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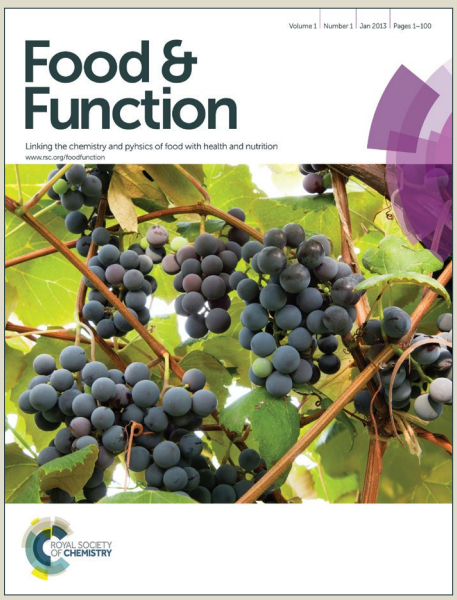

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Stability of polyphenols and carotenoids in strawberry and peach yoghurt throughout *in vitro* gastrointestinal digestion

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Abstract

The aim of this research was to evaluate the influence of the *in vitro* gastrointestinal digestion on the stability and bio-accessibility of phenolic compounds and carotenoids, as well as on the antioxidant activity in strawberry and peach enriched yoghurt.

The radical scavenging capacity of strawberry and peach yoghurt was 480 and 550% higher, respectively at the level of the intestine than in fruit yoghurt not submitted to digestion. In strawberry the amount of bio-accessible anthocyanins increased during gastric digestion and the transition to the intestinal compartment produced a decrease in all the analyzed classes of polyphenols, being more pronounced on pelargonidin-3-glucoside (65%) and pelargonidin-3-rutinoside (58%).

In peach (+)-catechin content strongly decreased (80%), neochlorogenic, chlorogenic acid, rutin and the carotenoid zeaxanthin decreased at lower levels, between 32-45%, while β-carotene was rather stable under gastric conditions (increased 12%) during intestinal digestion.

Despite the decrease in the concentration of these bioactive compounds after being subjected to the *in vitro* gastrointestinal digestion, results suggest that fruit yoghurt is an important source of bio-accessible polyphenols and carotenoids that despite some losses induced by digestion conditions, still release relevant amounts at the level of intestine to be absorbed and promote the health benefits.

Keywords: *Fragaria* × *ananassa, Prunus persica,* yoghurt, gastrointestinal system

1. Introduction

Consumption of fruits has been associated to their health benefits usually related to their vitamin, mineral or specific antioxidant compounds, in particular polyphenols. Among the principal issues concerning the beneficial effects of polyphenols, their bioavailability and metabolic fate must be considered. The bioavailability of a dietary compound is dependent on its digestive stability, its release from the food matrix (referred as bio-accessibility), and the efficiency of its transepithelial passage. Bioavailability differs greatly from one polyphenol to another, and for some compounds it depends on dietary source [1, 2].

Other factors involved in determining the bioavailability of polyphenols are the stability under gastrointestinal conditions and the release from the food matrix, especially from the solid one. For example, the very low bioavailability of anthocyanins can be attributed, at least partially, to the high instability of these molecules in the mild alkaline condition of the small intestine [1, 3].

Various studies report the effect of *in vitro* gastro-intestinal digestion on the stability and release of polyphenols from beverages as reported for anthocyanins release from red wine [4], from an extract made from raspberries [5], cocoa flavanols and procyanidins [6], anthocyanins and phenolic compounds from pomegranate juice [3], catechins from green and black tea [7] and polyphenols from chokeberry juice [8]. However, only few studies have been carried out on the solid food matrices. Saura-Calixto, et al. [9] studied the changes on total polyphenols of a Spanish Mediterranean diet, Vallejo, et al. [10] studied the availability of phenolic compounds, glucosinolates, and vitamin C of broccoli inflorescences submitted to digestion under *in vitro* gastrointestinal conditions and Serrano, et al. [11] studied carotenoids bio-accessibility from digested green leafy vegetables.

Researches concerning the bio-accessibility of polyphenols from the solid matrices are important, since only the compounds released from the food matrix are potentially bio-accessible and after gastrointestinal tract effect in condition to exert their beneficial effects [1].

Strawberry fruit, contains a large spectrum of phenolic components including not only the coloured anthocyanins, but also the colourless phenolics (particularly ellagic acid, quercetins, etc.) contributing to its high antioxidant activity [12, 13]. Peaches are rich in polyphenols (like chlorogenic acid, neochlorogenic acid, catechin, epicatechin and quercetin 3-rutinoside) [14, 15] and carotenoids (particularly rich in lutein, zeaxanthin, β-cryptoxanthin and β-carotene) [16, 17]. It has been proven that flavonols have protective effect against cardiovascular disease [18, 19] and reduction of digestive tract cancer risk [20] and that carotenoids are associated with protective effects against some types of cancer, age-related macular degeneration, and heart disease.

