Strawberry dietary fiber functionalized with phenolic antioxidants from olives. Interactions between polysaccharides and phenolic compounds.

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Abstract.

The interaction of strawberry cell wall with hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), two potent phenolic antioxidants naturally found in olive fruit with important biological properties, was investigated. The interaction occurred with drying and seemed to be more complex, strong and irreversible than a simple association. MALDI TOF-TOF analysis suggested covalent (ester bond) and non-covalent (strong hydrogen-bonding, mostly) interactions. The oxygen radical absorbance capacity (ORAC) assay confirmed that the phenols maintained partially their antioxidant activity after binding to the soluble dietary fraction. This soluble dietary fiber was obtained following digestion simulated in vitro with gastric and intestinal fluids. Although the antioxidant activity of HT and DHPG was affected by the dietary fiber interaction, this activity was restored when polysaccharide size was reduced by enzymatic treatment, suggesting that a similar process could occur in the colon. Thus, the use of this novel antioxidant-enriched soluble dietary fiber as a functional food ingredient could potentially promote intestinal health.

Keywords: Hydroxytyrosol; 3,4-dihydroxyphenylglycol; strawberry dietary fiber; phenol-polysaccharide complex; antioxidant activity;
1. Introduction.

Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) are the major antioxidant phenols found in olive fruit with potential health benefits that include antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic properties (Fernández-Bolaños, López, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2008). Furthermore, in 2012 the European Food Safety Authority (EFSA) endorsed the health claim that consumption of HT and its derivatives (>5 mg/day) protects the cardiovascular system, preventing the oxidation of LDL cholesterol by free radicals (EFSA NDA Panel, 2012). This bioactivity is due to the molecular structure of both compounds that contains an ortho-diphenolic group and an additional hydroxyl group in the β position in the case of DHPG. These phenolic compounds are easily assimilated by the human body, reaching blood plasma in 15 or 20 min and eliminated 6-8 h later by the renal and digestive system (Echevarría, Ortiz, Valenzuela, & Videla, 2017). In previous studies we have demonstrated the potential binding interactions between pectin and HT or DHPG and described the formation of complexes as an efficient system for the delivery of the phenolic antioxidants to the colon. This encapsulation system protects the compounds from degradation during gastrointestinal transit to the colon (Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017; Bermúdez-Oria, Rodríguez-Gutiérrez, Rodríguez-Juan, González-Benjumea, & Fernández-Bolaños, 2018).

Interactions between intracellular polyphenols and plant cell walls have been described. Specifically, interactions between procyanidins and apple cell wall and individual components, cellulose, hemicelluloses and pectin have been shown, with the highest affinity found for interactions with pectin (Le Bourvellec & Renard, 2005). The polyphenols bind quickly and spontaneously to cell wall polysaccharides of dietary-fiber-rich foods when they are released by the rupture of fruits and vegetables during eating (grinding, mastication) or processing (boiling, autoclaving or freeze-drying) (Liu, Martinez-Sanz, Lopez-Sanchez, Gilbert, & Gidley, 2017). Therefore, the bioaccessibility of polyphenols would be affected by these interactions.
An important amount of ingested polyphenols is not bioaccessible after gastric and small intestinal digestion as it is bound to cell wall material (Saura-Calixto, 2011). These bound polyphenols reach the colon where they are released and fermented by bacterial microflora into absorbable metabolites that may be important for maintaining good gut health (Pozuelo et al., 2012). Furthermore, the phenolic compounds associated with soluble and insoluble dietary fiber can scavenge free radicals, protecting cells against oxidative damage in the intestinal ecosystem (Pérez-Jiménez & Saura-Calixto, 2015).

In previous works we observed that the simple phenolic compounds HT and DHPG form a strong interaction with soluble polysaccharides (pectin) via a combination of covalent (ester) and non-covalent bonds (hydrogen bonding and/or electrostatic interactions) as suggested by MALDI TOF-TOF analysis (Bermúdez-Oria et al., 2017; Bermúdez-Oria et al., 2018). We also demonstrated that the phenols’ antioxidant activity was maintained after complexation and after digestion simulated *in vitro* with gastric and intestinal fluids (Bermúdez-Oria et al., 2018).

The present study was carried out to obtain a potential source of dietary fiber from strawberry dietary fiber with higher antioxidant properties due to the addition of two potent phenolic compounds present in olive fruit, HT and DHPG. The aim was to identify free as well as complexed HT and DHPG in the soluble and insoluble dietary fiber of strawberry dietary fiber, to characterize putative interactions by MALDI TOF-TOF, and to determine the *in vitro* antioxidant activity of such complexes.

### 2. Materials and methods.

2.1. Isolation of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from olive oil by-products.

HT and DHPG were extracted by hydrothermal treatment at 50–80 °C for 60 min from *alperujo*, a by-product of olive oil extraction using the two-phase separation system. After
treatment, a liquid phase rich in both compounds was obtained. HT and DHPG were purified by two different exchange chromatography system obtaining a 90-95 % of purity in both compounds. These processes have been described and patented by Fernández-Bolaños et al., (2013).

2.2. Preparation of strawberry cell wall material.

Alcohol-insoluble solids (AIS) from strawberry fruits were prepared according to the method of Renard, 2005. Briefly, strawberries with the calix or green hull removed into 2-3 pieces and directly grinded in a domestic blender in 70 % ethanol and filtered on nylon cloth. The solid was ground and washed repeatedly with 70 % ethanol until the filtrate became colorless. The final drying was performed by solvent exchange, 96% ethanol and acetone, and oven dried overnight at 40 ºC.

