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Protein-polyphenol-polysaccharide impact on (+)-catechin and cyanidin-3-glucoside bioaccessibility through in vitro model

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In vitro evaluation of the effects of protein-polyphenol-polysaccharide interactions on (+)-catechin and cyanidin-3-glucoside bioaccessibility

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Abstract

The bioaccessibility of cyanidin-3-glucoside and (+)-catechin in model solutions when β-lactoglobulin (β-LG) and pectin/chitosan were present was investigated using an *in vitro* model simulating gastrointestinal conditions.

On mouth, the free cyanidin content increased (+) 90 and 14% while the (+)-catechin content decreased (-) 23 and 13%, respectively for mixtures with -pectin and -β-LG-pectin. Under gastric conditions, cyanidin content decreased 85 and 28% for mixtures with -pectin and -β-LG-pectin. On contrary, after gastric digestion, (+)-catechin bioacessibility increased and kept values similar to the original samples for all the systems tested. The transition to the intestinal environment induced a significant alteration on both polyphenols and this effect was more marked for cyanidin. Systems with pectin allowed to obtain higher content of cyanidin bioaccessible. The gastric conditions promoted an increase in antioxidant capacity, followed by a decrease on the intestine.

Free (+)-catechin and cyanidin-3-glucoside content decreased when exposed to the gastrointestinal tract conditions. However, when incorporated in food matrix components, the gastrointestinal tract may act positively on the extraction of polyphenols, since they were progressively released from protein and polysaccharides bonds, being available for the absorption and to exert their biological effects.

Keywords: β-lactoglobulin, (+)-catechin, cyanidin-3-glucoside, pectin, high molecular weight chitosan, gastrointestinal system

1. Introduction

The diet is the principal human source of polyphenolic compounds. Polyphenols consumption has been related to several health beneficial effects, mainly related with chronic diseases such as reduced incidence of cancer and cardiovascular diseases [1]. The bioavailability of polyphenols depends on a variety of factors, including their release from the matrix during gastrointestinal digestion [2].

Several reports have described the interactions between proteins and many kinds of endogenous and exogenous agents such as dietary polyphenols [3] as well as polysaccharides. Under low pH, polysaccharides such as pectin stabilizes milk proteins namely caseins and β-lactoglobulin (β-LG) mainly via electrostatic forces [4]. The resulting complexes between protein and polysaccharide may be soluble or insoluble depending on the environmental conditions [5]. Proteins and polyphenols can also interact in solution by the formation of multiple weak interactions (mainly hydrophobic) hydrogen bonding, reinforcing and stabilizing the complexes between amino acid side chains and polyphenol aromatic rings [3]. Polyphenols are ingested as complex mixtures immersed in a food matrix, e.g. fruit, fruit juice, fruit yoghurt, which undergo a digestion process in the gut. It is important to determine how this digestion process affects polyphenols stability and consequently their bioaccessibility for uptake as well as their possible beneficial effects on the gut cells [6]. On dairy matrices is very common to see interactions between milk proteins (such as β-lactoglobulin), polyphenols from fruit and polysaccharides that are normally added as food stabilisers and which can influences the stability and structure of foods. These interactions may have a detrimental effect on the *in vivo* solubility of both phenolic and proteins [7]. Little is known about phenolics *in vivo* free content and antioxidant capacity in the presence of dietary factors that may interact with them interfering in their bioavailability [8, 9] and

consequently reducing their biological properties. Those interactions within matrix components could decrease degradation throughout the gastrointestinal tract and simultaneously reduce their bioacessibility.

Various studies reported the effect of *in vitro* gastrointestinal digestion on the stability of pure phenolic compounds [10] and on the stability and release of polyphenols from beverages and food matrices [2, 6]. During the gastrointestinal digestion bioactive compounds may be protected from degradation through the possible interaction that could occur with food components, becoming more available for absorption *in vivo*.

Strawberry yoghurt is one of the most consumed dairy products around the world. In previous studies we observed that bioaccessability of the anthocyanins decreased significantly possibly due to the interaction between the main reactive protein in the yoghurt (β-LG) and the polysaccharides (carrageenan) present in the fruit formulation [9]. To study the bioaccessibility of different classes of polyphenols, the (+)-catechin and cyanidin-3-glucoside were selected as representative from flavan-3-ols and anthocyanins, the main classes of polyphenols present in strawberry. This pH value was chosen because it is the typical pH of many food matrices[11-14.]

The aim of this work was to study the bioaccessibility of (+)-catechin/ cyanidin-3-glucoside using an *in vitro* model that simulated some chemical (pH, temperature and bile salts) and biological (gastric and pancreatic enzymes) gastrointestinal conditions where polyphenols were individually mixed with β-LG and pectin or chitosan at pH 4. Changes in the antioxidant activity during the digestion as well as the digestive stability of pure phenolic compounds were investigated.

2. Materials and methods

2.1. Reagents list

The acetic acid, sodium acetate, sodium carbonate, fluorescein, 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH) and 6-hydroxy-2, 5, 7, 8-tetramethylbroman-2-carboxylic acid (trolox), β-lactoglobulin (β-LG), pure (+)-catechin and high molecular weight chitosan (HMW) (624 kDa; >75% of deacetylation) were purchased from Sigma–Aldrich (Sintra, Portugal). Pure cyanidin-3-glucoside was purchased from Extrasynthése (Lyon, France).

The pectin from citrus peel type DF (70% esterification) was provided by CP Kelco (Lille Skensved, Denmark) was supplied by Sigma–Aldrich (Sintra, Portugal).