It is considered by nutritionists that yoghurt have a high nutritional value and positive bio-active effects, usually reinforced by the addition of prebiotic ingredients [21] and probiotic bacteria [22-24]. Fruit yoghurt is among the most common fermented dairy products consumed around the world [25]. To increase the functionality and antioxidant capacity of these dairy products, food ingredients such as fruit is commonly added [22, 26, 27].

Despite the interest in the health benefits of phenolics, little is known about their *in vivo* free content and antioxidant capacity in the presence of dietary factors that may interact with phenolics during digestion interfering in their bio-accessibility [28]. Information is available on the effect of protein on the antioxidant properties of phenolics through hydrogen bond and hydrophobic interactions [29, 30] and about the

effect of other components such as polysaccharides that can affect the interaction between polyphenols and proteins [31-35].

In the present study we investigated the bio-accessibility of the major classes of polyphenols from strawberry and peach preparates incorporated on a yoghurt matrix using an *in vitro* model that simulated some physical (temperature, and movements by agitation), chemical (pH, temperature and bile salts) and biological (gastric and pancreatic enzymes) gastro-intestinal conditions. In addition, changes in the antioxidant activity during the digestion were also investigated.

2. Materials and methods

2.1. Reagents list

The 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), formic acid, fluorescein, 6-hydroxy-2, 5, 7, 8-tetramethylbroman-2-carboxylic acid (trolox), α–amylase, methanol, pepsin, pancreatin were purchased from Sigma–Aldrich (Sintra, Portugal). Hydrochloric acid (HCl) was purchased on Merck (Algés, Portugal), calcium chloride ($CaCl_2$) and sodium hydrogencarbonate ($NaHCO_3$) on VWR International (Carnaxide, Portugal).

Polyphenol standards (+)-catechin, chlorogenic acid, ellagic acid, neochlorogenic acid, quercetin-3-rutinoside and β-carotene were obtained from Sigma–Aldrich (Sintra, Portugal), cyanidin-3-glucoside, pelargonidin-3-glucoside, pelargonidin-3-rutinoside and zeaxanthin were purchased from Extrasynthése (Lyon, France). Bile salts were purchased at Oxoid™, Hampshire, UK.

2.2. Preparation of strawberry and peach formulations

Individually quick frozen (IQF) strawberry (*Fragaria* × *ananassa* Duch.**)** was blended to purée (50%) and were mixed with sugar (27%), glucose and fructose syrup (8%) in a mixed reactor with jacket heating and cooling. Carrageenan (0.38%), starch (2%), cochineal carmine (0.0095%) and strawberry aroma (0.48%) were dispersed in cold water separately and these ingredients were added to the strawberry.

Peach (*Prunus persica* (L.) Batsch 'Diamond Princess') purée (50%) was mixed with, xanthan gum (0.05%), carrageenan (0.1%) and starch (2.3%) in a mixed reactor with jacket heating and cooling. Next, the flavors (0.19%), sweeteners (0.17%) and citric acid (0.07%) were added to the peach. All the ingredients were dispersed in cold water separately before addition to peach.

Both mixtures were pasteurized at 90 °C for 3 min.

2.3. Preparation of strawberry and peach yoghurt samples

Low fat plain yoghurts were acquired, at the beginning of their 30 d shelf-life, in a local market and were used to incorporate industrial strawberry preparations under aseptic conditions. Strawberry preparation was added in a proportion of 20% of the yoghurt weight. Fruit preparations are generally added to yoghurt products within the range of 10-20% level in the final product [36].

The yoghurt-fruit mixture was distributed in 100 mL sterile polypropylene containers and kept during 72 h at 2 ºC. Each sample was prepared in duplicate.

2.4. *In vitro* simulated gastrointestinal (GI) digestion

To monitor the release of individual polyphenols from strawberry and peach yoghurt matrices at different stages of digestion, samples of yoghurt were collected from mouth (ca. 20 mL), gastric digest (ca. 20 mL), intestinal digest (ca. 20 mL) and

used to make extracts to further analyse polyphenols and carotenoids. Two replicas from the GI system were made and two replicas of blanks were prepared with identical chemicals but without enzymes, and underwent the same conditions as the samples.

