Title: Bioaccessibility of rutin, caffeic acid and rosmarinic acid: influence of the *in vitro* gastrointestinal digestion models.

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ABSTRACT

The bioaccessibility and antioxidant activity of rutin, caffeic acid and rosmarinic acid were evaluated using three *in vitro* gastrointestinal digestion models: filtration, centrifugation and dialysis. At intestinal level, a significant degradation of all compounds was observed when results were expressed on concentration basis (mg/mg lyophilized sample), mainly due to the dilution effect that occurs during digestion. However, when results were expressed as absolute amounts (total mg in the digested fraction), this degradation was much lower, or even absent in the case of rutin. Moreover, bioaccessibility (in terms of total mg) was higher in filtration and centrifugation than in the dialysis method. A significant reduction of antioxidant activity was observed after intestinal digestion of the three standards, regardless of the method used. In conclusion, the methodology and units used to report results are two critical parameters to take into account in bioaccessibility studies.

KEYWORDS: phenolic compounds; bioaccessibility; dialysis; centrifugation; filtration.

Chemical compounds studied in this article:

Rutin (PubChem CID: 5280805); Caffeic acid (PubChem CID: 689043); Rosmarinic acid (PubChem CID: 5281792)

1. Introduction

Phenolic compounds are an extensive family of secondary plant metabolites with multiple biological properties. Several epidemiological studies have shown that dietary phenolic compounds play a role in the prevention of cancer, and cardiovascular and neurodegenerative diseases, among others (Del Rio et al., 2013; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). In order to exert their beneficial effects, phenolic compounds must be available to some extent in the target tissue, and this is dependent on their absorption in the gut (Saura-Calixto, Serrano, & Goñi, 2007).

In vitro digestion models are widely used in food and nutritional sciences for predicting compounds bioaccessibility due to several advantages in respect to the *in vivo* models, since they are relatively inexpensive and simple, more rapid, do not present ethical restrictions, conditions can be controlled, sampling is easy and results are reproducible (Minekus et al., 2014). Furthermore, the evaluation of bioaccessibility using this type of models is well correlated with the data obtained in animal and human studies (Biehler & Bohn, 2010). Bioaccessibility has been defined as the amount of an ingested nutrient that is released from a food matrix and is available for intestinal absorption (Parada & Aguilera, 2007). In the case of phenolic compound stability, different food matrices and vegetable extracts have been subjected to gastrointestinal digestion, allowing the assessment of their bioaccessibility. Nowadays, there are different ways of simulating the bioaccessible fraction of foods at intestinal level. The easiest approach is to analyse the resulting content of the entire intestinal fraction, just by its filtration to separate the soluble material (fraction available for uptake) (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007). In addition, dialysis and centrifugation are two common techniques that have been also used for simulating the bioaccessible fraction of food and extracts (Etcheverry, Grusak, & Fleige, 2012). In the dialysis model, the dialyzable fraction represents the sample that goes through the semi-

permeable membrane and is available for absorption; meanwhile the fraction outside the dialysis membrane represents the non-absorbable sample. In the solubility model, the intestinal sample is centrifuged to obtain a supernatant (soluble components that could be potentially absorbed) and a precipitate (unabsorbed compounds). Advantages and disadvantages of each model have been described. Separation by centrifugation or filtration, followed by analysis of soluble components has been reported as a good estimate of compounds available for transport across the intestinal epithelium (Bermúdez-Soto et al., 2007; Pinacho, Cavero, Astiasarán, Ansorena, & Calvo, 2015; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). In the case of dialysis, data should be carefully studied since parameters such as molecule dimensions, polymerization degree and presence of sugar in the molecule, or even the membrane washing procedure may modify the amount of sample able to permeate through the membrane (Bermúdez-Soto et al, 2007; Chiang, Chen, Jeng, Lin, & Sung, 2014). However, when undigested compounds form colloidal dispersions, dialysis may be the better choice since centrifugation will only separate the insoluble undigested material with sufficient density (Minekus et al., 2014). Moreover, dialysis could be a useful tool for coupling the dialyzable fraction with cell lines without further purification steps (Bouayed, Hoffmann, & Bohn, 2011).

Recent literature reports a diversity of studies where the effects of *in vitro* digestion on dietary polyphenols has been studied by using the three above-mentioned techniques: filtration (Chen et al., 2016; Pinacho et al., 2015), dialysis membrane (Carbonell-Capella, Buniowska, Esteve, & Frígola, 2015; Mosele, Macià, Romero, & Motilva, 2016), and centrifugation (He et al., 2016; Pineda-Vadillo et al., 2016). These different methods gave rise to different conclusions about the bioaccessibility of phenolic compounds.

Finally, another relevant methodological consideration is worthy to be mentioned. Although the stability of pure compounds may be not representative of their stability within a food

matrix (Bermúdez-Soto et al., 2007; Siracusa et al., 2011), working with standards allows studying digestive stability in a simplified way, avoiding interferences with other factors. The aim of this work was to determine the bioaccessibility of different standard phenolic compounds after an *in vitro* gastrointestinal digestion comparing different digestion models (filtration, centrifugation and dialysis membrane). In addition, evaluation of the evolution of the antioxidant activity during the digestion process was also performed.

2. Materials and methods

2.1. Materials

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97%), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), rutin, caffeic acid, rosmarinic acid, dialysis cellulose membrane (molecular weight cut-off of 14000), alpha-amylase from human saliva (A1031, 852 units/mg protein), pepsin from porcine gastric mucosa (P7000, 674 units/mg protein), pancreatin from porcine pancreas (P1750, 4 × United States Pharmacopeia specifications) and bile extract (B8631) were purchased from Sigma-Aldrich (Steinheim, Germany). All the solvents employed, analytical and HPLC grades were purchased from Panreac (Barcelona, Spain).

2.2. *In vitro* digestion

Three standard compounds were selected for the digestion model systems carried out in this work: rutin, caffeic acid and rosmarinic acid (Fig. 1). Rutin (quercetin-3-rutinoside) is a flavonoid glycoside present in many vegetables, fruits and plants; caffeic acid is the most abundant phenolic acid widely found in coffee beans and fruits; and rosmarinic acid is found in different aromatic herbs from the *Lamiaceae* family. They were chosen due to their well-recognized antioxidant capacity.