2.2- Preparation of protein-polyphenols-polysaccharide mixtures

The (+)-catechin, cyanidin-3-glucoside and β–LG powders were dissolved in acetate buffer (0.1 M; pH 4) under agitation (400 rpm) at room temperature. The pectin from citrus peel type DF (70% esterification) and the high molecular weight chitosan (HMW) were dissolved in acetate buffer upon heating (400 rpm, 30 min at 70 ºC) and then cooled down to room temperature before mixing. The pH value in each solution was adjusted at pH 4 with HCl 1 M.

The mixed systems were prepared by stirring (400 rpm, 30 min at room temperature) and stored 24 h at 2 ºC to ensure complete hydration. All samples were prepared in order to reach final concentrations of 0.2 mg/ mL for (+)-catechin and cyanidin-3-glucoside, 3 mg/ mL for β-LG and 3.8 mg/ mL wt for pectin and HMW chitosan.

The systems used for the study were polyphenol-protein, polyphenol-polysaccharide, protein-polysaccharide and polyphenol-protein-polysaccharide and they were prepared at 400 rpm for 30 min at room temperature and finally stored 24 h at 2 ºC.

125

126 **2.3. *In vitro* digestion**

127 The simulated gastrointestinal digestion study was performed with the technique
128 developed by Madureira, et al. [15]. For mouth digestion, a 0.6 mL of α–amylase solution
129 (100 U/mL) and incubation took place for 1 min at 37 ºC and 200 rpm.
130 For gastric digestion the pH was adjusted to 2.0 with concentrated HCl (1N) and the
131 mixture was incubated with pepsin (25 mg/mL) (from porcine stomach mucosa, pepsin
132 A, EC 3.4.23.1, Sigma, Steinheim, Germany) at a rate of 0.05 mL/mL of sample in a
133 shaking bath for 60 min at 37 ºC. For intestinal digestion the pH was adjusted to 6.0
134 with $NaHCO_3$ (1M) before addition of pancreatin (from porcine pancreas, 2 g/L, Sigma-
135 Aldrich Chemistry) and bile salts (12 g/L, Oxoid™, Hampshire, UK) at a ratio of 0.25
136 mL/mL of sample and further incubation of the mixture for an additional 120 min at 37
137 ºC.

138 Controls without added enzymes were run in parallel to differentiate the effects
139 due to the presence of enzymes from those that may be caused by the chemical
140 environment in the assays. For that the volume of enzyme added was replaced by the
141 solvent used in their dissolution. The $CaCl_2$ at 1 mM was used to replace α–amylase,
142 HCl 0.1 M as pepsin and $NaHCO_3$ at 0.1M for pancreatin and bile salts. A total of n=2
143 *in vitro* incubations with each mixture combination were carried out and values are
144 presented as means ± SD.
145 To avoid interference of protein or the complexes formed, at the end of each
146 gastrointestinal step digestion (mouth, gastric and intestinal), 2 mL of samples were
147 taken, filtered through a 3kDa membrane for analysis of the free phenolic compounds.

148

149 **2.4. Analysis of total antioxidant activity**

The Oxygen Radical Absorbance Capacity (ORAC-FL) assay was based on that proposed by [16] with modifications by [17]. Briefly, the reaction was carried out at 40 ºC in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 µL) contained fluorescein (70 nM), AAPH (14 mM), and antioxidant [Trolox (9.98×10^{-4} – 7.99×10^{-3} µmol/mL) or sample (at different concentrations)]. The fluorescence was recorded during 137 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The equipment was controlled by the FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Final ORAC-FL values were expressed as mg of Trolox equivalent/ mL.

2.5- HPLC-DAD analysis

The phenolic compounds in the current study were analysed according Silva, et al. [18] with some modifications. Briefly, the analysis of (+)-catechin and cyanidin-3-glucoside concentration was carried out with HPLC-DAD (Waters Series 600, Massachussets, USA). It was used a reverse phase Symmetry® C18 column (250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size) with a guard column containing the same stationary phase (Symmetry® C18).

The mobile phase for the polyphenols was composed of solvent A: water/methanol/formic acid (92.5:5:2.5 v/v/v) and solvent B: methanol/water (94:6 v/v).

The program began with a linear gradient starting at: 0 to 30% B in 10 min 30 to 50% B in 10 min 50 to 0% B in 5 min and kept at 0% B during 5 min.

The flow rate was 0.75 mL/min, the oven temperature was set as 25 ºC, the injection volume was 50 µL and the runs were monitored at 278 and 515 nm. Results were expressed as micrograms per mL.

2.6- Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows. Normality of data distribution was tested by Kolmogorov-Smirnov method.

Statistical significance values of the groups' means was made by one-way analysis of variance with Tuckey post hoc test to compare groups' means. The statistical analysis was performed for three replicates and were considered significant when $P < 0.05$.

3-Results and Discussion

In order to reproduce some of the real conditions present in yoghurt formulations, the individual concentration of (+)-catechin or cyanidin-3-glucoside was at 0.2 mg/ mL, β-LG at 3 mg/ mL and both polysaccharides (pectin and chitosan) at 3.8 mg/ mL. Natural yoghurt contains ca. 3 mg/ mL of proteins and a strawberry preparation can contain (+)-catechin concentrations varying approximately from 40 to 200 µg/ g depending on the variety and geographical conditions [19] and cyanidin-3-glucoside may be in the range of 3 to 7 mg/ 100 g [20, 21]. Polysaccharides are present in food products as stabilisers agents at concentrations that could vary between 2 and 7 mg/mL [22]. Ionic polysaccharides are usually more reactive and for that one anionic polysaccharide (pectin) and a cationic polysaccharide (chitosan) were selected in this study.