The simulated GI system was performed according Madureira, et al. [37] with some modifications. Mouth digestion was conducted with 0.6 mL of α–amylase solution (100 U/mL) and incubation took place for 1 min at 37 ºC and 200 rpm. For gastric digestion the pH was adjusted to 2.0 with concentrated HCl (1N) and the mixture was incubated with pepsin (25 mg/mL) (from porcine stomach mucosa, pepsin A) at a rate of 0.05 mL/mL of sample in a shaking bath for 60 min at 37 ºC. Intestinal digestion was performed by adjusting pH to 6.0 with $NaHCO_3$ (1M) before addition of pancreatin (from porcine pancreas, 2 g/L) and bile salts (12 g/L) at a ratio of 0.25 mL/mL of sample and further incubation of the mixture for an additional 120 min at 37 ºC.

The same procedure was applied to the mixtures without enzymes, where the volume of enzyme added was replaced by the solvent used in their dissolution. The $CaCl_2$ at 1 mM was used to replace α–amylase, 0.1N HCl as pepsin and $NaHCO_3$ at 0.1M for pancreatin and bile salts.

2.4. Extraction of polyphenols and carotenoids for chemical analyses

Strawberry and peach hydrophilic antioxidants were obtained according to Redeuil, et al. [38] with some modifications. Strawberry and peach yoghurt (20 g) was homogenised with 30 mL of methanol acidified with formic acid (9:1 v/v) using an ultra-turrax (IKA T18. Wilmington. USA) at 24000 rpm for 1 min. The homogenised sample was kept at -20 ºC during 1 h to allow protein precipitation. The slurry was then centrifuged at 4000 × *g* at 4 ºC for 10 min and the supernatant filtered through a 3 kDa cutoff membrane (Vivaflow® 50, Sartorius) to remove soluble proteins.

A 25-mL aliquot of the extract was evaporated to dryness in a RVC 2-18 speed-vacuum evaporator (Christ. Osterode am Harz. Germany) at 30 ºC and the residue dissolved in 2 mL of methanol to further analysis.

Carotenoids were extracted as described by Wright and Kader [39]. Briefly, 5 g of peach yoghurt were suspended in 5 mL of cold ethanol and homogenized at 24000 rpm for 3 min using an ultra-turrax. Hexane (4 mL) was added to the homogenate and the resulting mixture was homogenized for an additional 2 min before the slurry was centrifuged for 10 min at 4000 ×*g*. The hexane layer containing the carotenoids was transferred to a polypropylene tube and a solution of saturated sodium chloride (2.5 mL) and an additional 4 mL of hexane were added and the resulting mixture and homogenized for 1 min. The mixture was centrifuged as described above, and the hexane layer recovered for analyses. All extracts were performed in triplicate samples.

The results of each extract determination (on time zero of digestion (T_0), after mouth, gastric and intestinal digestion) were reported to the fresh weight of strawberry and peach purée concentrate used in 20% of yoghurt weight. Results as mg per gram of biomass was obtained to according Eq.(1).

$$C(mg/g biomass) = \frac{(mg/mL) * Extract\ volume\ (mL)}{g\ biomass} \quad \text{Eq.(1)}$$

2.5. Analysis of total antioxidant activity

The total antioxidant activity was measured by the Oxygen radical absorbance capacity (ORAC-FL) assay and was performed according that proposed by Contreras, et al. [40]. 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

199 FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm
200 excitation and 520 nm emission filters was used. The equipment was controlled by the
201 FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black
202 polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox
203 solutions were prepared daily and fluorescein was diluted from a stock solution (1.17
204 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in
205 duplicate and at least three independent runs were performed for each sample. Final
206 ORAC-FL values were expressed as mg of Trolox equivalent/ mL.
207
208 **2.5- HPLC-DAD analysis**
209 Profiles of polyphenols were determined by HPLC-DAD (Waters Series 600.
210 Mildford MA. USA). Separation was performed in a reverse phase Symmetry® C18
211 column (250 × 4.6 mm i.d.. 5 µm particle size and 125 Å pore size) with a guard
212 column containing the same stationary phase (Symmetry® C18).
213 Chromatographic separation of phenolic compounds from strawberry was carried
214 out with a solvent A-water/methanol/formic acid (92.5:5:2.5v/v/v) and solvent B-
215 methanol/water (94:6 v/v) under the following conditions: linear gradient starting at 0 to
216 30% B in 40 min, 30 to 50% B in 20 min and from 50 to 0% B in 10 min at 0.75 ml/min
217 with an injection volume of 50 µl. Detection was achieved by a diode array detector
218 (Waters, Milford, MA , USA) at wavelengths ranging from 200 to 600 nm in 2 nm
219 intervals. Absorbance was measured at 280 nm (flavan-3-ols), and 350 nm (flavonols).
220 Standards used were: (+)-catechin, quercetin-3-rutinoside, ellagic acid (Sigma, Sintra,
221 Portugal) expressed as µg/g fruit. Anthocyanins were separated with the same solvents
222 and with a linear gradient starting at: 15 to 30% B in 20 min, 30 to 35% B in 5 min, 35
223 to 0% B in 15 min and kept at 0% B during 5 min with flow rate of 0.75 ml/min.

