid phase extraction and the use of 4-*n*-octylphenol as the internal standard, is capable of operating with a detection limit of 4.99  $\mu\text{g}/\text{mL}$  for bisphenol and lower limits for metabolites in bacterial cells. The method was also successfully applied in the analysis of bacterial culture medium with a detection limit of 0.64  $\mu\text{g}/\text{mL}$  for bisphenol. The feeding experiments revealed that *L. lactis* is capable to uptake of bisphenol and contribute to its elimination from the culture medium. Further experiments are needed for the detection and quantification of metabolites as a function of culture time and administrated bisphenol concentration.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2297-8739/5/1/12/s1>, Figure S1: Blank chromatograms of *L. lactis* cells (A) and cell free bacterial medium (B).

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**Conflicts of Interest:** The authors declare no conflict of interest.

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