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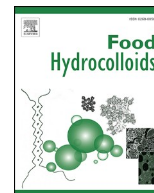

Confirmation by solid-state NMR spectroscopy of a strong complex phenol-dietary fiber with retention of antioxidant activity in vitro

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ABSTRACT

The aim of this study was to prepare a complex between the olive phenolic compounds, hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG) and their mixture, with the soluble and insoluble dietary fiber of apple cell wall. A strong interaction between phenols and the apple cell wall occurred during the drying phase and it was confirmed by ultraviolet–visible spectrometry, Fourier transform infrared spectrometry, differential scanning calorimetry, thermogravimetry and especially by solid-state ^{13}C NMR spectroscopy. The antiradical activity by DPPH, ABTS and ORAC assays confirmed that the simple phenolic HT/DHPG maintained in part their antioxidant activity after complexation with the apple cell wall. In addition, the HT/DHPG-soluble and insoluble fractions obtained after simulated gastrointestinal fluids retained this antioxidant activity. These complexes may be protected from absorption during gastrointestinal transit to reach the colon. In the case of the soluble dietary fiber, an enzymatic treatment, in a simulation of hydrolysis by colonic microflora, released oligomers with potential antioxidant activity from this complex. Therefore, the intake of HT/DHPG bound to the fiber of apple cell wall could provide many of the health benefits associated with dietary fiber, and be fermented by gut bacteria to contribute to a healthy antioxidant environment.

1. Introduction

Polyphenols and dietary fiber are two important nutritional components present in plant food, which are associated with the prevention of cancer and chronic diseases (Zhu, 2018). During the digestion or food processing, plant cells are ruptured and polyphenols are released from the vacuoles and come into contact with cell wall polysaccharides, the indigestible component of dietary fiber (Le Bourvellec, Le Quere, & Renard, 2007; Robles-Almazan et al., 2018). The interactions can occur spontaneously by the ability of polyphenols to bind to polysaccharides by hydrophobic and hydrogen bonding, and by covalent bonds (Renard, Watrelot, & Le Bourvellec, 2017). Several studies have confirmed that procyanidins and phenolic acid were able to bind to pectin and the cell wall of apple and to cellulose (Le Bourvellec, Guyot, & Renard, 2009; Phan, Flanagan, D'Arcy, & Gidley, 2017). These interactions may influence the bioavailability of the antioxidants, thus affecting their absorption, although polyphenols bound to dietary fiber, which survive upper gastrointestinal-tract digestion and reach the colon, may be important for maintaining good gut health (Saura-Calixto et al., 2010). The fiber, named "antioxidant dietary fiber" by Saura-Calixto (1998), combines the benefits of dietary fiber and natural antioxidants, and has shown promising results in relation to gastrointestinal health, including antioxidant and antiproliferative capacities as well as the prevention of cardiovascular disease (Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013).

In a recent work we studied the interaction between hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), two the most potent and abundant antioxidant phenols in the olive fruit with important biological activity (Wani et al., 2018), and strawberry cell wall during drying and revealed a strong and irreversible complex, especially with the soluble dietary fraction (Bermúdez-Oria, Rodríguez-Gutiérrez, Fernández-Prior, Vioque, & Fernández-Bolaños, 2019). Obtaining a dietary

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fiber associated with the phenolic compounds HT/DHPH, which exhibit numerous health-beneficial properties based on their potent ROS scavenging activity and their ability to modulate cellular response against oxidative stress (Robles-Almazan et al., 2018), could be useful for creating a novel tailor-made dietary supplement to promote intestinal health.

The present study focused on the development of a scalable, reproducible and simple complexation method with a different raw fiber material, such as apple dietary fiber, for the recovery of soluble and insoluble dietary fiber enriched with HT/DHPG. The procedure is based on the drying of cell wall material with an aqueous solution of HT and DHPG and including a two-step process for simulated gastrointestinal fluids without the addition of digestive enzymes. In addition, we investigated the chemical characteristics of HT/DHPG combined with the soluble and insoluble dietary fiber of apple cell wall by ultraviolet–visible (UV–vis) spectrometry, Fourier transform infrared spectrometry (FT-IR), thermogravimetry analysis (TGA), differential scanning calorimetry (DSC), and solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The in vitro antioxidant capacity of such complexes was also determined.

2. Material and methods

2.1. Purification of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from alperujo, a by-product of olive oil

HT and DHPG were purified from alperujo, the main by-product of olive oil extraction, using a chromatographic fractionation resulting in compounds with 90–95% purity by weight (Fernández-Bolaños et al., 2013).

2.2. Preparation of apple cell wall material

Alcohol-insoluble solids (AIS) from apple fruits were made using the method of Renard (2005). Briefly, apples were peeled and cut into 2–3 pieces. The pieces were directly ground in a domestic blender in 70% ethanol and subsequently filtered through a nylon cloth. The resulting solid was ground and washed repeatedly with 70% ethanol until the filtrate had no color. Drying was performed by solvent exchange (96% ethanol and acetone), and then overnight in an oven at 40 °C.

2.3. Preparation of the apple cell wall-HT/DHPG complex

Approximately 500 mg of apple cell wall alcohol-insoluble solid (AIS) were added to 10 mL of 10 mg/mL HT or DHPG solutions (corresponding to an initial amount of 200 mg bioactive compound (BC)/g cell wall) or a mixture of 5 mg/mL of HT and 5 mg/mL of DHPG solution (100 mg of HT: 100 mg of DHPH/g cell wall). After overnight swelling, the samples were dried in the oven for 72 h at 60 °C. After drying, the cell wall was rehydrated with water and washed copiously with 70% ethanol to remove the free phenols. The cell wall/phenol complex was then separated by filtration. The content of free HT and DHPG was measured by HPLC and the bound phenols were calculated by the difference.

