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In vitro digestion of dairy and egg products enriched with grape extracts: Effect of the food matrix on polyphenol bioaccessibility and antioxidant activity

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Abstract

The aim of this study was to evaluate the effect of the food matrix on polyphenol bioaccessibility and antioxidant activity during the *in-vitro* digestion of dairy and egg products enriched with grape extracts (GE). Four GE-enriched matrices produced under industrial conditions (custard dessert, milkshake, pancake and omelet) and the GE dissolved in water (control solution) were submitted to *in vitro* digestion and the bioaccessibility of the major classes of polyphenols as well as the evolution of the antioxidant activity of the matrices were monitored at oral, gastric and intestinal level. Apart from the digestion effect itself, the release, stability and solubility of polyphenols was governed by mainly two factors: 1- the composition and structure of the food matrices and 2- the class of polyphenol. Results showed that the inclusion of the GE extracts into the different egg and dairy food matrices greatly impacted the release and solubility of anthocyanins and proanthocyanidins during digestion, especially in the solid food matrices and during the oral and gastric phases of digestion. Also, the presence of the food matrices protected anthocyanin from degradation during the intestinal phase. However, if the total phenolic content is considered at the end of the whole digestion process, the proportion of soluble (bioaccessible) and insoluble phenolics delivered by the enriched-matrices was quite similar to that of the control solution. On the contrary, the food matrix effect did not affect the antioxidant activity of the matrices, which remained constant during the oral and gastric phases but greatly increased during the intestinal phase of digestion. Among the GE-enriched matrices, omelet presented higher recoveries of total phenolics and antioxidant activity at the end of digestion.

Keywords: *in vitro* digestion; bioaccessibility; food matrix; polyphenols; anthocyanins; proanthocyanidins.

1. Introduction

Grape extracts (GE), a by–product resulting from juice and wine making process, are very promising ingredients for the food industry. They are natural, inexpensive, can be obtained in considerable quantities and represent a very good source of phenolic compounds such as anthocyanins, proanthocyanidins, flavanols, flavonols, phenolic acids and stilbenes. The list of biological activities and health benefits associated with the intake of grape phenolic compounds is large. Antioxidant, cardioprotective, anti-carcinogenic, anti-inflammatory or anti-aging properties have been observed both *in vitro* and *in vivo* (Xia, Deng, Guo, & Li, 2010; Teixeira et al., 2014; Soares De Moura et al., 2002). Due to these myriad of healthy effects, polyphenol-rich grape products have been suggested as potential effective candidates in preventing metabolic diseases such as metabolic syndrome, a risk factor for cardiovascular disease and mortality that affects nearly one-fourth of the developed world's population (Chuang & McIntosh, 2011).

Absorption, metabolism and bioavailability, i.e. the proportion of a compound that reaches systemic circulation and remains available to be used by cells or tissues, are indicative concepts when assessing the beneficial effects of polyphenols. However, their measurement usually requires high cost and complicated *in vivo* studies. In addition, some health effects of polyphenols may not require their absorption through the gut barrier: anthocyanin antioxidant activity seems to protect against the oxidative damage implicated in many degenerative diseases of the gastrointestinal tract such as colorectal cancer or inflammatory bowel disease (D'evoli et al., 2013). On the contrary, the proportion of polyphenols released from the food matrix and solubilized into the digestive fluids (bioaccessibility) is a key step that has to be accomplished in all cases since only bioaccessible polyphenols can be further absorbed and remain bioavailable. Although it is difficult to exactly mimic the physiological conditions taking place *in vivo*, the use of *in vitro* digestion models is ideal for this kind of studies due to their simplicity, ease of application and low cost.

Structure and composition of the food matrix in which polyphenols are included are factors that can either enhance or prevent the release and stability of these compounds during digestion and hence, their effectiveness. The effect of the co-digestion of polyphenols with different food components, matrices or diets has been proven to affect their digestibility, bioaccessibility or antioxidant activity (Ribnicky et al., 2014; Sengul, Surek, & Nilufer-Erdil, 2014; Gordon J. McDougall, Dobson, Smith, Blake, & Stewart, 2005; Dupas, Marsset-Baglieri, Ordonaud, Ducept, & Maillard, 2006). The release from liquid or solid food matrices has been also studied but mainly in naturally enriched matrices like fruits and juices (Tagliazucchi, Verzelloni, & Conte, 2012; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). However, the inclusion, release and digestibility of polyphenols into and from non-naturally enriched food matrices have been scarcely studied to date.

On the other hand, dairy and egg products are excellent foods to be fortified: they have natural and great nutritional properties, are accepted worldwide by all age groups, can be eaten on a daily basis and exist under a great variety of forms and structures.

In the present study, we investigated the effect of the food matrix on polyphenol bioaccessibility and antioxidant activity during the *in-vitro* digestion of dairy and egg products fortified with grape extracts. For this purpose, four GE-enriched matrices produced under industrial conditions (custard dessert, milkshake, pancake and omelet) and a GE control solution (no matrix present) were submitted to an *in vitro* digestion model and the bioaccessibility of the major classes of polyphenols as well as the evolution of the antioxidant activity of the matrices were monitored at oral, gastric and intestinal levels.

2. Material and methods

2.1. Chemicals

All solvents (HPLC grade) and chemicals were purchased from Sigma Aldrich (St Louis, MO, USA) unless further specified. Type VI-B α-amylase from porcine pancreas (A3176), pepsin from porcine gastric mucosa (P6887), porcine bile extract (B8631), pancreatin from porcine pancreas 8xUSP (P7545), potato starch (S2004), 3,5 dinitrosalicylic acid (D0550), D(+) maltose monohydrate from potato (M5885), bovine blood hemoglobin (H2500), N-p-tosyl-L-arginine methyl ester hydrochloride (TAME) (T4626), N-benzozyl-L-tyrosine ethyl ester (BTEE) (B6125), aminoantipyrine (4-AP) (O6800), type II horseradish peroxidase (HRP) (P8250), catechin standard (43412), fluorescein sodium salt (F6377), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) (238813), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) (440914), 2,4,6- tripyridyl-S-triazine (TPTZ) (T1253), epicatechin standard (68097), epigallocatechin standard (E3768), epicatechin 3-O-gallate standard (E3893), phloroglucinol (P3502) and ascorbic acid (A1300000) were also supplied by Sigma Aldrich. Malvidin 3-O-glucoside (0911S) was purchased from Extrasynthese (Lyon, France). Bile salts quantification was performed using a DiaSys commercial kit (Cat. No. 1 2212 99 90 313).