The *in vitro* digestion model for each standard compound included three steps (oral, gastric and intestinal digestion) (Fig. 2) and it was based on the procedure described by Pinacho et

al. (2015) with some modifications. The amount of standard subjected to digestion was chosen taking into account the minimum average content of phenolic compounds in one serving of vegetable foods (Manach et al., 2004). 50 mg of standard were dissolved in 20 mL of distilled water, the mixture was then placed in a Falcon tube and warmed at 37 ºC (water bath) to initiate the simulated oral digestion. Then, 12.5 µL of alpha-amylase (1.3 mg/mL solution in 1 mM $CaCl_2$) was added. The pH was adjusted to 6.5 with 1 M $NaHCO_3$ and the sample was incubated in a water bath at 37 ºC for 2 min with magnetic stirring to complete the oral step. For the gastric digestion, on the same tube, 16.5 µL of pepsin (160 mg/mL solution in 0.1 M HCl) was added, pH was adjusted to 2.5 with 3M HCl and the incubation time was 2 h at 37 ºC.

In the simulated intestinal phase, three independent procedures (A, B and C) were carried out (Fig. 2). In digestion A, 125 µL of pancreatin-bile extract (4 mg of pancreatin + 25 mg of bile extract mL/solution in 0.1 M $NaHCO_3$) was added to the gastric mixture. The digestion continued for another 2 h at 37 ºC after adjusting the pH to 7.5 with 1 M or 0.1 M $NaHCO_3$. At this step, the sample is called intestinal fraction (IF) and was analysed as a whole. In the digestion B, after the intestinal digestion, samples were centrifuged (51070 g, 4 ºC, 60 min) to obtain the soluble fraction (SF) and the residual fraction (RF). In the digestion C, the intestinal phase was performed with a dialysis membrane. A segment of a dialysis membrane (5-8 cm), previously hydrated with deionized water for 3 hours, was filled bubble-free with the amount of $NaHCO_3$ (1 M or 0.1 M) required to reach pH 7.5 in the intestinal sample (postgastric mixture with pancreatin-bile extract). In the case of rutin, 1 mL 1 M $NaHCO_3$ was added, 3 mL for caffeic acid and 2 mL 0.1 M $NaHCO_3$ plus 0.3 mL 1 M $NaHCO_3$ for rosmarinic acid. As it can be seen, the different acid-base properties of the three standards caused a different response to the $NaHCO_3$ addition. The dialysis bag was placed inside the

digestion flask for 2 h at 37 ºC. Afterwards, the solution outside the dialysis membrane (OUT fraction) and the content of the dialysis membrane (IN fraction) were collected.

Each standard was digested in duplicate for each procedure (A, B and C). Individual tubes for oral, gastric and intestinal digestion were used to ensure homogeneity in the sample in each step. The stock solutions for all enzymes were freshly prepared. Samples obtained from each digestion step were lyophilized (Cryodos-50, Telstar, Barcelona, Spain), accurately weighed and then dissolved in methanol/water/formic acid (79.9/20/0.1; v/v/v) (González-Barrio, Borges, Mullen, & Crozier, 2010) for determining the remaining amount of standard after each digestion phase and also for the antioxidant activity analysis. Different dilutions were prepared to perform the tests.

2.3. DPPH assay

DPPH assay was performed as described by García-Herreros, García-Iñiguez, Astiasarán and Ansorena (2010) with slight modifications. Briefly, a DPPH solution (0.04 mg/mL) was prepared in methanol and diluted to obtain an absorbance of 0.8 ± 0.02 at 516 nm. Then, 1 mL of diluted sample was mixed with 1 mL of DPPH solution. Two controls were prepared: control 1 (1 mL of the sample solvent + 1 mL of DPPH) and control 2 (1 mL of sample solvent + 1 mL of methanol). After 30 min in the dark at room temperature, 200 µL of each solution (sample, control 1 and control 2) were transferred into a 96 well micro-plate and the absorbance was measured at 516 nm (FLUOStar Omega spectrofluorometric analyser, BMG Labtechnologies, Offenburg, Germany). The average absorbance for each sample was then calculated as the percentage of inhibition (% I) calculated according to the equation (1):

$$\% I = [[(Abs_{control\ 1} - Abs_{Control\ 2}) - (Abs_{sample} - Abs_{control\ 2})]/(Abs_{control\ 1} - Abs_{control\ 2})] \times 100 \quad (1)$$

Where $Abs_{control\ 1}$, $Abs_{control\ 2}$ and Abs_{sample} were the absorbance after 30 min incubation of the control 1, control 2 and sample, respectively. The percentage of inhibition versus the concentration of samples was then plotted. A calibration curve with Trolox was used for

obtaining the antioxidant capacity. Results were expressed as μg Trolox/mg lyophilized digested sample. Four measurements were done per replicate (n=8).

2.4. ABTS assay

ABTS assay was carried out following the procedure described by García-Herreros et al. (2010). Briefly, $ABTS^{\cdot+}$ chromogenic radical was generated by a chemical reaction mixing an aqueous solution of ABTS with potassium persulphate (140 mM). The mixture was kept in the dark for12 hours and its absorbance was adjusted with solvent sample to 0.7 ± 0.02 at 741 nm. In a 96 well micro-plate, $ABTS^{\cdot+}$ working solution (182 μL) was allowed to react with 18 μL of each dilution of the sample or control (sample solvent) for 6 min. The absorbance was then measured at 741 nm using a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). The decrease in absorbance was recoded as percentage of inhibition (% I), according to the equation (2):

$$\% I = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (2)$$

Where $Abs_{control}$ was the absorbance of the control and Abs_{sample} was the absorbance of the sample. The percentage of inhibition versus the concentration of the samples was plotted. A calibration curve with Trolox was used for calculating the antioxidant capacity. Results were expressed as μg Trolox/mg lyophilized digested sample. Four measurements were done per replicate (n=8).