2.4. Determination of cell wall composition

After hydrolysis with trifluoracetic acid (TFA) (2N, 121 °C, and 1 h), the hemicellulosic sugar composition was obtained from the reduction and acetylation of the solubilized sugars, and the acetate of alditol formed by gas chromatography (GC) was measured (Englyst & Cummings, 1984). The chromatographic parameters used were developed by Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, and Fernández-Bolaños (2012). The phenyl-phenol method was used for the determination of uronic acid after hydrolysis with sulfuric acid (Blumenkrantz & Asboe-Hansen, 1973).

2.5. Gastrointestinal digestion in vitro with simulated gastric and intestinal fluids

HT/DHPG-bound cell wall, 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 37 °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 present 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 37 °C with agitation. Thereafter, the samples were filtered to separate the soluble fraction from the insoluble fraction, and the free HT and DHPG in the soluble fraction were quantified and the bound phenols were calculated by the difference.

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

2.6.1. Antiradical activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

Free radical-scavenging capacity was determined using the DPPH method as described by Rodríguez et al. (2005). The results were expressed as the average of the ratios of the slopes of the lines obtained for each sample, using the Trolox calculation for the wavelengths. The results were expressed in terms of Trolox equivalent antioxidant capacity in µmol Trolox/g of sample.

In the case of insoluble material, the antioxidant activity was evaluated as described by Fuentes-Alventosa et al. (2009) with slight modifications (Bermúdez-Oria et al., 2019).

2.6.2. Antiradical activity: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The ABTS method was carried out following the modification made by Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, and Fernández-Bolaños (2012). The results were expressed in terms of Trolox equivalent antioxidant capacity in µmol Trolox/g of sample.

2.6.3. Antioxidant activity: Oxygen radical absorbance capacity (ORAC) method

The antioxidant capacity was measured by the ORAC method following the protocol of Ou, Hampsch-Woodill, and Prior (2001) with the modifications of Bermúdez-Oria et al. (2019). Results were expressed as µmol Trolox equivalents/g of sample.

2.7. Hydrolysis of HT/DHPG linked with soluble dietary fiber and ultrafiltration

The enzymatic hydrolysis of the soluble fiber-phenol complex was performed by a mixture of 4 µg/mL of endo- and exo-polygalacturonase and pectin esterase (Novo Nordisk, Bagsvaerd, Denmark) according to Bermúdez-Oria et al. (2019).

After hydrolysis, the sample was ultrafiltered by an Amicon 8400 stirred cell (Millipore Corporation, Bedford, MA, USA) using a molecular-weight cut-off of 3000 and 1000 Da, washing each retained fraction with water until 300 mL of permeate remained. The three fractions obtained were: the retained fractions over 3000 Da, a fraction over 1000 Da, and the eluted fraction over 1000 Da. All of them were analyzed for antiradical activity by the DPPH, ABTS and ORAC methods.

2.8. Characterization of cell wall-phenol complex

UV Spectrometry. UV spectroscopy was performed using a Coulter DU 800 UV/visible spectrophotometer (Beckman, USA) in the range 200–400 nm.

FT-IR Spectrometry. FT-IR spectra of samples were obtained using a FT-IR Bomem MB-120 spectrophotometer (ABB, Canada) in the range 4000-350 cm^{-1} by the KBr method.

Differential Scanning Calorimetry and thermogravimetry analysis. Measurements were carried out with a thermal analyzer (Q20 DSC, TA Instruments, New Castle, DE). The samples were heated from 50 to 275 °C at a heating rate of 10 °C min^{-1} in a nitrogen atmosphere. The analysis was performed using a Q600SDT (TA Instruments, New Castle, DE), with a temperature range between 50 °C and 500 °C at a heating rate of 5 °C min^{-1}, in a nitrogen atmosphere. For the thermogravimetric analysis a Q600SDT (TA Instruments, New Castle, DE) was used. Approximately 10 mg of sample material were heated from 50 °C to 500 °C at a heating rate of 5 °C min^{-1}, in a nitrogen atmosphere.

2.8.1. ^{13}C CP/MAS NMR spectroscopy

For the molecular characterization, solid-state ^{13}C nuclear magnetic resonance (NMR) spectra were acquired, using a Bruker Avance HD 400 MHz spectrometer operating at a^{13}C frequency of 100.6 kHz and applying the cross-polarization magic angle spinning (CP/MAS) technique. Zirkonium rotors with KELF-caps with an outer diameter of 4 mm were spun in a triple resonance probe at the magic angle with a spinning speed of 14 kHz. A ramped ^{1}H- pulse was applied during the contact time of 1 ms. Using a pulse delay time of 2 s between 15,000 and 81,000 scans were accumulated to achieve adequate signal-to-noise ratios of the spectra.

3. Results and discussion

3.1. Preparation of the complex hydroxytyrosol (HT) and 3-4-dihydroxyphenylglycol (DHPG) and cell wall (dietary fiber) of apple

The influence of drying on the formation of a complex of HT/DHPG-bound to the cell wall of strawberries was investigated in a previous study (Bermúdez-Oria, Rodríguez-Gutiérrez, Fernández-Prior, Vioque, & Fernández-Bolaños, 2019; Renard, Watrelot, & Le Bourvellec, 2017). To confirm that such a complex formation occurs with apple cell walls as well, we assayed the homogenization of apple cell wall, as an alcohol-insoluble solid, in the presence of 10 mg/mL HT and DHPG solutions and a mixture of HT:DHPG of 5 mg/mL each for further drying. The content of free HT and DHPG was measured by HPLC and the bound phenols were calculated by the difference (Fig. 1). There was a higher retention of HT and DHPG by apple cell wall (52 and 72%) than previously observed for strawberry cell wall (40 and 47%), respectively. In the case of the HT:DHPG mixture, the retention was intermediate between HT and DHPG, at 61.5%. Drying enhanced the binding of apple cell wall with HT/DHPG, as occurred with strawberry cell wall. It has been 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 surface area of the cell wall (Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012).