2.3. Proximate composition of cell wall.

Non-cellulosic sugar composition was determined by hydrolysis with 2 N trifluoracetic acid (TFA) at 121º C for 1 h. The released sugars were quantified after reduction and acetylation by gas chromatography (GC) (Englyst & Cummings, 1984). Cellulose was quantified from the glucose released after Saeman hydrolysis with sulfuric acid in two steps (Saeman, Moore, Mitchell, & Millet, 1954) and quantified as acetate of glucitol by gas chromatography, subtracting the glucose non-cellulosic material. Chromatographic conditions utilized were described by Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández-Bolaños, 2012). Klason lignin level was determined gravimetrically as the amount of acid-insoluble material remaining after sulfuric acid hydrolysis. Uronic acid was quantified using the phenyl-phenol method after sulfuric acid hydrolysis (Blumenkrantz & Asboe-Hansen, 1973).

2.4. Preparation of the strawberry dietary fiber -HT/DHPG complex.

Approximately 0.5 g of strawberry dietary fiber alcohol insoluble solid was added to 10 mL of 1–10 mg/mL HT or DHPG solutions. After overnight swelling, the samples were dried by two
methods: oven-drying for 72 h at 60 °C and 80 °C or freeze-drying (start temperature – 40 to 20 °C during 3 days).

2.5. Extraction and analysis of HT and DHPG from complex.

Extraction of HT/DHPG present in the soluble and insoluble strawberry dietary fiber complex was evaluated complex in various organic solvents including methanol:water (20, 40, 80%), acetone, and dimethyl sulfoxide (DMSO):water (10, 30, 60, 90%) of a volume of 25 mL using 0.1 g dietary fiber. In addition, the extraction was assayed using the protocol for extraction of ester-linked phenol to fiber as described by Jaramillo, Rodríguez, Jiménez, Guillén, Fernández-Bolaños, & Heredia (2007). The sample was treated with 2 N NaOH for 24 h at room temperature under nitrogen. The solution was acidified and extracted three times with ethyl acetate. Also the complex was subjected to acid hydrolysis according to the method of Graciani & Vázquez (1980) for the quantification of HT from oleuropein (an olive molecule containing HT linked by ester bond to elenolic acid). 0.1 g of dry beads/emulsion were treated with 10 mL of 3 N HCl and heated to 100 °C for 10 min and filtered. The insoluble dietary fiber complex was subjected to the procedure of extraction of nonextractable polyphenols with strong acidic treatment, with methanol/H2SO4 90:10 (v/v) at 85° C for 20 h, which releases hydrolysable polyphenols. An additional n-butanol/HCl 97.5:2.5 (v/v) treatment at 100° C for 60 min was used to release anthocyanins (Pérez-Jiménez & Saura-Calixto, 2018). In no cases it was possible to quantify the strongly bound HT or DHPG.

The quantities of HT or DHPG were determined by HPLC according to a previously published method (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007).

2.6. Effect of the phenolic concentration on the formation of HT/DHPG-dietary fiber complex.

The AIS material (0.5 g) was suspended in 10 mL of HT or DHPG solution to a concentration of 0.1, 0.5, 1.0, 1.5, 2.0, 5.0, 10, and 15 mg/mL. After soaking and hydration the
mixture was oven-dried at 60º C for 72 h. The solution of free phenol after re-hydration with water and 70% ethanol wash (200 mL) was separated from the dietary fiber material/phenol complexes by filtration under vacuum. The content of free HT or DHPG was measured by HPLC. The amount of phenolic compound bound to the dietary fiber was determined by subtracting the amount in the filtrate from that of the initial solution.

2.7. In vitro gastrointestinal digestion.

*Simulated gastric and intestinal fluids (process 1)*

HT/DHPG-bound dietary fiber, free of soluble phenols, was immersed in 100 mL 0.1 M HCl solution at pH 1.2 (simulated gastric fluid) and incubated with gentle shaking in a water bath at 37º C for the first 2 h. After incubation, the sample was filtered with filter paper, and the filtrate used for the quantification of HT and DHPH delivered in gastric fluid. The insoluble fraction was adjusted to pH 6.8 with the addition of 100 mL phosphate buffer solution (simulated intestinal fluid). The samples were incubated for another 3 h in a water bath at 37º C with agitation. After incubation, the samples were filtered and the soluble fraction was separated from the insoluble fraction, and the HT/DHPG was quantified.

*In vitro digestion with artificial gastrointestinal juices and enzymes (process 2)*

Simulated in vitro gastric and intestinal digestion by addition of artificial gastrointestinal juices and gastrointestinal enzymes was carried out following the procedures described by Epriliati, D’Arcy, & Gidley, (2009) and Padayachee et al., (2013) with slight modifications.

**Simulated digestion - mouth**

The pH of the sample (0.5 g HT/DHPG-dietary fiber) was adjusted to pH 6.9 with the addition of 5 mL diluted phosphate buffer containing α-amylase (1 g/L), 1.336 mM Ca₂Cl, 0.174 mM MgSO₄, 12.8 mM KH₂PO₄, and 23.8 mM NaHCO₃ to simulate digestion in the mouth. The suspension was incubated at 37º C for 5 min with continuous agitation.

