**Total Antioxidant Capacity of New Natural Powdered Seasonings after Gastrointestinal and Colonic Digestion**

Running title:

**Total Antioxidant Capacity of New Natural Seasonings after Digestion**

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**Abstract**

New powdered seasonings, rich in natural antioxidant compounds, have successfully been applied recently in different food matrices. Once ingested, the antioxidants contained in these seasonings may exert protective effects against oxidative stress along the gastrointestinal tract. This fact was evaluated by submitting the different seasonings under study to simulated digestion followed by assessing the reducing and antiradical capacities of the digested fractions. Enzymatic gastrointestinal digestion enhanced 2-3 times both antioxidant activities and colonic fermentation increased more than 10-fold the radical scavenging ability of digested fractions compared with undigested seasonings. Digested fractions derived from the seedless wine pomace seasoning presented generally the highest antioxidant properties. The results were evaluated considering bioaccessibility factors to have a more realistic overview of the potential antioxidant capacities of the seasonings and of the probable beneficial effects of their consumption on the prevention of oxidative damage along the gut.

*Keywords:* ABTS; Antioxidant capacities; Colonic microbial fermentation; Enzymatic gastrointestinal digestion; Folin index; QUENCHER; Wine pomace; Seasoning.

*Abbreviations and nomenclature:* **ABTS**, 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid; **CF**, colonic fermented; **CFr**, colonic fermented residue; **CFs**, colonic fermented supernatant; **FC**, Folin-Ciocalteu; **GAR**, global antioxidant response; **GID**, gastrointestinal digested; **GIDD**, gastrointestinal digested+dialyzed; **Q-**, QUENCHER; **RWPS**, red wine pomace seasoning; **Sd-S**, seasoning obtained from seeds; **Sk-S**, seasoning obtained from seedless red wine pomace, in which grape skins are the main component; **TAC**, total antioxidant capacity; **W-S**, seasoning obtained from whole red wine pomace.

## 1. Introduction

Epidemiological studies and associated meta-analyses strongly suggest that long term consumption of fruits and vegetables plays a pivotal role in the prevention against numerous chronic diseases such as cancer (Pandey & Rizvi, 2009; Sun, Chu, Wu, & Liu, 2002). In the gastrointestinal tract, these health-protective effects are partially attributed to their antioxidant properties (Halliwell, Zhao, & Whiteman, 2000), which have been associated with their high phytochemical (mainly phenolic compounds and carotenoids) and antioxidant dietary fibre contents (Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013; Saura-Calixto et al., 2010).

An adequate bioavailability of bioactive substances is a prerequisite for potential systemic effects *in vivo* (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). However, many antioxidants remain in the intestinal luminal contents and may exert a local beneficial effect within the gut by protecting possible oxidisable molecules and the intestinal epithelium from oxidative damage occurring during digestion (Goñi & Serrano, 2005; Halliwell et al., 2000). In this regard, the chemical alterations and the bioaccessibility of antioxidant compounds in the gastrointestinal tract are key aspects that determine their bioavailability (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014; Rein et al., 2013), especially for those foods rich in antioxidant dietary fibre due to its low digestibility (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011). Gastrointestinal digestion is able to release, from food matrices, some entrapped antioxidants that might be absorbed in the small intestine, whereas other antioxidants remain enclosed in the indigestible fraction and reach the large intestine (Scalbert & Williamson, 2000). These bioactive substances and the metabolites formed after their fermentation by gut microbiota could exert their antioxidant activity *in situ* or, to some extent, be absorbed in the lower regions of the colon (Delgado-Andrade, Conde-Aguilera, Haro, Pastoriza de la Cueva, & Rufián-Henares, 2010; Saura-Calixto et al., 2010). Similarly, the insoluble matter in ingested food, which remains in the gastrointestinal tract for a long time, may help to counteract the free radicals that are continuously formed in the gut (Pérez-Jiménez et al., 2013; Tabernero, Venema, Maathuis, & Saura-Calixto, 2011).

In view of the above, the effects of the digestive process on the phytochemicals contained in foods, and on their antioxidant activity, have attracted great attention from the scientific community over the last years (Heim, Tagliaferro, & Bobilya, 2002; Rein et al., 2013). Thus, several *in vitro* digestion models to assess

bioaccessibility that allow the study of changes in dietary components during the gastric and intestinal stages have been implemented (Carbonell-Capella et al., 2014; Hur, Lim, Decker, & McClements, 2011; McDougall, Fyffe, Dobson, & Stewart, 2005). Despite the limitations of *in vitro* digestion models, especially those comprising only a static simulated digestion, the good correlation of the results obtained with those from several animal and human studies has been established (Alminger et al., 2014; Saura-Calixto et al., 2010). Furthermore, the combination of *in vitro* digestion models with total antioxidant capacity (TAC) assays for the digested fractions obtained has been suggested as a first approach to predict the *in vivo* antioxidant activity of foods (Goñi, Martín, & Saura-Calixto, 2005; Rufián-Henares & Delgado-Andrade, 2009). Consequently, Delgado-Andrade et al. (2010) have proposed a methodology to determine the global antioxidant response (GAR) of food, which is defined as the sum of the antioxidant activities of the soluble and insoluble fractions obtained after a simulated gastrointestinal digestion. According to this method, the TAC of these digested fractions is measured separately, using classical and QUENCHER (Gökmen, Serpen, & Fogliano, 2009) assays, respectively, and then combined to estimate the GAR of foodstuffs. Thus far, several plant-based foods have been assessed following the GAR method, and important variations in the antioxidant activities exhibited by the different food matrices tested have been detected (Papillo, Vitaglione, Graziani, Gokmen, & Fogliano, 2014; Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011).

The promising use as food ingredients of new seasonings obtained from red wine pomace (RWPSs) has recently been demonstrated (García-Lomillo, González-Sanjosé, Del Pino-García, Rivero-Pérez, & Muñiz, 2014). The new powdered vegetal seasonings are antioxidant-rich products, containing mainly phenolic compounds, which may contribute to the intake of exogenous natural antioxidants and reinforce the endogenous redox environment once ingested. In this regard, it has been suggested that consumption of wine pomace may help prevent colon cancer (López-Oliva, Agis-Torres, García-Palencia, Goñi, & Munoz-Martínez, 2006) and its high antioxidant content certainly play an important role in this protective effect. On the basis of the previous considerations, the present study was conducted to evaluate the effects of the digestive process on the antioxidant activity of three of these new seasonings, targeting the antioxidant capacities of digested fractions which can mimic those produced in the small and the large intestine after intake of each studied seasoning. For this purpose, the TAC of *in vitro* digested fractions (including both gastrointestinal and colonic phases) was measured using QUENCHER methodologies.