2.5. High-performance liquid chromatography (HPLC)

The amount of phenolic standard compounds present after each digestion step and after the three different intestinal digestion conditions tested (A, B or C) were determined by HPLC. 20 μL of sample properly diluted and filtered through a 0.45 μm membrane filter were injected in a HPLC unit model 1200 (Agilent Technologies, Palo Alto, CA, USA) coupled to a diode array detector (DAD). Chromatographic separation was performed using a Kinetex 5

µm RP 250 x 4.6 mm reversed phase column (Phenomenex, Macclesfield, UK). The mobile phase was acetonitrile (solvent A) and 0.1% aqueous formic acid (solvent B). Gradient elution was 5-40% of solvent A over 55 min and finally increased to 90% for 10 min as reported by Juániz et al. (2016). The flow rate was 1 mL/min. Detection was performed by DAD at 360 nm for rutin and at 325 nm for caffeic acid and rosmarinic acid. A calibration curve was obtained from each phenolic compound to quantify the amount of each standard in the samples. Standards were diluted in methanol/water/formic acid (79.9/20/0.1; v/v/v), and concentrations ranged from 0.005 to 0.3 mg/mL for rutin, from 0.01 to 0.44 mg/mL for caffeic acid, and from 0.02 to 0.3 mg/mL for rosmarinic acid. The results were expressed both as concentration (w/w) in mg/mg lyophilized digested sample and as total mg of standard in the digested sample. Total mg of standard in the digested fraction was obtained multiplying the concentration determined by HPLC (mg/mg lyophilized digested sample) by the lyophilized sample weight. These data were used to calculate the bioaccessibility. All samples were injected in duplicate.

2.6. Bioaccessibility

The bioaccessibility (amount of compound available for absorption) was determined in the three intestinal methodologies assayed: filtration, centrifugation and dialysis that resulted in obtaining the IF (intestinal fraction), the SF (soluble fraction) and the IN fraction, respectively. In order to compare the bioaccessibility results using data expressed as A) total mg in the digested fraction or B) concentration (mg/mg lyophilized digested sample), two formulas were used:

A) Bioaccessibility (%) = (total mg of standard in the digested fraction/ initial amount of standard) x 100 (3)

B) Bioaccessibility (%) = (concentration of standard in the digested fraction/ initial concentration of standard) x 100 (4)

IF, SF and IN samples were considered as the bioaccessible fractions and the RF and OUT fraction as the non-absorbable fractions (D'Antuono, Garbetta, Linsalata, Minervini, & Cardinali, 2015; McDougall, Dobson, Smith, Blake, & Stewart, 2005; Pinacho et al., 2015).

2.7. Statistical analysis

Mean and standard deviation of data obtained from each replicate of digestion were calculated. One-way analysis of variance (ANOVA) followed by Scheffé *post hoc* test ($p < 0.05$) was applied to evaluate the statistically significant differences of the samples along the digestion process. Pearson correlation coefficients between DPPH and ABTS and between antioxidant activity and amounts of phenolic compounds detected by HPLC were calculated. All statistical analyses were performed using Stata12 (StataCorp LP, Texas, U.S.A.).

3. Results and discussion

Compound stability

The effect of different *in vitro* gastrointestinal digestion methods on the stability and bioaccessibility of three different phenolic compounds was evaluated: a flavonol (rutin) and two hydroxycinnamic acids (caffeic acid and rosmarinic acid).

Table 1 shows the evolution of the amount of the three compounds (total mg of standard) during the different digestion steps. No remarkable differences were observed between the initial amount subjected to digestion and the amounts recovered during the oral and gastric steps, showing that these processes hardly altered the stability of the three phenolic compounds studied. These results are in agreement with previous studies where compounds present in foods (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martin-Belloso, 2013) , plant extracts (Pinacho et al., 2015) and in pure phenolic compounds (Bermúdez-Soto et al.,

2007; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010), such as phenolic acids and flavonols, demonstrated their stability under gastric conditions. Table 1 also shows the evolution of the three compounds expressed as mg/mg lyophilized digested sample. In this case, after the oral and gastric steps, the concentration of caffeic and rosmarinic acids decreased significantly ($p < 0.05$) although the one of rutin did not. This decrease could be due to the $NaHCO_3$ used to adjust the pH in the oral digestion of both phenolic acids. In these cases, the lyophilized sample, in addition to the remaining phenolic standard, contained the $NaHCO_3$, leading to a certain "dilution effect" or a lower proportion of the standard in the analysed sample. It means that, per gram of lyophilized sample, lower amount of standard is found. Indeed, this effect was clearly shown when the results were calculated as % of the initial sample. Using absolute amounts of standards in the digested samples, rutin and caffeic acid showed recoveries around 100% and rosmarinic acid around 85-92%. However, when the results were referred to mg/mg lyophilized digested sample, the remaining % of sample decreased to 75% and 78% (oral and gastric, respectively) in the case of caffeic acid, and 67% and 68% (oral and gastric, respectively) for rosmarinic acid, maintaining the 100% in the case of rutin.

Even though some studies have also described significant losses of some phenolic compounds during salivary and gastric digestion (Kamiloglu et al., 2016; Siracusa et al., 2011; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004), most phenolic compounds remain stable. In fact, the oral step lasts a few minutes, and polyphenol degradation is usually a time-depending process. Moreover, it has also been described that acid pH during the gastric step protects polyphenols against degradation (Pineda-Vadillo et al., 2016).

Our results point to a higher compound degradation at intestinal level. Furthermore, some differences were found depending on the methodology (A, B or C) used. For method A

(filtration to obtain the IF), when results were expressed as concentration (mg/mg lyophilized digested sample), a significant decrease in the amount of rutin, caffeic acid and rosmarinic acid was observed, recovering only 37%, 8% and 27% of the initial concentration, respectively. This loss of phenolic compounds after gastro-intestinal digestion is supported by several studies (Bermúdez-Soto et al., 2007; Celep, Charehsaz, Akyuz, Acar, & Yesilada, 2015; Chiang et al., 2014; Siracusa et al., 2011). These compounds are highly sensitive to the mild alkaline conditions present at the small intestine where most dietary polyphenols are degraded or transformed into other compounds (Bermúdez-Soto et al., 2007). Nevertheless, other studies have reported high stability after *in vitro* pancreatic digestion of compounds such as rosmarinic acid (Costa, Grevenstuk, da Costa, Gonçalves, & Romano, 2014), pure quercetin and catechin (Tagliazucchi et al., 2010), ellagic acid (Gil-Izquierdo, Zafrilla, & Tomás-Barberán, 2002) or ferulic acid (Kamiloglu et al., 2016). In general, the differences among studies may result from the effect of the food matrix and also from the different experimental conditions applied.