When the HT/DHPG-bound cell wall (dietary fiber) was digested in vitro using simulated gastric fluid (pH 1.2, 0.1 M HCl solution, 2 h) with

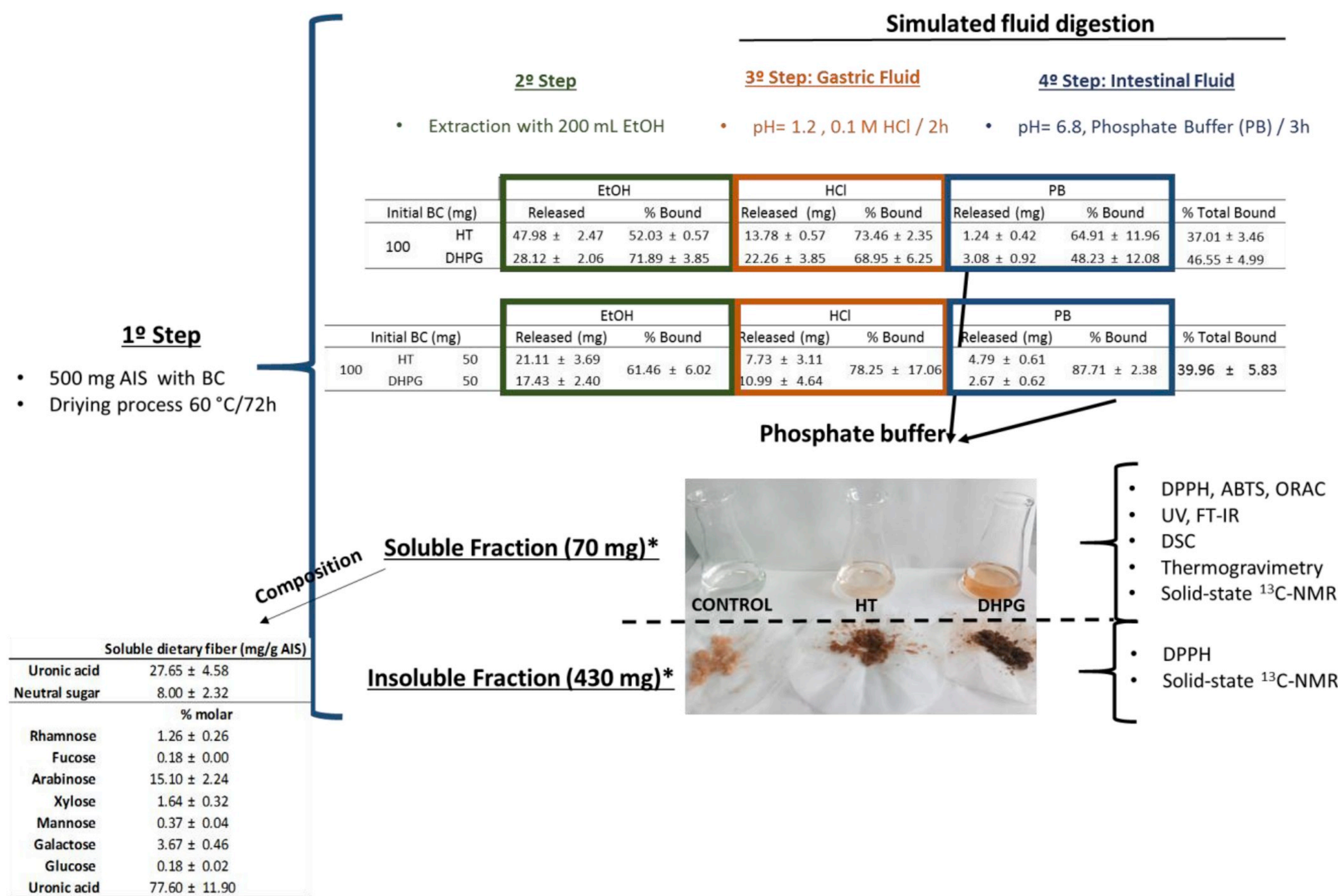

Fig. 1. Scheme of the in vitro simulated gastrointestinal digestion process of HT/DHPG or their mixture HT:DHPG bound to apple dietary fiber. Percentage of bioactive compounds (BC) bound to AIS during the drying process (oven-dried), after release with ethanol, and bound in each step of digestion simulation with respect to the bound BC. *Represents the average weight of the insoluble and soluble fractions obtained from the digestion of HT/DHPG-bound cell wall (n = 3 for HT and n = 3 for DHPG). The table lists the composition (mg/g AIS) of the HT/DHPG-soluble dietary fiber complex. Each value is the average of four replicates using two HT and two-DHPH-soluble fiber complex samples. Molar percentage of uronic acid and neutral sugar. The antioxidant used and characterization method are also indicated. BC: bioactive compound; PB: phosphate buffer; AIS: alcohol insoluble solid.

a subsequent pH change, and simulating intestinal fluid (pH 6.8 phosphate buffer, 3 h), the amount of HT and DHPG released was very high, in contrast to the result for strawberry cell wall, from which there was practically no release. In this case, the interactions between BC and the apple cell wall seemed much weaker once oven-dried and were released in gastric and intestinal conditions more easily to give a final result of 37 and 47% total retention of HT and DHPG, respectively, which coincides with the retention in the case of the strawberry cell wall (Bermúdez-Oria et al., 2019). The 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 brown color, which came from the initial compounds and was retained in both fractions (Fig. 1).

