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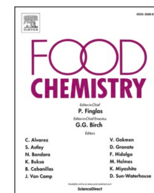

# Study on the chemical behaviour of Bisphenol S during the *in vitro* gastrointestinal digestion and its bioaccessibility

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A R T I C L E I N F O

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A B S T R A C T

This study evaluated the chemical behaviour of Bisphenol S (BPS) and determined its bioaccessibility after human ingestion using a standardised *in vitro* gastrointestinal digestion protocol and an analytical method based on high-pressure liquid chromatography coupled with a photodiode array and tandem mass spectrometry. The effects of different factors such as gastric pH, enzymes, and food matrix on the solubility and chemical stability of BPS were studied to evaluate their contribution to its bioaccessibility. The results highlighted that BPS was available at the end of the digestion process in the range of 50–80%, and was susceptible to absorption at the intestinal level. The effect of pH was not significant as a single factor. The presence of enzymes slightly decreased the bioaccessibility of BPS in the intestinal phase with gastric pH increase. Additionally, a soy drink reduced BPS bioaccessibility by up to 5% after oral intake. Finally, a few BPS degradation products were found in non-bioaccessible fractions at different pH values.

## 1. Introduction

Bisphenol A (BPA) has been gradually replaced by other analogues of the bisphenol family, such as Bisphenol S (BPS), because it belongs to the group of endocrine-disrupting chemicals (EDCs). The European Food Safety Authority (EFSA) defines an EDC as any chemical that can interact directly or indirectly with the endocrine system and, subsequently, lead to an alteration in the hormonal balance and cause adverse effects on target organs and tissues (EFSA, 2010). However, the data available regarding the safety of the bisphenol analogues are limited; thus the possibility that they can produce similar adverse effects as BPA cannot be excluded (Russo, Barbato, & Grumetto, 2016). A recent study performed by Thoene et al. (2020) highlighted that BPS was more toxic to the reproductive system than BPA and it promoted certain hormonal breast cancers at the same rate as BPA.

Bisphenol S or 4,4́-sulfonyldiphenol, which contains two hydroxyl groups joined by a sulfone group, is a main substitute of BPA. BPS is stronger than other bisphenols in terms of acidity and is more stable than BPA (Wu et al., 2018). The chemical structure and physicochemical properties of BPS are listed in Table 1 (ChemSpider, Royal Society of Chemistry, 2020).

BPS is used as a monomer in synthetic polymers as well as in epoxy, and is frequently found in a wide variety of consumer products, such as plastics and food packaging. In addition, the recycled paper and plastic, especially thermal paper, is a significant source of BPS in consumer-related products (Wu et al., 2018). BPS has also been detected by other studies in recycled cardboard and paper intended for use in food packaging (Liao, Liu, & Kannan, 2012, Vázquez Loureiro, Rodríguez-Bernaldo De Quirós, & Sendón, 2018). Similar to BPA, BPS can migrate from the contact material to the food, representing a risk to human health when ingested through the diet. Dermal exposure of BPS through contact is also possible in humans in addition to oral exposure (Liao, Liu, & Kannan, 2012).

The use of BPS as a monomer or starting substance in the manufacturing of plastic materials intended to come into contact with foodstuffs is currently permitted according to Regulation (EU) No 10/2011. BPS has a specific migration limit (SML) of 0.05 mg/kg of foodstuff (Commission Regulation (EU) No 10/2011), but this restriction is not applicable for other non-plastic food contact materials (Vázquez Loureiro et al., 2018). According to a report by the Scientific Cooperation Working Group of the EFSA, there is no other national legislation on BPS in the member states of the European Union (EFSA, 2011).

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EFSA is in close contact with the European Chemicals Agency (ECHA) regarding the ongoing proposal for the classification of BPS under the Reproductive Toxicant Category 1B for any adverse effects on development, sexual function, and fertility. However, on 16 April 2020, the EFSA published a technical report assessing two newly published studies on BPS. The new data from the investigations suggest that BPS is rapidly metabolised and eliminated from rats. Based on these two studies, the EFSA concluded that the lowest NOAEL (No Observed Adverse Effect Level) of 20 mg/kg body weight per day neither affects the current specific migration limit (SML) for BPS of 0.05 mg/kg food nor the current authorization under Regulation (EU) No 10/2011 of BPS (EFSA, 2020).

The use of sensitive and specific methods are mandatory to efficiently determine BPS in food-contact materials as well as in food samples. The methodologies recently used to determine BPS are liquid chromatography coupled to a fluorescence detector (HPLC-FLD) (Russo et al., 2016), a triple quadrupole mass spectrometer (LC-MS/MS) with ionization by electrospray ionisation (ESI) (Vázquez Loureiro et al., 2018, Xian et al., 2017), and atmospheric pressure chemical ionisation (APCI) (Eichman, Eck, & Lagalante, 2017).

The relationship between a food contaminant and its presence in the human body is complex. Several factors can influence its bioaccessibility (fraction released from the food matrix in the gastrointestinal tract which becomes available for absorption) and the bioavailability (fraction of the compound that reaches the systemic circulation) of food contaminants, such as the type of food matrix, the route of food contamination (internal or superficial), chemical properties of the contaminant, and cooking preparations (Cunha et al., 2017).

The estimated daily intakes (EDIs) of BPS were calculated in a study based on a simple pharmacokinetic approach, assuming similar pharmacokinetics for BPS and BPA. According to this model, the EDIs of BPS (mean values) were estimated to be 3.47, 1.48, and 0.707 μg/person/day in Japan, U.S.A., and China, respectively. The BPS EDIs were also calculated in another study based on bisphenol concentrations measured in food samples combined with estimated consumption patterns. According to this model, the EDIs of BPS were predicted to be 9.55 and 1.31 ng/kg body weight/day, for the Chinese and American adults, respectively (Geueke, 2014).

Despite advances in research on BPS in recent years, there are many gaps in knowledge regarding its chemical behavior in the human body after oral intake. There is a need to study the influence of the food matrix and the effects of luminal factors (such as pH and enzymes) on the potential of BPS to be absorbed by the human body and the possible interactions between BPS and other food components, (Cunha et al., 2017). To the best of our knowledge, no published information is available in literature regarding the bioaccessibility of BPS.

This study focuses on assessing the chemical behaviour of BPS and its potential degradation products at different gastric pH values, and the effect of enzymes during the different phases of a simulated gastrointestinal digestion process, with the objective of evaluating the bioaccessibility of BPS. The INFOGEST *in vitro* digestion model that was designed and published by Minekus et al. (2014), which was recently updated and published in Nature protocols (Brodkorb et al., 2019), was used in this study, and the analytical method for the determination of BPS was performed as described by Vázquez Loureiro et al. (2018) with slight modifications. The *in vitro* digestion process was carried out with a soy drink, a real food sample, since it is a typical hot beverage consumed in a disposable paper packaging that may contain BPS, to evaluate the effect of the food matrix on the bioaccessibility of BPS.