Table 2 summarizes some published studies in which the stability of commercial phenolic standards was assessed after *in vitro* digestion processes. Despite all of them work with pure compounds, the data showed high variability. For instance, the % of loss after intestinal digestion for rutin was found to be from only 3% (Bermúdez-Soto et al., 2007) to total loss (Siracusa et al., 2011). In the case of chlorogenic acid, it was from 44% (D'Antuono et al., 2015) to 95.7% (Siracusa et al., 2011), and for quercetin, from 5.8% (Tagliazucchi et al., 2010) to total loss (Siracusa et al., 2011). Therefore, the digestion methodology seems to be a key factor for assessing bioaccessibility. As described by Minekus et al. (2014) there is significant variation in the use of *in vitro* parameters between the individual models described in the literature, hardening the comparison of results among studies. Apart from that, we also

observed that there was no uniformity in the units used to express the results, contributing to the heterogeneity in the data.

Interestingly enough, in our work, expressing HPLC results as total mg of standard measured in the digested sample (Table 1), we observed no effect on rutin by the simulated digestion, reaching a recovery of approximately 100% during all the digestive phases, in comparison to the recovery 60% and 76% in the IF for caffeic acid and for rosmarinic acid, respectively. This information highlights the importance of the units used to express the results of this type of experiments. When our results were expressed as concentration (mg standard/mg lyophilized digested sample), the observed decrease in the presence of rutin, caffeic acid and rosmarinic acid might be due to the previously mentioned dilution effect, but do not imply a real loss of the compound. In fact, this is in agreement with Bermúdez-Soto et al. (2007) where the results were expressed as total mg and only 3% loss were reported for pure rutin.

Bioaccessibility

A prerequisite for bioavailabilty of any compound is their bioaccessibility in the gut (Holst & Williamson, 2008). Normally, dietary polyphenols are poorly absorbed (in a range of 2-20%) (Hu, 2007), therefore the analysis of how digestion affects their stability is crucial for understanding their absorption and metabolism. Although it is difficult to exactly mimic the physiological conditions of the *in vivo* digestion, the potential bioaccessible phenolic compounds fractions after the complete digestion process were obtained by different approaches. IF was obtained using the easiest approach (filtration), IN fraction was obtained by applying the dialysis method and SF was recovered using the centrifugation method. Fig. 3 compares the three different fractions for determining the bioaccessibility. In Fig. 3A (expressed as total mg of standard in the corresponding fraction) the lowest amount of the phenolic compounds was detected in the IN fraction, being rutin the least dialyzable compound (1.65%), followed by caffeic acid (7.69%) and rosmarinic acid (7.92%). The

recovery of phenolic compounds in the IF and in the SF was significantly higher than in the IN fraction, since not every soluble compound was able to penetrate the membrane. The bioaccessibility of rutin in these two conditions was 100%, without significant differences between the IF and the SF. Caffeic and rosmarinic acids, behaved similarly, being the bioaccessibility in IF significantly higher (60% and 76%, respectively) than in SF (46% and 69%, respectively). Our results correlate with previous studies that show lower recovery of phenolic compounds using dialysis membrane (Bermúdez-Soto et al., 2007). For instance, Pinacho et al. (2015) analysed the soluble intestinal fraction after filtration and the recovery of caffeic acid was very high (only 5% loss) and rutin was only partially degraded (40% loss). Bermúdez-Soto et al. (2007) also found a high recovery of flavonols, ranging between 70% and 85% and also for caffeic acid derivatives (72-76%), using filtration as methodology. However, phenolic acids and rutin were not detected in the dialyzed fraction in Rodríguez-Roque et al. (2013) and total dialyzable polyphenols were lower than total soluble polyphenols in the intestinal phase in Bouayed, Deusser, Hoffmann and Bohn (2012). The low recovery by dialysis matches the low bioavailability of phenolic compounds, although many factors affect the process and not all of them can be associated with the absorption of the compound. Some hypothesis to explain the low recovery of standards in the IN fraction could be the formation of complexes of phenolic compounds with digestive enzymes, giving rise to higher molecular weight compounds, not able to pass through the dialysis membrane. In addition, it has to be considered that the inside volume of the dialysis bag is small, affecting the osmotic distribution, and probably limiting the pass of the compounds through the membrane. Namely, the amount of compound (total mg) that passes through the membrane is dependent on the volume of the dialyzed fraction.

In order to determine the amount of compound that reaches the intestine, the use of concentration units is quite common in the literature (Bouayed et al., 2012; Pinacho et al.,

2015; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). Fig. 3B shows the bioaccessibility calculated as concentration (mg/mg lyophilized digested sample), instead of a whole (total mg in the digested fraction). In this case, the most bioaccessible compound was rosmarinic acid (27-46% of recovery), the less stable was caffeic acid (7-11%) and in a middle position rutin (32-39%). The differences observed between the two different ways for reporting bioaccessibility highlight again the importance of how to express the results. When the results were expressed as concentration, the % of recovery rose significantly in the dialyzable fraction (IN), showing rosmarinic acid the highest recovery. This could be explained by the osmotic equilibrium on both sides of the membrane that occurs during dialysis, compensating the concentration of the compound on both sides. In this sense, % of bioaccessibility in mg/mg lyophilized digested sample was reduced in IF and SF in comparison with % bioaccessibility in absolute values (total mg in the digested fraction). This implies that the soluble fraction is not purely phenolic compound, pointing to a dilution of the sample along the digestion which might be responsible for the decrease in the concentration.

The bioaccessibility of a compound is influenced by many factors, such as the chemical state of the compound, the food matrix, interactions with other components or the presence of suppressors or cofactors (Parada & Aguilera, 2007). All these variables, as well as the methodology to obtain the bioaccessible fraction could explain the wide variability of results present in the literature. Nevertheless, the existing data are not conclusive enough to recommend which method is the most appropriate for the assessment of bioaccessibility in polyphenols (Etcheverry et al., 2012).

The presence of a significant amount of phenolic compounds, (more than 50% of the initial amount for caffeic and rosmarinic acids and practically the totality of rutin) in the OUT fraction and in the case of rutin, also in the RF implies a large amount of available sample

that can reach the colon. This means that unabsorbed compounds may be metabolized by the microflora, increasing polyphenol bioavailability and possibly, their presumed biological activity (Tuohy, Conterno, Gasperotti, & Viola, 2012). In fact, polyphenol glycosides such as rutin, require a hydrolysis by intestinal or microbial enzymes for their absorption *in vivo* (Manach et al., 2004).