## 2. Materials and methods

### *2.1. Chemicals*

Ammonium bicarbonate ($NH_4HCO_3$), 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), porcine bile extract, calcium chloride dihydrate ($CaCl_2.2H_2O$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), L-cysteine hydrochloride, gallic acid, hydrochloric acid (HCl), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), maleic acid, porcine pancreas pancreatin, potassium chloride (KCl), potassium phosphate monobasic ($KH_2PO_4$), resazurin sodium salt, sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), sodium phosphate dibasic ($Na_2HPO_4$), sodium phosphate monobasic ($NaH_2PO_4$), sodium sulphide nonahydrate ($Na_2S.9H_2O$), Tris hydrochloride (Tris), tryptone, enzymes used in enzymatic digestion α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3), lipase (EC 3.1.1.3), and pepsin (E.C 3.4.23.1), and cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Folin-Ciocalteu (FC) reagent and sodium carbonate ($Na_2CO_3$) were purchased from Panreac Química S.L.U. (Barcelona, Spain).

### *2.2. Materials*

The seasonings used in this study were made in the pilot plant of the Food Technology Department of University of Burgos (Spain) as previously described (García-Lomillo et al., 2014), applying the process patented by González-Sanjosé, García-Lomillo, Del Pino-García, Muñiz-Rodríguez, & Rivero-Pérez (2013). Three different types of powdered seasonings were used, which were labelled as W-S, corresponding to the seasonings obtained from whole red wine pomace; Sk-S, representing the seasonings obtained from seedless red wine pomace, in which grape skins are the main component; Sd-S, referring to the seasonings obtained from the seeds separated from red wine pomace. Three different batches of each type of RWPS were used in this study.

The inoculums used for colonic fermentation were obtained at the animal-housing unit of the University Hospital of Burgos (Spain) by mixing the caecal content from 5 male Wistar rats (body weight of 250 ± 5

g) fed with standard maintenance diet. All aspects of this procedure were conducted in accordance with the guidelines established by the Ethics Committee at both the University Hospital of Burgos and the University of Burgos.

*2.3. In vitro gastrointestinal digestion and colonic fermentation of the seasonings*

Simulated complete digestion of the three RWPSs (Sk-S, W-S, and Sd-S) was performed according to the method describe by Saura-Calixto, Serrano, & Goñi, (2007), with only slight modifications in the dialysis step. This *in vitro* static digestion model allows the estimation of the bioaccessibility of dietary antioxidants, and mainly comprises two consecutive stages, an enzymatic gastrointestinal digestion first phase, followed by a colonic microbial fermentation phase (**Figure 1**). Briefly, each powdered seasoning, labelled as "undigested" (UD), was successively incubated with digestive enzymes, as described in detail by Saura-Calixto et al. (2007), yielding the so-called "gastrointestinal digested" (GID) fractions. Each GID was centrifuged (3,000 g, 15 min, 25 ºC) to separate the supernatant and the solid residue. This centrifugation was repeated twice, washing the residue with 5 mL of Milli-Q water. All the supernatants obtained were then combined, transferred into cellulose membrane dialysis tubing, and dialyzed against a total of 2 L of water for 24 h (changing the water twice). The dialysis retentate was mixed with the GID solid residue to obtain the so-called "gastrointestinal digested+dialyzed" (GIDD) fraction, which contained the compounds hypothetically non-absorbed in the small intestine that may reach the large intestine. The GIDD fraction was the substrate for the action of colonic microbiota, obtaining the "colonic fermented" (CF) fraction. Finally, the CF fraction was centrifuged (2,500 g, 10 min, 25 ºC) to collect the supernatant (CFs) and the residual solid (CFr) respective fractions. All fractions isolated along the simulated digestive procedure were lyophilized, weighed, and stored at -20 ºC until their analysis.

Each digested fraction (GID, GIDD, CF, CFs, and CFr) were obtained by triplicate from each batch of each type of RWPSs under study.

*2.4. Yields (%, w/w) of the in vitro digested fractions*

The yields of each of the digested fractions (GID, GIDD, CF, CFs, and CFr) were expressed as percentages (%, w/w) with respect to the corresponding initial quantity of each UD seasoning.

Taking into account that the chemicals, enzymes, and colonic inoculums added to perform the *in vitro* pre- and post-colonic digestion also contributed to the mass of the lyophilized digested fractions, control digestive procedures were run in parallel in absence of seasonings. As such, digested control fractions were also obtained in triplicate. The mass of these control fractions was used to calculate the "real" yield of each corresponding digested fraction.

### *2.5. Total antioxidant capacity (TAC)*

QUENCHER (Q-) versions of two classical total antioxidant capacity (TAC) assays (Folin-Ciocalteu index and ABTS) were selected to evaluate the TAC of such fractions.

#### *2.5.1. QUENCHER Folin-Ciocalteu assay (Q-FC)*

A sample mass of each lyophilized digested fraction (1 ± 0.005 mg) was mixed with 0.2 mL of Milli-Q water and 0.2 mL of FC reagent, and allowed to react for 5 min. A 4 mL aliquot of 0.7 M $Na_2CO_3$ solution was then added and the mixture made up to a final volume of 10 mL with MQ water. After incubation for 1 h in the dark with continuous stirring, the supernatant was separated and the absorbance at 750 nm was measured in an UV-vis spectrophotometer U-2000 (Hitachi, Ltd., Hubbardston, MA, USA) (Del Pino-García, García-Lomillo, Rivero-Pérez, González-Sanjosé, & Muñiz, 2015). A dose-response curve was plotted using different quantities of gallic acid as the standard.

#### *2.5.2. QUENCHER ABTS assay (Q-ABTS)*

A sample mass of each lyophilized fraction (1 ± 0.005 mg) was weighed and mixed with 10 mL of the $ABTS^{\cdot+}$ working solution, which was prepared in Milli-Q water following the procedure described by Del Pino-García et al. (2015). After incubation for 30 min in the dark in an orbital shaker, the supernatant was separated and the absorbance at 734 nm was measured. A linear calibration curve was obtained with different amounts of Trolox as the standard.