2.2. Grape extract

The GE used as source of polyphenols, registered as Eminol®, was provided by Grupo Matarromera. The extract, which is obtained by means of a patented extraction process (ES 2 309 032), comes 100% from red grapes (*Vitis vinifera*, variety Tempranillo) harvested from vineyards located in the Designation of Origin Ribera de Duero (Castilla and León, Spain).

The phenolic composition of the GE (Table 1) was determined after extraction with a methanol/water/ acetone/TFA (25/3045//0.037) solution as described by Mané et al., (2007). Phenolic and hydroxycinnamic acids, tryptophan, flavonols, anthocyanins and flavan-3-ol monomers

and dimers were analyzed at 35°C using a Waters Acquity UPLC-DAD system (Milford, MA) equipped with an Acquity BEH C18 column (150 × 1 mm i.d., 1.7 µm; Waters, Milford, MA). Mobile phase consisted of water/formic acid (99/1, v/v) (eluent A) and methanol/formic acid (99/1, v/v) (eluent B). Flow rate was 0.08 mL/min. The elution program was as follows: isocratic for 1 min with 2% B, 2-18% B (1-7 min), isocratic with 18% B (7-9 min), 18-30% B (9-12 min), isocratic with 30% B (12-14 min), 30-75% B (14-27 min), 75-95% B (27-32 min), isocratic with 95% B (32-37 min). Identification was achieved on the basis of UV-visible and mass spectra. ESI-MS/MS analyses were performed with a Bruker Daltonics Amazon (Bremen, Germany) mass spectrometer equipped with an electrospray source and an ion trap mass analyzer. The spectrometer was operated in the positive ion mode (capillary voltage: 2.5 kV; end plate off set: -500V; temperature: 200°C; nebulizer gas: 10 psi dry gas: 5L/min) and negative mode (capillary voltage: -4.5 kV; end plate off set: -400 V; temperature: 200°C; nebulizer gas: 10 psi and dry gas: 5 L/min). Collision energy for fragmentation used for MS2 experiments were set at 1.

Concentrations were calculated from peak areas at 520 nm for anthocyanins, at 360 nm for flavonols, at 320 nm for hydroxycinnamic acids and at 280 for flavan-3-ols, tryptophan, and gallic acid, using external calibrations. Malvidin 3-glucoside, quercetin 3-glucoside and caffeic acid were used as standards for quantification of anthocyanins, flavonols and hydroxycinnamic acids, respectively.

2.3. Enriched food matrices and control solution

Two liquid (milkshake and custard dessert) and two solid (pancake and omelet) enriched food matrices were produced and supplied by ADEXGO Ltd (Balatonfüred, Hungary). Milkshake was provided as a packed-powder that had to be rehydrated in water before use. Custard dessert was provided as a combined product in which the GE was provided in an independent polyethylene bag and had to be added and mixed with a commercial custard dessert before use. GE enriched pancakes and omelets were provided frozen in modified atmosphere trays. The control solution was prepared by directly dissolving the GE (provided in an independent polyethylene bag) in water at 40 mg/ml. Food matrices detailed composition and production flow-charts are presented in Fig. 1.

2.4. In vitro oro-gastro-intestinal digestion

The control GE solution and the GE-enriched matrices were subjected to successive oral, gastric and intestinal digestion following a new standardized static digestion method based on physiologically relevant conditions developed by Minekus et al., (2014). This model was developed by the COST action INFOGEST (www.cost-infogest.eu).

Before digestion, all the matrices were prepared/defrosted and enough quantity of each one was freeze dried for further polyphenol extraction and quantification. In addition, the enzymatic activities of individual enzymes and pancreatin, as well as the bile salt concentration in the porcine bile extract were determined following the protocols proposed by Minekus et al., (2014).

Custard dessert, milkshake and control solution were not subjected to mastication due to their liquid structure. For pancake and omelet, mastication was simulated by using a manual mincer (Eddington's Mincer Pro. Product code 86002, Berkshire, UK). Then, 25 g of the liquid or minced matrices were mixed with 17.5 ml of simulated salivary fluid electrolyte stock solution (SSF), 125 µl of 0.3 M $CaCl_2$, 4.875 ml of water and 2.5 ml of α- amylase solution in SSF (1,500U/ml), all of them pre-warmed at 37°C. The mix was thoroughly mixed and incubated under stirring for 2 min at 37°C. Gastric digestion continued by the immediate addition to the oral bolus of 37.5 ml of simulated gastric fluid electrolyte stock solution (SGF), 25 µl of 0.3 M $CaCl_2$ and enough volume of 1 M HCl to adjust the pH to 3. After the addition of 10 ml of porcine pepsin solution made up in SGF (20,000 U/ml) and distilled water to a final volume of 100 ml, the mix was thoroughly mixed and incubated under stirring for 2 h at 37°C. At the end of gastric digestion, intestinal digestion was mimicked by the addition of 55 ml of simulated intestinal fluid electrolyte stock solution (SIF), 200 µl of 0.3 M $CaCl_2$ and 12.5 ml of 160 mM bile extract solution in SIF. After adjusting the pH to 7 with 1M NaOH, 25 ml of a pancreatin solution made up in SIF (800 U/ml, based on trypsin activity) and distilled water to a final volume of 200 ml were added. The final mix was then digested under stirring for 2 h at 37°C.

Instead of withdrawing aliquots from the reaction vessel at the end of the oral, gastric or intestinal step, individual digestions were carried out for each phase of digestion. Also, in order to ensure the stability of the phenolic compounds, the oral and intestinal samples were acidified to pH 2 right after their digestion. Finally, all digestions were immediately centrifuged at 21,000*g* and 5°C for 20 min, and the supernatants and pellets collected, freeze dried and stored until further used for polyphenol extraction.

2.5. Polyphenol extraction

Extractions from the freeze dried digested fractions and matrices were performed in triplicate following the protocol developed by Mané et al., (2007). Briefly, 200mg of the freeze dried samples were suspended in 8 ml of methanol and stirred during 2 min. Then, 24ml of an acetone/water/TFA mixture (60/40/0.05) were added and stirred during 1h at room temperature. Finally, after a 15min centrifugation step at 10,000*g* and room temperature, 1.5ml of supernatant was taken from each sample and fully evaporated in a Savant SVC200H Speedvac concentrator

(Thermo, NY, USA). The remaining polyphenol and antioxidant analyses were performed on these pellets.