## 2. Materials and methods

### 2.1. Chemicals and analytical standards

*Solvents:* Acetonitrile (ACN) LC-MS grade, methanol (MeOH) LC-MS grade and absolute ethanol for analysis were obtained from Merck (Darmstadt, Germany). Ultrapure type I water was obtained from an Autwomatic Plus purification system (Wasserlab, Navarra, Spain).

*Analytical standards and sample preparation reagents*: Bisphenol S (BPS, CAS: 80–09-1) 98%, and potassium hexacyanoferrate(II) trihydrate ≥ 99.5% ($C_6FeK_4N_6$, Carrez I, CAS: 14459–95-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$, Carrez II, CAS: 7446–20-0) was obtained from Merck.

*Digestion reagents*: Calcium chloride dihydrate ≥ 99% ($CaCl_2(H_2O)_2$, CAS: 10035–04-8), potassium chloride ≥ 99% (KCl, CAS: 7447–40-7), magnesium chloride hexahydrate ≥ 99% ($MgCl_2(H_2O)_6$, CAS: 7791–18-6), and ammonium carbonate ≥ 30% ($(NH_4)_2CO_3$, CAS: 506–87-6) were purchased from Sigma-Aldrich, potassium dihydrogen phosphate 98–102% ($KH_2PO_4$, CAS: 7778–77-0) was purchased from Panreac (Barcelona, Spain), sodium bicarbonate > 99.7% ($NaHCO_3$, CAS: 144–55-8) was purchased from Probus (Badalona, Spain). Sodium chloride ≥ 99.5% (NaCl, CAS: 7647–14-5), hydrochloric acid 37% (HCl, CAS: 7647–01-0) and sodium hydroxide ≥ 99% (NaOH, CAS: 1310–73-2) were obtained from Merck (Darmstadt, Germany).

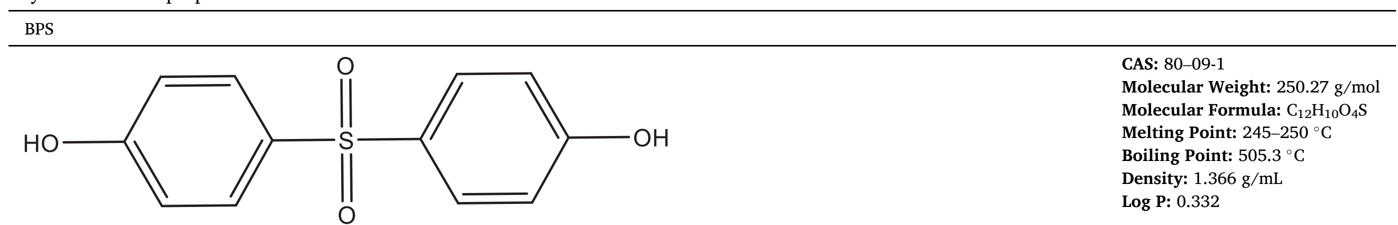
*Digestive enzymes:* α-amylase obtained from *Bacillus* sp. (CAS: 9000–90-2, 50 units (U) $mg^{-1}$ solid), pepsin obtained from porcine gastric mucosa (CAS: 9001–75-6, ≥ 250 U $mg^{-1}$ solid), pancreatin obtained from porcine pancreas (CAS: 8049–47-6, 8 USP; Lipase Activity: ≥ 8 U $mg^{-1}$; Amylase Activity: ≥ 100 U $mg^{-1}$; Protease Activity: ≥ 100 U $mg^{-1}$), and bovine bile (CAS: 8008–63-7) were purchased from Sigma-Aldrich.

A stock solution of BPS was prepared at a concentration of 1000 mg/L in ethanol. An intermediate solution with a concentration of 500 mg/L was prepared in ethanol to perform spiking of the sample. BPS concentrations higher than those usually found in food and food contact materials were used for the quantification of possible BPS degradation products after the resulting dilution at each stage of digestion. Ethanol minimises the impact on the viability of enzymes during protocol development (the final content of ethanol in the samples was 10%). Calibration solutions, in the concentration range of 0.1 to 50 mg/L, were prepared in 10% ethanol ($EtOH:H_2O$, 10:90 v/v). The stock solution was maintained at −30 °C, while the intermediate solutions were stored at 4 °C until analysis.

The Carrez I solution was prepared by dissolving 15 g of potassium hexacyanoferrate (II) trihydrate in 100 mL of water, while Carrez II was prepared by dissolving 30 g of zinc sulfate heptahydrate in 100 mL of water. Both solutions were maintained in a refrigerator until analysis.

**Table 1**
Physical-chemical properties of BPS.

BPS




**CAS:** 80–09-1
**Molecular Weight:** 250.27 g/mol
**Molecular Formula:** $C_{12}H_{10}O_4S$
**Melting Point:** 245–250 °C
**Boiling Point:** 505.3 °C
**Density:** 1.366 g/mL
**Log P:** 0.332

### 2.2. Food sample

A sample of soy drink was purchased from a local supermarket in Santiago de Compostela (Spain) and analysed to study the bioaccessibility of BPS in a real food sample. The ingredients mentioned in the label of the purchased soy drink were water, soybeans, and sea salt. The fat content was 2 g/100 mL (saturated 0.3 g/100 mL), the protein content was 3.6 g/100 mL, and 22 mg/100 mL of isoflavones were also present.

### 2.3. *In vitro* simulation of human gastrointestinal digestion (INFOGEST Protocol)

#### 2.3.1. Preparation of the fluids of each digestion phase and enzymes

The digestion protocol consists of three phases (oral, gastric, and intestinal) with their corresponding fluids: Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The fluids of each digestion step were prepared with ultrapure type I water, according to the methodology described by Minekus et al. (2014), recently improved by Brodkorb et al. (2019). Each digestion fluid had a different electrolyte composition, and the pH was adjusted to 7 in SSF, to pH 1, 2, and 3 in SGF and pH 7 in SIF using 1 M HCl or 1 M NaOH with a pH meter (Mettler Toledo, SevenCompact). The fluids were prepared every two days to avoid the pH variations over time and the potential growth of microorganisms favoured by the environment.

To carry out the digestion assay, α-amylase, pepsin and pancreatin from porcine, and the bile salts of bovine origin were used because of their low cost and their similarity to human enzymes in terms of function. The enzymes and the bile salts were prepared with ultrapure "type I" water to reach the final concentrations of 75 U/mL for α-amylase in the final fluid mixture of oral phase, 2000 U/mL for pepsin in the final fluid mixture of the gastric stage, and 100 U/mL for pancreatin in the final solution of the intestinal phase. Similarly, the bile salt mixture used during the intestinal phase was prepared to reach a final concentration of 10 mM in the final solution of the digestion process. The enzyme solutions and bile salts were prepared on the same day as the digestion protocol and kept during the entire procedure in a container with ice to retain enzyme stability and avoid activity losses.