Antioxidant activity

DPPH and ABTS assays were performed to measure the free radical scavenger ability in the samples during the digestion process (Table 3 and Table 4). Results were expressed as µg Trolox/ mg lyophilized digested sample in order to know the antioxidant activity in each step, regardless of the compound or compounds responsible for this activity. Although ABTS values were higher than the ones of DPPH, a good correlation was observed between both techniques (rutin, r= 0.97; caffeic acid, r=1; rosmarinic acid, r=0.96; $p < 0.001$). High correlation coefficients were also found between the antioxidant activity of each compound during the digestion and their concentration, which was evaluated by HPLC. These results confirm that the measured antioxidant activity was dependent on the phenolic compounds concentrations (DPPH *vs* rutin r= 0.95; ABTS *vs* rutin r= 0.96; DPPH *vs* caffeic acid r= 1; ABTS *vs* caffeic acid r= 1; DPPH *vs* rosmarinic acid r= 0.99; ABTS *vs* rosmarinic acid r= 0.95; $p < 0.001$).

After the oral and gastric phases, a slight decrease in the antioxidant activity for caffeic acid and rosmarinic acid was observed (19-12% and 36-24%, respectively), whereas rutin showed no loss in ABTS values and even higher antioxidant activity assessed by DPPH compared to the initial sample. However, after intestinal digestion the decrease in antioxidant activity was significant in the three compounds, especially in the case of caffeic acid. Similar trend (loss in antioxidant capacity during intestinal digestion) was previously reported in the digestion of different foods (Carbonell-Capella et al., 2015; Celep et al., 2015; Rodríguez-Roque et al.,

2013; Rodríguez-Roque et al., 2014). However, other studies have indicated a high antioxidant capacity of intestinal digested samples (Oliveira & Pintado, 2015; Pineda-Vadillo et al., 2016; Tagliazucchi et al., 2010), attributed to the deprotonation of the hydroxyl moieties present on the aromatic ring of the phenolic compounds due to the mild alkaline intestinal environment (Bouayed et al., 2011) or to the formation of derived products with a higher antioxidant capacity than that of their precursors (Pineda-Vadillo et al., 2016). Regarding the bioaccessible fractions (IF, SF and IN), there were no significant differences in the antioxidant activity among samples in the case of caffeic acid. Nevertheless, rosmarinic acid showed high DPPH and ABTS values in the IN fractions, in contrast to rutin, which presented the lowest values in this fraction. Therefore, the antioxidant activity found in the each bioaccessible fraction was mostly dependent on the compound itself rather than on the methodology used.

Moreover, antioxidant activity values were also considerable in the OUT and the RF samples. This fact could lead to a discussion in which the unabsorbed fractions could also play an important role in protecting the gastrointestinal tract from reactive oxygen species (ROS) generated during digestion processes (Bouayed et al., 2011).

4. Conclusions

Bioaccessibility studies are difficult to be compared due to the many variables that may influence the gastrointestinal digestion, such as the fraction used for their quantification and the units used for reporting the results. Therefore, the information obtained from the *in vitro* digestion processes should be carefully analysed. In particular, expression the results as concentration of a compound in the digested fractions could be compromised by a dilution effect. Consequently, it would be very interesting to give also the absolute amount of this compound. Regarding the bioaccessible fraction, we have demonstrated that it its strongly

affected by the methodology used at the intestinal level. Results obtained with the filtration and centrifugation techniques were quite similar and gave rise to higher values than those obtained by a dialysis membrane. Despite the fact that the antioxidant activity in the bioaccessible fractions decreased significantly, a remarkable antioxidant activity could still reach the colon and protect the gastro-intestinal tract from oxidative damage.

5. Abbreviations

DAD, diode array detector; HPLC, high-performance liquid chromatography; IF, intestinal fraction; IN, dialyzed fraction; RF, residual fraction; OUT, non-dialyzed fraction; SF, soluble fraction; w/w, weight/weight.

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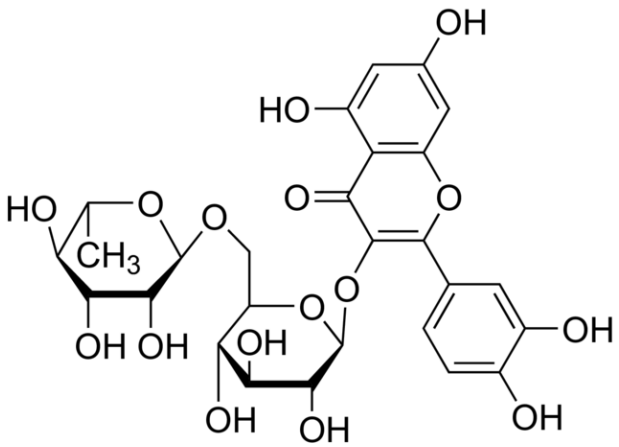
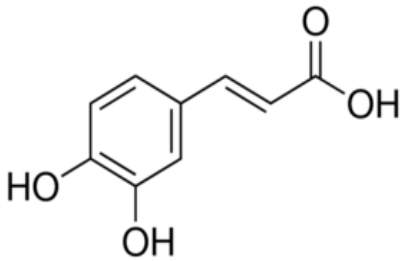
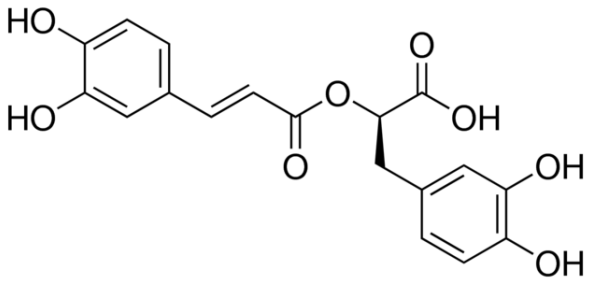
Figure 1. Chemical structures of rutin, caffeic acid and rosmarinic acid



Rutin (Quercetin 3-O-rutinoside)



Caffeic acid (3,4-dihydroxycinnamic acid)



Rosmarinic acid

Source: Sigma-Aldrich.

Figure 2. Scheme of the three procedures of *in vitro* gastrointestinal digestion applied to phenolic compounds standards. IF: intestinal fraction; SF: soluble fraction; RF: residual fraction; OUT: non-dialyzed fraction; IN: dialyzed fraction.