2.6. Total phenolic quantification

Although widely used to report total phenolic content, the Folin-Ciocalteu method has substantial interferences with many other non-phenolic molecules such as ascorbic acid, reducing sugars, peptides and purines (Singleton, Orthofer, & Lamuela-Raventos, 1999; Slinkard & Singleton, 1977). Since many of these compounds were present in our matrices or could be produced during the course of digestion, a more specific enzymatic method developed by Stevanato et al., (2004) was used. The method, based on the oxidation of phenols to phenoxyl radicals by the horseradish peroxidase enzyme (HRP) has already been used in samples after *in vitro* digestion (Tagliazucchi et al., 2010; Tagliazucchi, Verzelloni, & Conte, 2012). Briefly, 0.1 ml of each resolubilized pellet or catechin standard solution was added to 3 ml of 0.1 M potassium phosphate-buffered solution, pH 8, containing 3 mM 4-AP, 2 mM H_2O_2 and 10 U of HRP. The absorbance value was read at 500 nm after exactly 15 min incubation. Catechin standard solutions in water ranged from 2 to 600 µg/ml. Each sample was quantified in triplicate. Results were expressed in milligrams of catechin equivalents per 100 grams of matrix.

2.7. Anthocyanin quantification

Total anthocyanin content in the matrices and *in vitro* digestion fractions were quantified by RP-HPLC. After redissolution of the pellets in H_2O/methanol/formic acid (75/11.25/13.75 v/v) and filtration through 0.2µm cellulose filters (Sartorius ministart RC4 17821), anthocyanins were separated on a Grace/Vydac 201TP C18 column (250x4.6mm particle size 5 µm particle size, 4.6mm by 250mm length) connected to an Agilent 1100 HPLC system provided with a photo diode array detector (Agilent technologies, Massy, France). Elution was performed according to a previous method with slight modifications (Sanza, Domínguez, & Merino, 2004). The chromatographic conditions were: 30°C; 50µl injection volume; 0.5 ml/min flow-rate; eluent A was methanol; eluent B was methanol/water/formic acid (45/45/10, v/v), and eluent C was formic acid/water (15/85, v/v). Zero-time conditions were A/B/C (0/25/75); at 25 min the pump was adjusted to A/B/C (0/80/20) and kept at such for 10 min; at 38–43 min the conditions were A/B/C (100/0/0). At 45 min the initial conditions were reached again and maintained during 15 min before the next injection. Absorbance was measured at 528nm and quantification (calculated as mg of malvidin-3-O-glucoside equivalents (M3OGE) /100g of food matrix) was carried out by means of an external calibration method and by measurement of each peak area.

2.8. Proanthocyanidin analysis

Proanthocyanidin composition and mean degree of polymerization (mDP) were determined after phloroglucinolysis as described by Fournand et al., (2006). After dissolution of the pellets in 250µL of methanol-HCl 0.2N containing phloroglucinol (50g/L) and L-ascorbic acid (10g/L), samples were heated in a water bath at 50°C for 20min. Then, the phloroglucinolysis reaction was stopped by adding an equal volume of ammonium formate 200mM and the reaction medium was analyzed by UPLC-DAD-ESI-IT-MS as previously describe in section 2.2.

The concentration of each unit released after phloroglucinolysis was calculated from its peak area at 280 nm (i.e. flavan-3-ols from terminal units and the corresponding phloroglucinol derivatives from extension and upper units), using the calibration curve established for the corresponding standard, either commercial ((+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-gallate) or purified in the laboratory (phloroglucinol derivatives).The mDP of proanthocyanidins, which took into consideration the free flavan-3-ol monomers present in the sample before depolymerization, was calculated as follows:

$$mDP = \sum Total\ unit\ concentration / \sum terminal\ units\ concentration$$

2.9. Calculations

For each phenolic compound analyzed, the proportion released from the food matrices or control solution into the digestive fluids (bioaccessibility) and the proportion that remained insoluble (non bioaccessible) during the different phases of digestion was calculated as follows:

$$Bioaccessibility(\%) = (Qtt\ supernatant / Qtt\ digested) \times 100$$

$$Insoluble\ (\%) = (Qtt\ pellet / Qtt\ digested) \times 100$$

where $Qtt\ supernatant$ is the compound quantity (in mg) in the supernatant at the end of the corresponding phase of digestion, $Qtt\ pellet$ is the compound quantity (in mg) in the pellet at the end of the corresponding phase of digestion and $Qtt\ digested$ is the compound quantity (in mg) that was submitted to digestion (based on the results obtained after the chemical extraction in the control/matrices). Finally, the total recovery for each class of polyphenol was calculated as follows:

$$Total\ recovery = Bioaccessibility(\%) + Insoluble\ (\%)$$

2.10 Antioxidant activity

2.10.1. ORAC –Fluorescein (ORAC-FL) method

ORAC– FL assays were carried out in 75 mM phosphate buffer pH 7.4 (PBS) following the protocol of Dávalos et al. (2003) which was developed from the original ORAC-FL assay of Ou et al.

(2001). Briefly, 20 µl of resolubilized pellet were placed in triplicate into the microplate. After the addition of 120 µl of a 116.6 nM fluorescein solution in PBS, the plate was incubated for 15 min at 37°C. Then 60 µl of a 14 mM AAPH solution in PBS were rapidly added and the plate was immediately placed in a SAFAS Monaco FLX-Xenius spectrofluorometer; fluorescence was recorded every min for 80 min. In order to compare different plates, the photomultiplier voltage of the first plate was automatically adjusted and then kept fixed during the following readings. A blank (fluorescein + AAPH) using PBS buffer instead of the antioxidant solution and ten calibration solutions using Trolox (1.12-15 µM, final concentration) as antioxidant were also carried out in each assay. Antioxidant curves (fluorescence *vs* time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor fluorescence $_{blank,t=0}$/fluorescence $_{sample,t=0}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + \sum_{i=1}^{i=80} fi/fo$$

where f_0 is the initial fluorescence reading at 0 min and fi is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. ORAC-FL values were expressed as mmol of Trolox equivalents per 100 g of food matrix by using the standard curve calculated for each assay.

2.10.2. FRAP method

The reducing ability of samples by single electron transfer was determined by the ferric reducing ability of plasma (FRAP) assay (Benzie & Strain, 1996). In brief, 3 ml of freshly prepared FRAP reagent (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ at a ratio of 10:1:1) were added to 0.1 ml of each resolubilized pellet. After exactly 8 min, the absorbance was read at 593 nm. Ascorbic acid (vitamin C) solutions ranging from 0.58 to 150 mg/ml were used to create a calibration curve. The results were expressed as milligrams of vitamin C equivalent antioxidant activity (VCEAC) per 100 g of food matrix.