**Simulated digestion - stomach**
The pH was readjusted to pH 2 with 5 mL of a solution of 5 mM KCl, 130 mM NaCl and 0.5 N HCl, which was added to the sample to simulate the lowest pH of the gastric environment. 0.5 mL pepsin solution (0.2 g pepsin from porcine gastric mucosa/5 mL 0.1 M HCl) was added and the mixture incubated for 1 h in a water bath at 37º C with agitation.

Simulated digestion - intestine

The pH of the sample was gradually increased to pH 6–7 by the addition of 0.1 M NaHCO₃, followed by the addition of 2.5 mL pancreatin-bile salts solution (10 mg pancreatin from porcine pancreas and 50 mg porcine bile salts per 2.5 mL 0.1 M NaHCO₃). Sample was incubated for 2 h in a water bath at 37º C with agitation. After incubation, the enzyme activity was terminated by the addition of 6 M HCl to pH 2, and the samples were centrifuged. The HT and DHPG content of the supernatant were quantified using HPLC.

2.8. Antioxidant activity measured by DPPH, ABTS and ORAC assays.

*Antioxidant activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH).*

Antioxidant activity was measured using the DPPH method as described previously (Rodríguez et al., 2005). The radical-scavenging capacity of each antioxidant was expressed as percent DPPH’ remaining in solution after 30 min of reaction, calculated by the equation:

\[
\text{DPPH’ remaining (\%)} = \left(\frac{A_1 - A_2}{A_0}\right) \times 100
\]

where \(A_0\) is the absorbance of the control, 100% of DPPH in methanol with water added instead of the sample, \(A_1\) is the absorbance of the sample, and \(A_2\) is the absorbance of blank sample, sample in methanol without DPPH’ reactive.

In the case of insoluble material, the antioxidant activity was evaluated as described Fuentes-Alventosa et al., (2009) with slight modifications. Briefly, 2–6 mg of insoluble material was suspended in 1 mL of DPPH’ reagent (3.8 mg/50 mL methanol). After 30 min of continuous agitation, the material was centrifuged, and the absorbance of the supernatant was measured at 490 nm.
Antioxidant activity, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The ABTS assay was performed according to the method of Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, & Fernández-Bolaños (2012). This assay is based on the scavenging of ABTS radical (ABTS⁺) by antioxidants. Results were expressed as percent ABTS’ remaining in solution, calculated by the equation:

\[ \text{ABTS’ remaining (\%)} = \frac{(A_1 - A_2)}{A_0} \times 100 \]

where \( A_0 \) is the absorbance of the control (water instead of the sample solution), \( A_1 \) is the absorbance of the sample, and \( A_2 \) is the absorbance of the sample under identical condition as \( A_1 \) with ethanol instead of ABTS’ solution.

Oxygen radical absorbance capacity (ORAC) assay.

The ORAC assay is based upon the inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH). The reactive oxygen species (ROS) generated from this thermal decomposition quenches the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples were assayed according to Ou, Hampsch-Woodill, & Prior (2001) with minor modifications. Sample was diluted with sodium phosphate buffer (10 mM, pH 7.4) and 25 µL of sample was transferred to a microplate. The blank well received 25 µL phosphate buffer while standards received 25 µL trolox solutions (10–140 µM). Then 150 µL of 1 µM fluorescein was added to all wells. After incubation (37º C, 15 min), 25 µL AAPH (250 mM) was added to each well to initiate the reaction and a reading taken every 5 min for 90 min (Ex. 485 nm, Em. 538 nm) in a microplate reader (Fluoroskan Ascent™, Thermo Scientific™). Results were calculated using the difference of areas under the fluorescein decay curve between the blank and the sample and expressed as µmol Trolox equivalents/g of sample.

2.9. Enzymatic hydrolysis of HT/DHPG-bound soluble dietary fiber and ultrafiltration.
The hydrolysis of soluble complex was carried out using a mixture of pectinolytic enzymes (4 µg/mL) including endo- and exo-polygalacturonase and pectin esterase (Novo Nordisk, Bagsvaerd, Denmark). The mixture (50 mL) was incubated at 37º C for 24 h and the hydrolysis was terminated by heating to 100º C for 10 min.

The hydrolyzed fraction was subjected to successive ultrafiltration using an Amicon 8400 stirred cell (Millipore Corporation, Bedford, MA, USA) through a molecular weight cut off of 5000, 3000, and 1000 Da. Each retained solution was washed with water until 300 mL of permeate was collected. Four fractions were obtained: the retained fractions > 5000 Da, > 3000 Da, and > 1000 Da, and the eluted fraction < 1000 Da. All fractions were analyzed for antioxidant activity by ORAC assay as above.


The MALDI-TOF TOF mass spectra were acquired in positive ion mode over a mass-to-charge ratio (m/z) range of 150–2000 Da using an UltraFleXtreme Bruker mass spectrometer Smartbeam-II laser. The instrument was operated at an accelerating voltage of 26.45kV with an extra voltage of 13.399 kV. Each spectrum was produced by accumulating data from 1000–2000 laser shots. The matrix solution of HCCA (alpha-cyano-4-hydroxycinnamic acid) was prepared in 10mg/mL ACN:H₂O:TFA (50:47.5:2.5) (v/v/v) in the presence of sodium trifluoroacetate. The samples were desalted by dialysis during 72 h against deionized water in a dialysis tube of theoretical porosity of 12 KDa (Sigma-Aldrich, St Louis, MO, USA).