199

200 **3.1-*In vitro* cyanidin-3-glucoside bioacessibility**

201 The cyanidin-3-glucoside bioacessibility was determined by HPLC-DAD before
202 and throughout the *in vitro* simulated gastrointestinal system on samples where protein
203 (β–LG) and two polysaccharides (pectin/ chitosan) were present.

204 Significant ($P < 0.05$) differences were observed between the mixtures of
205 polyphenol-protein-polysaccharide tested, possibly due to the interactions that may
206 occur between the components. The cyanidin-3-glucoside presented a decrease of (-)
207 73%, when it was mixed with pectin and a decrease of (-) 23% in the presence of β–LG-
208 pectin (Fig. 1.A). Chitosan, however, did not cause any interference in cyanidin-3-
209 glucoside bioacessibility, even in mixtures with protein.

210 During the *in vitro* simulation of human digestion an increase ($P < 0.05$) in
211 cyanidin content (90%) was observed after mouth digestion for the mixture of cyanidin-
212 pectin and a slight increase (14%) on the ternary mixture of cyanidin-β–LG-pectin (Fig.
213 1.A). One of the mechanisms reported by Mateus, et al. [23] was that competition between
214 polyphenol and carbohydrate for binding sites in the protein could occur preventing the
215 formation of aggregates. The cyanidin increase after mouth digestion could be the result
216 of it's released from pectin complexes due to competition mechanisms with the protein,
217 α–amylase. According with Soares, et al. [24] the presence of polysaccharides in solution
218 affects the interaction between tannins and proteins. Pectins have a higher ionic
219 character capable of establishing hydrophilic interactions [24].

220 On the simulated system where no enzyme was used an increase of (+) 58 and
221 8%, was observed respectively for the mixtures of cyanidin-pectin and cyanidin-β–LG-
222 pectin (Fig. 1.B). The increase in cyanidin content could be the result of its release from
223 the complexes formed with pectin.

After stomach digestion, cyanidin content decreased (-) 85 and 28% respectively for the mixtures of cyanidin-pectin and cyanidin-β–LG-pectin (Fig. 1.B). The reduction of cyanidin bioacessibility could be related with an increased interaction between pectin and cyanidin due to the low pH. Dongowski, et al. [25] observed that rats fed with diets rich in pectin, no distinct depolymerisation or demethoxylation occur in the upper part of the gastrointestinal tract, however, a partial degradation seems to be possible under the physicochemical conditions of the stomach and small intestine. As the pH is lowered, from mouth (pH 4) to stomach (pH 2), ionisation of the carboxylate groups is suppressed, resulting in a hydration reduction of the carboxylic acid groups. As a result of reduced ionisation, the polysaccharide molecules no longer repel each other over their entire length, and as a result, they can associate and form a gel. Gel formation is caused by hydrogen bonding between free carboxyl groups on the pectin molecules and the hydroxyl groups of neighbouring molecules [26]. It is known that pectin has the ability to develop a gel-like network, forming hydrophobic pockets able to encapsulate procyanidins [27]. As reported for procyanidins, the cyanidin molecules may be entrapped in the pectin net promoting interaction between cyanidin-pectin.

Pure cyanidin decreased (-) 13% after pepsin digestion when compared with concentrations detected on mouth. Similar variation was observed on the simulated gastric digestion without enzyme (Fig. 1.B), revealing that exposure to pH and temperature conditions (pH 2, 37 ºC) for 60 min could be the main reason for the cyanidin content decrease. Other researchers found that flavylium cation of anthocyanins increased as a result of the low pH (pH 2) found on stomach[28, 29].

Under the pancreatin bile salt digestion (simulation of small intestine digestion), a significant decrease in cyanidin-3-glucoside was observed for all the mixtures. By comparison with results obtained on gastric digestion, the intestinal conditions induced

a decrease of (-) 90, 89, 85, 59 and 80%, respectively on cyanidin alone, cyanidin-β–LG, cyanidin-chitosan, cyanidin-β–LG-pectin and cyanidin-β–LG-chitosan, while cyanidin increased (+) 199% when mixed with pectin (Fig. 1.A). Previous studies have shown that pectin interacts with various food and intestinal components such as bile salts [30-32]. This interaction could be stronger than the interaction between pectin and cyanidin inducing a consequent release at the level of the intestine. Contrarily, in chitosan mixture, in the passage from stomach to intestine, a higher decrease in the cyanidin-3-glucoside content was observed than in mixtures with pectin. At the final pH of the digestion model (pH 7.5), the chitosan molecules should have lost their electrical charge since the pKa value of the amino groups is around 6.5, and would therefore not be able to promote bridging flocculation of the droplets [30]. If chitosan allowed some protection to cyanidin, at the level of intestine, chitosan may disaggregate and left cyanidin more exposed to degradation.

Intestinal digestion conditions without enzymes resulted in a decrease between (-) 98-99% for the mixtures of cyanidin alone, cyanidin-β–LG, cyanidin-chitosan, cyanidin-β–LG-pectin and cyanidin-β–LG-chitosan and an increase of (+) 18% on cyanidin-pectin mixture (Fig. 1.B). Increased cyanidin-3-glucoside accessibility for the last mixture could be explained by the alkaline pH-values found on intestine (pH 7), where pectin is rapidly de-esterified and can be degraded even at room temperature. The un-esterified carboxyl groups are partially ionised salts producing a negative charge on the molecule, which together with the hydroxyl groups induces attraction of water. The repulsive forces between these groups, due to their negative charge, can be sufficiently strong to prevent the formation of a pectin network [26].