2.11. Statistical assays

Polyphenol extraction in the matrices and *in vitro* digestion fractions were performed in triplicate. Anthocyanin quantification, polyphenols quantification analyses and both antioxidant assays were performed in all the extracted samples (n=3) while proanthocyanidin analysis were performed in only one of the extracts (n=1). Results were expressed in means ± standard deviation (SD). Correlations were established by Pearson regression analysis at a 95% significance level. Comparison of polyphenols' bioaccessibilities/recoveries between the different matrices and steps of

digestion were studied by a post one-way ANOVA Tukey's test at α= 0.01. All statistical analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA).

3. Results

3.1. Evaluation of the food matrix effect on the stability and solubility of phenolic compounds during in vitro digestion

The first part of the study was focused on the evaluation of the food matrix effect on the stability and solubility of phenolic compounds during the different phases of *in vitro* digestion. In order to achieve this goal, the quantification of total phenolic content and of the two most abundant groups of polyphenols present in the GE used in the study, namely anthocyanins and proanthocyanidins (Table 1), was carried out in the soluble and insoluble fractions of the oral, gastric and intestinal phases of *in vitro* digestion. It must be underline that the quantified polyphenols only constituted the 10% of the GE.

3.1.1 Anthocyanins

The results reported in Fig.2 show that, when digested in the absence of any food matrix, anthocyanin stability was not affected by the oral nor the gastric phases of digestion (total AC recoveries in these phases were 96 and 101 %, respectively). In addition, most anthocyanins remained soluble, as indicated by the small proportion (around 10%) recovered in the pellet/insoluble fraction. On the other hand, the transition from the acid gastric phase to the neutral/basic intestinal phase produced big changes in anthocyanin stability and solubility: after 2h of digestion only 55 % of total anthocyanins could be detected: 30% bioaccessible and 25 % in the insoluble fraction.

In custard dessert and milkshake, the presence of the food matrix did not greatly affect anthocyanin stability and solubility with respect to the control solution. The main differences were found in the oral phase, where the percentage of insoluble anthocyanins was higher (24 and 30% in custard and milkshake, respectively, *vs* the 12% in the control), and in the intestinal phase, where the percentage of soluble anthocyanins was significantly higher (39 and 47% in custard and milkshake, respectively, vs 30% in the control). Among the two liquid matrices, milkshake presented slightly higher values of total recovery during the whole digestion.

On the other hand, anthocyanins included into the pancake and omelet did behave differently from those of the control solution and the liquid food matrices. During the oral phase, a very high proportion of anthocyanins remained insoluble (70 and 82% in pancake and omelet, respectively). The release of anthocyanins occurred during the subsequent gastric phase. At the end of it, around 60% of anthocyanins were already soluble. Surprisingly, the final incubation with

pancreatic solution did not have any significant effect ($p<0.01$) and total anthocyanin content remained constant in both matrices with respect to the previous gastric phase. In addition, the proportion of soluble/insoluble anthocyanins did not change after the 2h of intestinal digestion. Among the two solid matrices, omelet presented slightly higher values of total recovery than pancake during the whole digestion.

3.1.2. Proanthocyanidins

As shown in Fig. 3, proanthocyanidin stability and solubility during the digestion of the control solution was similar to that of anthocyanins, particularly during the oral and gastric steps of digestion. During these phases, no proanthocyanidins were degraded (total recovery values were over 100% in both cases) and, except for a small proportion of proanthocyanidins that precipitated during the gastric phase, most of them remained soluble (88% and 77 % in the oral and gastric steps, respectively). During the intestinal phase, although the degradation of proanthocyanidins was less pronounced than that of anthocyanins (83% were recovered), most of them were recovered in the insoluble fraction at the end of digestion (57%). Finally, mDP analyses showed that proanthocyanidin mDP was always higher in the insoluble fraction than in the soluble ones and 2- the mDP of both fractions was much lower after the final step of digestion.

The inclusion of the GE in the custard dessert and milkshake did not affect proanthocyanidin stability during digestion, as indicated by the total recovery values obtained at the end of the whole digestion process (95% and 117%, respectively). However, it considerably affected their solubility during the oral and gastric phases; contrary to the control solution, most proanthocyanidins remained insoluble at the end of both steps of digestion whereas the highest solubility values were obtained at the end of the intestinal phase. In particular, while only 25% and 33% of proanthocyanidins were soluble in custard and milkshake, respectively, their solubility increased during te intestinal digestion, up to 60 and 83%, respectively. The exact opposite behavior was observed for the control solution, in which proanthocyanidin solubility was 88 and 26% at the end of the oral and intestinal phases, respectively. In terms of mDP, the tendency observed in these matrices was the same as in the control solution. Actually, their degree of depolymerization at the end of digestion was almost identical to that of the control solution: 1.1 for the soluble fractions and around 3.5 for the insoluble ones.

In omelet and pancake, proanthocyanidins behave in a similar way than in the liquid matrices, i.e. their solubility increased along digestion. However, the proportion of insoluble proanthocyanidins was much higher. In fact, except during the intestinal digestion of the omelet, where a significant proportion of proanthocyanidins were resolubilized into the liquid phase (47%), proanthocyanidin solubility never exceeded 18%. Regarding stability, despite the low recoveries

found during the oral and gastric phases, all proanthocyanidins were recovered at the end of digestion in omelet (101%). In pancake, on the other hand, a small proportion of them seemed to have been degraded or irreversibly absorbed (total recovery after digestion was 77%). In terms of degree of polymerization, the mDP values were higher in the insoluble fraction. However, the mDP values found in the solid matrices were generally lower than those of the control solution and liquid food matrices, particularly during the oral and gastric phases.

3.1.3. Total phenolics

The evolution of the total phenolic recoveries in the soluble and insoluble fractions of the control and GE- enriched matrices during *in vitro* digestion is presented in Fig. 4. In the control solution, phenolic compounds remained stable during digestion as indicated by the high recovery rates, close to 100% or higher. Regarding solubility, most phenolics remained soluble during the oral and gastric phases (87% and 80%, respectively). On the contrary, most of them precipitated during the final intestinal phase of digestion (75%).

In custard and milkshake, although phenolic solubility had the same tendency than in the control solution, i.e. decreasing during the course of digestion, a large proportion of phenolic compounds remained insoluble (from 34 to 75 %). Except for the intestinal phase of the custard dessert where total phenolic recovery was close to 100%, total phenolic recoveries were around 10 to 30% smaller in these liquid matrices than in the control solution. In pancake and omelet, although some soluble phenolic compounds were progressively released from the solid matrices during digestion, most of the phenolic compounds remained mainly insoluble all along digestion. Regarding stability, it can be noticed that omelet presented the highest total phenolic recoveries compared to the other food matrices.