Injection volume was 50 µl and the UV–vis detector was set at 510 nm. Pure standards used were cyanidin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside (Extrasynthése, Lyon, France) expressed as µg/g strawberry. The analyses were made in triplicates from each extract performed for each condition analysed.

For polyphenols from peach yoghurt the elution of extracted compounds follows a linear gradient starting with 0% solvent B which increased to 30% B in 40 min, 30 to 50% B in 20 min and from 50 to 0% B in 10 min at a flow rate of 0.75 ml/min. Retention times and spectra of compounds were analysed by comparison with pure standards and quantification performed by the calibration curves of (+)-catechin, chlorogenic acid, neochlorogenic acid and quercetin-3-rutinoside and expressed as µg/g peach. Carotenoids from peach yoghurt extracted as described above were eluted using the mobile phase composed by acetonitrile (55%), methanol (22%), dichloromethane (11.5%) and hexane (11.5%). Ammonium acetate was added at 0.02% to stabilize carotenoids under isocratic conditions at 1.0 mL/min flow rate during 20 min, at 25 ºC with an injection volume of 40 µL. β-Carotene and zeaxanthin were quantified using a calibration curve built with pure standards and expressed as µg/g peach.

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.

Statistically significant values of the groups' means were determined by one-way analysis of variance with Tuckey post hoc test to compare groups' means. The statistical analyses performed were considered significant when $P < 0.05$.

3. Results and Discussion

3.1. Effect of simulated GI digestion on total antioxidant capacity of fruit yoghurt

A strawberry methanolic extract made from 20% fruit enriched yoghurt prepared as described in 2.2 and 2.3 presented an antioxidant capacity determined by oxygen radical absorbance capacity (ORAC) of ca. 45 mg trolox equivalents per gram of strawberry. During the passage throughout simulated GI system an overall increase in the radical scavenging capacity of strawberry incorporated on yoghurt matrix was observed. In mouth, antioxidant capacity was the same as the observed for the strawberry yoghurt before initiate the digestion. When the yoghurt mixture was submitted to stomach digestion ORAC values increased to 182 mg trolox/g strawberry corresponding to an increase of 300%. Under intestinal conditions the antioxidant capacity continues to increase until reach 261 mg trolox/ g strawberry being 43% higher than in the stomach. It was possible to observe that the antioxidant capacity of strawberry increased 480% on the level of the simulated intestinal digestion (Figure 1). In the case of simulated GI system without enzymes (only pH and bile salts) the highest ($P < 0.05$) antioxidant capacity values were obtained on the stomach digestion presenting more 100% than that measured at the level of mouth or intestine (Figure 1). So, the increase of antioxidant capacity previously described on the intestine may be related with enzymes action.

Peach methanolic extract made from peach enriched yoghurt presented an antioxidant capacity of 23 mg trolox/ g peach. On mouth digestion no significant differences were observed, however, when submitted to stomach conditions a 417% increase on ORAC was observed. From stomach to intestinal compartment antioxidant capacity increased 26% (Figure 2). Similarly to strawberry yoghurt, peach incorporated on yoghurt matrix presented on the intestine more 550% of radical scavenging capacity than yoghurt not submitted to critical digestion conditions. The simulated system

without digestive enzymes presented the same result as strawberry, where on stomach antioxidant activity was only 40% higher ($P < 0.05$) than that observed for mouth or intestine digestion (Figure 2).

For both strawberry and peach, the increased antioxidant capacity on stomach seemed to be a result of pH lowering effect together with enzymes action leading to higher extractability of polyphenols due to acidification and enzymatic release from protein/ polysaccharide matrix on stomach. According to Baublis, et al. [41], GI pH conditions caused a dramatic increase in antioxidant activity of aqueous extracts of wheat-based ready-to-eat (RTE) breakfast cereals, since gastric conditions may influence phenolic compositions esterified to sugars or acids. Higher antioxidant capacity can be also a result from anthocyanins content increase since their stability under acidic conditions of stomach was already reported by Bermúdez-Soto, et al. [8] on chokeberry, on raspberry [5] and on pomegranate [3]. In fact anthocyanins increase after stomach *in vitro* digestion was attributed to the lower pH of the sample, which renders an increase of the flavylium cation in the solution [3].

It is known from the literature that the radical scavenger activity of polyphenols is strongly pH-dependent, where usually higher pH values lead to an increase of this capacity. This increase has been attributed to the deprotonation of the hydroxyl moieties present on the aromatic rings of the phenolic compounds. Theoretically, upon deprotonation of a hydroxyl moiety, the additional negative charge generated in the molecule may decrease the energy required for homolytic O-H bond dissociation, and, thus, facilitate hydrogen atom donation reactions [42, 43]. However, our results are not in accordance with those reports since the increase of pH from stomach to intestine lead to a decrease in the antioxidant capacity, when GI pH conditions were simulated (Figure 1 and 2).