#### 2.3.2. Static *in vitro* simulation of gastrointestinal food digestion

The standardised *in vitro* digestion simulation protocol carried out in this work was based on an international consensus developed by INFOGEST, and its methodology was designed to be used with standard laboratory equipment (Brodkorb et al., 2019). This method consists of simulating a static digestion, which is, reproducing a digestion process in which constant proportions of food are added to enzymes and electrolytes within a constant pH for each digestive phase. Falcon tubes spiked with BPS were prepared in triplicate for each stage of digestion (oral, gastric, and intestinal), and each gastric pH and its corresponding tubes with intestinal fluid (pH 1, 2, and 3). A blank control test tube was prepared without the BPS. The parameters used, such as the volume of electrolytes, enzymes, bile, pH, and time of digestion, were based on standard physiological data given by the INFOGEST protocol (Brodkorb et al., 2019). The pH of each fluid was checked and monitored during the digestion process that was performed in a water bath preheated to 37 °C with rotary shaking (GFL 1083). Oral digestion was performed at pH 7 for 2 min with amylase, the gastric digestion was performed at pH 1, 2, and 3, for 2 h with pepsin, and finally, the intestinal phase was carried out at pH 7 for 2 h by using pancreatin and bile salts. A non-enzyme assay and a test without the soy drink samples were also carried out to observe the effects of pH and the food matrix, respectively. For these assays, enzymes and the food matrix were both replaced by ultrapure Type I water.

Samples were collected after each phase and subjected to subsequent analysis by UHPLC-PDA-MS/MS. For the assays performed with enzymes, the samples were placed on crushed ice to stop or minimize enzyme activity at the end of each digestion step.

#### 2.3.3. Treatment of the sample for subsequent analysis by UHPLC-PDA-MS/MS

Once the digestion process was complete and the samples were collected, the Falcon tubes were centrifuged at 3992 × g for 10 min at 0 °C (Hettich Centrifuge Universal 320 R) to separate the supernatant and pellet. This procedure was done to verify the bioaccessibility of BPS and see if it is soluble in the medium or if the non-bioaccessible BPS precipitates and remains in the pellet. An aliquot of the supernatant (5 mL) was transferred to another Falcon tube and mixed with 500 µL of Carrez I and 500 µL of Carrez II (VELP Scientifica vortex mixer, Italy) to precipitate the soluble carbohydrates and proteins. The test tubes were then centrifuged again, and the supernatant was filtered under vacuum with a Phenomenex membrane of nylon with a diameter of 47 mm and a pore size of 0.45 µm. Finally, an aliquot of the extracted supernatant was filtered through a 0.20 µm membrane filter and introduced into the vial for UHPLC-PDA-MS/MS analysis.

The pellets obtained after the two steps of centrifugation (the first was the non-bioaccessible pellet, and the second was the bioaccessible pellet from the initial supernatant) were extracted with 10 mL of ACN. The sample was vortexed and placed in an ultrasonic bath (P. Selecta, Spain) for 10 min. Then, an aliquot of 2 mL was filtered using a membrane filter with a 0.20 µm pore size and evaporated until dryness using a stream of nitrogen at a temperature of 40 °C (RapidVap Vertex Evaporator, Labconco). Finally, the dry pellet residue was resuspended in a test tube with 1 mL of 10% ACN (ACN:$H_2O$, 10:90 v/v), mixed well in an ultrasonic bath for 10 min, and filtered again with a 0.20 µm filter before being introduced into the vial for subsequent chromatographic analysis.

### 2.4. UHPLC-PDA-MS/MS analysis instrumentation

The analytical method used for the determination of BPS was that described by Vázquez Loureiro et al. (2018), with some modifications. The system used for the identification and quantification of BPS was an ultra-high-performance liquid chromatography (UHPLC) system composed of an Accela autosampler, an Accela 1250 pump fitted with a degasser, and a column thermostatted system coupled with a photodiode detector (PDA), and a triple quadrupole mass spectrometer TSQ Quantum Access MAX. The software used for the acquisition of the chromatograms was Xcalibur version 2.1.0 (Thermo Fisher Scientific, San José, CA, USA).

For the chromatographic separation of BPS, a Kinetex Polar C18 100 Å column (100 mm × 2.1 mm internal diameter, 2.6 µm particle size) was used, with a pre-column from Phenomenex (Torrance, CA, USA). MeOH and water were used as mobile phases. The flow rate remained constant at 0.4 mL/min, and the injection volume was 10 µL. The chromatographic separation conditions were as follows: A mixture of MeOH:$H_2O$ (15:85 v/v) for the first minute in the isocratic mode, then the concentration of MeOH gradually increased to reach 60% at minute 8, followed by another gradient to 100% water at minute 12, which was held constant until 20 min. Finally, the method returned to the same initial conditions at minute 27.

Scanning in the PDA detector was performed continuously at wavelengths between 200 and 600 nm, and the total capture time was 20 min. Quantification was performed using the external standard method with a calibration curve at 260 nm. The mass spectrometer was operated in the negative ESI mode. The optimised settings of the MS/MS detector were as follows: Spray voltage, 2500 V; vaporizer temperature, 340 °C; capillary temperature, 350 °C. Nitrogen was used as the sheath gas (pressure 35 psi) and auxiliary gas (pressure 10 arbitrary units), while argon was used as the collision gas (1.5 mTorr). MS data were acquired in selected ion monitoring (SIM) mode, including the $m/z$ corresponding to BPS ($m/z$ 249) and also possible degradation products described in the bibliography: 73, 77, 78, 92.1, 93, 97.1, 106, 123, 125,

127, 129.1, 130, 137, 141, 143, 149, 155.9, 156.9, 157.1, 165.1, 173.1, 183.9, 187.1, 195.1, 199, 207, 217.1, 227, 233.1, 241, 255.1, 265, 271.1, 283, 317, 318, 338, 339, 351, 385, and 497 (Gao et al., 2018; Lu et al., 2019; Shao et al., 2017; Sun et al., 2019).

### 2.5. Statistical analysis

The contents of BPS during the different stages of digestion and under diverse test conditions are presented as the mean ± standard deviation. Data were compared by one-way ANOVA, and significant differences were assessed by Duncan's post hoc test at a 95% confidence level using the IBM SPSS Statistics software for Mac OS X (version 24.0; StatSoft, Inc., Tulsa, OK, USA).