#### *2.5.3 Total antioxidant capacity expressions*

The Q-TAC values were expressed in two different ways:

- *Absolute approach*: in this case Q-TAC values were expressed with respect to the mass of each fraction analysed. Thus, final values were expressed as µmol of standard equivalents (GAE or TE) per gram of GID, GIDD, CF, CFs, and CFr fractions, and per gram of UD seasonings.

As mentioned above, chemicals, enzymes, and colonic inoculums used for the *in vitro* digestion protocol may contribute to the mass of each digested fraction. Similarly, they can contribute to the antioxidant activity measured on the lyophilized digested fractions. So, "real" TAC values (absolute approach) for each studied fraction were calculated considering both the Q-TAC values and the mass contribution of the respective digested control fractions.

- *Relative approach*: in this case Q-TAC values of each of the digested fraction (GID, GIDD, CF, CFs, and CFr) were expressed as µmol of standard equivalents (GAE or TE) per gram of undigested seasoning. These values were calculated taking into account the "real" yields of such fractions. In this way, the relative approach tries to give a more accurate estimation of the potential TAC of each seasoning after intake, considering the potential bioaccessibility of their bioactive compounds along the gastrointestinal tract.

### *2.6. Data presentation and statistical analysis*

The results were expressed as mean ± standard deviation (n=3). Real replicates from three different batches of each seasoning were used. Furthermore, analytical parameters were measured also in triplicate. Statistical data analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). A one-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was applied to establish significant differences among the values of each digested fraction for the same seasoning, and among the values of each studied seasonings for the same fraction. Significance level of $p < 0.05$ was considered.

## 3. Results and Discussion

A methodology that combines a complete *in vitro* pre- and post-colonic digestion of foodstuffs with the analysis of the total antioxidant capacity of the obtained digested fractions by QUENCHER assays is proposed.

This methodology has been used to study the potential antioxidant capacities of three new seasonings derived from red wine pomace, which have been satisfactorily applied in food matrix. Yields of each simulated digestion phases were determined, and the potential antioxidant ability of the obtained fractions was estimated applying Q-TAC assays. Since few data are available about the effects of the digestive process on the antioxidant activity of wine pomace and related products, this study can contribute to the state of the art and point out new interesting data.

*3.1. Yields of each "in vitro" digested fractions*

Digestive and colonic fermentative processes lead to the release and modification of food components, thus determining their bioaccessibility and bioavailability. The extent, intensity and location of these transformations depend, among other parameters, on the foodstuff composition (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Palafox-Carlos et al., 2011; Rein et al., 2013). This fact was evidenced by the differences observed among the obtained yields for each seasoning (Sk-S, W-S, and Sd-S) (Table 1). Fractions obtained after submitting plant food to such gastrointestinal digestive phase contain different types of soluble and insoluble compounds (Saura-Calixto et al., 2007): bioaccessible and absorbable molecules (which are known as bioavailable compounds), bioaccessible but non-absorbable molecules, and non-bioaccessible particles that remain attached to the food matrices. GID products represent all these potentially bioactive compounds which derive primarily from several precursors of higher size (oligomers and polymers) present in the studied seasonings, such as phenolic compounds, proteins, and the different constituents of the dietary fibre fraction (García-Lomillo et al., 2014).

The observed decrease in the yields of the GIDD fractions compared to those of GID fractions noted that significant amounts of compounds were solubilized during the enzymatic gastrointestinal digestion, and some of them were able to diffuse out of the dialysis tubing. Although only mechanical forces are considered in the simulated digestion (Alminger et al., 2014), in a simplified manner, the dialyzed compounds could represent the constituents of the seasonings hypothetically bioavailable in the small intestine. The significantly higher decrease from GID to GIDD fractions detected for Sk-S than for W-S and Sd-S pointed out the higher digestibility and putative bioavailability of the compounds contained in the first RWPS. This fact was evidenced by comparing, in percentage, the amount of GIDD fractions with respect to the corresponding GID fractions (Sk-S: 73.9%, W-S: 77.4%, and Sd-S: 93.2%), with the higher

percentage indicating the lower estimated digestibility and bioavailability. In general terms, the yields obtained for GIDD fractions were in agreement with the values reported by Bravo & Saura-Calixto (1998) and Goñi et al. (2005), who found that the indigestible fraction of grape pomace represented around 80% of dry matter, with dietary fibre (including associated non-extractable polyphenols, being the major constituent of this fraction (ranging from 72-79%). Consequently, the lower dietary fibre content in Sk-S (48.6%) than in Sd-S (58.9%) (García-Lomillo et al., 2014) might partly explain the higher digestibility of Sk-S and the lower yields of its GIDD fraction.

The yields obtained for CF fractions were similar or higher than for GIDD fractions. Concretely, significant increases were detected in W-S and Sd-S, but not in Sk-S. Previous research has reported that grape seed flavan-3-ol monomers can promote the growth of certain beneficial gut bacterial groups (Cueva et al., 2013). As W-S and Sd-S contain wine pomace seeds and are richer in flavan-3-ol derivatives than Sk-S (García-Lomillo et al., 2014), it is possible that the release of some monomers during the fermentation of their GIDD fractions exerted a positive effect on the growth of colonic microbiota. In this case, the higher microbial population in CF fractions of W-S and Sd-S could explain the observed increase in their mass.

The overall fermentability of each RWPS by colonic microbiota was estimated by comparing, in percentage, the amount of solubilized colonic fermented compounds (CFs fractions) with respect to the corresponding GIDD fractions. In this way, interferences due to the possible different amount of gut microbiota acting in each case were partially eliminated. Lower quantity of CFs products was obtained after colonic fermentation of GIDD fractions derived from Sk-S (51.0%) than from W-S and Sd-S (58.1% and 59.2%, respectively). Bravo & Saura-Calixto (1998) reported that insoluble dietary fibre represented more than 90% of the total dietary fibre in grape pomace, with no important differences between grape skin- or seed-enriched samples. Therefore, taking into account the low colonic fermentability of insoluble dietary fibre (Bravo, Abia, & Saura-Calixto, 1994; Saura-Calixto et al., 2010), the results of the current study may be explained considering other compounds present in the GIDD fraction. Goñi et al. (2005) noted that proteins are the second highest constituent of the indigestible fraction of grape pomace (about 14%), which is due to their low digestibility (about 12%, with no differences between grape pomace constituents). However, proteins are metabolized to a large extent in the colon, with higher yields observed for seed proteins (around 70%) than for skin proteins (around 60%). In light of the above, and considering that the protein content of the RWPSs was rather similar, ranging from 12-14% (García-Lomillo et al.,

2014), the fermentation of proteins might play an important role to explain the estimated lower fermentability of Sk-S compared with W-S and Sd-S.