299 However, when enzymes were present antioxidant capacity increased from
300 stomach to intestine. The antioxidant activity of extracts may be produced from the
301 combined action of phenolic constituents and other compounds such as extractable
302 proteins [44], hemicellulose, amino acids, peptides, soluble sugars [45]. Cereal proteins have
303 been known to exert strong antioxidant properties [46] and hence some water soluble
304 proteins as well as phenolics might be present in the extracts, which could contribute to
305 the antioxidant activity observed mainly at the level of intestine, where the antioxidant
306 capacity was higher.
307
308 **3.2. Effect of simulated digestion on the profile of polyphenols from strawberry**
309 **yoghurt**
310 The strawberry extract made in each compartment of the simulated GI system
311 with and without enzymes was analysed by HPLC-DAD and the compounds variations
312 identified are listed on Table 1.
313 The (+)-catechin content presented no significant changes from the beginning of
314 digestion to stomach. However, on the level of the intestine it was possible to observe a
315 significant decrease ($P < 0.05$) of 47%. On the simulated GI system without enzymes
316 only a decrease of 14% was observed in catechin content (Table 1). Tagliazucchi, et al. [1]
317 found on pure flavonoids (catechin and quercetin) that they were only slightly degraded
318 in the mild alkaline environment. In contrast, Bermúdez-Soto, et al. [8] found a loss of
319 58% of catechin when incubated in simulated intestinal fluid. The interactions between
320 catechin and digestive enzymes could mask catechin and make it undetectable with
321 HPLC analysis. Laurent, et al. [47] found a decrease of 41% in catechin after intestinal
322 digestion. Besides phenolics they also reported decrease of some cells enzyme
323 activities, such as alkaline phosphatase, sucrase-isomaltase and aminopeptidase N as a

result of interaction with polyphenols. It is well known that phenolic compounds can have strong affinities with proteins and particularly with human salivary proline rich proteins and histatins [48-51] to form both non-covalent and covalent associations according to the phenolic compound size. According to Arts, et al. [52] and de Freitas and Mateus [48] the (+)-catechin and (-)-epicatechin were able to interact with proline rich proteins such as β-casein. Rohn, et al. [53] reported a loss of pancreatic α-amylase and trypsin activities in the presence of phenolic compounds.

Quercetin-3-rutinoside presented a decrease of 40% along the GI system while in the control digestion without enzymes a decrease of 88% was detected. These results make evidence that quercetin content was more strongly affected by the alkaline pH on intestine than by the presence of the enzymes. Bermúdez-Soto, et al. [8] reported a higher stability on pure quercetin-3-rutinoside (3% losses) under gastric and intestinal digestion. The difference between results obtained and reported could be explained by the matrix, since quercetin-3-rutinoside can bind to milk proteins becoming less bio-accessible and prone to degradation [54].

The ellagic acid was very stable along with the simulated GI system, presenting variations of 3%, while under the simulated conditions of pH, the ellagic acid presented an increase of 19% after the entire digestion system, proving that pH had an important role in ellagic acid release. Free ellagic acid could be the result of release from the ester form in the ellagitannins during the alkaline treatment leading to an increase of this compound during digestion [55].

Concerning the anthocyanins content the higher variation was observed on the intestine compartment. Mouth digestion only affected in a significant way the pelargonidin-3-glucoside, promoting a decrease of 28%. The amount of bio-accessible anthocyanins on the level of stomach increased, being just significant for pelargonidin-

3-glucoside (55%). The transition from the acidic gastric to the mild alkaline intestinal environment caused a decrease in the amount of bio-accessible anthocyanins. At the end of the entire phase of digestion the bio-accessible anthocyanins content were 46, 65 and 58% lower for cyanidin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside, respectively (Table 1). Anthocyanins are highly unstable and easily susceptible to degradation. In foods, the stability of anthocyanins is affected by pH, temperature, light, and oxygen, as well as by the presence of proteins. Association between anthocyanins and milk proteins can allow protection from degradation during the GI system. Viljanen, et al. [56] observed that in aqueous phase 20% of anthocyanins can be associated with proteins. It was reported that β-LG can form non-disulphide covalent linkages with sour cherry anthocyanins [57].