Once released from the protein/polysaccharide systems, the cyanidin-3-glucoside decrease could be partially explained by the transformation of the flavylium

cation to the colourless chalcone at the pH value of the medium (pH 7). The formation of chalcone and its subsequent fission between the B and C rings is also favored by elevated temperature of 37 ºC and results in the destruction of the anthocyanin chromophore [10, 28]. Anthocyanins loss can also be in part a result from the metabolization to some non-coloured forms, oxidized, or degraded into other chemicals, which would escape from the detection under the present conditions [28]. Chalcones generally are not detected on analysis that involves acidification and measurement by HPLC that are specific for the flavylium cations [29].

Analysing the entire simulated gastrointestinal system the cyanidin-3-glucoside presented decreases higher than 80% for all systems except for cyanidin-pectin and cyanidin-β–LG-pectin, which decreased only (-) 17 and 67%, respectively. These results revealed that pectin allowed more protection along the gastrointestinal system releasing more cyanidin on the intestinal digestion than systems that use chitosan and/ or β-LG. Pillay and Fassihi [33] described that drug release rates from calcium–alginate–pectinate systems increases with an increase in media pH values. They described an increase of drug content from (+) 3 to 16% from pH 1.5 to 4 and more than 97% when pH increased up to 6.6. This may be attributed to both matrix structure and drug solubility with increasing pH.

When comparing the content of cyanidin available after 60 min of intestinal digestion, all the systems tested presented higher content when compared with cyanidin alone: cyanidin-β–LG (42%), cyanidin-pectin (174%), cyanidin-chitosan (75%), cyanidin-β–LG-pectin (214%) and cyanidin-β–LG-chitosan (139%) (Fig. 1.A).

The conditions of pH were very important on cyanidin stability along digestion, since when analysing the simulated conditions of pH and temperature, cyanidin content

298 decreased (-) 98-99% for all mixtures, except cyanidin-pectin, which decreased (-) 66%
299 (Fig. 1.B).

300 Those results allowed concluding that enzymes had significant effect on
301 cyanidin-3-glucoside release from the cross-linkings established with protein and
302 polysaccharide allowing increased availability after the digestion. Nevertheless, the pH
303 changes combined with the higher temperature of 37 ºC also had an important impact on
304 cyanidin-3-glucoside stability.

305

306 **3.2-Cyanidin-3-glucoside antioxidant capacity along gastrointestinal system**

307 The cyanidin-3-glucoside content was also monitored throughout gastrointestinal
308 system and the antioxidant capacity conveyed by this flavonoid was also measured by
309 the ORAC method.

310 The variations observed for cyanidin content were reflected in its antioxidant capacity,
311 which was increased after mouth digestion of the mixtures of cyanidin-pectin (129%)
312 and cyanidin-β–LG-pectin (123%) due to the cyanidin-3-glucoside concentration
313 increase. On simulated conditions of mouth without α–amylase, the ORAC values
314 increased only for the mixture of cyanidin-pectin (142%).

315 On gastric digestion pure cyanidin content have decreased, however, the
316 antioxidant capacity increased: cyanidin alone (28%), cyanidin-β–LG (46%), cyanidin-
317 chitosan (40%) and cyanidin-β–LG-chitosan (3%) and a decrease was registered for
318 cyanidin-pectin (57%) and cyanidin-β–LG-pectin (52%) (Fig.2.A). Anthocyanin
319 increase after stomach *in vitro* digestion was previously attributed to the lower pH of
320 the sample, which renders an increase of the flavylium cation in the solution [28].
321 Comparing previous results with HPLC quantifications the interaction between cyanidin
322 and pectin were favoured by the lower pH, since the cyanidin content as well its

antioxidant capacity was significantly ($P < 0.05$) reduced. The hydrogen bond interactions between anthocyanins sugars at position 3 of the heterocyclic ring, the OH groups of the B ring and the planarity of the molecule could induce the formation of ππ bonds and produce effects with adsorbents like lignin, as well as forming weak interactions or the inclusion of anthocyanin in the pores of the adsorbent [11]. Padayachee, et al. [34] observed ionic charge interactions between the anthocyanins and pectin on purple carrot juice concentrate at pH 4. Under those conditions anthocyanins are not completely deprotonated and at this pH, pectin is slightly negatively charged [35].

On the gastric system without enzymes, the pH lowering promoted significant decrease only when cyanidin interacts with pectin (90%) and with β–LG-pectin (45%) (Fig. 2.B).

The intestinal digestion decreased the antioxidant capacity when cyanidin was alone (41%), since its content had also decreased. However, on combinations of cyanidin-pectin, cyanidin-β–LG-pectin and cyanidin-β–LG-chitosan the antioxidant capacity increased (+) 195, 483 and 413%, respectively (Fig. 2.A). After the simulated gastrointestinal system the total balance of antioxidant capacity was negative for the cyanidin alone under digestion conditions, while the remaining systems presented an increase, mainly for the ternary mixtures.

In the simulated system where enzymes were absent an increase on antioxidant capacity was observed for cyanidin alone, cyanidin-pectin, cyanidin-chitosan and cyanidin-β–LG-pectin, while significant decrease was registered for cyanidin-β–LG and cyanidin-β–LG-chitosan (Fig. 2.B).

The decrease observed in the antioxidant capacity of cyanidin alone could be related with the action of enzymes that could have affected the cyanidin structure, because in the absence of enzymes the antioxidant capacity increased.