3. Results and discussion.

3.1. Preparation of hydroxytyrosol (HT) and 3-4-dihydroxyphenylglycol (DHPG)-bound to strawberry dietary fiber.
Components of dietary fiber (polysaccharides) have the ability to bind phenolic compounds (Saura-Calixto, 2011); (Liu et al., 2017) and in some cases such complexes present an important antioxidant capacity (Wu et al., 2011); (Pérez-Jiménez & Saura-Calixto, 2015). We suggested in previous studies that the natural phenols present in olive fruit, HT and DHPG, interact with pectin to form complex antioxidants (Bermúdez-Oria et al., 2017; Bermúdez-Oria et al., 2018). In this work, we investigate if the interaction between these compounds with high antioxidant activity, and dietary fiber of strawberry or some components of the cell wall may occur. Such an interaction would possibly enhance the functional properties of dietary fiber by increasing its antioxidant activity.

Since fruit processing often involves tissue disruption and the polyphenols leave vacuolar organelles and bind quickly and spontaneously to the cell wall (Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012), we assayed the homogenization of strawberry fruits in the presence of HT and DHPG solutions (1 mg/mL) in a domestic blender. However, after trying different contact times (1–24 h), the blended mixture was centrifuged and the totality of HT and DHPG was recovered in the supernatant. From an accidental discovery we observed that there was considerable retention when the mixture was fully dried at room temperature. Drying may cause irreversible modification to the cell wall with the collapse of the cell wall material (Le Bourvellec & Renard, 2005). It is also documented that boiling and drying decreases the binding affinity of apple cell walls for procyanidins due to pectin solubilization and degradation, and by altering the cell wall surface area (Le Bourvellec et al., 2012);(Liu et al., 2017). However, drying seemed to enhance the binding of strawberry dietary fiber with HT/DHPG in our case.

To further evaluate the influence of drying on the cell wall’s capacity to interact with HT and DHPG, cell wall material from strawberry was prepared as an alcohol insoluble solid. In addition, the influence of the main components of the cell wall, cellulose and pectin, were assayed. The cell wall material and their highly hydrophilic components were swollen in the presence of aqueous solutions of HT and DHPG (1 mg/mL) and then fully dried for 72 h at 60 °C
and 80 °C in an oven or by lyophilization. After drying, the cell wall and their components were rehydrated with water and then washed copiously with ethanol 70%, and the dietary fiber/phenol complex separated by filtration. The content of free HT and DHPG was measured by HPLC and the bound phenols calculated by the difference (Figure 1a). There was a slightly higher retention, statistically significant (p < 0.05), of HT to dried strawberry dietary fiber at 60° than 80° C, but this was not the case for DHPG, where no significant difference between the two drying temperatures was observed. Cellulose and pectin also showed a high binding affinity for HT and DHPG. In the case of cellulose, DHPG was retained in a higher proportion when dried at 80° C, while for pectin was no significant difference between the two drying temperatures. Freeze-drying showed a considerable retention for HT in cell wall while this retention was null in the case of DHPG. However, for cellulose and pectin, there were no significant differences between freeze- and oven-drying for HT, while DHPG presented lower retention following freeze-drying in the case of pectin. All further studies of the cell wall interaction with HT/DHPG employed oven drying at 60° C.

The composition of the native strawberry cell wall material and the oven-dried (72 h at 60° C) dietary fiber with phenol (HT) showed no significant differences (Table 1a). The two main sugars were glucose, essentially from cellulose and galacturonic acid from pectin, while other neutral sugars such as arabinose, xylose and galactose were also predominant, consistent with the sugar composition reported in the literature (Marlett & Vollendorf, 1994).

3.2. Effect of the phenolic concentration on the formation of HT/DHPG-dietary fiber complex.

The effect of increasing the HT and DHPG concentration in the solution on the amount of phenols bound to the same amount of strawberry dietary fiber after drying was investigated. Figure 1b shows the experimental, which represents the amount of bound HT or DHPG as a function of the free phenol concentration after re-hydration and 70% ethanol wash. For example, the amount of bound DHPG increased steadily at low concentrations but showed steeper increases of bound
compound at higher concentrations until the amount of DHPG released was the same. The HT binding was similar although higher concentrations were required to see the steep increase at higher concentrations. However, as shown in the Figure 1b, the results studied do not reach equilibrium.

3.3. Behavior of HT/DHPG-bound dietary fiber in digestive conditions.

The HT/DHPG-bound dietary fiber was digested in two different in vitro simulated gastrointestinal media. In the first, the dietary fiber was digested in simulated gastric fluid (pH 1.2, 0.1 M HCl solution, 2 h) and next in simulated intestinal fluid (pH 6.8 phosphate buffer, 3 h). The dissolution of phenolic compound was monitored in each step. The second simulation of the digestion conditions of the gastrointestinal tract was carried out with the addition of digestive enzymes. The amount of phenols that remained bound to dietary fiber was determined for the final digestion process.