Based on the monosaccharide analysis of the soluble fraction and its uronic acid content (Fig. 1), the predominant component of the HT/DHPG-bound complex was an arabinose-rich pectin. This is in agreement with our previous reports of strong binding between HT and DHPG with pectinate beads (Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017) or with the soluble dietary fiber of strawberry (Bermúdez-Oria, Rodríguez-Gutiérrez, Rodríguez-Juan, González-Benjumea, & Fernández-Bolaños, 2019). Therefore, soluble fibers, such as pectin, with associated antioxidant compounds, could be of interest in the food industry due to their health benefits and potential technological applications (Dranca & Oroian, 2018; Naqash, Masoodi, Rather, Wani, & Gani, 2017; Park & Yoon, 2015).

3.2. Physical and chemical characterization of the HT/DHPG complex with apple dietary fiber

3.2.1. UV absorption spectrum

Since there were no free phenolic compounds present, the UV absorption of HT/DHPG was clearly affected by the dietary fiber interaction. In the absorption spectra of the complex of HT and DHPG in the soluble fraction (Fig. 2a), a small band was found at 280 nm (π-π* transition of the phenolic group), and was slightly more pronounced in the case of DHPG, but not present in the control spectrum (soluble fraction obtained from apple cell wall with no addition of BC). This slight increase in signal intensity indicated that the phenols had been incorporated into the soluble fraction, presumably to soluble polysaccharide constituents of apple dietary fiber, likely through of combination of hydrogen bonding and hydrophobic interaction (Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017; Bermúdez-Oria, Rodríguez-Gutiérrez, Rodríguez-Juan, González-Benjumea, & Fernández-Bolaños, 2018).

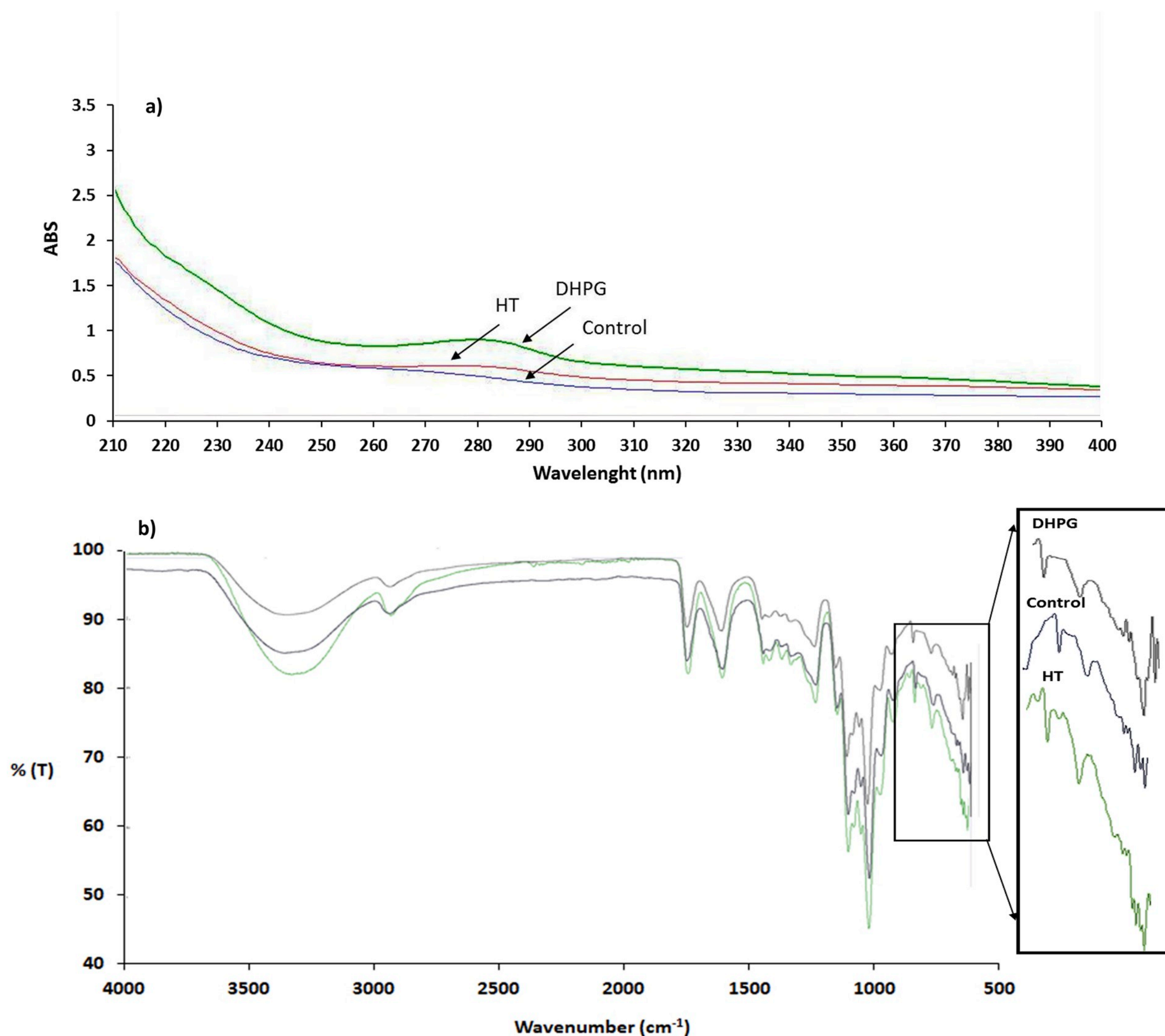

Fig. 2. Ultraviolet–visible (UV) (a) and Fourier transform infrared (FT-IR) (b) spectra of HT/DHPG–soluble dietary fiber complex and comparison with control (soluble dietary fiber with no BC).