## 3. Results and discussion

### 3.1. Analytical performance

The quantification of BPS was performed using a UHPLC-PDA-MS/MS method previously developed and validated by Vázquez Loureiro et al. (2018) with some modifications. The analytical performance of the selected method was evaluated for the linearity, sensitivity, and precision. BPS quantification was done using the external standard method with a calibration line of eight points in the concentration range of 0.1–50 mg/L at a wavelength of 260 nm. Each concentration was injected in triplicates. The calibration line fitted to the linear equation $y = a \times + b$, where $a$ is equal to 101,786 and $b$ is 24691. The correlation coefficient, $R^2$, was 0.9990, indicating good linearity. The example of a UV chromatogram at 260 nm and an MS chromatogram extracting the $m/z$ 249 from a BPS standard at 5 mg/L is shown in Fig. 1.

Sensitivity was evaluated based on the limits of detection (LODs) and quantification (LOQs). The quantification and detection limits were estimated as the lowest concentration that provided a signal-to-noise ratio higher than ten or three, respectively. The method performed in this study showed a good sensitivity with an LOD of 0.001 mg/L and an LOQ of 0.1 mg/L, which corresponds to the lowest calibration level of the calibration curve. The precision, expressed as the relative standard deviation (RSD %), was determined in terms of repeatability analysis, where standard solutions at all concentration levels were evaluated inter-day (n = 8). Good repeatability was demonstrated with RSD lower than 8% in terms of peak areas and retention time.

The extraction method was examined in terms of the recovery percentage. Triplicate Falcon tubes were prepared and spiked with BPS at a concentration of 50 mg/L per phase of digestion (oral, gastric, and intestinal) and at each gastric pH (pH 1, 2, and 3). The recoveries obtained were in the range of 70–109%, except those in the intestinal phase at pH 3 in the presence of enzymes (see Table 2).

### 3.2. Effect of pH on the solubility of BPS in the absence of enzymes

Since the pH of the gastric lumen in humans is quite variable, different pH values were tested within the range of 1–3 to cover all the possible pH values (Scott, Weeks, Melchers, & Sachs, 1998) and evaluate the possible effects of the acidic pH on the solubility and chemical stability of BPS in this study.

Table 2 shows the results of the BPS quantities (in μg), expressed as the mean of three replicates with the standard deviation, and the corresponding recovery obtained at each phase and pH for all conditions. The percentage of bioaccessible BPS which was soluble in the supernatant, and of non-bioaccessible BPS insoluble in the pellet, were calculated based on the ratio of milligrams of BPS in the sample to the milligrams expected (Table 2).

The results revealed that in the absence of both enzymes (Non-Enz) and food matrix (Fig. 2a), most of the BPS was soluble in the supernatant and, therefore, could be bioaccessible (B). The results indicated that there was no significant difference between the three pH values tested ($p > 0.05$), since the recovery percentage of the BPS remained around 80% in the gastric phase (see Fig. 2a), except for that in the intestinal phase, which slightly decreased. However, the recovery percentage of BPS in the supernatant and in the pellet was slightly higher at acidic pH values (in the gastric phase) than at pH 7 (intestinal phase), as seen in Table 2. There were contrasting results for the assay performed in the soy drink

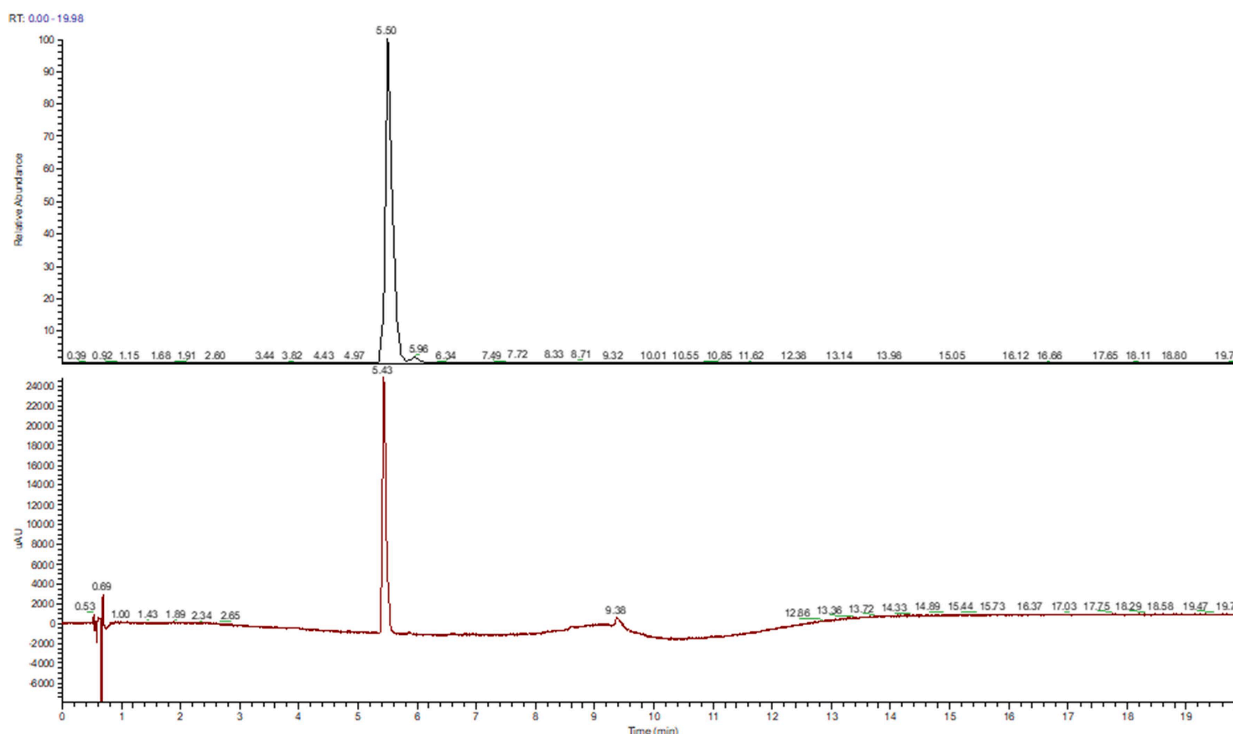



**Fig. 1.** MS chromatogram extracting the $m/z$ 249 (top) and UV chromatogram at 260 nm (bottom) from a standard of BPS at 5 mg/L.