348 The pH was shown to be a dominant factor in the ultimate radical scavenging
349 capacity of the wine anthocyanins as observed by Borkowski, et al. [36], with increasing
350 pH significantly increased the capacity for radical scavenging. The pH-dependent
351 increase in the radical scavenging capacity of hydoxyflavones was previously attributed
352 to an effect on hydroxyl moiety deprotonation [37]. Part of the pH-dependent increase in
353 anthocyanins radical scavenging capacity is related to the conversion of the flavylium
354 cation, known to be the dominant form at low pH values, to quinoidalbase, carbinol
355 pseudobase, and E-chalcone with an increasing pH. Theoretical calculations
356 corroborated that upon deprotonation of the flavylium cation towards any of the
357 possible neutral forms, the bond dissociation energy decreases reflecting higher radical
358 scavenging capacity. Upon formation of quinoidal-base, carbinol pseudobase, and E-
359 chalcone, the compounds become better radical scavengers [36].
360

361 **3.3-*In vitro* (+)-catechin bioacessibility**

362 The mouth digestion promoted a (-) 70% decrease in the original content when
363 (+)-catechin was alone (Fig. 3.A). In the remaining systems with protein-polysaccharide
364 the higher decrease was observed for catechin-chitosan (40%) and catechin-β-LG-
365 chitosan (38%), while the presence of -β-LG, -pectin and -β-LG-pectin induced a
366 decrease of (-) 28, 23 and 13%, respectively (Fig. 3.A). On simulated system without
367 enzymes the pure (+)-catechin presented a decrease of (-) 31%, while the remaining
368 systems presented variations lower than 10% (Fig. 3.B). Those results revealed that α-
369 amylase had an important role in the decrease of (+)-catechin bioacessibility.

370 After the gastric digestion, (+)-catechin bioacessibility increased resulting in
371 concentrations similar to those obtained on the original samples, however, besides the

372 increase observed, solution of (+)-catechin presented less 20% of its content than before
373 digestion.
374 The decrease in (+)-catechin bioacessibility on mouth and then an increase on stomach
375 could be the result of complexes formed between (+)-catechin and α–amylase, which in
376 the presence of low pH (pH 2) found on stomach the interactions with α–amylase was
377 reduced and (+)-catechin became more available. It was proved by other authors that
378 polyphenols were capable of binding to human salivary α-amylase through hydrogen
379 bonds with the polar groups of proteins promoting their precipitation [38, 39].
380 According with Rodríguez-Roque, et al. [40] gastric digestion improves the release of
381 phenolic compounds from the soymilk matrix (total phenolic acids increased by 70%
382 and total flavonoids by 33%). This fact was attributed to hydrolysis of phenolic
383 compounds bound to food constituents due to the acidic pH and enzymatic activity.
384 Similarly, an increase in phenolic compounds extractability from the food matrix due to
385 gastric conditions was suggested by Baublis, et al. [41] on wheat-based ready-to-eat (RTE)
386 breakfast cereals. Saura-Calixto, et al. [42] reported that phenols linked to high molecular
387 weight compounds, such as proteins and carbohydrates, may be released by digestive
388 enzyme action, leading to a significant increase in their concentrations after gastric
389 digestion.
390 The simulated gastric conditions with the absence of enzymes also revealed no
391 interactions between (+)-catechin and pectin that could provide any kind of protection
392 from degradation. The factor that showed the greatest effect on recovery of catechin
393 after gastric digestion was the exposure for 60 min to 37 ºC, which promoted a decrease
394 of (-) 55 and 34%, respectively for the systems: catechin-pectin and catechin-β-LG-
395 pectin (Fig. 3.A). When procyanidins were submitted to digestion together with
396 carbohydrate-rich food, the amount of (+)-catechin and (-)-epicatechin present in the

digestion mixture after the gastric step was significantly higher, revealing that the presence of carbohydrate-rich food, showed a significant effect on the digestibility of procyanidins in the *in vitro* digestion model [43]. Also Zhu, et al. [44] have found high stability on (+)-catechin and (-)-epicatechin when they were subjected to simulated gastric juice (pH 1.8) and Record and Lane [45] found that flavan-3-ols from both green and black tea present high gastric stability (>80%).

The intestinal step of the gastrointestinal digestion was the responsible for the major decrease on (+)-catechin bioacessibility. The higher decrease was observed for pure (+)-catechin (90%) followed by catechin-β-LG (67%), catechin-pectin (67%), catechin-chitosan (49%), catechin-β-LG-pectin (48%) and catechin-β-LG-chitosan (61%) (Fig. 3.A). The (+)-catechin decrease was mainly promoted by the action of pancreatin and bile salts, because when they were not present, the pH increase (pH 7) promoted lower decreases on (+)-catechin alone (42%), catechin-β-LG (24%), catechin-chitosan (33%) and catechin-β-LG-chitosan (17%) and an increase was observed for catechin-pectin (59%) and catechin-β-LG-pectin (18%) (Fig. 3.B). In these two last systems the increase in accessibility of (+)-catechin could have been promoted by its release from pectin when pH increased. It is known that pectin degrades in alkaline solutions (pH 6) due to a β-elimination cleavage of the glycosidic bonds adjacent to an esterified carboxyl group [46].

These results are in agreement with previous studies in model systems that have reported catechins to be stable in acidic conditions, while being unstable in a variety of solutions at near-neutral or greater pH [47, 48].