### *3.2. Potential antioxidant activities of each "in vitro" digested fractions*

The ability of the different RWPSs, once ingested, to reduce reactive species (Q-FC assay) and to quench free radicals (Q-ABTS assay) along the gastrointestinal tract was estimated from the Q-TAC data of the fractions isolated throughout the *in vitro* digestion procedure. These Q-TAC values were evaluated under two points of view, considering the antioxidant capacity of each gram of the digested fractions (absolute approach), and regarding the antioxidant capacity expressed per gram of UD seasonings (relative approach). Furthermore, two comparative studies were carried out, one among seasonings (Sk-S, W-S, and Sd-S), and other among digested fractions (GID, GIDD, CF, CFs, and CFr), considering also the UD seasonings in the case of absolute approach data.

#### *3.2.1. Total antioxidant capacity of each digested fractions: absolute approach*

The results obtained from the Q-FC and Q-ABTS assays (**Figure 2**, **A** and **B**, respectively) showed significant variations regarding the two factors under study, the seasonings and the digested fractions analysed.

Concerning the differences among the three RWPSs before digestion (UD seasonings), Sk-S and W-S exhibited greater antioxidant capacity than Sd-S. These results were almost certainly due to the differences among wine pomace constituents in terms of content, antioxidant capacity, hydrophilicity, solubility, and accessibility of their antioxidant compounds (Del Pino-García et al., 2015). GID fractions showed similar Q-TAC values for the different seasonings. Therefore, despite the lower digestibility of Sd-S (*Section 3.1.*), a higher increase in the "absolute" antioxidant activity displayed by Sd-S than by Sk-S and W-S was observed following enzymatic gastrointestinal digestion. The rest of digested fractions (GIDD, CF, CFs, and CFr) generally showed a similar trend between the three RWPSs as found prior digestion. Thus, after the liberation and absorption of bioavailable antioxidants in the small intestine, the net antioxidant capacity displayed by the compounds that reach the colon might again be higher for Sk-S and W-S than for Sd-S, both before and after the action of colonic microbiota. In addition, it must be noted that Sk-S gave the

highest Q-TAC values in CFs products, despite the slightly lower fermentability estimated for this RWPS (*Section 3.1.*).

Regarding the effects of the simulated digestion, in general, the different digested fractions showed significantly higher Q-TAC values than their respective undigested seasonings. Concretely, the Q-FC and Q-ABTS values for GID fractions were around two- and three-fold higher than for UD seasonings, respectively. Therefore, the enzymatic gastrointestinal digestion phase produced a marked increase in the antioxidant capacity of the RWPSs. These results generally agree with those reported by Rufián-Henares & Delgado-Andrade (2009). These authors demonstrated that *in vitro* gastrointestinal digestion was essential to allow the release of a large quantity of antioxidant compounds, showing an increase of up to four-fold in the ABTS values for the soluble digested fraction with respect to the original products. However, the small variations detected between the TAC values of GID and GIDD fractions suggested that the absorption of compounds in the small intestine did not severely affect the "absolute" antioxidant capacity of GIDD fractions. The observed opposite tendencies between the values of both Q-TAC assays could be explained considering the possible elimination, during dialysis, of compounds with free radical scavenging capacities, as well as with capacity to interfere in the measure of the FC index.

The colonic fermentation phase led to marked increases in the Q-ABTS values, but less noticeable effects on the Q-FC results. Concretely, the values of $ABTS^{\cdot+}$ scavenging capacity displayed by CF fractions exceeded that obtained for UD seasonings by almost 10-fold. These results appear to point out that the action of colonic microbiota induced important chemical changes on the compounds retained in GIDD fractions, releasing metabolites with high free radical scavenging ability but not with significant reducing properties. The Q-TAC values for CFs fractions appear to show that metabolites solubilized during the colonic fermentation had higher antioxidant capacity than the global CF fractions. These compounds had both reducing and free radical scavenging capacities. On the contrary, CFr products gave marked lower Q-FC and Q-ABTS values than their respective CF fractions. These findings suggest that the soluble antioxidant compounds generated and released due to the action of gut microbiota were the main responsible for the results obtained in CF fractions, which was especially important with regard to the potential antiradical capacity of the digested seasonings.

Differences observed between the absolute antioxidant capacities of the digested fractions seem to be, at least in part, due to the structural changes occurring in the RWPS matrices during digestion. These

alterations might lead to the exposure of functional groups on the surface or somewhere inside the solid matrices, thus improving the accessibility for the radicals (Rufián-Henares & Delgado-Andrade, 2009). This fact could partially explain the increased antioxidant capacity of the digested fractions in comparison with the UD seasonings.

In parallel, it must be noted that the type, amount, and activity of any antioxidant compound present in the digested fractions may greatly differ from the initial situation of their precursors in the seasonings. Indeed, some antioxidants, such as anthocyanins and other phenolic compounds, might be lost or transformed (isomerized, hydrolysed, etc.) by gastrointestinal enzymes, or due to their instability under the pH conditions of the simulated digestion (McDougall et al., 2005; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010; Velderrain-Rodríguez et al., 2014). In contrast, different new bioactive molecules with antioxidant properties can be simultaneously formed in the gastrointestinal tract. Some of them may consist on bioactive metabolites generated from modifications of antioxidant compounds, and others become from metabolic reactions associated to other food components.