**Table 2**
Average quantity values (n = 3) of spiked samples (μg) before digestion ($C_i$ = 250 μg) and relative recoveries of BPS in both fractions obtained from the different digestion phases, bioaccessible (B) and non-bioaccessible (NB), for the four assays performed in presence and absence of enzymes and food matrix.

| *In vitro* Digestion phases | | Absence of enzymes | | Presence of enzymes | | Sig. |
|---|---|---|---|---|---|---|
| | | Soy drink | Without soy drink | Soy drink | Without soy drink | |
| **Oral** | | | | | | |
| **pH 7** | **B** (μg) | 186.7 (±1.4)$^{a}$ | 182.4 (±10.6)$^{a}$ | 187.4 (±0.9)$^{a}$ | 197.2 (±3.4)$^{a}$ | n.s. |
| | **NB** (μg) | 15.2 (±2.2)$^{a}$ | 2.4 (±1.0)$^{c}$ | 11.4 (±1.1)$^{b}$ | 1.9 (±0.3)$^{c}$ | *** |
| | **Recovery** (%) | 81 (±1.1)$^{C}$ | 74 (±4.3) | 80 (±0.5)$^{C}$ | 80 (±0.4)$^{A}$ | n.s. |
| **Gastric** | | | | | | |
| **pH 1** | **B** (μg) | 193.0 (±4.7)$^{a}$ | 200.1 (±17.3)$^{a}$ | 197.4 (±6.9)$^{a}$ | 202.8 (±7.8)$^{a}$ | n.s. |
| | **NB** (μg) | 13.6 (±1.2)$^{b}$ | 1.5 (±0.4)$^{c}$ | 40.6 (±2.9)$^{a}$ | 3.3 (±0.8)$^{c}$ | *** |
| | **Recovery** (%) | 83 (±1.4)$^{bBC}$ | 81 (±0.6)$^{b}$ | 95 (±3.8)$^{aB}$ | 82 (±3.4)$^{bA}$ | * |
| **pH 2** | **B** (μg) | 197.1 (±0.9)$^{b}$ | 196.0 (±13.7)$^{b}$ | 216.8 (±7.4)$^{a}$ | 201.7 (±1.2)$^{b}$ | * |
| | **NB** (μg) | 11.8 (±1.1)$^{b}$ | 1.4 (±0.1)$^{c}$ | 20.2 (±6.9)$^{a}$ | 2.3 (±0.8)$^{c}$ | ** |
| | **Recovery** (%) | 84 (±0.4)$^{bAB}$ | 79 (±1.0)$^{b}$ | 95 (±5.7)$^{aB}$ | 82 (±0.5)$^{bA}$ | ** |
| **pH 3** | **B** (μg) | 200.7 (±1.1)$^{a}$ | 202.3 (±7.6)$^{a}$ | 156.6 (±13.6)$^{b}$ | 195.4 (±3.4)$^{a}$ | *** |
| | **NB** (μg) | 13.8 (±0.9)$^{b}$ | 1.5 (±0.2)$^{c}$ | 115.1 (±4.3)$^{a}$ | 3.2 (±0.4)$^{c}$ | *** |
| | **Recovery** (%) | 86 (±0.6)$^{bA}$ | 82 (±0.6)$^{bc}$ | 109 (±4.7)$^{aA}$ | 79 (±1.2)$^{cA}$ | *** |
| **Intestinal** | | | | | | |
| **pH (1) 7** | **B** (μg) | 196.9 (±4.8)$^{a}$ | 180.4 (±4.2)$^{b}$ | 200.5 (±10.7)$^{a}$ | 207.4 (±5.9)$^{a}$ | ** |
| | **NB** (μg) | 6.3 (±0.3)$^{a}$ | 2.0 (±0.3)$^{b}$ | 1.0 (±0.1)$^{c}$ | 0.5 (±0.2)$^{d}$ | *** |
| | **Recovery** (%) | 81 (±1.1)$^{aBC}$ | 73 (±1.8)$^{b}$ | 81 (±1.1)$^{aC}$ | 83 (±2.4)$^{aA}$ | * |
| **pH (2) 7** | **B** (μg) | 197.3 (±4.3)$^{a}$ | 189.2 (±1.8)$^{a}$ | 203.9 (±7.7)$^{a}$ | 197.9 (±2.9)$^{a}$ | n.s. |
| | **NB** (μg) | 4.5 (±0.2)$^{a}$ | 1.7 (±0.6)$^{b}$ | 1.0 (±0.34)$^{b}$ | 1.6 (±0.9)$^{b}$ | ** |
| | **Recovery** (%) | 81(±1.8)$^{aC}$ | 76 (±0.7)$^{b}$ | 82 (±3.1)$^{aC}$ | 80 (±0.8)$^{aA}$ | ** |
| **pH (3) 7** | **B** (μg) | 181.6 (±3.1)$^{a}$ | 175.3 (±10.8)$^{a}$ | 119.3 (±4.1)$^{b}$ | 114.6 (±3.3)$^{b}$ | *** |
| | **NB** (μg) | 4.2 (±0.8)$^{ab}$ | 1.3 (±0.4)$^{c}$ | 6.7 (±2.9)$^{a}$ | 1.9 (±0.1)$^{bc}$ | ** |
| | **Recovery** (%) | 74 (±1.0)$^{aD}$ | 71 (±4.3)$^{a}$ | 51 (±1.1)$^{bD}$ | 47 (±1.3)$^{bB}$ | *** |
| **Sig.** | | *** | n.s. | *** | *** | |

B: bioaccessible fraction; NB: non-bioaccessible fraction.
Data are expressed as the mean values ± standard deviation (n = 3). Means followed by different lowercase superindexes indicate significant difference at $p < 0.05$ among the BPS amounts for the NB and B fractions and total recovery percentages, within each assay in the absence and presence of enzymes and food matrix (same row); means followed by different uppercase superindexes indicate significant differences at $p < 0.05$ for the recovery percentages of the different digestion phases and pH values tested within the same assay (same column); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

without enzymes (Fig. 2b). The solubility of BPS among the different digestion stages varied significantly ($p < 0.001$), with the highest percentage of soluble BPS obtained at pH 3 of the gastric phase, while the lowest was at pH 7, in both the intestinal (after pH 3 at the gastric stage) and oral steps.

The effect of pH, as a single factor, on BPS solubility observed at different stages of digestion was not significant. However, the pH effect, together with other variables, such as the presence of a food matrix or enzymes, could significantly impact the bioaccessibility of BPS and will be further discussed.

### 3.3. Effect of enzymes on the bioaccessibility of BPS at different phases of digestion

The results showed that similar percentages of bioaccessible BPS were obtained in the oral and gastric phases on comparing the test performed without enzymes and the assay performed with the enzymes and without the food matrix (Fig. 2a). However, significant differences in the percentage of bioaccessible BPS were observed that were dependent on the pH in the intestinal phase. The bioaccessibility of BPS increased significantly for the samples obtained from the gastric phase set at pH 1 and pH 2 ($p < 0.01$ and $p < 0.05$, respectively), compared to the assay performed without enzymes. However, the bioaccessibility of BPS significantly decreased at pH 7 to < 50% ($p < 0.01$) for the samples obtained from the gastric phase at pH 3.