Record and Lane [45] reported that individual flavan-3-ols were less stable in intestinal conditions (pH 7.5, no enzymes) with recoveries for green teas of 1% for (-)-epigallocatechin gallate, 8% for epigallocatechin, 59% for epicatechin gallate, and 71%

for (-)-epicatechin. These results suggested that (+)-catechin degradation may be driven by auto-oxidation at near-neutral or greater pH (common in the small intestine), by the presence of digestive secretions (bile, etc.), or dissolved O_2 and presence of reactive oxygen species (ROS) [49].

The content of available (+)-catechin on the mixture with protein and polysaccharides presented variations lower than 10% (Fig. 3.A). When the (+)-catechin alone was submitted to the gastrointestinal system it presented a total decrease of (-) 92%. Also Bermúdez-Soto, et al. [6] observed a 58% decrease in (+)-catechin after *in vitro* digestion of chokeberry juice.

Making the global balance the (+)-catechin alone when submitted to the gastrointestinal system presented a decrease of (-) 92% while in the remaining systems where protein or polysaccharides were present the decreases were between 60 and 70%.

3.4-(+)-Catechin antioxidant capacity along gastrointestinal system

The antioxidant capacity determined by the ORAC revealed that mouth digestion promoted a general decrease in antioxidant capacity of (+)-catechin between (-) 30-66% in all systems tested (Fig. 4.A).

When catechin was exposed to gastric conditions the antioxidant capacity increased in all systems and then decreased after exposure to intestinal digestion conditions. The (+)-catechin content changes along the gastrointestinal system was reflected in the radical scavenging capacity in the same way.

After the passage throughout the *in vitro* simulated gastrointestinal system, (+)-catechin alone decreased (-) 49%, while in the mixtures with protein and polysaccharide the catechin content increased for most of the cases (8-21%) (Fig. 4.A).

The pH variations and temperature used in the simulated system without pancreatin and bile salts induced a significant decrease in antioxidant capacity for all systems, where (+)-catechin alone and catechin-β-LG were the systems presenting higher decrease, (-) 62 and 68%, respectively (Fig. 4.B).

The antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure [50] and, as described above, oxidations reactions at near-neutral pH (pH 7) on the intestine could promote decrease on (+)-catechin antioxidant capacity. The higher preservation of antioxidant capacity on the gastrointestinal system under the action of enzymes could be associated with a higher release of polyphenol from the complexes formed within protein/ polysaccharide promoted by their action.

4-Conclusions

Cyanidin-3-glucoside and (+)-catechin interacts with pectin and β-LG forming complexes. Those complexes allowed protection of polyphenols from gastrointestinal enzymes action and pH variation, resulting in higher bioaccessibility. Gastric digestion in general leads to an increase of (+)-catechin accessibility for all the mixtures, while the cyanidin-3-glucoside content decreased for mixtures with pectin. The passage to the intestinal environment caused a decrease in both polyphenols for all mixtures tested. Antioxidant activity increased with gastric pH decrease (pH 2), followed by a decrease on intestine, where pH strongly increase (pH 7). In the end of the gastrointestinal system the radical scavenging capacity was higher for all the mixtures of polyphenols, protein and polysaccharides than undigested mixtures.

Pure (+)-catechin and cyanidin-3-glucoside mixed with β-LG and pectin or chitosan presented higher resistance to degradation imposed by the gastrointestinal tract

conditions. Pectin was the polysaccharide that allowed higher bioaccessibility of cyanidin-3-glucoside to be absorbed or to exert its biological effects. This work allowed understanding that some types of polysaccharides (pectin) can be used in food formulations, allowing higher polyphenols protection and availability after the gastrointestinal system. Future work should be done with different polyphenols, polysaccharides and with caseins, as representative of different food matrices and assuming the specificity of such reactions.

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List of Figures

Figure 1- Quantitative analysis of cyanidin-3-glucoside by liquid chromatography (HPLC-DAD) on each mixture with β-LG, pectin and chitosan and recovered after mouth, gastric and intestine *in vitro* digestion with enzymes (A) and without enzymes (B). Cyanidin-3-glucoside (■), cyanidin-3-glucoside-β-LG (■), cyanidin-3-glucoside-pectin (■), cyanidin-3-glucoside-chitosan (■), cyanidin-3-glucoside-β-LG-pectin (■), cyanidin-3-glucoside-β-LG-chitosan (□). [a] Different letters represent significant differences ($P < 0.05$) between samples in each digestion phase.

Figure 2- Antioxidant activity (ORAC) of cyanidin-3-glucoside (mg trolox/mL) on each mixture with β-LG, pectin and chitosan after mouth, gastric and intestine *in vitro* digestion with enzymes (A) and without enzymes (B). Cyanidin-3-glucoside (■), cyanidin-3-glucoside-β-LG (■), cyanidin-3-glucoside-pectin (■), cyanidin-3-glucoside-chitosan (■), cyanidin-3-glucoside-β-LG-pectin (■), cyanidin-3-glucoside-β-LG-chitosan (□). [a] Different letters represent significant differences ($P < 0.05$) between samples in each digestion phase.