3.2.2. FT-IR analysis

The FT-IR spectra of the soluble fraction confirmed the characteristic absorption bands of pectin at 3000-3800 cm^{-1} and 2900 cm^{-1}, which were attributed to OH- and C–H stretching vibrations, respectively (Fig. 2b). The peaks at 1100, 1700, and 1745-1760 cm^{-1} were assigned to –C-O-C-, C=O stretching, and ester carbonyl stretching vibrations, respectively. No significant differences were observed in the corresponding spectra of the complex of HT/DHPG with the soluble fraction compared to the control FT-IR spectrum. A total disappearance of the characteristic bands of phenolic compounds was observed in the complex spectra, which may be attributed to the complexation and, partially, to the low loading content of BC in relation to the pectin macromolecule content. Only a slight difference in the zone of 600–670 cm^{-1}, associated with C–H bonds of flexion out-of plane of the aromatic compounds, was observed. This is a reliable indicator of the incorporation of HT/DHPG into the complex, although this result did not indicate the type of binding involved.

3.2.3. Thermogravimetric (TGA) and differential scanning calorimetry (DSC) analysis

TGA analysis was performed on the control and the two complexes containing BC samples. TGA plots showed three regions at 25–100 °C, 150–350 °C and 350–500 °C (Fig. 3a), as reported by other authors for pectin (Combo et al., 2013). The first region (25–100 °C) was attributed to the water loss during the temperature rise, and the second region (150–350 °C) corresponded to a rapid loss in mass due to the polysaccharide decomposition, with complete decomposition of the pectin at 240 °C, which could be attributed to the splitting of the saccharide rings. The third region (350–500 °C), the only region that showed differences between the control and HT/DHPG complex samples, showed a slow loss in mass after the volatilization and thermal decomposition of other components with the subsequent formation of solid char and various gaseous products. Decomposition of the BC linked to the soluble fraction was observed above 400 °C. The thermal stability of pectin was practically unaltered by the introduction of phenolic compounds and only a very slight loss in weight occurred in both phenols from 400 to 500 °C.

The control's DSC thermogram showed an endothermic peak at around 215 °C which corresponded to the melting temperature and was not observed in the DSC thermogram of HT and DHPG complexes in the scanned range of the assay conditions (Fig. 3b). Furthermore, the DSC plot revealed a slight exothermic reaction in the control and HT/DHPG samples, corresponding to the degradation of the polysaccharide, which began at around 230 °C (Fig. 3c), with a maximum at 260 °C and a slight shift at a higher temperature was observed for the HT-bound complex. In addition, the intensity of the heat flow observed in the HT and DHPG soluble complexes was several times higher than for the control, revealing significant changes due to the presence of BC at these high temperatures.