The percentage of bioaccessible BPS decreased as the pH (after the gastric phase) increased (pH 1 > pH 2 > pH 3) in the intestinal phase. This effect could be due to the fact that trypsin precipitated BPS in the intestinal phase at its optimal pH and a poor extraction was obtained with the method used to break these interactions, as described by Wang and Zhang (2014). A similar effect was observed in the gastric phase, where the percentage of bioaccessible BPS decreased as the pH increased. This could be due to the fact that at pH 1 and 2, the enzyme pepsin reduces its active site, while pepsine is close to its isoelectric point at pH 3 and could bind to BPS and precipitate it. Wang and Zhang (2014) showed that the BPS could affect the secondary and tertiary structures of both enzymes (trypsin and pepsin). The previous study also observed that the activity of pepsin decreased with increasing concentrations of BPS, while trypsin activity did not change remarkably. However, the concentrations of BPS used by these authors were higher, and the amount of enzymes used was lower than that used in the present study. Moreover, in this work, pancreatin was used for the intestinal phase in this study. Pancreatin is a mixture of several digestive enzymes, including trypsin, amylase, and lipase, among other components. This mix of enzymes, together with the bile salts, could contribute to the reduction of BPS bioaccessibility at the intestinal level after the gastric phase at pH 3. Other studies have shown that BPS can also inhibit the activity of other proteins, such as alpha-amylase and serum albumin (Mathew, Sreedhanya, Manoj, Aravindakumar, & Aravind, 2014; Yang, Hou, Zhang, Ju, & Liu, 2017), as well as alter the microenvironment around cells and induce oxidative stress (Zhang et al., 2016).

The remaining percentage of BPS that could not be determined in both the supernatant and pellet of the intestinal phase might be due to the change in pH from acidic to neutral at the end of the gastric phase. Moreover, the addition of electrolytes at the beginning of the intestinal phase could also favour the degradation or possible polymerization reaction or the aggregate formation of BPS products that can be insoluble in the medium, which cannot be determined under the test conditions used in this study.

Thus, it is possible that BPS might undergo some chemical transformation into possible degradation products considering that 100 % of the BPS added to the initial solution was not recovered in the samples (especially in the intestinal phase). However, a limited number of chemical species were detected during the UHPLC-MS/MS analysis.

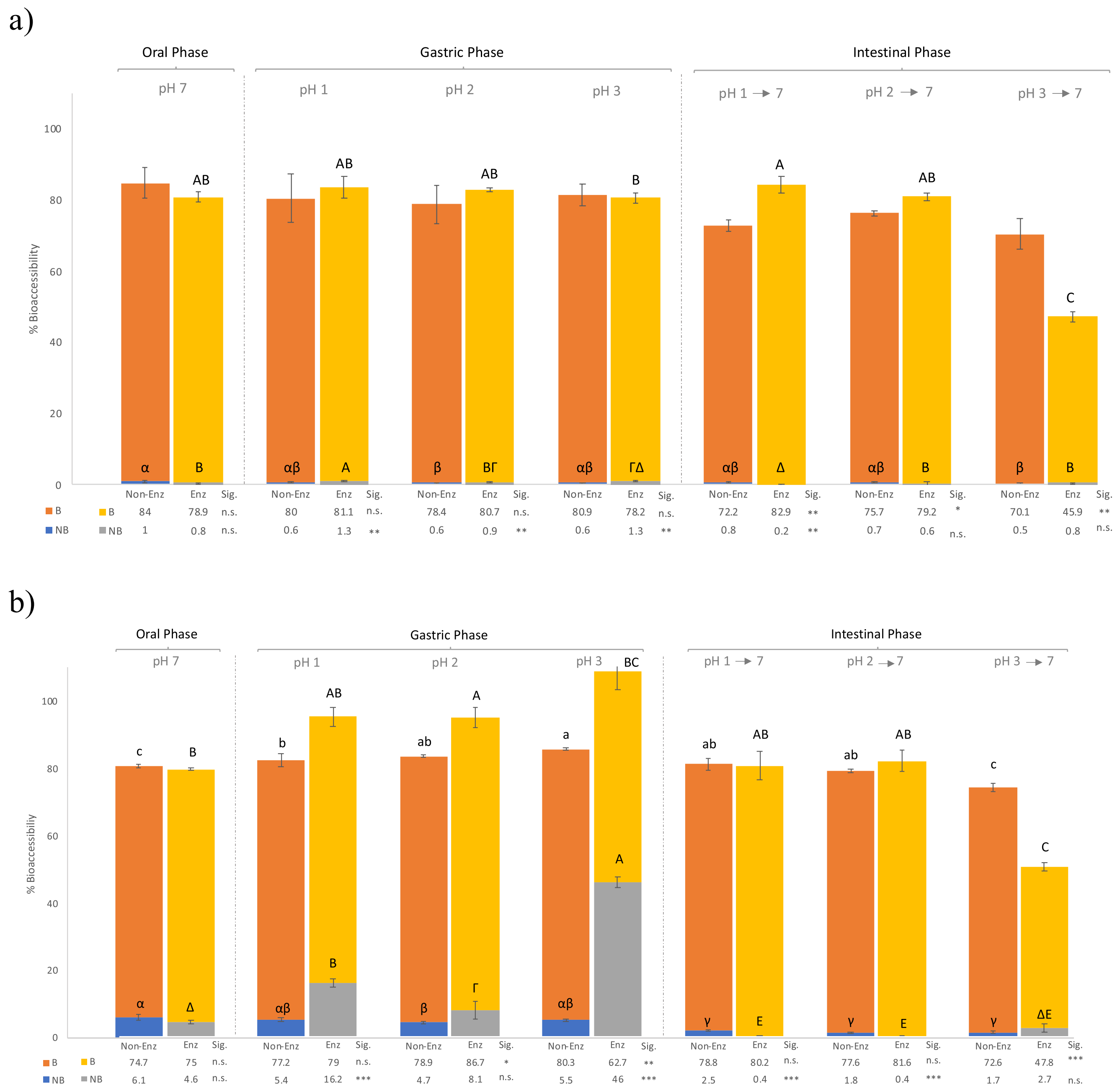



**Fig. 2.** Effect of the presence (Enz) and absence (Non-Enz) of enzymes on the solubility and bioaccessibility (B) and non-bioaccessibility (NB) of BPS during the different stages of gastrointestinal digestion at different pH values evaluated in a) The absence of food matrix (replaced by water) and b) The presence of food matrix (soy drink). The lowercase letters indicate significant difference at $p < 0.05$ among BPS percentages for the NB and B fractions for the assays in the absence of enzymes; while different uppercase letters indicate significant difference at $p < 0.05$ for different digestion phases and pH values tested for the assays with enzymes; Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant. Greek alphabet letters were used to indicate significant differences among NB fractions and Latin alphabet for B fractions.