Figure 3- Quantitative analysis of (+)-catechin by liquid chromatography (HPLC-DAD) on each mixture with β-LG, pectin and chitosan and recovered after mouth, gastric and intestine *in vitro* digestion with enzymes (A) and without enzymes (B). Cyanidin-3-glucoside (■), cyanidin-3-glucoside-β-LG (■), cyanidin-3-glucoside-pectin (■), cyanidin-3-glucoside-chitosan (■), cyanidin-3-glucoside-β-LG-pectin (■), cyanidin-3-

618 glucoside-β-LG-chitosan (□). [a] Different letters represent significant differences ($P <$
619 0.05) between samples in each digestion phase.
620
621 **Figure 4-** Antioxidant activity (ORAC) of (+)-catechin (mg trolox/mL) on each mixture
622 with β-LG, pectin and chitosan after mouth, gastric and intestine *in vitro* digestion with
623 enzymes (A) and without enzymes (B). Cyanidin-3-glucoside (■), cyanidin-3-glucoside-
624 β-LG (■), cyanidin-3-glucoside-pectin (■), cyanidin-3-glucoside-chitosan (■), cyanidin-
625 3-glucoside-β-LG-pectin (■), cyanidin-3-glucoside-β-LG-chitosan (□). [a] Different
626 letters represent significant differences ($P < 0.05$) between samples in each digestion
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Figure 1