3.2. Evaluation of the food matrix effect on the antioxidant activity during in vitro digestion

The second part of the study was focused on the evaluation of the food matrix effect on the antioxidant activity of the enriched egg and dairy products during digestion In order to get information about the two main antioxidant mechanisms of polyphenols, the antioxidant activity of the samples was measured by ORAC and FRAP methods. While ORAC method measures the antioxidant ability to quench free radicals by hydrogen atom transfer, FRAP method is based on single electron transfer mechanism.

3.2.1. FRAP

As presented in Fig. 5A, the antioxidant activity of the control solution and the GE-enriched matrices was not strongly affected during digestion when measured by the FRAP method. Except for

the intestinal digestion of the omelet, where the antioxidant activity increased until almost double the initial antioxidant value (187%), in the rest of the matrices it remained constant and very close to their initial antioxidant activity. At the end of the whole digestion process, the remaining antioxidant activity was 91%, 112%, 113% and 125% in the control, custard dessert, milkshake and pancake, respectively.

The correlations between on the one hand the FRAP antioxidant activity, and on the other hand total phenolic content (0.910; $p<0.0001$), anthocyanin content (0.987; $p<0.0001$) and proanthocyanidin content (0.981; $p<0.0001$), were very high and similar. Thus, in the control solution and liquid food matrices, most of the antioxidant activity was measured in the soluble fractions, especially during the oral and gastric phases of digestion. On the contrary, in the solid matrices, the main contribution to the total antioxidant activity was provided by the insoluble fractions.

3.5.2. ORAC

As it can be seen in Fig. 5B, during the oral and gastric phases of digestion, the antioxidant activity of the control and enriched matrices measured by the ORAC method was quite similar to those measured by FRAP method, and close to 100%. Only after the gastric digestion of the liquid matrices, the total antioxidant activities were slightly higher than those measured by FRAP method (135% *vs* 97.9% for the custard dessert, and 128% *vs* 111% for the milkshake). On the contrary, the intestinal phase of digestion dramatically increased (2 to 3-fold) the initial antioxidant activity of all samples, up to 269% in control solution, 225% in custard, 251% in milkshake, 254% in pancake, and 333% in omelet.

When measured by ORAC, the correlations of the antioxidant activity with the different polyphenols classes were slightly smaller for anthocyanins (0.771; $p<0.0001$) and proanthocyanidins (0.811; $p<0.0001$) than for total phenolic content (0.912; $p<0.0001$).

4. Discussion

In the present study, four dairy and egg products enriched with grape extracts were submitted to *in vitro* digestion to determine the food matrix effect on their antioxidant activity and on anthocyanin, proanthocyanidin and total phenolic bioaccessibility. Since some polyphenols from the GE such as anthocyanins could be efficiently absorbed across the oral and gastric mucosa (Talavéra et al., 2003; Talavéra et al., 2004), a recently standardized model comprising the three phases of digestion (oral, gastric and intestinal) was used. At the end of each digestion step, two fractions were collected and analyzed separately: the soluble fraction and the insoluble one. The soluble fraction would be the one that could be available for absorption into the systemic circulation after active or passive transport through the digestive tract i.e. bioaccessible. On the contrary, the

insoluble fraction would comprise the non bioaccessible compounds that would reach the successive compartment of digestion (or large intestine at the end of the intestinal phase). Despite dialysis membranes have been widely used to separate such fractions, many factors can actually interfere during dialysis, thus leading to non-reliable results (Bermúdez-Soto et al., 2007). Hence, in the present study, separation was finally done by a centrifugation step. Finally, it must be stressed that, since the total phenolic quantification is based on the oxidation of phenol groups rather than in a molecular quantification of them, phenolic bioaccessibility here measured is more related to phenol activity than to phenol molecular concentration.

4.1. In the absence of food matrix, polyphenols are stable and mainly soluble during oral and gastric digestion, but undergo degradation and precipitation during the intestinal phase

In order to discriminate the food matrix effect from the *in vitro* digestion effect, the latter was independently studied by submitting a water solution of the GE (control solution) to the *in vitro* digestion procedure. The results obtained, which were in line with those of other authors, showed that most polyphenols remained stable and soluble during the oral and gastric steps of digestion. By contrast, many of them were extensively modified and/or precipitated during the intestinal step of digestion.

During the oral phase, it is very likely that most polyphenols precipitated due to the ability of proteins to interact and form insoluble aggregates with them , especially the high-molecular-weight proanthocyanidins (Sarni-Manchado et al., 1999). At this step of digestion, since any salivary proline-rich proteins were added, the most abundant protein was the added α-amylase. A previous study performed with extracts from the same source (*Vitis vinifera*) and at the same pH (7), proved the formation of insoluble aggregates between α- amylases and proanthocyanidins (Gonçalves et al., 2011). Similarly, the interaction of polyphenols with gastric and intestinal enzymes has been previously described (Gu et al., 2011; He et al., 2007). In terms of stability, the high polyphenol recoveries found in the control solution after the oral step can be explained by the fact that, although many polyphenols of the GE such as proanthocyanidins, flavonoids or anthocyanins are not stable under neutral and/or basic pH conditions (Tagliazucchi et al., 2010 ; Kay et al., 2009, Serra et al., 2010; Fernández et al., 2013), their degradation is usually a time-depending process and the oral step lasted only 2 min. During the gastric step, the well-known high stability of polyphenols against degradation under the acidic gastric media (McDougall et al., 2005; Tagliazucchi et al., 2010) maintained anthocyanin, proanthocyanidin and total phenolic recovery practically unaltered. Finally, the degradation of anthocyanins as well as the degradation and precipitation of proanthocyanidins during intestinal digestion is also in line with previously published studies (Serra et al., 2010; Fernández et al., 2013;McDougall et al., 2005; Podsędek et al., 2014). The decrease observed on

proanthocyanidin mDP during the intestinal phase of digestion was likely caused by oxidation reactions (which are favored at higher pH values) rather than to depolymerization ones, which usually take place under acidic conditions. Indeed, the formation of acid-resistant inter and intramolecular bonds during oxidation leads to lower proanthocyanidins recovery and errors in the estimation of their mDP by phloroglucinolysis (Poncet-Legrand et al., 2010).