### *3.4. Effect of the food matrix on the bioaccessibility of BPS*

The studies available so far regarding the effects on BPS are in an environmental context, using water or residual water as a sample (Kovačič, Gys, Kosjek, Covaci, & Heath, 2019). The tests performed in this study without a food matrix would be the equivalent of the previous studies. To the best of our knowledge, this is the first study in which a soy drink was used as the food matrix. The selection of this food sample was based on its packaging material, where BPS was previously detected (Vázquez Loureiro et al., 2018), as it is a hot beverage consumed in a takeaway paper packaging.

As shown in Fig. 3, the data were reorganised to compare and evaluate the effect of the food matrix on the bioaccessibility of BPS without enzymes (Fig. 3a) and with enzymes (Fig. 3b).

Regarding the solubility and stability of BPS on the food matrix without enzymes (Fig. 3a), the results showed that the bioaccessibility of BPS changed significantly ($p < 0.001$) among the different phases of the digestion process, contrary to that observed for the assay performed in water ($p > 0.05$). The solubility of BPS decreased in the intestinal phase at pH 7 after the gastric phase at pH 3. However, the comparison between soy drink and water samples at the same stage of digestion and pH values of the assay revealed no significant differences ($p > 0.05$) among almost all cases regarding the bioaccessibility of BPS. On the contrary, the differences were more significant ($p < 0.001$) for the non-

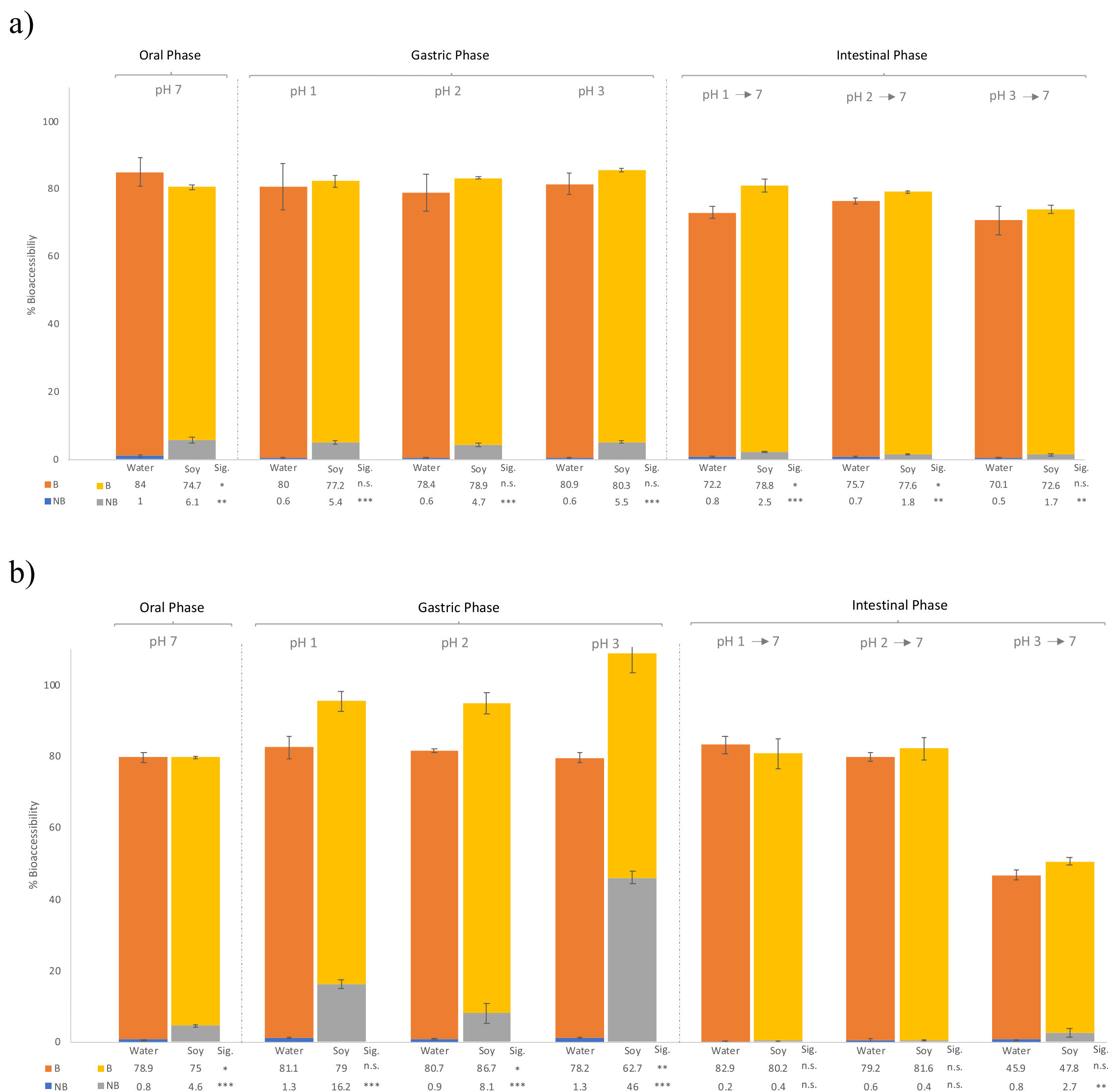



**Fig. 3.** Effect of the food matrix, soy drink, (soy) versus its replacement by water (water) on the solubility and bioaccessibility (B) and non-bioaccessibility (NB) of BPS during the different stages of gastrointestinal digestion at different pH values evaluated in a) absence of enzymes and b) in the presence of enzymes. Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant.

bioaccessible (NB) fractions in the oral and gastric phases with high levels of insoluble BPS in the pellets of the soy drink samples.

The percentage of bioaccessible BPS at pH 1 and 2 was maintained compared to the oral and gastric phase (see Fig. 3b) in the test with the soy drink sample performed with enzymes. However, the BPS solubility decreased significantly ($p < 0.001$) in the gastric phase set at pH 3, having a greater impact on the final bioaccessibility after complete digestion (50% bioaccessible in the intestinal phase). This effect was also observed in the assay performed without the food matrix and could be due to the presence of the enzymes (Fig. 2a), as explained in section 3.3.

A different pattern was observed in the enzyme test performed with the soy drink sample, where the percentage of non-bioaccessible BPS increased (Figs. 2b and 3b). This effect could be due to the possible interactions of some of the sample components (such as proteins and isoflavones) with the BPS molecule. The possibility of BPS binding or biotransformation into degradation products favours the precipitation. This effect was more pronounced in the gastric phase, especially at pH 3.