The samples of AIS (0.5 g) mixed with HT/DHPG solutions (1, 2, and 10 mg/mL) were fully dried for 72 h at 60°C by oven- or freeze-dried and further re-hydrated and washed with ethanol (70%) to recover the unbound phenolic compound. The amount of HT and DHPG bound to the dietary fiber is presented in Figure 2. For 1 mg/mL of initial bioactive compounds (BC) solution (20 mg/ g AIS), 70 % of HT and 40% of DHPG were bound to the dietary fiber when oven-dried and there was practically no release by gastric and intestinal conditions (Process 1, simulated gastric and intestinal fluids). An increase of retention of BCs was even observed using the enzymatic method (77% for HT and 66% for DHPG, Process 2), whereas the further dissolution of the phenolic compounds would be expected after the addition of digestive enzymes, resulting in enzymatic hydrolysis of protein, carbohydrates, and lipids. However, for simulated fluid digestion, while the retention of HT decreased to approximately 40% when the initial HT amount dispersed in the dietary fiber was increased to 40 and 200 mg/ g AIS, the retention of DHPG remained fairly constant, at 40–50%. In the case of freeze-drying, the interactions between BC and dietary fiber
were much weaker, with only 35 % of HT bound to dietary fiber, half the amount following oven-drying, and no retention in the case of DHPG.

For the simulated gastric fluid, the binding interaction was very strong resulting in a minimal extraction of BC and practically no release was observed with the further pH change, simulating intestinal fluid (90–99 % of total retention). In fact, even after the assay, extraction with organic solvent or alkaline and acid hydrolysis in severe conditions resulted in no extraction of HT and DHPG from the strongly bound dietary fiber (data not shown).

Dissolved fraction of phosphate buffer was separated from the insoluble fraction, with both fractions showing a high retention of HT and DHPG, confirmed by the color that comes from the initial compounds which was retained in both fractions (Figure 2). This means that a high amount of the phenolic antioxidants in the soluble and insoluble fraction may be protected from absorption during gastrointestinal transit and this may reach the colon in significant amounts for fermentation by gut bacteria.

3.4 Antioxidant activity of the insoluble and soluble fractions.

The antioxidant activity of the soluble and insoluble dietary fiber fraction linked to HT and DHPG was studied by the DPPH method in comparison with the corresponding soluble and insoluble fraction (control) obtained from the dietary fiber of strawberry but without the addition of BCs. The results obtained for the insoluble fraction of 40 mg BC/g AIS and 200 mg BC/g AIS complexes are presented in Figure 3. The assayed fibers were phenol-free, so their antioxidant activity is exclusively due to that corresponding to the fiber fraction or complexed BC. Indeed, no differences were found between the samples with HT or DHPG added and the control. This is indicative that strawberry fiber has an important amount of phenol linked to fiber (0.44 g/100 g dry matter for a total of dietary fiber of 24.9/100 g dry matter), as has been reported to contribute to the fiber fraction’s antioxidant activity (Saura-Calixto, 2011). These compounds are called non-extractable polyphenols or macromolecular antioxidants, and include fundamentally
proanthocyanidins and hydrolysable tannins, which remain in the insoluble dietary fiber of strawberry and reach the colon with promising results in relation to gastrointestinal health (Pérez-Jiménez & Saura-Calixto, 2015). However, the HT and DHPG linked to the insoluble fraction did not maintain their antioxidant properties.

The antioxidant activity of the soluble fraction was studied by three different methods (DPPH, ABTS and ORAC assays) and the results for two concentrations of HT and DHPG added to the mixture with the AIS (40 and 200 mg/g AIS) are presented in Figures 4a and 4b. In the case of the lower concentration (40 mg/g AIS), no differences were found between BC-bound soluble dietary fiber and the control for both the DPPH and ABTS assays. However, for the sample of soluble fraction with a higher concentration (200 mg/g AIS) of linked-DHPG, more free radical scavenging activity was observed for both methods. This confirmed that the DHPG linked to soluble dietary fiber maintains some antioxidant activity. It is possible that the additional –OH group of DHPG with respect to HT helps to provide greater availability of the catechol group, which is responsible for the BCs’ antioxidant activity, a result that is in agreement with a previous report on DHPG-pectin complex formation via encapsulation (Bermúdez-Oria et al., 2018).

The ORAC determinations revealed significant differences between HT and the control as well as DHPG and the control, with a particularly significant increase in activity for the higher concentrations of BC in the complex (Figure 4c). However, with this assay, no differences were observed between HT and DHPG, which does not support the hypothesis that the hydroxyl group in the ortho position of DHPG allows the compound to act as an antioxidant in contrast to HT. Nevertheless, this finding is consistent with a previous study, which reported an interaction between polyphenols from tea and β-glucan, and displayed oxygen scavenging-activity indicative of the complex’s antioxidant behavior (Wu et al., 2011).

Since the soluble dietary fiber control did not present antioxidant activity or was very low, whereas the insoluble fiber control did, we can conclude that very little polyphenols were associated to the soluble dietary fiber of strawberry, although there has been a report of the
presence of a small fraction of flavonoids and phenolic acid (Saura-Calixto, 2011). Nevertheless, the two potent phenolic antioxidants HT and DHPG form a strong complex with the soluble polysaccharides of strawberry dietary fiber and impart their antioxidant properties to the complex.

The finding of soluble polysaccharides with antioxidant activity (BC-bound soluble dietary fiber) that are indigestible in the upper gastrointestinal tract but fermented in the large intestine make this complex an interesting putative dietary supplement because it may promote intestinal health. The combination of the properties of both components would provide a single material capable of scavenging free radicals and counteracting the effect of dietary pro-oxidants, as well as the potential beneficial systemic effects due to generation of short chain fatty acids (acetic, propionic, butyric) by fermentative microflora (Saura-Calixto, 2011).