In terms of antioxidant activity, the very large increase of the radical scavenger capacity measured by ORAC during the intestinal phase seems to be caused by the formation of new oxidation products with a higher antioxidant activity than that of their precursors. Another reason could be the unmasking of a previously sterically impeded pool of hydroxyl radicals as a consequence of conformational changes undergone by polyphenols. In any case, the antioxidant activity of polyphenols is a complex phenomenon that relies on many other factors such as the assay performed or the solubility of polyphenols (Plumb et al., 1998; Dangles, 2012). Finally, although ORAC only measures antioxidant activity mediated by hydrogen transfer, it is very likely that these derived products could also quench radicals by single electron transfer since, in general, deprotonation increases the electron-donating capacity of polyphenols (Prior, Wu, & Schaich, 2005).

4.2. In the GE-enriched foods, the composition and structure of the matrix, and the class of polyphenols govern polyphenol stability and bioaccessibility

In the liquid matrices (custard dessert and milkshake), due to their immediately solubilization into the digestive fluids, the only parameter affecting polyphenol stability and solubility during the different phase of digestion was the matrix composition, i.e., the interactions of polyphenols with the food matrix components. Many of the ingredients of our matrices, such as casein, lactose, starch or fructose have already proven to decrease phenolic bioaccessibility during *in vitro* gastric and intestinal digestion. (Sengul et al., 2014). In pancake and omelet, in addition to the composition, an extra parameter influenced the release and solubilization of polyphenols during digestion: the solid structure of the matrices. As a result, in pancake and omelet most anthocyanins, proanthocyanidins and total phenolics were recovered in the insoluble fraction during the oral and gastric phases. It is interesting to stress that the presence of the food matrix, far from being and impediment for anthocyanin stability and solubility during the intestinal phase, actually protected anthocyanins from degradation, especially in omelet and pancake.

Total recovery rates of both anthocyanins and proanthocyanidins were similar values. However, the capacity of proanthocyanidins to interact with other food components and form insoluble aggregates during the oral and gastric phases of digestion was much higher than that of anthocyanins. In addition, not only during the digestion of the GE-enriched matrices but also during

the digestion of the control solution, the mDP of the insoluble proanthocyanidins were always higher than that of the soluble ones. This indicates that long-chain proanthocyanidins are more prone than the smaller ones to interact and form insoluble complexes with macromolecules such as proteins (Sarni-Manchado et al., 1999) and plant cell wall material (Le Bourvellec et al., 2004). As the result of these two factors, as it can be seen in Fig. 2, 3 and 4, the quantities and the proportion of each polyphenol that were bioaccessible or not varied greatly from one matrix to another during the different phases of digestion.

4.3. The four GE-enriched dairy and egg matrices delivered similar proportions of bioaccessible polyphenols than the control solution at the end of digestion and did not affect the antioxidant activity

Since monomeric and low molecular weight polyphenols are primarily absorbed in the upper small intestine and most of the higher molecular-weight polymers such as proanthocyanidins are usually absorbed in the large intestine after being metabolized by the colonic microbiota, it would be more suitable and relevant to do the comparison of the matrices among themselves and with respect to the control solution at the end of the intestinal digestion. As mentioned above, the polyphenols that remained soluble at the end of this step would be the ones that could be absorbed by the small intestine and those that remained insoluble would be the ones to reach the large intestine for colonic fermentation. Thus, it can be concluded that the four enriched dairy and egg products tested in our study could deliver similar proportions of soluble and insoluble phenolics at the end of the intestinal digestion than the control solution. Among the different matrices omelet presented the highest recovery values. Although the assessment of bioactivity requires relevant assays and cannot be predicted in any way from bioaccessibility assays, the results here obtained look promising since some clinical studies have already proven the beneficial effects of the GE used in our study (Eminol®). In particular, a study performed by Yubero et al., in 2013, proved that the ingestion of 700mg of encapsulated Eminol® significantly lowered the plasmatic LDL-cholesterol and oxidation levels in healthy volunteers. Since the capsules of the study were degraded in the stomach, the behavior and quantities of polyphenols liberated during digestion of the Eminol should have been equivalent to that of the control solution of our study. These quantities could be easily delivered by the matrices here studied.

As mentioned in the results section, the inclusion of the GE in the different matrices did not affect its antioxidant activity, which remained unaltered during the oral and gastric phases and greatly increased during the intestinal phase especially in the omelet. The results obtained are in line with those of Oliveira et al., (2015) in which, the radical scavenging capacity of strawberry and peach enriched yoghurts increased by 480 and 550% respectively during the intestinal phase of *in vitro*

digestion. Although according to the latest clinical trials performed to test the benefits of dietary antioxidants, the relationship between the antioxidant capacity of foods or their products of digestion (especially if measured *in vitro*) and its beneficial effect on humans after ingestion cannot be establish, it seems quite probable that the GE-enriched matrices could effectively exert some beneficial effects in the gastrointestinal lumen before absorption such as the protection of other food components (such as polyunsaturated fatty acids) and/or intestinal cells from oxidative stress.

5. Conclusion

In conclusion, the inclusion of the GE extracts into the different egg and dairy food matrices greatly impacted the release and solubility of anthocyanins and proanthocyanidins during digestion, especially in the solid food matrices and during the oral and gastric phases of digestion. Also the presence of the food matrices protected anthocyanin from degradation during the intestinal phase. However, if the total phenolic content is considered at the end of the whole digestion process, the proportion of soluble and insoluble phenolics delivered by the enriched-matrices was quite similar among them and with respect to the control solution. On the other hand the food matrix effect did not affect the antioxidant activity of the matrices, which remained constant during the oral and gastric phases but greatly increased during the intestinal phase of digestion. Among them, omelet presented higher total phenolic and antioxidant activity recoveries. Although most assays should be done in order to check their bioactivity, the fortification of dairy and eggs products with GE seems a feasible strategy to develop polyphenol-enriched foods.

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Figure captions

Fig. 1 Production flow-chart and composition in % (w/w) of the control solution and GE-enriched matrices. * Time of cooking for the first side of the pancake/omelet. ** Time of cooking for the second side of the pancake/omelet. *** Only milk quantity was expressed in the packaging of the commercial custard dessert.

Fig. 2 Evolution of anthocyanin recovery in the soluble (Sol)(□) and insoluble (Insol.)(■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE-enriched matrices. Data are means ± SD (n=3). Significant differences between matrices for the same step of digestion and fraction are denoted by different letters superscripts after one-way ANOVA and Tukey' test at $p<0.01$.

Fig. 3 Evolution of proanthocyanidin recovery and their mean degree of polymerization (mDP) in the soluble (Sol.)(□) and insoluble (Insol.)(■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE- enriched matrices. mDP of proanthocyanidins are displayed in bold.