Saura-Calixto et al. (2007) estimated that around 32% of the ingested fruit-derived phenols were bioaccessible in the small intestine, and around 56% became bioaccessible in the large intestine. The most hydrophilic phenolic forms, such as glycosylated flavonols or quinic acid derivatives of hydroxycinnamic acids, may readily solubilize in the aqueous phase in the upper gastrointestinal tract, whereas less soluble flavonoid aglycones or procyanidins may be strongly bound to dietary fibre and proteins (Le Bourvellec & Renard, 2012; Palafox-Carlos et al., 2011; Rein et al., 2013). In the case of wine pomace, certain complex phenolics, such as polymeric proanthocyanidins bound to antioxidant dietary fibre, represent about 15-30% of dry matter (Bravo & Saura-Calixto, 1998). In addition, acidic pH and proteolytic enzymes (such as pepsin) in the gastric tract play an important role in polyphenols bioaccessibility by releasing phenolic compounds bound to solid matters (Alminger et al., 2014; Rufián-Henares & Delgado-Andrade, 2009; Tagliazucchi et al., 2010). This fact is in agreement with the increase of Q-TAC values observed after the enzymatic gastrointestinal digestion phase in the present study. Furthermore, according to the high Q-ABTS values observed after the colonic fermentation phase, the action of microbial enzymes (such as esterase and xylanase) must be regarded as another factor that could contribute to increase the pool of free phenolic acids, thereby enhancing the antioxidant capacity of the fermented fractions (Acosta-Estrada et al., 2014; Kroon, Faulds, Ryden, Robertson, & Williamson, 1997). Similarly, the action of β-glucosidases of

enteric bacterial origin might hydrolyse phenolic glycosides, thus liberating the corresponding aglycones which usually display higher antioxidant activity than the glycoside forms (Aura et al., 2005).

Finally, it should be noted that, in the large intestine, several antioxidant phenolic compounds may be newly formed as a result of the extensive transformation of phenols retained into the residual undigested fractions by gut microbiota. Simple soluble compounds can thereby be generated, being phenylacetic, phenylpropionic and benzoic acid derivatives the main phenolic bacterial metabolites (Acosta-Estrada et al., 2014; Fernández-Panchón, Villano, Troncoso, & García-Parrilla, 2008; Pérez-Jiménez et al., 2013; Sánchez-Patán et al., 2012). Consequently, non-extractable phenolic compounds that reach the large intestine may significantly contribute to the antioxidant capacity in the colonic contents (Touriño et al., 2011).

*3.2.2. Total antioxidant capacity of each digested fractions: relative approach*

The results of the relative Q-TAC values of each digested fractions evidence that RWPS intake may considerably increase the pool of exogenous antioxidants in the entire gastrointestinal tract and, thereby, contribute to the antioxidant capacity of the intestinal luminal contents. Relative Q-TAC values of the gastrointestinal digested fractions showed the highest Q-FC values, while colonic fermentation fractions exhibited the highest Q-ABTS values (**Figure 3, A** and **B**, respectively). Furthermore, Q-TAC values of CFr fractions were the lowest, although the Q-TAC values of these fractions indicated certainly important role in the antioxidant capacity of the colonic contents.

Independently of the Q-TAC methodology used, the results showed that most of the digested fractions may display considerably higher antioxidant capacities once they are present in the small or large bowels than before being ingested.

With regard to differences between the seasonings (Sk-S, W-S, and Sd-S), similar antioxidant capacity profiles were obtained for the three RWPSs in each Q-TAC assay. A slight tendency toward lower values was observed in the products derived from Sd-S, but differences were statistically significant in only a few cases.

A significant decrease of about 11% between the Q-FC values of GID and GIDD fractions of the seasonings with higher digestibility (Sk-S and W-S) was observed. However, marked falls (ranged from 27-36%) in the Q-ABTS values were detected for the three seasonings. This finding suggests a higher

antiradical activity than reducing capacity of those compounds potentially absorbable through the intestinal barrier in the small intestine. Nevertheless, the most notable difference between the values obtained by the two Q-TAC assays was found comparing the values after the colonic fermentation. Significant decrease (about 8-19%) between the Q-FC values of GIDD and CF fractions were observed. These results contrasted with the marked increase (around 3.6-fold) showed by the Q-ABTS values of these fractions. The soluble compounds in the large intestine (CFs fractions) represented around 70-76% of the reducing capacity of CF fractions, and about 73-81% of their $ABTS^{\cdot+}$ scavenging ability, whereas the insoluble compounds that remained in the residue (CFr fractions) represented around 32-36% of the Q-FC indexes obtained for CF fractions but only about 10-11% of their Q-ABTS values. As these contributions clearly show, the Q-ABTS values for CF fractions are higher than those obtained when adding the values for their soluble and insoluble components, which represent 91.4% (Sk-S), 84.3% (W-S), and 91.0% (Sd-S) of all colonic fermented products. This finding indicates that possible synergistic interactions might take place between soluble and insoluble antioxidants in CF fractions, which has previously been suggested (Çelic, Gökmen, & Fogliano, 2013). Moreover, these synergisms appear to be specially marked in W-S, suggesting that the presence of compounds from both wine pomace skins and seeds might promote the synergistic effects. However, these interactions do not appear to occur with regard to the reducing power of CF fractions. Compounds contained in the CFr fractions showed the lowest potential antioxidant capacities of all digested products analysed, although they still retained around half of the Q-TAC exhibited by the UD seasonings.

However, a higher antioxidant capacity in the colonic residual contents can be expected following the consumption of RWPSs than of other plant-based foods with lower quantities of highly polymerized phenolic compounds bound to and/or entrapped in the food matrices. The easier digestibility of such foods certainly results in higher bioaccessibility of their antioxidant compounds in the upper intestine (Carbonell-Capella et al., 2014; Papillo et al., 2014). In this regard, the higher Q-FC index obtained for CFr fractions derived from Sk-S and W-S than from Sd-S must finally be pointed out. These results can be partly explained by the much higher degree of polymerization of the proanthocyanidins present in skins than seeds of *Vitis vinífera* L. cv. Tempranillo grapes (Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo Da Silva, 2003), which might restrict their fermentability (Serrano, Puupponen-Pimiä, Dauer,