Other studies reported the high instability of anthocyanins at neutral or slightly basic pH and is attributable to the formation of the colourless chalcone pseudobase resulting in the destruction of anthocyanin chromophore [3-5, 8]. Our results support these previous findings suggesting that strawberry anthocyanins are highly stable in the acidic conditions of the stomach while they are degraded in the alkaline conditions of the intestine. The pH influence on anthocyanins was corroborated in this study by the result obtained on the major strawberry anthocyanin (pelargonidin-3-glucoside) content, which increased 88% on simulated conditions on stomach without enzymes (Table 1).

The control digestions carried out without mouth, gastric and pancreatic enzymes showed significant decreases on some compounds indicating that the extraction of polyphenolic compounds during the *in vitro*, gastric and pancreatic digestions was also a result of the chemical conditions, such as pH values. The significant variations ($P < 0.05$) observed were obtained for the content of (+)-catechin

(14%), quercetin-3-rutinoside (88%), pelargonidin-3-glucoside (41%) and pelargonidin-3-rutinoside (46%) (Table 1).

The higher decrease in polyphenols content in simulated system could indicate that enzymes could be promoting the release of polyphenols from interactions with matrix becoming more bio-accessible and consequently more susceptible to be degraded by the action of chemical (pH) and temperature conditions. Kırca and Cemeroğlu [58] reported a very fast degradation of anthocyanins in coloured juices and nectars stored at 37 ºC and [59] reported blackberry anthocyanins degradation when juice and concentrate were stored between 5-37 ºC.

3.3. Effect of simulated digestion on the profile of polyphenols and carotenoids from peach yoghurt

Peach extracts obtained from peach yoghurt submitted to the GI system presented a significant ($P < 0.05$) decrease in the content of the polyphenols (+)-catechin (80%), chlorogenic acid (45%), neochlorogenic acid (39%), quercetin-3-rutinoside (32%) (Table 2).

Other studies supported these findings; Tarko, et al. [60] reported a 30% decrease in black chokeberry in neochlorogenic and chlorogenic acids after the digestion. Bermúdez-Soto, et al. [8] reported a decrease in the levels of pure chlorogenic acid and the formation of neochlorogenic acid during the pancreatic incubation period, which occurred with a concurrent increase of the final pH of the incubation mixture (from 7.5 to 8.5).

The phenolic instability under alkaline pH suggests that these compounds undergo several chemical reactions, mainly oxidation and polymerization, leading to the formation of other phenolic derivatives, such as chalcones, which are not available for absorption because of their high molecular weight and low solubility [61]. In fact, it has

been reported that alkaline conditions transform 50–60% of flavanones into chalcones [62]. Furthermore, some dietary constituents such as fiber, proteins, and iron reduce the solubility and availability of phenolic compounds [9, 28].

Concerning carotenoids, only zeaxanthin decreased (38%) in a significant way, but β-carotene was very stable during all the simulated system (Table 2).

Rodríguez-Roque, et al. [61] observed a decrease of 31 and 69% on blended fruit juice (orange, pineapple, and kiwi) carotenoids in the small intestinal digestion when compared to gastric digestion. Hedren, et al. [63] showed that 3% of β-carotene from raw carrot pieces was bio-accessible and an increase of β-carotene bio-accessibility was observed in the presence of oil. Food matrix has a significant influence on the bio-accessibility of carotenoids, such as β-carotene, lutein, and β-cryptoxanthin, when a blended juice was combined with whole milk (up to 148%) than with skimmed or soy milk (up to 63 and 38%, respectively) [64].

The β–carotene was very stable along all the *in vitro* GI system. It was previously observed that the fat present in food like chicken probably promoted micellarization of β–carotene from mango and the fat emulsification could be stabilised by protein [65].

On the level of mouth and stomach no significant differences were observed, however, a slight increase in compounds content was observed on the level of stomach digestion. These results suggest that gastric digestion improves the release of carotenoid compounds from the yoghurt matrix. Stomach plays a significant role in the bioavailability of carotenoids by initiating their transfer from the vegetable matrix to the fat phase of the meal [66]. This fact could be mainly attributed to the acidic pH and enzymatic activity during this digestive phase, which can induce the hydrolysis of some phenolic compounds bound to other food constituents such as proteins [41, 67, 68]. Saura-

Calixto, et al. [9] reported that phenols linked to high molecular weight compounds, such as proteins and carbohydrates, may be released by digestive enzyme action, leading to a significant increase in their concentrations after gastric digestion.

The intestinal digestion was the main step responsible for the foremost polyphenolic and carotenoids changes.

Along the GI system without enzymes carotenoids presented a significant decrease mainly on zexanthin ($P < 0.05$, 42%) and a slight and non-significant decrease on β–carotene (8%) when exposed to pH conditions of the stomach. Carotenoids are known to be unstable in acidic media, because they are susceptible to oxidation owing to the numerous double bonds of their chemical structure [63]. Considering peach carotenoids under intestine conditions, only zeaxanthin decreased (87%), significantly. This decrease was higher than that observed in the presence of enzymes and the same was observed by Hedren, et al. [63] who observed that the amount of released β-carotene decreased by 80% when bile salts were omitted from the digestion mixture and was probably dissolved within lipid droplets originating from the carrot mixture.