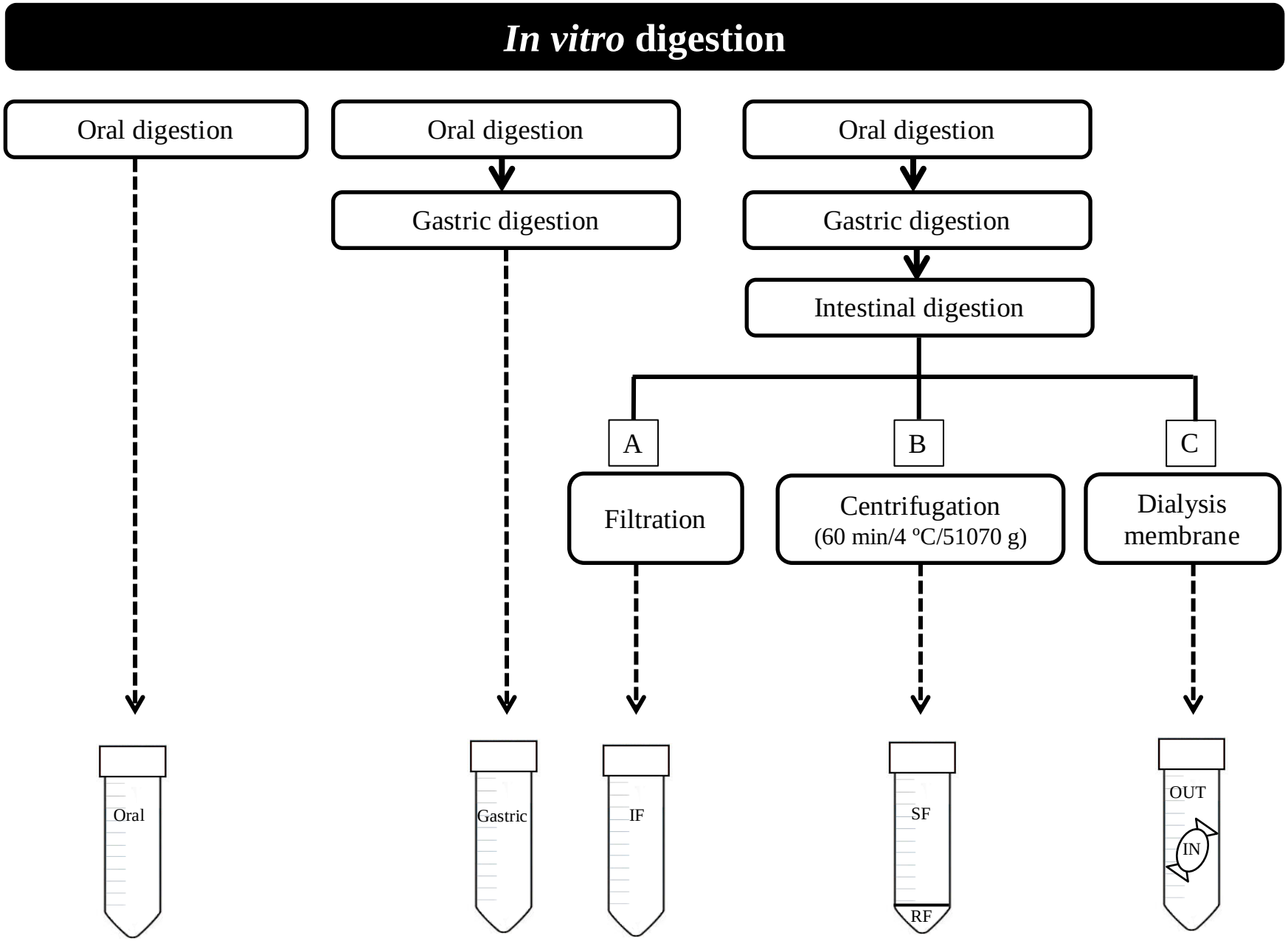
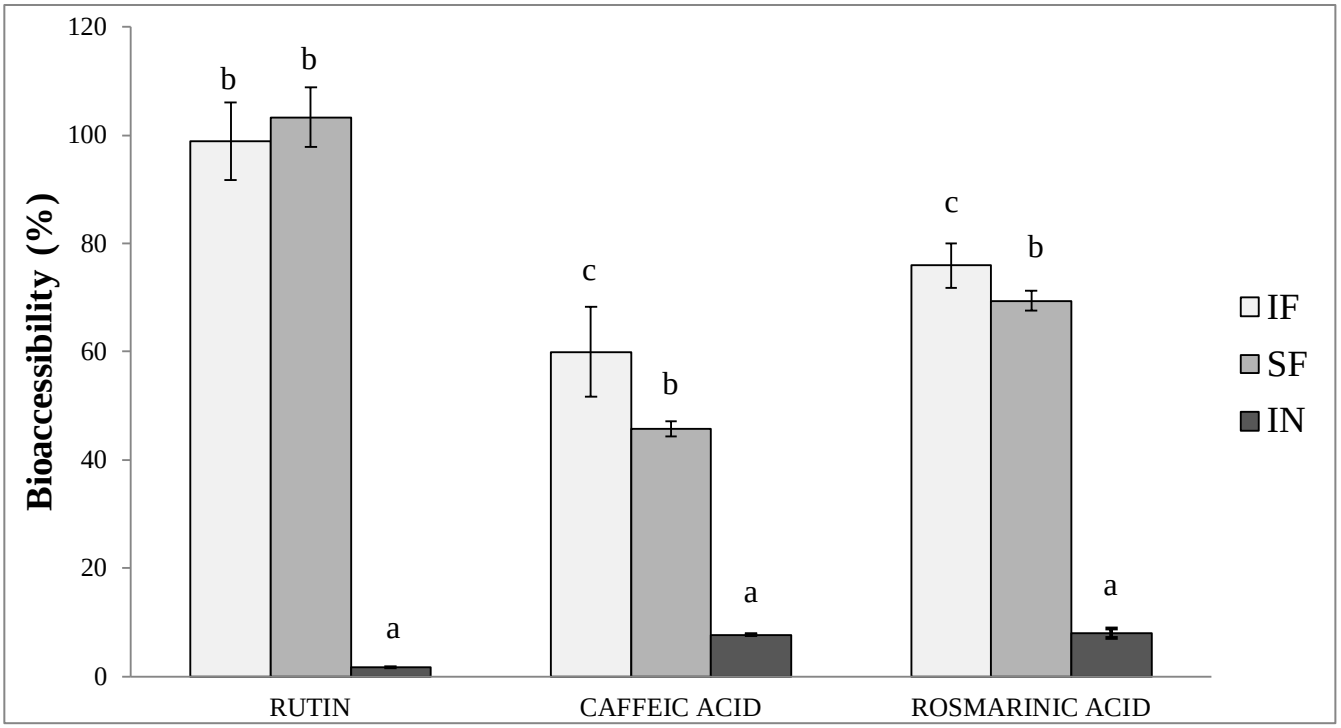