418 Aura, & Saura-Calixto, 2009) and contribute to the higher reducing capacity exhibited by those compounds
419 retained in the colonic contents after intake of RWPSs containing wine pomace skins.
420
421 **4. Conclusions**
422 Gastrointestinal digestion and colonic fermentation may certainly produce important positive effects on the
423 total antioxidant capacities of seasonings obtained from red wine pomace (RWPSs). This fact is evidenced
424 by the considerably higher antioxidant activities exhibited by most of the digested fractions isolated
425 throughout *in vitro* digestion.
426 Enzymatic gastrointestinal digestion enhanced both the reducing and the antiradical activities, whereas
427 colonic fermentation produced a marked increase in the free radical scavenging capacity, mainly due to the
428 contribution of the colonic fermented solubilized compounds. However, insoluble residues that might pass
429 thought the gut still retained considerable antioxidant capacity, so they may help to counteract the effects of
430 dietary pro-oxidants in the gastrointestinal tract.
431 A general tendency to higher antioxidant capacity was observed for the digested fractions of the seasonings
432 obtained from seedless and whole wine pomace (Sk-S and W-S, respectively) than for those of the
433 seasonings derived from seeds (Sd-S). Furthermore, the higher digestibility of the former seasoning
434 enabled the release of large quantities of bioaccessible antioxidants which could possibly be absorbed in
435 the small intestine. This fact, in addition to the slightly lower fermentability of Sk-S, may balance the
436 antioxidant activity that the different seasonings evaluated might potentially display along the
437 gastrointestinal tract.
438
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443
444 **Conflict of interest**
445 The authors have no conflicts of interest to disclose.
446

447 **References**


448 Acosta-Estrada, B. A., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bound phenolics in foods, a
449 review. *Food Chemistry*, *152*, 46–55.

450 Alminger, M., Aura, A. M., Bohn, T., Dufour, C., El, S. N., Gomes, A., … Santos, C. N. (2014). *In Vitro*
451 Models for Studying Secondary Plant Metabolite Digestion and Bioaccessibility. *Comprehensive*
452 *Reviews in Food Science and Food Safety*, *13*, 413–436.

453 Aura, A.-M., Martin-Lopez, P., O'Leary, K. A., Williamson, G., Oksman-Caldentey, K.-M., Poutanen, K.,
454 & Santos-Buelga, C. (2005). *In vitro* metabolism of anthocyanins by human gut microflora. *European*
455 *Journal of Nutrition*, *44*, 133–142.

456 Bravo, L., Abia, R., & Saura-Calixto, F. (1994). Polyphenols as Dietary Fiber Associated Compounds.
457 Comparative Study on in Vivo and in Vitro properties. *Journal of Agricultural and Food Chemistry*,
458 *42*, 1481–1487.

459 Bravo, L., & Saura-Calixto, F. (1998). Characterization of Dietary Fibre and the *In Vitro* indigestible
460 Fraction of Grape Pomace. *American Journal of Enology and Viticulture*, *49*, 135–141.

461 Carbonell-Capella, J. M., Buniowska, M., Barba, F. J., Esteve, M. J., & Frígola, A. (2014). Analytical
462 Methods for Determining Bioavailability and Bioaccessibility of Bioactive Compounds from Fruits
463 and Vegetables: A Review. *Comprehensive Reviews in Food Science and Food Safety*, *13*, 155–171.

464 Çelic, E. E., Gökmen, V., & Fogliano, V. (2013). Soluble antioxidant compounds regenerate the
465 antioxidants bound to insoluble parts of foods. *Journal of Agricultural and Food Chemistry*, *61*,
466 10329–10334.

467 Cueva, C., Sánchez-Patán, F., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., …
468 Moreno-Arribas, M. V. (2013). *In vitro* fermentation of grape seed flavan-3-ol fractions by human
469 faecal microbiota: changes in microbial groups and phenolic metabolites. *FEMS Microbiology*
470 *Ecology*, *83*, 792–805.

471 Del Pino-García, R., García-Lomillo, J., Rivero-Pérez, M. D., González-Sanjosé, M. L., & Muñiz, P.
472 (2015). Adaptation and Validation of QUick, Easy, New, CHeap, and Reproducible (QUENCHER)
473 Antioxidant Capacity Assays in Model Products Obtained from Residual Wine Pomace. *Journal of*
474 *Agricultural and Food Chemistry*, *63*, 6922–6931.

475 Delgado-Andrade, C., Conde-Aguilera, J. A., Haro, A., Pastoriza de la Cueva, S., & Rufián-Henares, J. Á.

(2010). A combined procedure to evaluate the global antioxidant response of bread. *Journal of Cereal Science*, *52*, 239–246.

Fernández-Panchón, M., Villano, D., Troncoso, A., & García-Parrilla, M. (2008). Antioxidant Activity of Phenolic Compounds: From *In Vitro* Results to *In Vivo* Evidence. *Critical Reviews in Food Science and Nutrition*, *48*, 649–671.

García-Lomillo, J., González-Sanjosé, M. L., Del Pino-García, R., Rivero-Pérez, M. D., & Muñiz, P. (2014). Antioxidant and Antimicrobial Properties of Wine By-products and their Potential Uses in the Food Industry. *Journal of Agricultural and Food Chemistry*, *62*, 12595–12602.

Gökmen, V., Serpen, A., & Fogliano, V. (2009). Direct measurement of the total antioxidant capacity of foods: the "QUENCHER" approach. *Trends in Food Science & Technology*, *20*, 278–288.

González-Sanjosé, M. L., García-Lomillo, J., Del Pino-García, R., Muñiz-Rodríguez, P., & Rivero-Pérez, M. D. (2013). Universidad de Burgos. ES Patent 2524870B2.

Goñi, I., Martín, N., & Saura-Calixto, F. (2005). *In vitro* digestibility and intestinal fermentation of grape seed and peel. *Food Chemistry*, *90*, 281–286.

Goñi, I., & Serrano, J. (2005). The intake of dietary fiber from grape seeds modifies the antioxidant status in rat cecum. *Journal of the Science of Food and Agriculture*, *85*, 1877–1881.

Halliwell, B., Zhao, K., & Whiteman, M. (2000). The gastrointestinal tract: A major site of antioxidant action? *Free Radical Research*, *33*, 819–830.

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry*, *13*, 572–584.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry*, *125*, 1–12.

Kroon, P. A., Faulds, C. B., Ryden, P., Robertson, J. A., & Williamson, G. (1997). Release of Covalently Bound Ferulic Acid from Fiber in the Human Colon. *Journal of Agricultural and Food Chemistry*, *45*, 661–667.

Le Bourvellec, C., & Renard, C. M. G. C. (2012). Interactions between Polyphenols and Macromolecules: Quantification Methods and Mechanisms. *Critical Reviews in Food Science and Nutrition*, *52*, 213–248.

López-Oliva, M. E., Agis-Torres, A., García-Palencia, P., Goñi, I., & Munoz-Martínez, E. (2006).