Fig. 4 Evolution of total phenolic recovery in the soluble (Sol)(□) and insoluble (Insol.)(■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE-enriched matrices. Data are means ± SD (n=3). Significant differences between matrices for the same step of digestion and fraction are denoted by different letters superscripts after one-way ANOVA and Tukey' test at $p<0.01$.

Fig. 5 Evolution of the initial antioxidant activity measured by FRAP (A) and ORAC (B) in the soluble (Sol.)(□) and insoluble (Insol.)(■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE- enriched matrices. Data are means ± SD (n=3). Significant differences between matrices for the same step of digestion and fraction are denoted by different letters superscripts after one-way ANOVA and Tukey' test at $p<0.01$

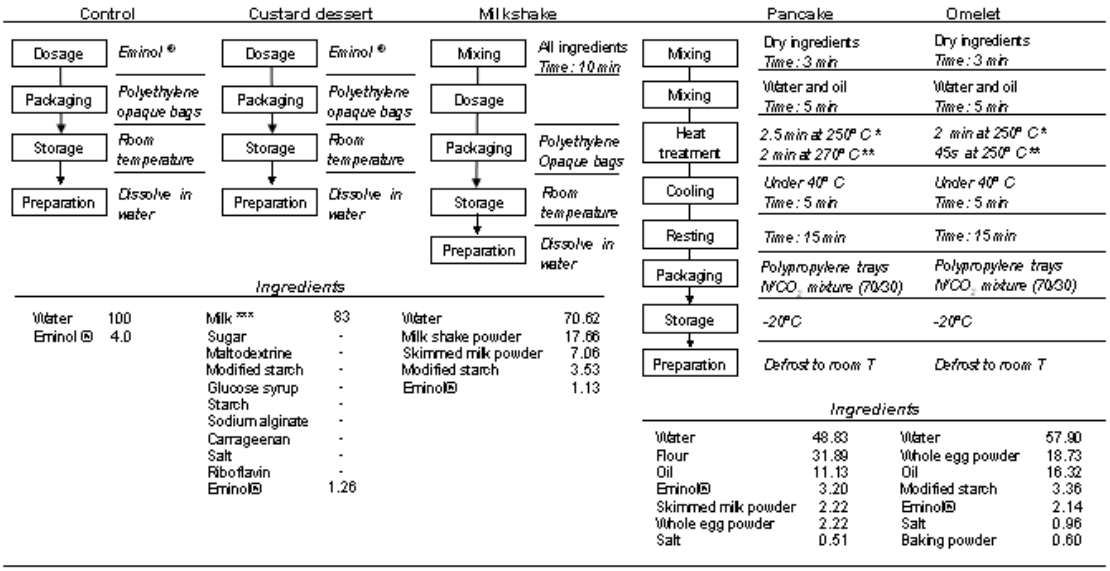
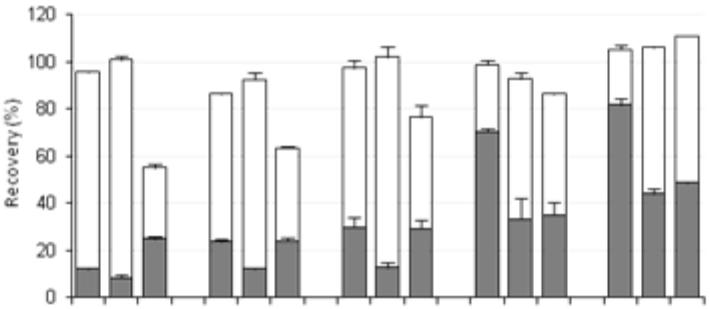
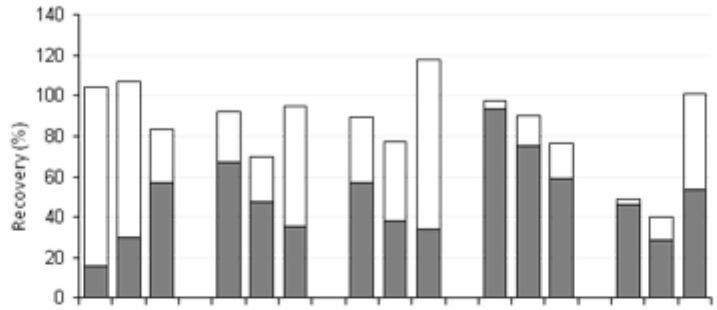


Fig. 1. Production flow-chart and composition in % (w/w) of the control solution and GE-enriched matrices. * Time of cooking for the first side of the pancake/omelet. ** Time of cooking for the second side of the pancake/omelet. *** Only milk quantity was expressed in the packaging of the commercial custard dessert.



	Control			Custard			Milkshake			Pancake			Omelet		
Sol.	83^{a}	92^{a}	30^{d}	63^{b}	80^{b}	39^{c}	67^{b}	89^{a,b}	47^{bc}	28^{c}	59^{c}	51^{b}	23^{c}	62^{c}	62^{a}
Insol.	12^{d}	9^{c}	25^{bc}	24^{c}	12^{c}	24^{c}	30^{c}	13^{c}	29^{bc}	70^{b}	33^{b}	35^{b}	82^{a}	44^{a}	49^{a}
Total	96^{a,b}	101^{a}	55^{d}	86^{b}	92^{a}	63^{cd}	97^{a,b}	102^{a}	76^{bc}	99^{a,b}	93^{a}	86^{b}	105^{a}	106^{a}	111^{a}

Fig. 2. Evolution of anthocyanin recovery in the soluble (Sol) (□) and insoluble (Insol.) (■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE- enriched matrices. Data are means ± SD (n=3). Significant differences between matrices for the same step of digestion and fraction are denoted by different letters superscripts after one- way ANOVA and Tukey' test at $p<0.01$.