Fig. 3 Bioaccessibility (%) in the intestinal fraction (IF), soluble fraction (SF) and dialyzed fraction (IN) for rutin, caffeic acid and rosmarinic acid. A) Bioaccessibility (%) calculated with data expressed as total mg in the digested fraction. B) Bioaccessibility (%) calculated with data expressed as concentration (mg/mg lyophilized digested sample).

A)



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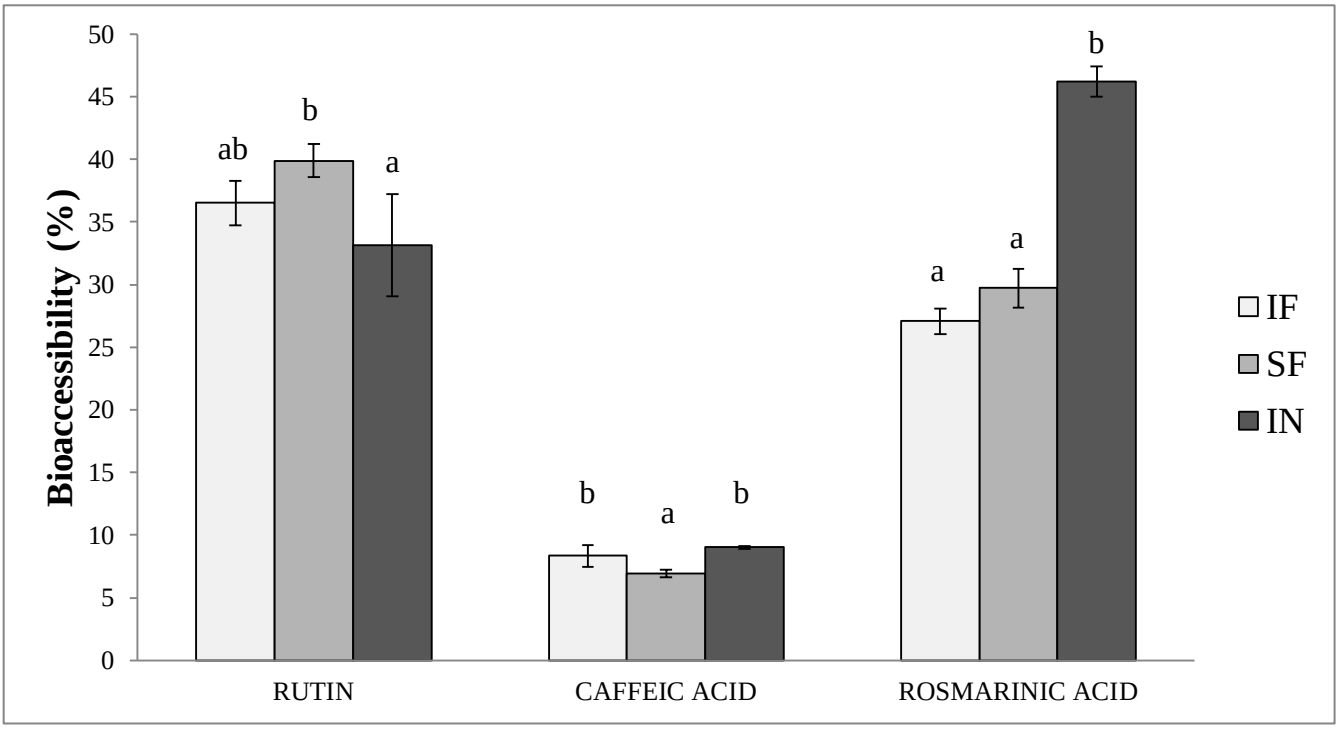

Within each standard, different letters indicate significant differences ($p < 0.05$) among fractions. Vertical bars indicate standard deviation.

Table 1. Evolution of rutin, caffeic acid and rosmarinic acid (mean ± standard deviation) determined by HPLC during *in vitro* gastrointestinal digestion. Results are expressed either as total mg in the digested sample or as concentration (mg/mg lyophilized digested sample).

Samples		Rutin		Caffeic acid		Rosmarinic acid	
		Total mg	mg/mg lyophilized digested sample	Total mg	mg/mg lyophilized digested sample	Total mg	mg/mg lyophilized digested sample
Initial		50.25 ± 0.36^{b}	1.00 ± 0.06^{c}	49.93 ± 0.54^{de}	1.15 ± 0.11^{c}	50.53 ± 0.67^{e}	1.01 ± 0.07^{e}
Oral		48.90 ± 1.05^{b}	1.02 ± 0.01^{cd}	47.52 ± 2.30^{d}	0.85 ± 0.03^{b}	42.95 ± 0.84^{cd}	0.68 ± 0.02^{d}
Gastric		51.50 ± 0.61^{bc}	1.05 ± 0.01^{cd}	54.22 ± 1.00^{e}	0.91 ± 0.03^{b}	46.39 ± 3.38^{de}	0.69 ± 0.05^{d}
Intestinal fractions:							
(A) Filtration	**IF**	49.93 ± 2.72^{b}	0.37 ± 0.02^{a}	29.80 ± 3.75^{c}	0.10 ± 0.01^{a}	37.97 ± 2.13^{bc}	0.27 ± 0.01^{a}
(B) Membrane	**IN**	0.83 ± 0.03^{a}	0.33 ± 0.04^{a}	3.77 ± 0.038^{a}	0.10 ± 0.001^{a}	4.09 ± 0.43^{a}	0.47 ± 0.01^{c}
	OUT	55.34 ± 0.45^{c}	0.45 ± 0.03^{b}	26.36 ± 1.69^{bc}	0.12 ± 0.005^{a}	35.04 ± 2.42^{b}	0.37 ± 0.02^{b}
(C) Centrifugation	**SF**	52.11 ± 3.30^{bc}	0.40 ± 0.01^{ab}	23.09 ± 0.94^{b}	0.08 ± 0.003^{a}	34.83 ± 0.78^{b}	0.30 ± 0.02^{a}
	RF	3.71 ± 0.20^{a}	1.09 ± 0.06^{d}	--	--	--	--

Different letters in the columns indicate significant differences ($p < 0.05$) among samples. IF: intestinal fraction; SF: soluble fraction; RF: residual fraction; OUT: non-dialysed fraction; IN: dialysed fraction. A, B and C indicate the intestinal methodology applied in each case.