Induction of epithelial hypoplasia in rat cecal and distal colonic mucosa by grape antioxidant dietary fiber. *Nutrition Research*, *26*, 651–658.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition*, *79*, 727–747.

McDougall, G. J., Fyffe, S., Dobson, P., & Stewart, D. (2005). Anthocyanins from red wine - Their stability under simulated gastrointestinal digestion. *Phytochemistry*, *66*, 2540–2548.

Monagas, M., Gómez-Cordovés, C., Bartolomé, B., Laureano, O., & Ricardo Da Silva, J. M. (2003). Monomeric, Oligomeric, and Polymeric Flavan-3-ol Composition of Wines and Grapes from *Vitis vinifera* L. cv. Graciano, Tempranillo, and Cabernet Sauvignon. *Journal of Agricultural and Food Chemistry*, *51*, 6475–6481.

Palafox-Carlos, H., Ayala-Zavala, J. F., & González-Aguilar, G. A. (2011). The Role Dietary Fiber Bioaccesibility and Bioavailability Fruit and Vegetable Antioxidants. *Journal of Food Science*, *76*, R6–R15.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*, 270–278.

Papillo, V. A., Vitaglione, P., Graziani, G., Gokmen, V., & Fogliano, V. (2014). Release of Antioxidant Capacity from Five Plant Foods during a Multistep Enzymatic Digestion Protocol. *Journal of Agricultural and Food Chemistry*, *62*, 4119–4126.

Pastoriza, S., Delgado-Andrade, C., Haro, A., & Rufián-Henares, J. A. (2011). A physiologic approach to test the global antioxidant response of foods. the GAR method. *Food Chemistry*, *129*, 1926–1932.

Pérez-Jiménez, J., Díaz-Rubio, M. E., & Saura-Calixto, F. (2013). Non-extractable polyphenols, a major dietary antioxidant: occurrence, metabolic fate and health effects. *Nutrition Research Reviews*, *26*, 118–129.

Rein, M. J., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. K., & da Silva Pinto, M. (2013). Bioavailability of bioactive food compounds: A challenging journey to bioefficacy. *British Journal of Clinical Pharmacology*, *75*, 588–602.

Rufián-Henares, J. A., & Delgado-Andrade, C. (2009). Effect of digestive process on Maillard reaction indexes and antioxidant properties of breakfast cereals. *Food Research International*, *42*, 394–400.

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., …

Bartolomé, B. (2012). Gut microbial catabolism of grape seed flavan-3-ols by human faecal microbiota. Targetted analysis of precursor compounds, intermediate metabolites and end-products. *Food Chemistry*, *131*, 337–347.

Saura-Calixto, F., Pérez-Jiménez, J., Touriño, S., Serrano, J., Fuguet, E., Torres, J. L., & Goñi, I. (2010). Proanthocyanidin metabolites associated with dietary fibre from *in vitro* colonic fermentation and proanthocyanidin metabolites in human plasma. *Molecular Nutrition and Food Research*, *54*, 939–946.

Saura-Calixto, F., Serrano, J., & Goñi, I. (2007). Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chemistry*, *101*, 492–501.

Scalbert, A., & Williamson, G. (2000). Dietary Intake and Bioavailability of Polyphenols. *The Journal of Nutrition*, *130*, 2073S–2085S.

Serrano, J., Puupponen-Pimiä, R., Dauer, A., Aura, A. M., & Saura-Calixto, F. (2009). Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition and Food Research*, *53*, S310–S329.

Sun, J., Chu, Y. F., Wu, X., & Liu, R. H. (2002). Antioxidant and Antiproliferative Activities of Common Fruits. *Journal of Agricultural and Food Chemistry*, *50*, 7449–7454.

Tabernero, M., Venema, K., Maathuis, A. J. H., & Saura-Calixto, F. (2011). Metabolite Production during in Vitro Colonic Fermentation of Dietary Fiber: Analysis and Comparison of Two European Diets. *Journal of Agricultural and Food Chemistry*, *59*, 591–598.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). *In vitro* bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry*, *120*, 599–606.

Touriño, S., Mateos-Martín, M. L., Fuguet, E., Vinardell, M. P., Cascante, M., & Torres, J. L. (2011). Metabolites in Contact with the Rat Digestive Tract after Ingestion of a Phenolic-Rich Dietary Fiber Matrix. *Journal of Agricultural and Food Chemistry*, *59*, 5955–5963.

Velderrain-Rodríguez, G. R., Palafox-Carlos, H., Wall-Medrano, A., Ayala-Zavala, J. F., Chen, C.-Y. O., Robles-Sánchez, M., … González-Aguilar, G. a. (2014). Phenolic compounds: their journey after intake. *Food & Function*, *5*, 189–197.

**Figure captions**

**Figure 1. Diagram of the main steps performed during the complete *in vitro* digestion of the seasonings.** The different fractions under study were: GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue.

**Figure 2. Antioxidant activities of the *in vitro* digested fractions derived from red wine pomace seasonings: absolute approach.** Total antioxidant capacities determined using the Q-FC (Folin-Ciocalteu) (**A**) and Q-ABTS (**B**) assays. Sk-S: seasoning obtained from seedless red wine pomace; W-S: seasoning obtained from whole red wine pomace; Sd-S: seasoning obtained from seeds. UD: undigested; GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue. Q-TAC values are expressed as mean ± standard deviation (n = 3). GAE: gallic acid equivalents. TE: Trolox equivalents. Significant differences ($p < 0.05$) among fractions (UD, GID, GIDD, CF, CFs, and CFr) for each seasoning are indicated by Roman letters. Significant differences ($p < 0.05$) among seasonings (Sk-S, W-S, and Sd-S) for each digested fraction are indicated by Greek letters.

**Figure 3. Antioxidant activities of the *in vitro* digested fractions derived from red wine pomace seasonings: relative approach.** Total antioxidant capacities determined using the Q-FC (Folin-Ciocalteu) (**A**) and Q-ABTS (**B**) assays. Sk-S: seasoning obtained from seedless red wine pomace; W-S: seasoning obtained from whole red wine pomace; Sd-S: seasoning obtained from seeds. UD: undigested; GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue. Q-TAC values are given as mean ± standard deviation (n = 3). GAE: gallic acid equivalents. TE: Trolox equivalents. Significant differences ($p < 0.05$) among fractions (UD, GID, GIDD, CF, CFs, and CFr) for each seasoning are indicated by Roman letters. Significant differences ($p < 0.05$) among seasonings (Sk-S, W-S, and Sd-S) for each digested fraction are indicated by Greek letters.