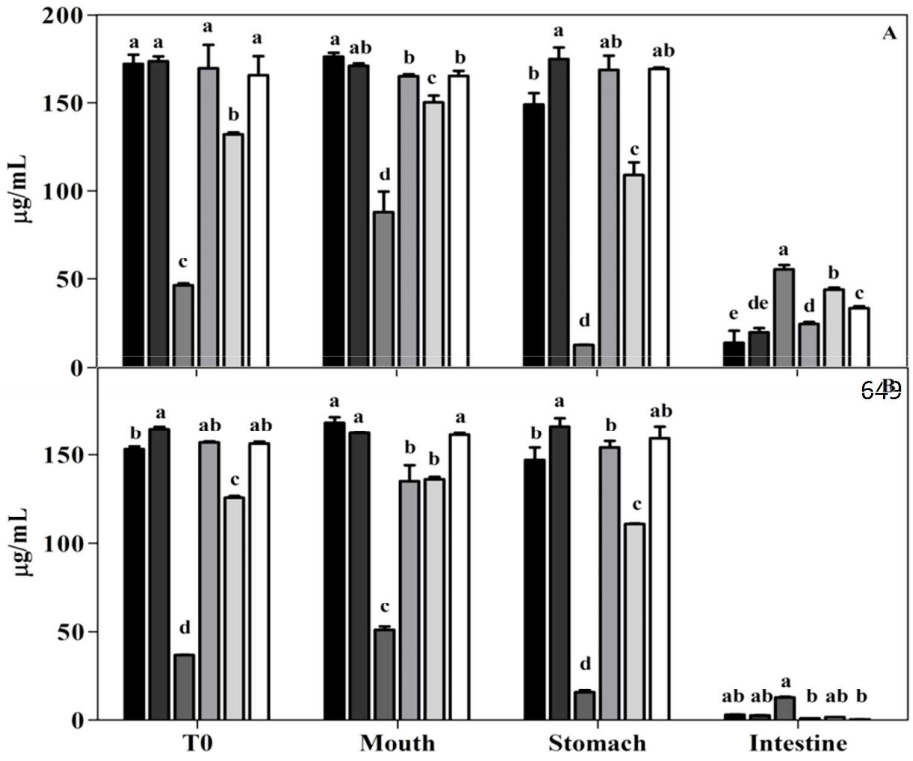


Figure 2

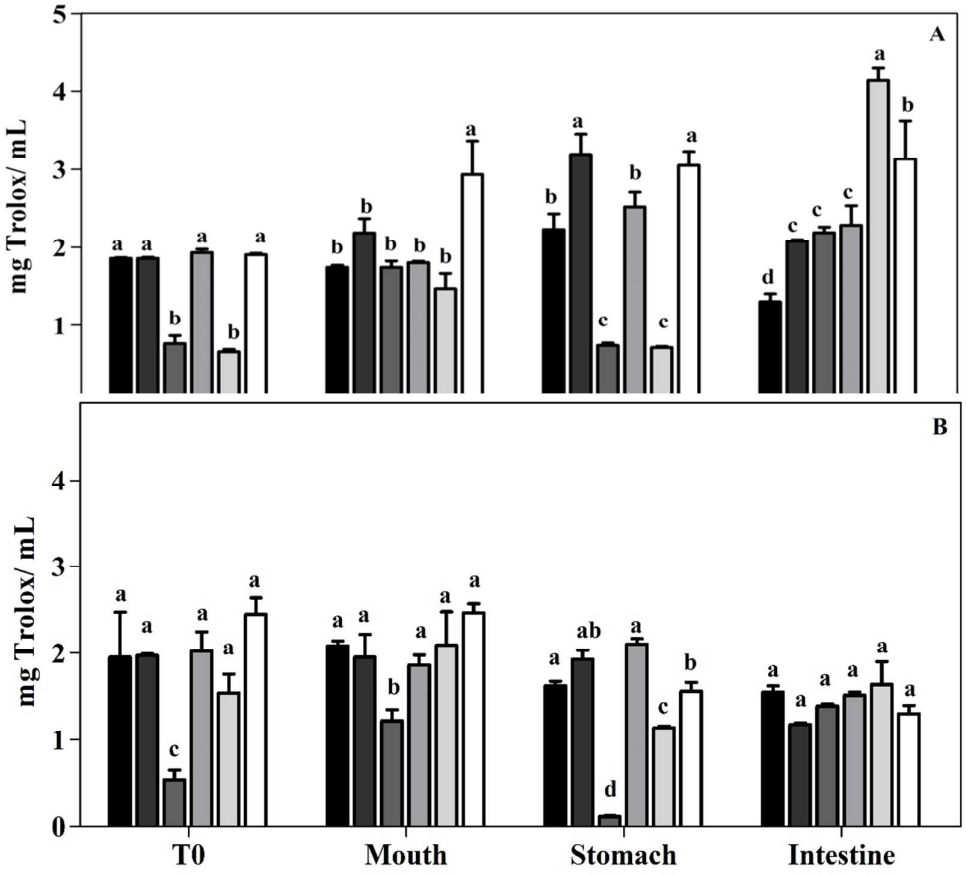
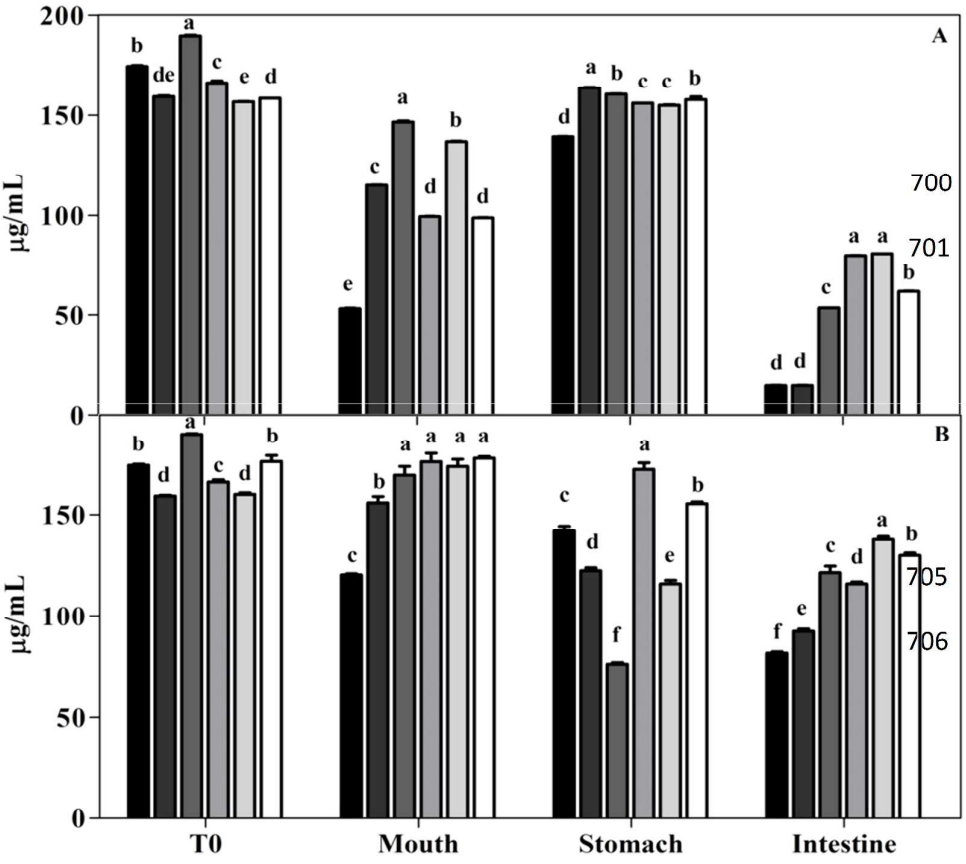


Figure 3



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723 **Figure 4**

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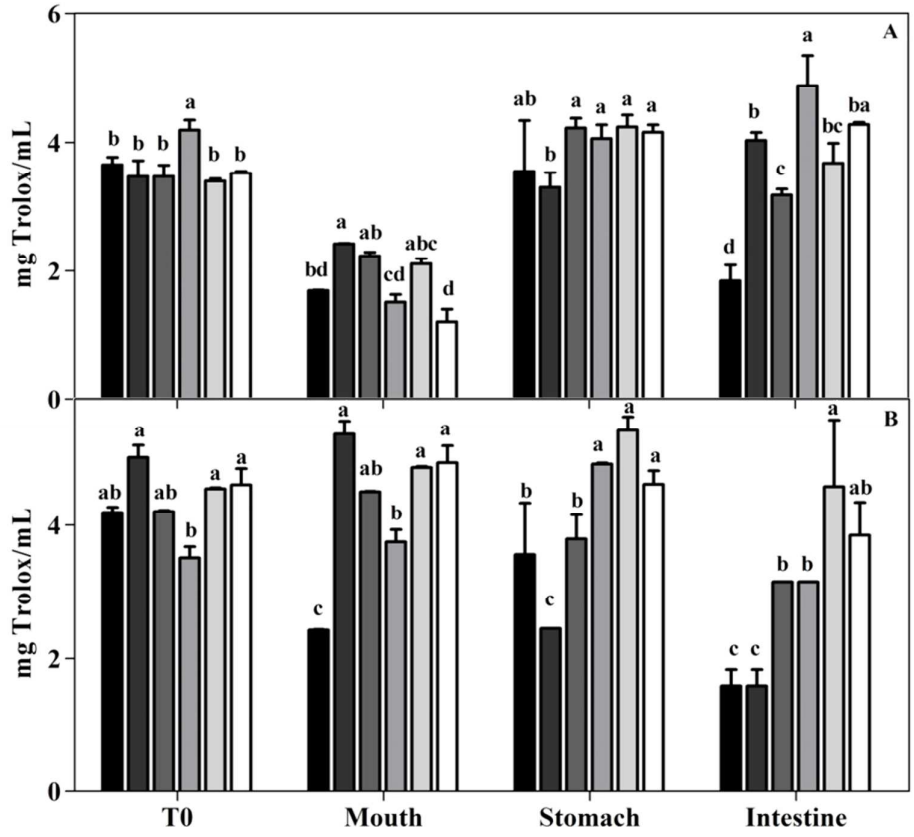

Graphical Abstract