In the simulated GI system without enzymes only (+)-catechin increased 67%, while the remaining compounds analysed presented no significant changes in their content (< 30%).

To have a better understanding about the potential effect of the available fruit phenolics after interaction with yoghurt components and after human digestion further studies *in vivo* should be performed. Some *in vivo* studies have described the potential of developing functional foods such as Morato, et al. [47] which described the benefits omega-3 enriched chocolate milk in reducing damaged muscle of post-exercised rats and Lollo, et al. [48] described that the benefits of probiotic fermented milk in the immune system of rats exercised to exhaustion .

Conclusions

Results obtained in this research revealed the amount of bioactive compounds from a strawberry and peach yoghurt that could be released from the yoghurt matrix and could be available for absorption *in vivo*. Therefore, *in vitro* methodologies, allow rapid progress in understanding physicochemical changes, interactions, and bio-accessibility of bioactive compounds.

The antioxidant capacity from the both fruits added to yoghurt increased by influence of intestinal digestion, probably due to chemical changes on polyphenols and carotenoids structure, like hydroxylation processes. In strawberry individual compounds presented a significant decrease with pelargonidin-3-glucoside presenting the highest variation. The pH had an important role in strawberry anthocyanins content variation. In peach (+)-catechin was the polyphenol with the highest variation, while the remaining polyphenols and zeaxanthin content decreased in lower extent.

The ellagic acid in strawberry and β-carotene in peach were the most stable compounds along entire *in vitro* GI system. Results suggest that, despite the significant decrease in the concentration of bioactive compounds, matrix allowed to protect some compounds from degradation, being now bio-accessible to be absorbed and utilized.

The results obtained in this research should be compared with additional *in vivo* studies to correlate the bio-accessibility of bioactive compounds between *in vivo* and *in vitro* methodologies.

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Table 1- Polyphenols quantitative profile (obtained by HPLC-DAD) of extracts prepared from strawberry enriched yoghurt (μg/mL) during a simulated gastrointestinal digestion with and without enzymes.

Table 2- Polyphenols quantitative profile (obtained by HPLC-DAD) of extracts prepared from peach enriched yoghurt (μg/mL) during a simulated gastrointestinal digestion with and without enzymes.

Table 1

	Concentration (µg/ g biomass)				
	Original	**Mouth digestion**	**Gastric digestion**	**Intestinal digestion**	**% variation**
With enzymes					
(+)-Catechin	534.8±37.9[a]	426.3±10.1[a]	476.6±29.8[a]	283.3±2.8[b]	(-) 47
Quercetin-3-rutinoside	11.0±0.9[a]	7.11±0.4[b]	9.0±0.3[ab]	6.6±0.6[b]	(-) 40
Ellagic acid	8.6±0.7[a]	7.9±2.7[a]	8.0±0.2[a]	8.3±0.6[a]	(-) 3
Cyanidin-3-glucoside	6.5±0.3[a]	6.6±0.2[a]	6.7±0.5[a]	3.5±0.9[b]	(-) 46
Pelargonidin-3-glucoside	70.6±0.3[b]	50.8±0.7[c]	78.8±0.2[a]	24.5±2.9[d]	(-) 65
Pelargonidin-3-rutinoside	6.7±0.3[ab]	5.5±0.2[b]	8.5±0.6[a]	2.8±0.2[c]	(-) 58
Without enzymes					
(+)-Catechin	469.4±23.0[a]	423.8±26.2[a]	492.0±63.8[a]	404.3±27.3[a]	(-) 14
Quercetin-3-rutinoside	13.4±2.0[a]	6.3±1.0[b]	8.4±0.4[b]	1.5±0.1[c]	(-) 88
Ellagic acid	14.6±2.3 [a]	3.9±1.2 [b]	6.7±0.6 [b]	17.4±2.5 [a]	(+) 19
Cyanidin-3-glucoside	4.8±0.3 [a]	3.0±0.9 [a]	4.9±0.5 [a]	4.8±0.1 [a]	0
Pelargonidin-3-glucoside	63.9±3.2 [b]	41.2±21.1 [b]	120.0±25.7 [a]	37.5±4.8 [b]	(-) 41
Pelargonidin-3-rutinoside	8.0±1.4 [a]	5.8±0.7 [a]	9.5±1.6 [a]	3.7±0.0 [a]	(-) 46

Values are shown as means ±SD (n = 6).
Percentage of variation at the end of the digestion process, where negative signal represents decrease and positive represent increase.
[a] Different letters represent significant differences ($P < 0.05$) in comparison to the original content