The non-bioaccessible BPS (see Fig. 3b) which was approximately 5% higher in the presence of the soy drink sample is interesting from a health point of view, because lower amounts of BPS are available to be absorbed at the intestinal level. The solubility of BPS was mostly affected by the gastric pH followed by the intestinal phase. The food matrix led to BPS precipitation in the pellet. It was observed that a higher amount of BPS (compared to the intestinal stage) tends to precipitate and therefore is not soluble in the supernatant in the oral and gastric phase in the test with soy drink sample. This effect could be due to food components. The effect of the food matrix as a single factor was significant for the NB fractions, and the combination of the food matrix and enzymes resulted in the most significant differences in the gastric phase BPS bioaccessibility (at pH 2 and 3) and NB fractions throughout the digestion process, except for that in the intestinal phase after gastric phases at pH 1 and 2 ($p > 0.05$).

In a study performed by Khmiri et al. (2020), the oral bioavailability of deuterated BPS in human volunteers after the ingestion of BPS-spiked cookies was approximately 62%. The bioaccessibility values obtained in the present study (50–80%) are in agreement with the previous findings since the absorption at the intestinal level might not be complete and reduce the BPS bioavailability in the blood circulation. The BPS bioaccessibility was lower than that observed for BPA (an average of 92%) in canned samples, taking into account the whole digestion process, in another study described by Cunha et al. (2017). Additionally, those authors observed different solubility behaviours among the digestive

compartments. The BPA bioaccessibility values were higher in the intestinal phase than in the gastric or oral phases, which is the opposite of that observed for the BPS. These results highlighted that the bioaccessibility of BPA might depend on the food matrix used, for example, due to its lipid content (Cunha et al., 2017). For this reason, a large number of different foodstuffs must be investigated in future studies to evaluate BPS bioaccessibility.

### 3.5. Degradation products

The presence of intermediate products was investigated, and several compounds were found at some stages of the digestion process (Fig. 4).

The fragment *m/z* 187, which corresponds to 1,2,3,4-tetrahydrodibenzo[*b*,*d*]thiophene, present in the non-bioavailable gastric pellet at pH 1, results from a molecular reorganization of BPS in a tricyclic structure with the loss of all oxygen atoms, including the two phenolic groups. Moreover, the fragment *m/z* 125, which corresponds to 4-mercaptophenol, present in the same extract, results from the reduction of BPS to 4,4́-thiodiphenol and the subsequent loss of a benzene ring and water (Sun et al., 2019).

The fragment *m/z* 271, present in the non-bioavailable pellet of the intestinal phase at the three different pH values tested, results from the loss of an oxygen atom from the BPS molecules and the subsequent substitution of a hydrogen atom of one of the phenol groups by a potassium atom (Shao et al., 2017).

## 4. Conclusions

This work is the first approach to evaluate the chemical behaviour of BPS (stability and solubility) and to assess the bioaccessibility of this molecule after ingestion or intake of a soy drink sample using an *in vitro* simulated gastrointestinal digestion model. The methodology used in our study, with the analytical method based on HPLC-PDA-MS/MS, is appropriate to evaluate the chemical behaviour of BPS. The BPS bioaccessibility values at the end of the digestion process were in the range of 50–80%; thus, BPS is susceptible to absorption at the intestinal level. However, the presence of enzymes slightly decreased the BPS bioaccessibility in the intestinal phase with the increase in the gastric pH, which may be due to the binding of the compound with enzymes and its subsequent precipitation. Therefore, humans with a higher gastric pH would be less exposed to the BPS risks because the bioaccessibility of this compound at pH 3 was reduced to 50%. These results also highlighted that a combination of the three factors (pH, enzymes, and to a lesser extent, the food matrix) affected BPS bioaccessibility, despite when considered independently, did not have a significant effect on the stability and solubility of this compound. Therefore, a larger number of different foodstuff matrices should be investigated in the future studies to consolidate the findings reported in our study and obtain more data on BPS bioaccessibility.

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## CRediT authorship contribution statement

**Antía Lestido Cardama:** Methodology, Investigation, Writing - original draft, Visualization. **Beatriz Millán Sánchez:** Methodology, Investigation. **Raquel Sendón:** Conceptualization, Validation, Writing - review & editing, Resources, Funding acquisition, Visualization. **Ana Rodríguez Bernaldo de Quirós:** Conceptualization, Validation, Writing - review & editing, Resources. **Letricia Barbosa-Pereira:** Conceptualization, Formal analysis, Validation, Supervision, Writing - review & editing, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

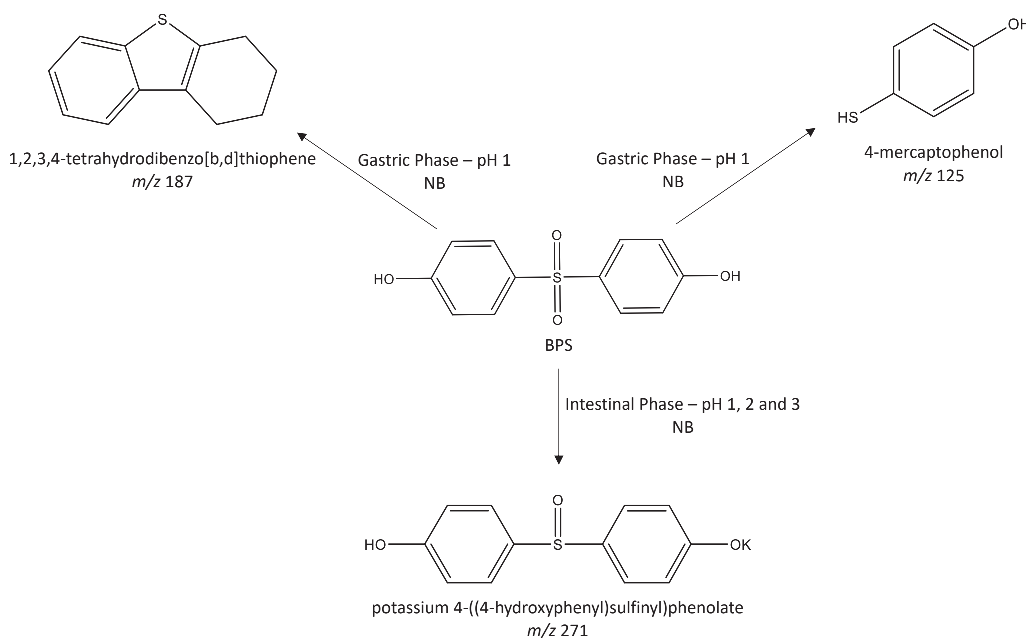


**Fig. 4.** Tentative identification by LC-MS/MS of degradation products of BPS found in some of the non-bioaccessible fractions at different stages of gastrointestinal digestion process with the soy drink samples.

the work reported in this paper.

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