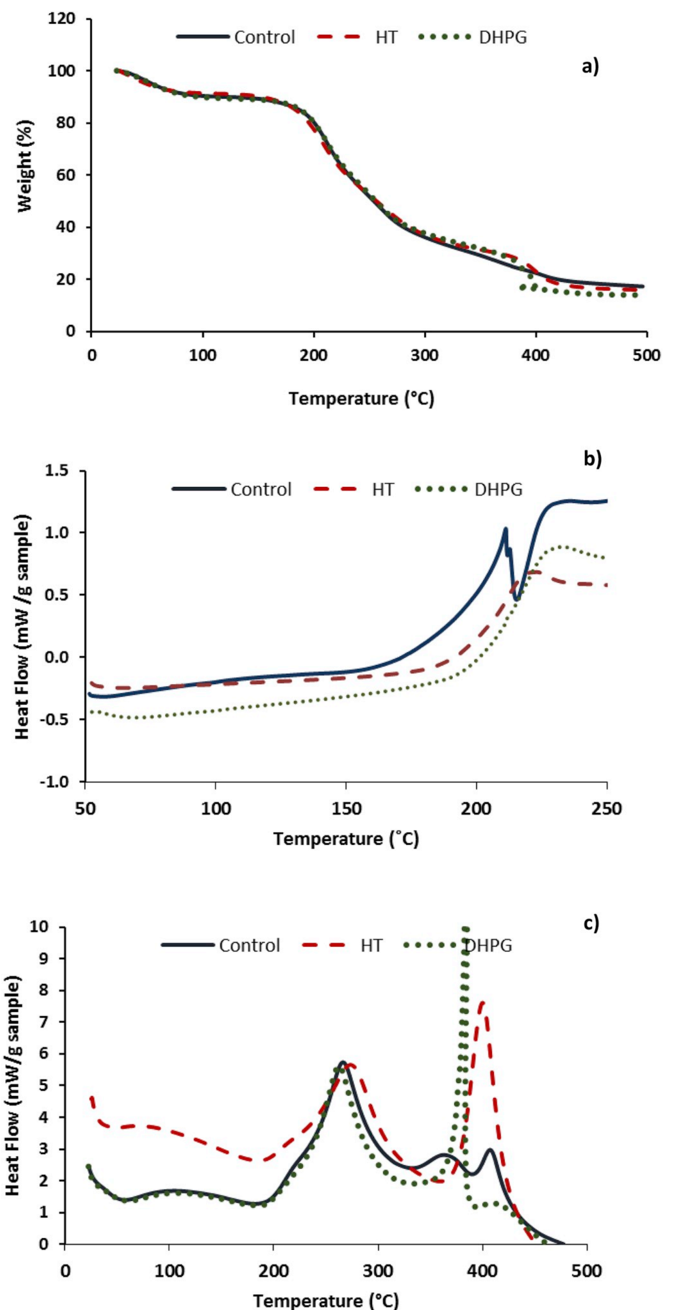

Fig. 3. Thermogravimetry analysis (TGA) (a) and differential scanning calorimetry (DSC) (b and c) thermograms of HT/DHPG–soluble dietary fiber complex and comparison with control (soluble dietary fiber with no BC).

3.2.4. Solid-state ^{13}C NMR spectroscopy

The solid-state ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance CP/MAS NMR technique was used to investigate interactions among the added phenols, HT and DHPG, and the soluble and insoluble dietary fiber of apple. For the dry soluble dietary fraction (Fig. 4), the resonance lines between 60 and 100 ppm were caused by the C1–C5 of the pectin backbone (Synytsya, Copikova, & Brus, 2003; Marcon, Carneiro, Wosiacki, Beleski-Carneiro, & Petkowicz, 2005; Ng et al., 2014). The peaks at 174 ppm and 170 ppm can be attributed to esterified and non-esterified carboxyl C (C6) of esterified and non-esterified galacturonic acid, respectively. The signal at 53 ppm corresponds to metoxyl C at C-6 (Fig. 4) (Synytsya, Copíková, & Brus, 2003; Ng et al., 2014). The additional peaks detected between 110 ppm and 160 ppm in the NMR spectra of freeze-dried complexes of HT/DHPG-soluble dietary fiber could be assigned to carbons in the aromatic ring. The signals between 130 and 145 ppm, present in the spectra of the antioxidants HT and DHPG (Fig. 4, assigned data), suggested that phenols were bound to the soluble dietary fiber. In addition, the signals between 31 and 37 ppm appeared more pronounced in samples with HT and DHPG incorporated. These could be assigned to C-2 of the ethyl chain of HT (38.4 ppp) or DHPG (68.7 ppm), which showed a downward shift probably due to a shielding effect produced by the polysaccharides, as occurs in the modification of C2 resonance of derived hydroxytyrosyl alkyl ethers (Madrona et al., 2009) and the alkylnitrohydroxytyrosyl ethers (Gallardo et al., 2016; Madrona et al., 2009). Since no major changes in the chemical shifts of the pectin signals due to the addition of HT/DHPG were observed, it may be concluded that complexation caused no major conformational changes in polysaccharides, although probably some chemical shift changes in the antioxidants.

The ^{13}C CP/MAS NMR spectra of the complex of HT and DHPG with

Soluble fiber

Sample		Chemical shift (ppm)
Pectin	C6 (COOCH3)	174.8
	C6 (COO-)	170.5
	C1	100.4
	C4	79.3
	C2.3.5	68.0
	O-Me	52.9
HT/DHPG	C-aromatic	38.4
		144.8
		143.2

Insoluble fiber

Sample		Chemical shift (ppm)
Pectin	C6 (COO-)	170.6
Cellulose	C1	104.4
Pectin	C4	83
Pectin	C2.3.5	71.5
Cellulose	C6	61.7
Pectin	O-Me	52.9
HT/DHPG	C-aromatic	38.4
		144.8
		143.2

Fig. 4. Solid-state ^{13}C NMR spectra of HT/DHPG–soluble and insoluble dietary fiber complex and comparison with a soluble and insoluble dietary fiber with no BC added (Control). Peak assignments are based on previously reported data.* ^{13}C NMR chemical Shifts (ppm) for solution of HT (125.8 MHz, DMSO-d_6, 303K) δ: 144.8 (C-5), 143.2 (C-6), 130.1 (C-3), 119.3 (C-8), 116.2 (C-4), 115.3 (C-7), 62.5 (C-1), 38.4 (C-2) (Gallardo et al., 2016), and for solution of DHPG (151 MHz, CD3OD) δ: 146.1 (C-5), 145.7 (C-4), 134.7 (C-3), 119 (C-8), 116.1 (C-4), 114.6 (C-7), 75.7 (C-1), 68.7 (C-2) (Kalampaliki, Giannouli, Skaltsounis, & Kostakis, 2019).

the insoluble fraction did not show significant differences compared to the spectrum of the insoluble fraction of the control (Fig. 4), a result which coincided with the findings of Phan et al. (2017) for apple cell walls. In our case, the spectrum was dominated by signals from cellulose, and other neutral polysaccharides, as well as pectic polysaccharides (Fig. 4). However, the lowest intensity signal was observed in the chemical shift region assigned to carboxyl C, indicating minor contributions of galacturonic acid, when compared to soluble dietary fiber. Although the spectrum of the insoluble fraction showed some weak intensity in the chemical shift region of phenol C, new resonance lines and a slight increase in signal intensity within this region were observed for the spectra of the complex insoluble material, allowing for the conclusion that complexed HT and DHPG were present (Fig. 4).