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693 **Table 2**

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	Concentration (µg/ g biomass)				
	Original	**Mouth digestion**	**Gastric digestion**	**Intestinal digestion**	**% variation**
With enzymes					
(+)-Catechin	35.5±1.9 [a]	34.13±0.4 [a]	28.2±2.6 [a]	7.1±0.9 [b]	(-) 80
Neochlorogenic acid	50.4±1.3 [a]	51.3±9.3 [a]	54.7±4.0 [a]	27.7±1.1 [b]	(-) 45
Chlorogenic acid	46.4±0.5 [a]	49.1±6.5 [a]	51.0±3.8 [a]	28.5±1.1 [b]	(-) 39
Quercetin-3-rutinoside	7.7±0.7 [a]	6.9±0.2 [ab]	8.3±0.9 [a]	5.2±0.0 [b]	(-) 32
Zeaxanthin	6.3±0.7 [b]	6.2±0.6 [b]	7.5±0.5 [a]	3.9±0.5 [c]	(-) 38
β-Carotene	4.1±0.3 [a]	4.5±0.6 [a]	4.9±1.3 [a]	4.6±0.3 [a]	(+) 12
Without enzymes					
(+)-Catechin	42.8±7.6 [b]	50.9±1.9 [b]	47.8±7.2 [b]	71.4±1.3 [a]	(+) 67
Neochlorogenic acid	66.1±10.2 [a]	68.1±2.8 [a]	40.8±8.8 [a]	57.5±3.9 [a]	(-) 13
Chlorogenic acid	60.2±10.9 [a]	63.3±3.5 [a]	40.7±7.5 [a]	54.0±1.6 [a]	(-) 10
Quercetin-3-rutinoside	8.4±1.4 [a]	9.7±0.4 [a]	7.1±0.5 [a]	8.8±0.1 [a]	(+) 4
Zeaxanthin	7.1±0.0 [b]	9.8±0.6 [a]	4.1±0.4 [c]	0.9±0.2 [d]	(-) 87
β-Carotene	3.8±0.9 [a]	4.9±0.3 [a]	3.5±0.3 [a]	2.7±0.6 [a]	(-) 29

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696 Values are shown as means ±SD (n = 6).
697 Percentage of variation at the end of the digestion process, where negative signal represents decrease and
698 positive represent increase.
699 [a] Different letters represent significant differences ($P < 0.05$) in comparison to the original content

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Figure 1

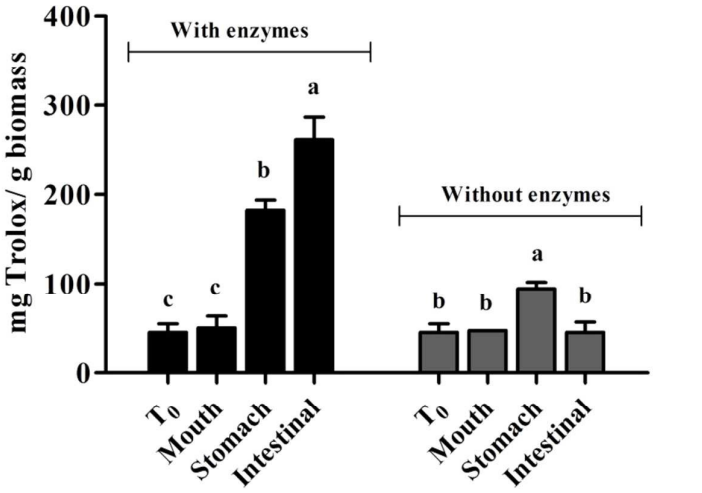
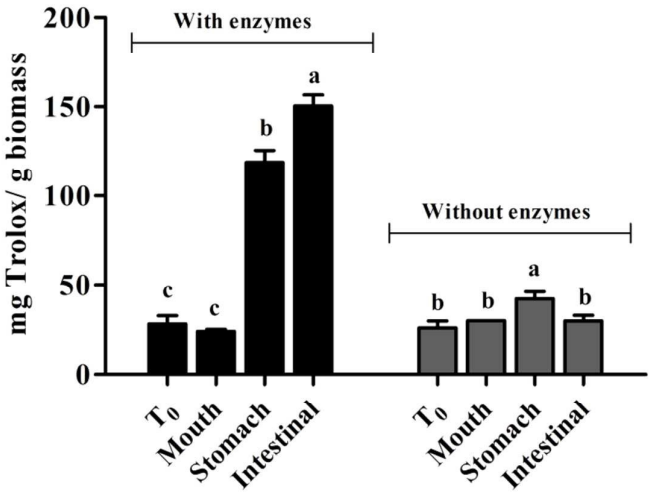


Figure 2



Graphical Abstract