3.5. Soluble dietary fiber composition

Based on monosaccharide analysis of the soluble fraction and its uronic acid content (Table 1b), pectin was observed to be the predominant component of the HT/DHPG-bound complex. This is in agreement with our previous reports where the strong binding of HT and DHPG with pectinate beads was reported (Bermúdez-Oria et al., 2017; Bermúdez-Oria et al., 2018). Therefore, soluble fibers such as pectin with associated antioxidant compounds could be of interest to the food industry due to its health benefits (Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006) and technological applications (Thakur, Singh, Handa, & Rao, 1997).


A mixture of pectinolytic enzymes was added to reduce the molecular size of the HT/DHPG-soluble dietary fiber complex in order to provide information about the effect that pectinase, produced by the colonic microflora, may have on the antioxidant properties of the complex. The solubilized fraction after enzymatic digestion was subjected to sequential ultra-filtration through a 5000, 3000, and 1000 Da molecular weight cut-off membrane. The corresponding eluted and
retained fractions were analyzed for antioxidant activity by the ORAC assay (Figure 4b). The antioxidant activity of the fraction below 1000 Da was similar to that obtained above 5000 Da in the case of the complex with HT. For the DHPG-bound complex, the activity appeared relatively high in all fractions. In addition, the antioxidant activity of both fractions > 5000 Da were similar to the activity of the initial fractions of HT/DHPG linked to soluble dietary fiber (Figure 4c). Together these results indicate that although the antiradical activities of HT and DHPG seem to be directly or indirectly affected by their interaction with polysaccharides, mostly pectin, their antioxidant activity is restored when the size of the polysaccharides is reduced. The reduction in size of the oligomers or the actual hydrolytic process of soluble dietary fiber or pectin by colonic bacterial enzymes could differ to the outcomes obtained in this study; however, this result suggests that the hydrolytic process in the colon releases oligomers with potential antioxidant activity. This activity could help to prevent certain kinds of degenerative or chronic diseases such as colon cancer or inflammatory bowel disease (IBD) (Saura-Calixto, 2011), although future research is needed to verify this hypothesis.

3.7. Investigation of the interaction between HT/DHPG and the soluble fraction.

The presence of a complex between the soluble dietary fiber fraction and HT/DHPG, which is not hydrolysable, even by the most severe methods applied to non-extractable polyphenols or macromolecular antioxidants (Pérez-Jiménez & Saura-Calixto, 2015), and gives no detectable UV absorption signal (data not shown), was confirmed by MALDI TOF-TOF analysis.

The MALDI TOF-TOF mass spectra of HT and DHPG-bound to the soluble fraction in positive ion mode (Figures 5a and 5b) showed a diversity of masses that was not present in the corresponding control without phenolic compound (data not shown). After studying possible combinations of different oligosaccharides with galacturonic acid units (194 Da), including methyl-ester groups (208 Da), pentoses (150 Da), hexoses (180 Da), and a fragment described by Domon & Costello (1988) that corresponds to a cross-ring of 60 Da from the non-reducing end,
134 Da if containing the reducing end of a galacturonic acid-type, or 120 Da of a hexose, and the corresponding possible adduct of sodium, did not found values m/z without phenol. It is important to remember that sugars (including uronic acid) lose 18 Da (H₂O) per glycosidic bond. In all cases the addition of one or two molecules of HT (154 Da) or DHPG (170 Da) was necessary to obtain coincidence with m/z values of the HT/DHPG-oligosaccharide complex. Several tentative structures taking into account all the possible combinations are proposed in Figures 5a and 5b. However, unequivocal evidence of non-covalent interactions (electrostatic and dipolar forces, hydrogen binding, hydrophobic interactions and/or van der Waals attractions) between HT/DHPG with soluble dietary fiber is difficult to find. Similarly, finding evidence to support the formation of covalent bonds between oligosaccharides of galacturonic acid and HT or DHPG as an ester that would explain the irreversible complexes that maintain in part the antioxidant activity of the BCs has proven very difficult.

The development of strong H-bonds between polyphenol derived from tea and β-glucan (Wu et al., 2011) or strong hydrophobic H-bonds between polysaccharides, mainly pectin, and polyphenols during wine making (Renard, Watrelot, & Le Bourvellec., 2017) have been reported in the literature. Furthermore, staining colorant, formed mainly by purple-blue anthocyanins and used during centuries as a natural cotton fabric dye, have been hypothesized to form H-bonds with cellulose fibers (Saura-Calixto, 2011). Also, more recently, electrostatic interactions were shown to be important in the interaction between polyphenols and cellulose-based composite and apple cell walls (Phan, Flanagan, D’Arcy, & Gidley, 2017). However, clear evidence of a HT/DHPG-soluble dietary fiber binding mechanism is still lacking. The formation of irreversible complexes between polyphenols and polysaccharides are frequently observed in the formation of pomace during juice extraction. Covalent interactions may be mediated by the oxidation of procyanidins, with the subsequent formation of highly electrophilic ortho-quinones mediated by polyphenoloxidase, which can lead to the formation of covalent bonds with macromolecules (Renard et al., 2017). In our case, the antioxidant activity found in the complex excludes the complete oxidation of ortho-
diphenols to quinone and the formation of such a covalent interaction. For the signals at \( m/z \) 895 there are two possibilities of non-covalent or covalent binding of HT (Figure 5a). The mass difference of 160 \( m/z \) units between the signals 895, 1057 and 1219 corresponds to the loss of hexose units, probably galactose. Also for \( m/z \) 1026, two possibilities were found for the same signal, with a combination of covalent and non-covalent in both possibilities. For DHPG, the ions at \( m/z \) 833 were attributed to a combination of tetramers bound to DHPG by an ester bond and by non-covalent bonds (Figure 5b). The mass difference between the signals 833, 995, 1157, 1319, 1481, and 1643, were 160 \( m/z \) units corresponding to the loss of hexoses, probably galactose, from a nonamer.