3.3. Antioxidant activity of the insoluble and soluble fractions in vitro

The insoluble apple fiber fraction showed very little radical scavenging activity in the assay conditions (Fig. 5); whereas the fiber mixed with BC (200 mg BC/g cell wall) showed certain antioxidant activity. This activity was highest for linked-DHPG, followed by the mixture HT:DHPG (100:100 mg/g cell wall), and lowest for HT, which required more fiber (up to 15 mg of insoluble fraction) to obtain an appreciable

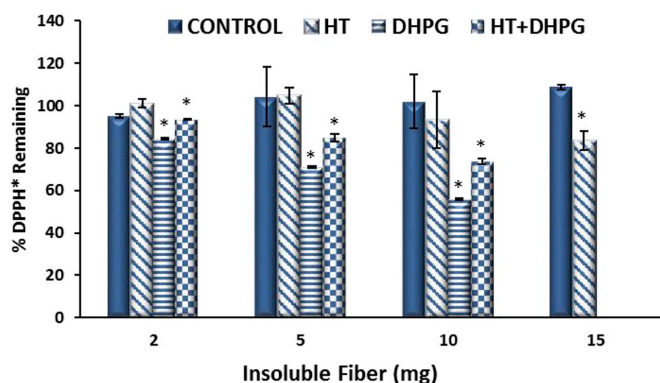

Fig. 5. DPPH scavenging activity of insoluble dietary fiber bound to HT (2, 5, 10 and 15 mg) and DHPG or the mixture HT:DHPG (2, 5 and 10 mg), expressed as percent DPPH remaining. Comparison with control (insoluble dietary fiber with no BC). Each bar represents the average value of three replicates. Error bars represent standard deviation (n = 3). * indicate statistical significance with respect to control fiber ($p < 0.05$).

de-coloration of the DPPH free radical. These results with apple cell wall were very different from those obtained in our previous work with insoluble strawberry fiber, in which there was no difference between the samples with HT- or DHPG-added and the control, although the control did present certain antioxidant activity (Bermúdez-Oria et al., 2019). We concluded that negligible amounts of polyphenols were associated with the insoluble dietary fiber of apple, although the addition of the potent phenolic antioxidants HT and DHPG or their mixture led to the formation of a strong complex with the insoluble material of apple cell wall.

The antioxidant activity of the *soluble cell wall* fraction was measured by three different methods (DPPH, ABTS and ORAC) with the addition of 200 mg of HT and DHPG, and their mixture (100:100 mg of HT:DHPG) added to a 1.0 g cell wall (Fig. 6a and b). In the case of HT-bound soluble dietary fiber or the soluble dietary fiber control, no activity was found using the ABTS and DPPH assays, although activity was observed using the ORAC assay, with significant differences between HT and the control. In contrast, the DHPG-bound soluble fraction and the HT:DHPG mixture, free radical scavenging activity was observed in all three methods. These results were in agreement with previous reports on DHPG-pectin complex formation via encapsulation (Bermúdez-Oria et al., 2017) and DHPG-bound soluble dietary fiber complex from strawberry cell wall (Bermúdez-Oria et al., 2019), which confirmed that the DHPG-linked complexes maintained more antioxidant activity than the HT-linked ones. An additional –OH group of DHPG with respect to HT may allow for greater availability of the catechol group, which is responsible for the antioxidant activity of the BC (Spizzirri et al., 2009). Curiously, the soluble fraction sample with the mixture of HT and DHPG (100:100 mg/g cell wall) showed promising antiradical activity (122 and 403 μmol of Trolox/g sample for DPPH and ORAC assays, respectively), whereas the sum of activity of HT and DHPG for DPPH and ORAC were only 47 and 357 μmol of the Trolox/g sample, respectively, using twice the amount of BC for the formation of the complex. These results suggest a possible synergistic effect between HT and DHPG.

Therefore, the two potent phenolic antioxidants HT and DHPG form a strong complex with the insoluble and soluble polysaccharides of apple cell wall and impart their antioxidant properties to the complex.