Table 2. Stability of commercial standards subjected to an *in vitro* gastrointestinal digestion: compilation of published research papers.

Reference	Method	Compounds	Units used for reporting results	After gastric digestion % of loss	After intestinal digestion % of loss
Costa et al. (2014)	F	**Rosmarinic acid**	µg/mL	**12-28**	**- (0.93-1.7)**
Siracusa et al. (2011)	F	Chlorogenic acid	µg/mL	58.10	95.70
		Rutin		**88.11**	**Total**
		Quercetin 3-O-glucoside		Total	Total
		Quercetin		Total	Total
Bermúdez-Soto et al. (2007)	F	Cyanidin 3-rutinoside	Total mg	- 6	9.1
		Quercetin-3-rutinoside (Rutin)		**0**	**3.1**
		(+)Catechin		3.1	58.0
		Chlorogenic acid		- 4.2	5.1
Boyer et al. (2005)	F	Quercetin	µg/g non-digested sample		25.7
		Quercetin-3-glucoside			13.6
D´Antuono et al. (2015)	C	Chlorogenic acid	µg/mL		44.3
		1,5-O-Dicaffeoylquinic acid			34.6
		3,5-O-Dicaffeoylquinic acid			45.7
Işik et al. (2014)	C	Quercetin-4´-O-glucoside	µg/mL	- 3	- 5
		Phloretin-2´-O-glucoside		0	- 5
Tagliazucchi et al. (2010)	C	Gallic acid	Absorbance at the wavelength of maximum absorption	4.6	43.3
		Caffeic acid		**0.1**	**24.9**
		Catechin		0.7	7.2
		Quercetin		0.9	5.8
		Reveratrol		- 2.3	69.5

Negative values indicate an increment in the compound. The standards highlighted in bold are the same as those used in our study. F: Filtration; C: Centrifugation

Table 3. Evolution of rutin, caffeic acid and rosmarinic acid antioxidant activity (DPPH assay) during *in vitro* gastrointestinal digestion.

DPPH		**Rutin**		**Caffeic acid**		**Rosmarinic acid**	
Samples		µg Trolox/mg lyophilized digested sample	% of AA	µg Trolox/mg lyophilized digested sample	% of AA	µg Trolox/mg lyophilized digested sample	% of AA
Initial		261.03 ± 17.3^{c}	100	971.53 ± 22.06^{d}	100	951.64 ± 37.90^{e}	100
Oral		291.65 ± 10.63^{d}	112	854.29 ± 13.52^{c}	88	741.25 ± 40.32^{d}	78
Gastric		326.86 ± 0.93^{e}	125	858.31 ± 15.78^{c}	88	724.80 ± 3.49^{d}	76
Intestinal fractions:							
(A) Filtration	**IF**	102.08 ± 3.79^{b}	39	171.06 ± 8.40^{ab}	18	258.50 ± 0.03^{a}	27
(B) Centrifugation	**SF**	104.90 ± 0.14^{b}	40	134.84 ± 1.36^{a}	14	270.66 ± 10.10^{a}	28
	RF	242.67 ± 0.97^{c}	93	--	--	--	--
(C) Membrane	**IN**	57.54 ± 4.00^{a}	22	154.72 ± 3.33^{ab}	16	459.27 ± 4.72^{c}	48
	OUT	127.46 ± 2.79^{b}	49	180.77 ± 3.53^{b}	19	373.35 ± 12.27^{b}	39

Data are expressed as mean ± sd and as %. Antioxidant activity before digestion was considered as 100%. Different letters in the columns indicate significant differences ($p < 0.05$) among samples. IF: intestinal fraction; SF: soluble fraction; RF: residual fraction; OUT: non-dialysed fraction; IN: dialysed fraction. A, B and C indicate the intestinal methodology applied in each case. AA: antioxidant activity

Table 4. Evolution of rutin, caffeic acid and rosmarinic acid antioxidant activity (ABTS assay) during *in vitro* gastrointestinal digestion.

ABTS		**Rutin**		**Caffeic acid**		**Rosmarinic acid**	
Samples		µg Trolox/mg lyophilized digested sample	% of AA	µg Trolox/mg lyophilized digested sample	% of AA	µg Trolox/mg lyophilized digested sample	% of AA
Initial		471.11 ± 37.54^{c}	100	1826.99 ± 4.97^{e}	100	1871.72 ± 70.18^{e}	100
Oral		488.61 ± 0.69^{c}	104	1485.85 ± 11.53^{c}	81	1530.66 ± 26.76^{d}	82
Gastric		472.57 ± 10.37^{c}	100	1526.43 ± 14.77^{d}	84	1193.23 ± 89.29^{c}	64
Intestinal fractions:							
(A) Filtration	**IF**	151.60 ± 7.82^{a}	32	303.24 ± 5.68^{a}	17	657.17 ± 20.37^{a}	35
(B) Centrifugation	**SF**	151.56 ± 2.73^{a}	32	297.21 ± 10.01^{a}	16	602.58 ± 13.62^{a}	32
	RF	392.32 ± 21.08^{b}	83	--	--	--	--
(C) Membrane	**IN**	131.56 ± 4.34^{a}	28	319.48 ± 6.24^{a}	17	850.44 ± 17.48^{b}	45
	OUT	167.27 ± 6.92^{a}	36	352.72 ± 3.73^{b}	19	933.69 ± 73.18^{b}	50

Data are expressed as mean ± sd and as %. Antioxidant activity before digestion was considered as 100%. Different letters in the columns indicate significant differences ($p < 0.05$) among samples. IF: intestinal fraction; SF: soluble fraction; RF: residual fraction; OUT: non-dialysed fraction; IN: dialysed fraction. A, B and C indicate the intestinal methodology applied in each case. AA: antioxidant activity