**Tables**

**Table 1**. **Yields of each digested fraction obtained after *in vitro* pre- and post-colonic digestion of the studied seasonings**.

| Digested fractions [a] | RWPSs[b] | | |
|---|---|---|---|
| | Sk-S | W-S | Sd-S |
| GID | 92.5 ± 1.4 d / α | 92.5 ± 1.1 d / α | 88.1 ± 1.3 bc /α |
| GIDD | 68.4 ± 0.7 c / α | 71.6 ± 1.5 b / β | 82.1 ± 0.9 b / γ |
| CF | 69.3 ± 2.2 c / α | 77.6 ± 1.5 c / α | 89.4 ± 7.6 c / β |
| CFs | 34.9 ± 0.8 a / α | 41.6 ± 0.5 a / β | 48.6 ± 1.9 a / γ |
| CFr | 40.8 ± 0.8 b / α | 43.1 ± 1.2 a / α | 47.9 ± 2.1 a / β |

Yields (%, w/w) with respect to undigested seasonings (100%, w/w) are given as the mean ± standard deviation (n = 3).

*a)* Digested fractions: GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue.

*b)* Red wine pomace seasoning (RWPSs) which were obtained from: seedless wine pomace (Sk-S); whole wine pomace (W-S); and seeds (Sd-S).

Significant differences ($p < 0.05$) among fractions (UD, GID, GIDD, CF, CFs, and CFr) for each seasoning are indicated by Roman letters. Significant differences ($p < 0.05$) among seasonings (Sk-S, W-S, and Sd-S) for each digested fraction are indicated by Greek letters.

**Figure 1**. (One-column figure)

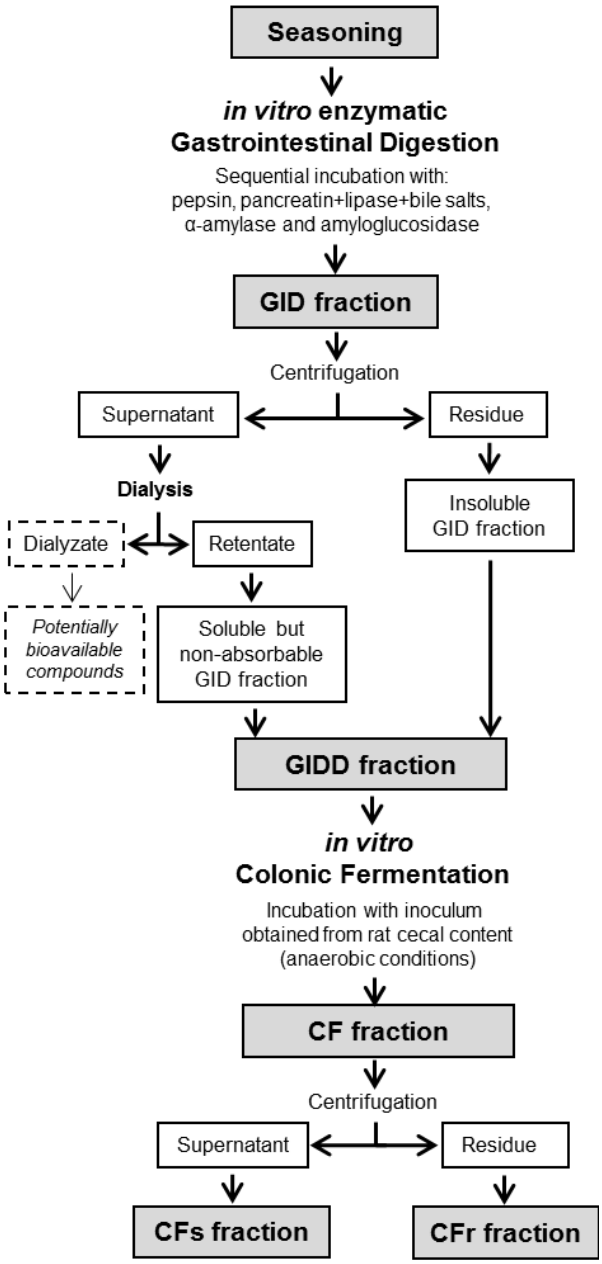

**Figure 2**. (Two-column figure)

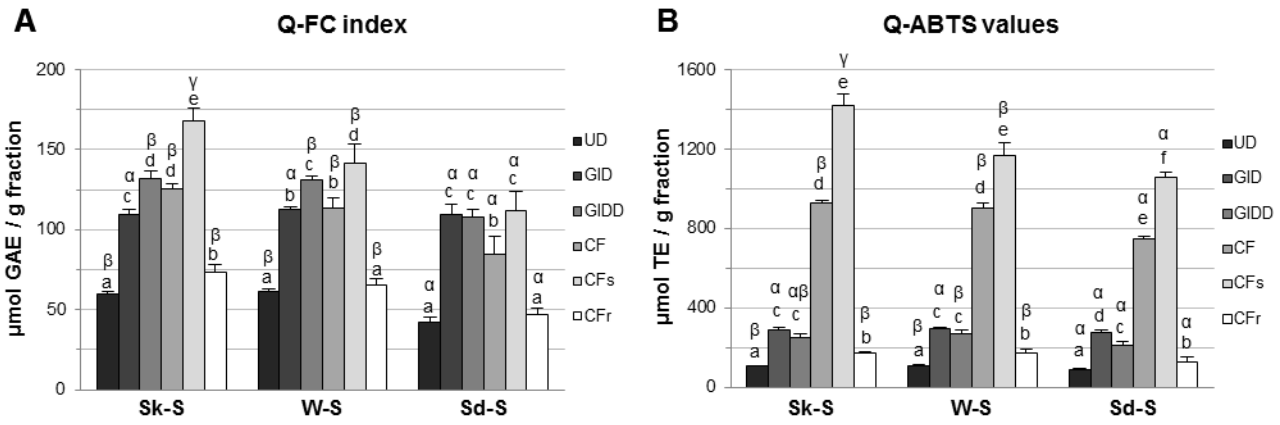

**Figure 3**. (Two-column figure)