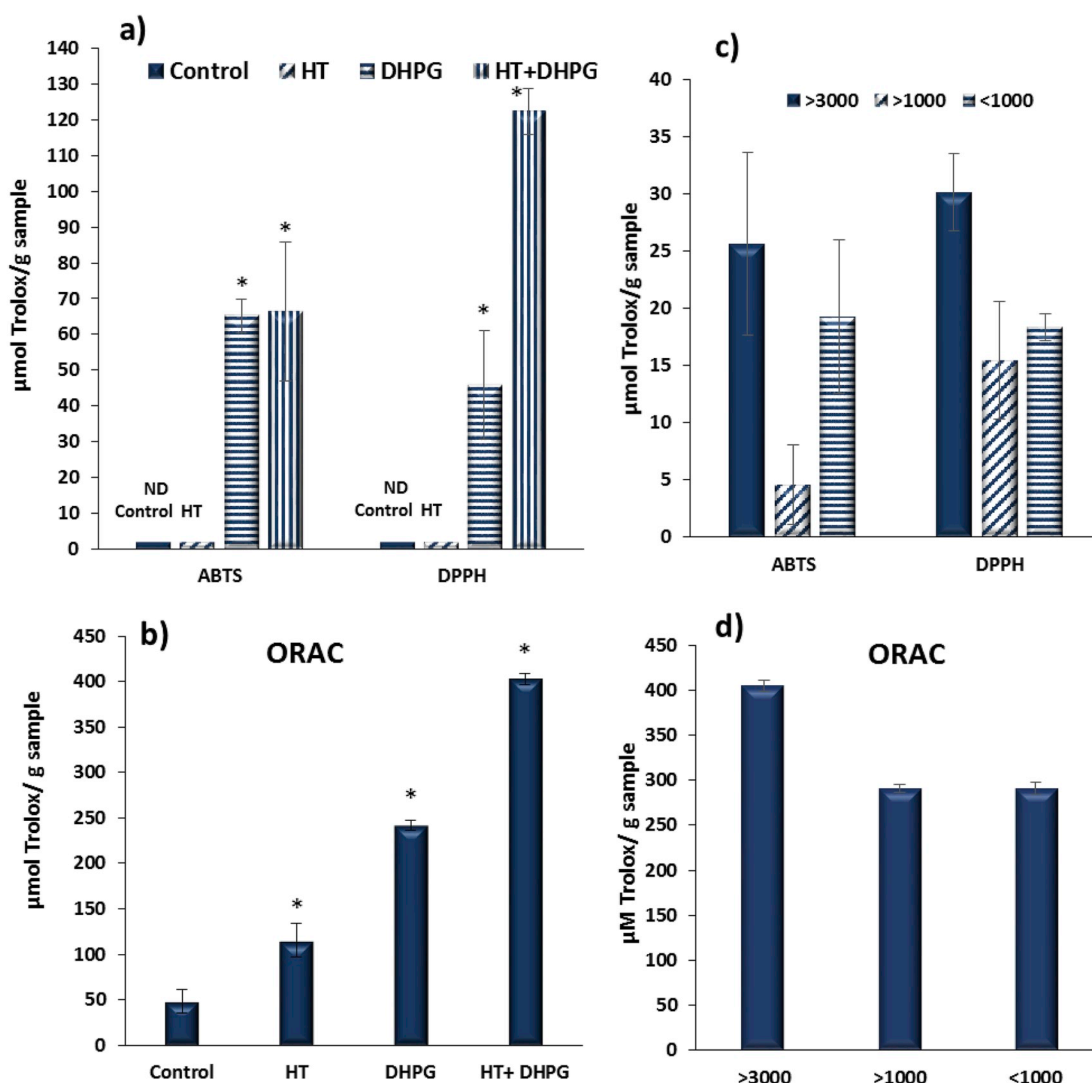

Fig. 6. ABTS and DPPH scavenging activity (a) and oxygen radical capacity (ORAC) (b) of the HT/DHPG- and their mixture (HT:DHPG)-soluble dietary fiber complex. The three assays are expressed as μmol Trolox/g sample. Antiradical capacity measured by ABTS and DPPH (c) and by ORAC (d) of the pectin fragments obtained from the HT:DHPG-soluble dietary fiber complex treated with a mixture of pectinolytic enzymes and recovered by a sequential ultra-filtration through 3000 and 1000 Da molecular weight cut off-membranes. Error bars represent standard deviation (n = 3). * indicate statistical significance with respect to control fiber ($p < 0.05$).

3.4. Release of pectin fragments with antioxidant activity from the HT: DHPG-soluble dietary fiber complex

In a previous study, when the size of the polysaccharides in the complex with strawberry cell wall was reduced, the antiradical activity was restored (Bermúdez-Oria et al., 2019). Therefore, a mixture of pectinolytic enzymes was added to reduce the molecular size of the HT: DHPG mixture-soluble dietary fiber complex to investigate its effect on the complex's antiradical activity. After enzymatic digestion the solubilized fraction was subjected to sequential ultra-filtration through a 3000 and 1000 Da molecular weight cut-off membrane and the corresponding eluted and retained fractions were analyzed for antiradical activity by DPPH, ABTS and ORAC assays (Fig. 6c and d). With the ORAC assay, the antioxidant activity of the fraction >3000 Da (405 μmol of Trolox/g sample) was similar to the activity of the initial fraction of the HT:DHPG mixture linked to soluble dietary fiber (Fig. 6d). In contrast, the antioxidant activity was high in the fractions of smaller molecular size, with values of 290 and 291 μmol of Trolox/g for the <1000 Da and 3000-1000 Da fractions, respectively. The increase in antioxidant activity in complexes of smaller molecular size indicated that although the antiradical activities of HT and DHPG seem to be directly or indirectly affected by their interaction with polysaccharides, mostly pectin, their antiradical activity is partially restored when the size of the polysaccharides is reduced.

The reduction in size of the oligomers or the hydrolytic process of soluble dietary fiber or pectin by colonic bacterial enzymes could change the outcomes obtained in this study; however, this result suggests that the hydrolytic process releases oligomers with potential antioxidant activity from this complex. This activity could help to prevent certain kinds of degenerative or chronic diseases such as colon cancer or inflammatory bowel disease (IBD) (Saura-Calixto, 2012), although future research is needed to verify this hypothesis.

4. Conclusions

This study confirmed the formation of phenol-polysaccharide complexes via drying. We provided experimental evidence that the complexation of HT/DHPG with soluble and insoluble apple cell wall retained antioxidant activity in vitro after exposure to simulated gastrointestinal fluid and may be protected from absorption during gastrointestinal transit to reach the colon. Enzymatic treatment and the reduction in size of the polysaccharides in the soluble dietary fiber complex, as a simulation of hydrolysis by colonic microflora, released oligomers with antioxidant activity and partially restored the activity of HT/DHPG affected by the interaction with components of the cell wall. As such, the complexes formed between HT and DHPG, which are two natural phenols present in olive fruit, with the cell wall of apple fruits could be optimized as a novel bioactive ingredient in functional food formulations to promote intestinal health.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Alejandra Bermúdez-Oria: Investigation, Methodology, Writing - original draft. **Guillermo Rodríguez-Gutiérrez:** Formal analysis. **África Fernández-Prior:** Resources, Validation, Investigation. **Heike Knicker:** Supervision, Visualization. **Juan Fernández-Bolaños:** Conceptualization, Writing - review & editing.

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