	Control			Custard			Milkshake			Pancake			Omelet		
Sol.	88	77	26	25	22	60	33	39	83	4	15	18	3	11	47
	5.1	5.2	1.5	6.3	3.9	1.1	4.1	2.9	1.1	2.9	2.6	1.5	1.8	1.7	1.9
Insol.	16	30	57	67	48	35	57	38	34	93	75	59	46	29	54
	8.4	15	3.5	10	7.6	3.5	8.0	13	3.6	7.2	8.6	5.9	4.2	5.0	3.1
Total	104	107	83	92	70	95	89	77	117	98	90	77	49	40	101
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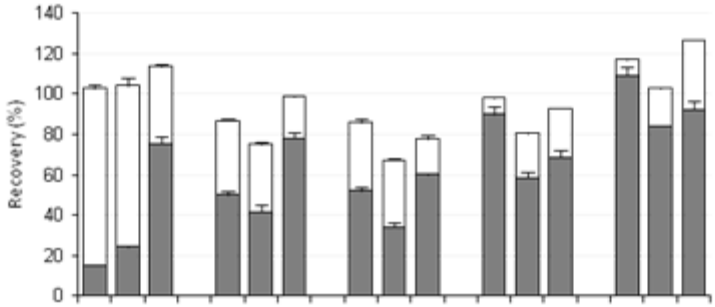
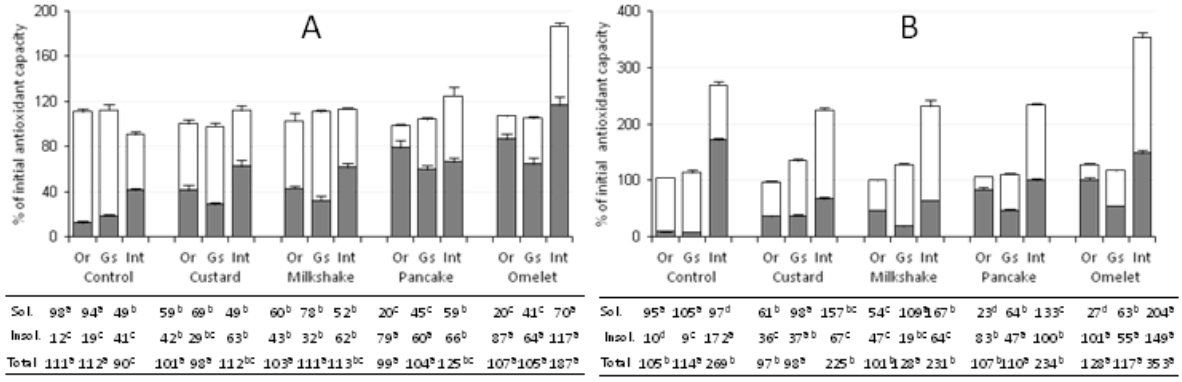
Fig. 3. Evolution of proanthocyanidin recovery and their mean degree of polymerization (mDP) in the soluble (Sol.) (□) and insoluble (Insol.) (■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE- enriched matrices. mDP of proanthocyanidins are displayed in bold.



	Control			Custard			Milkshake			Pancake			Omelet		
Sol.	87^{a}	80^{a}	38^{a}	37^{b}	33^{b}	21^{bc}	34^{b}	33^{b}	17^{c}	8^{c}	22^{c}	24^{b}	8^{c}	18^{c}	34^{a}
Insol.	15^{d}	25^{d}	75^{b}	50^{c}	42^{c}	75^{b}	52^{c}	34^{c}	60^{c}	90^{b}	58^{b}	69^{bc}	109^{a}	84^{a}	92^{a}
Total	103^{b}	104^{a}	114^{a}	87^{c}	75^{bc}	99^{b}	86^{c}	67^{c}	78^{c}	98^{bc}	80^{b}	93^{b}	117^{a}	103^{a}	127^{a}

Fig. 4. Evolution of total phenolic recovery in the soluble (Sol)(□) and insoluble (Insol.)(■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE- enriched matrices. Data are means ± SD (n=3). Significant differences between matrices for the same step of digestion and fraction are denoted by different letters superscripts after one-way ANOVA and Tukey' test at $p<0.01$.



	Control			Custard			Milkshake			Pancake			Omelet		
Sol.	98^{a}	94^{a}	49^{b}	59^{b}	69^{b}	49^{b}	60^{b}	78^{b}	52^{b}	20^{c}	45^{c}	59^{b}	20^{c}	41^{c}	70^{a}
Insol.	12^{c}	19^{c}	41^{c}	42^{b}	29^{bc}	63^{b}	43^{b}	32^{b}	62^{b}	79^{a}	60^{a}	66^{b}	87^{a}	64^{a}	117^{a}
Total	111^{a}	112^{a}	90^{c}	101^{a}	98^{a}	112^{bc}	103^{a}	111^{a}	113^{bc}	99^{a}	104^{a}	125^{bc}	107^{a}	105^{a}	187^{a}

	Control			Custard			Milkshake			Pancake			Omelet		
Sol.	95^{a}	105^{a}	97^{d}	61^{b}	98^{a}	157^{bc}	54^{c}	109^{a}	167^{b}	23^{d}	64^{b}	133^{c}	27^{d}	63^{b}	204^{a}
Insol.	10^{d}	9^{c}	172^{a}	36^{c}	37^{a,b}	67^{c}	47^{c}	19^{bc}	64^{c}	83^{b}	47^{a}	100^{b}	101^{a}	55^{a}	149^{a}
Total	105^{b}	114^{a}	269^{b}	97^{b}	98^{a}	225^{b}	101^{b}	128^{a}	231^{b}	107^{b}	110^{a}	234^{b}	128^{a}	117^{a}	353^{a}

Fig. 5. Evolution of the initial antioxidant activity measured by FRAP (A) and ORAC (B) in the soluble (Sol.) (□) and insoluble (Insol.) (■) fractions during *in vitro* oral (Or), gastric (Gs) and intestinal (Int) digestion of the control solution and GE- enriched matrices. Data are means ± SD (n=3). Significant differences between matrices for the same step of digestion and fraction are denoted by different letters superscripts after one-way ANOVA and Tukey' test at $p<0.01$

Table 1.
Eminol® composition. mDP = mean degree of depolymerization

Polyphenol	mg/g	mDP
Gallic acid	0.23	-
Tryptophan	0.85	-
Flavonols	3.26	-
Hydroxycinnamic acids	11.56	-
Anthocyanins	23.79	-
Proanthocyanidins (after depolymerization)	60.30	5.53

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FOOD RESEARCH INTERNATIONAL

Title: In vitro digestion of dairy and egg products enriched with grape extracts: Effect of the food matrix on polyphenol bioaccessibility and antioxidant activity

Highlights

- *In vitro* polyphenol bioaccessibility depends on the food matrix in which it is embedded
- Food matrices protect anthocyanins from degradation during intestinal phase
- Inclusion in food matrices increases *in vitro* anthocyanin and proanthocyanidin bioaccessibility in most matrices
- Antioxidant capacity of the enriched matrices is not affected by the presence of the food matrices which greatly increases during intestinal digestion
- At the end of digestion, the enriched matrices deliver similar proportions of soluble and insoluble phenolic compounds than the control