Although the possibility of covalent bonds, such as ester bonds, was found for HT and DHPG by MALDI TOF-TOF mass spectroscopy analysis, the release of these compounds by acid or alkali hydrolysis was not achieved. Yet alkaline hydrolysis generally hydrolyzes ferulic acids ester bonds when applied to cereal products or sugar-beet cell walls (Ralet, Thibault, Faulds, & Williamson, 1994). Also, the formation of covalent adducts between cell-wall and procyanidin due to the impact of thermal treatment has been recently described. During canning of pear piece, a neo-formed pink color was produced, which was not re-extractable with solvent or with enzymes and persisted in the residues, leading to the suspected formation of covalent adducts (Le Bourvellec, Gouble, Bureau, Loonis, Plé, & Renard, 2013). In our case, due to the effect of drying, we presume that a combination of non-covalent and covalent bonds, probably in the form of covalent adducts, were responsible for the strong binding of HT/DHPG to the dietary fiber.


Interaction between HT and DHPG, two natural phenols present in olive fruit, and the dietary fiber of strawberry fruits occurred after drying but did not occur after contact between suspended cell wall and phenols in solution. The phenolic compounds also bound to commercial pectin and cellulose after drying. The binding seemed very strong and non-reversible. From
MALDI TOF-TOF analysis we suggested that this strong interaction was due to a combination of covalent (ester bond) and non-covalent bonds (hydrogen bonding, electrostatic interaction). Further work to isolate and characterize the nature of these bonds is in progress. The effect of simulated gastric and small intestinal digestion on HT/DHPG-bound to dietary fiber material was also examined. The dissolved fraction of phosphate buffer was separated from the insoluble fraction and both fractions showed a high retention of HT and DHPG, confirmed by the color imparted by the initial compounds and retained in both fractions. Moreover, HT/DHPG-soluble and insoluble dietary fiber complexes showed antioxidant activity. Therefore, a high amount of antioxidants in the soluble and insoluble dietary fiber fraction may be protected from absorption during gastrointestinal transit to reach the colon. However, the potent phenolic antioxidants HT and DHPG only retained antioxidant properties after forming a strong complex with the soluble polysaccharides of strawberry dietary fiber. Interestingly, when the size of the polysaccharide was reduced by enzymatic treatment, the antioxidant activity of HT/DHPG affected by the interaction with components of cell wall was restored, which suggests that a similar process could occur in the colon. From a technological point of view, we present a simple method for the recovery of soluble dietary fiber supplemented or enriched with antioxidant compounds. The use of this new fiber combining the functional properties of both soluble dietary fiber and antioxidant could to be an interesting dietary supplement to promote intestinal health. Future research on the health benefits of dietary fiber linked to HT and DPHH are necessary to attest their functionality (Granato et al., 2018).

ACKNOWLEDGEMENTS

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6. References.


composites during simulated gastric and small intestinal digestion. *Food & Function*, 4, 906-916. doi: 10.1039/c3fo60091b

Pérez-Jiménez, J., & Saura-Calixto, F. (2015). Macromolecular antioxidants or non-extractable polyphenols in fruit and vegetables: Intake in four European countries. *Food Research International*, 74, 315–323. doi:10.1016/j.foodres.2015.05.007


Figure 1. Percentage of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) released after interaction with strawberry cell wall, cellulose and pectin following different drying conditions (60°C and 80°C oven-drying or freeze-drying). Initial concentration of bioactive compounds (BCs) 1 mg/mL; cell wall, cellulose and pectin 50 mg/ml. Error bars represent standard deviations (n = 3). *p < 0.05 and #p < 0.05 indicate significant differences between drying conditions for the bioactive compound and the matrix.

Figure 2. Effect of the bioactive compounds (BC) (0.1, 0.5, 1.0, 1.5, 2.0, 5.0, 10, and 15 mg/mL) on the formation of HT/DHPG-dietary fiber complex of strawberry.
Figure 3. Scheme of the two in vitro simulated gastrointestinal digestion process of HT/DHPH binding to strawberry cell wall. Percentage of bioactive compounds (BC) bound to AIS during drying process (oven-dried and freeze-dried) and bound in the final simulation digestion process respect to the BC bounded.*Represent the average weight of the insoluble and soluble fraction obtained from digestion process of HT-bound cell wall for an initial concentration of HT of 2 mg/ml (10 mL of solution on 500 mg of cell wall and oven-dried at 60 °C for 72 h) (n = 3 BC: bioactive compound; BP: phosphate buffer; AIS: alcohol insoluble solid
Figure 4. Antiradical capacity of insoluble fiber (2, 3, 4 or 6 mg) bound to HT/DHPG obtained from an initial bioactive compound (BC) concentration of (a) 2 mg/mL (40 mg BC/g CW) (b) and 10 mg/mL (200 mg BC/g CW) (b). Comparison with insoluble dietary fiber without BC added (Control). Antiradical capacity is expressed as percentage DPPH* remaining in solution after 30 min of reaction. Each bar is the average value of three replicates. Error bars represent standard deviations (n = 3). CW: cell wall
**DPPH**

![DPPH graph](image)

**ABTS**

![ABTS graph](image)
Figure 5. Antiradical capacity of the HT/DHPG-soluble dietary fiber complex obtained from two different initial concentrations of bioactive compound (BC), 2 mg /mL (40 mg of BC/ g CW) and 10 mg /mL (200 mg BC/g CW). Comparison with soluble dietary fiber without BC added (Control). a) Antiradical capacity is expressed as percent DPPH* remaining. b) Antiradical capacity is expressed as percent ABTS* remaining. c) Oxygen radical capacity (ORAC) is expressed as µMol Trolox / g extract. Each bar is the average value of three replicates. The error bars represent standard deviations (n = 3).

Figure 6. Antioxidant activity measured by oxygen radical capacity (ORAC) of recovered fractions after a sequential ultrafiltration process (5000, 3000 and 1000 Da) obtained from
HT/DHPG-soluble dietary fiber complex treated with a mixture of pectinolytic enzymes. Results are expressed as μmol Trolox / g extract. Error bars represent standard deviations (n = 3).
Figure 7. MALDI-TOF TOF mass spectra (positive mode) of dialyzed HT/DHPG-soluble dietary fiber complex. A) Tentative structures of the $m/z$ signal at 895 and 1027 for HT complex (b). Tentative structures of the signal $m/z$ at 833 for DHPG complex. The mass difference between other signals was of 162 m/z units that may correspond to hexose moieties.
Table 1. Composition (mg/g cell wall) of native cell wall material (AIS) and the oven-dried cell wall with HT added (HT-cell wall dried, 60° C/72 h) isolated from strawberries by the alcohol insoluble solid method. Each value is the average of three replicates ± SD. Percentage molar of uronic acid and neutral non-cellulosic sugars are listed.

<table>
<thead>
<tr>
<th></th>
<th>Native cell wall</th>
<th>HT-cell wall dried</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellulose</strong></td>
<td>104.03 ± 3.72</td>
<td>93.85 ± 4.08</td>
</tr>
<tr>
<td><strong>Lignin</strong></td>
<td>198.01 ± 8.06</td>
<td>256.38 ± 16.23</td>
</tr>
<tr>
<td><strong>Uronic acid</strong></td>
<td>149.07 ± 14.14</td>
<td>99.81 ± 15.12</td>
</tr>
<tr>
<td><strong>Hemicellulose</strong></td>
<td>100.27 ± 12.63</td>
<td>120.16 ± 7.15</td>
</tr>
<tr>
<td><strong>(non-cellulosic sugars)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Moisture</strong></td>
<td>101.12 ± 5.38</td>
<td>98.37 ± 2.75</td>
</tr>
</tbody>
</table>

% molar of non-cellulosic sugar

<table>
<thead>
<tr>
<th></th>
<th>Native cell wall</th>
<th>HT-cell wall dried</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhamnose</strong></td>
<td>2.01 ± 0.43</td>
<td>2.22 ± 0.78</td>
</tr>
<tr>
<td><strong>Fucose</strong></td>
<td>1.15 ± 0.09</td>
<td>0.97 ± 0.38</td>
</tr>
<tr>
<td><strong>Arabinose</strong></td>
<td>10.23 ± 0.64</td>
<td>10.92 ± 0.95</td>
</tr>
<tr>
<td><strong>Xylose</strong></td>
<td>17.50 ± 2.72</td>
<td>16.83 ± 2.88</td>
</tr>
<tr>
<td><strong>Mannose</strong></td>
<td>1.37 ± 0.14</td>
<td>1.72 ± 0.45</td>
</tr>
<tr>
<td><strong>Galactose</strong></td>
<td>9.28 ± 0.95</td>
<td>10.71 ± 1.52</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>2.69 ± 0.46</td>
<td>2.95 ± 0.85</td>
</tr>
<tr>
<td><strong>Galacturonic acid</strong></td>
<td>55.76 ± 4.71</td>
<td>40.28 ± 10.33</td>
</tr>
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</table>

Table 2. Composition (mg/g cell wall) of the HT/DHPG-soluble dietary fiber complex. Percentage molar of uronic acid and neutral non-cellulosic sugars are listed. Each value is the average of four replicates using two HT and DHPG-soluble fiber complex samples.

<table>
<thead>
<tr>
<th></th>
<th>HT/DHPG-soluble dietary fiber complex (mg/g cell wall)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uronic acid</strong></td>
<td>115.72 ± 6.38</td>
</tr>
<tr>
<td><strong>Neutral sugars</strong></td>
<td>84.62 ± 12.85</td>
</tr>
<tr>
<td>Sugar</td>
<td>% molar</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>1.48 ± 0.73</td>
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<tr>
<td>Fucose</td>
<td>0.16 ± 0.06</td>
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<tr>
<td>Arabinose</td>
<td>5.56 ± 1.00</td>
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<tr>
<td>Xylose</td>
<td>0.98 ± 0.25</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Galactose</td>
<td>4.24 ± 0.98</td>
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<tr>
<td>Glucose</td>
<td>1.10 ± 0.48</td>
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<tr>
<td>Galacturonic</td>
<td>90.36 ± 4.99</td>
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