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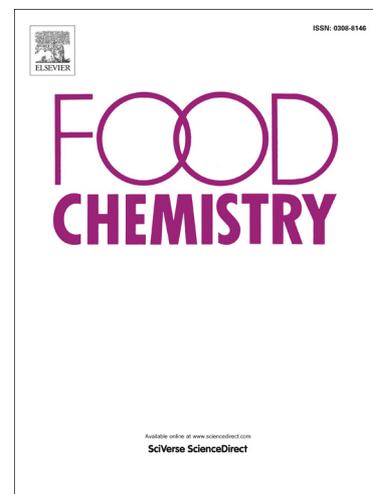
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**The food matrix affects the anthocyanin profile of fortified egg and dairy matrices during processing and *in vitro* digestion**

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## Abstract

The aim of the present study was to understand to what extent the inclusion of anthocyanins into dairy and egg matrices could affect their stability after processing and their release and solubility during digestion. For this purpose, individual and total anthocyanin content of four different enriched matrices, namely custard dessert, milkshake, pancake and omelette, was determined after their manufacturing and during *in vitro* digestion. Results showed that anthocyanin recovery after processing largely varied among matrices, mainly due to the treatments applied and the interactions developed with other food components. In terms of digestion, the present study showed that the inclusion of anthocyanins into food matrices could be an effective way to protect them against intestinal degradation, and also the incorporation of anthocyanins into matrices with different compositions and structures could represent an interesting and effective method to control the delivery of anthocyanins within the different compartments of the digestive tract.

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

## 1. Introduction

Anthocyanins are a very large group of natural pigments that belongs to the flavonoid subclass of polyphenols. Interest in anthocyanins has progressively increased during the last two decades due to their beneficial health effects. Ingestion of anthocyanins and anthocyanin-rich foods is associated with a lower risk of hypertension and type 2 diabetes mellitus (T2DM) (Muraki, 2013; Cassidy et al., 2011; Wedick et al., 2012); it is also directly correlated with a decrease in central systolic blood pressure (Jennings et al., 2012), lower peripheral insulin resistance (Jennings, Welch, Spector, Macgregor, & Cassidy, 2014) and the improvement of the lipid blood profile by increasing HDL-cholesterol and decreasing LDL-cholesterol (Qin et al., 2009; Zhu et al., 2011). Due to this myriad of healthy effects, anthocyanin-rich foods have been suggested as potential effective candidates for the prevention of metabolic disorders, 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)

The average daily intake of anthocyanins in some European countries, the United States or China (Wu et al., 2006; Zamora-Ros et al., 2011; Knaze et al., 2012; Li et al., 2013) could be sufficient to exert some beneficial effects according to some epidemiological studies, which established that 22.3 mg of anthocyanins/day lowers the risk of T2DM (Wedick et al., 2012). However, most of the beneficial effects observed during human intervention studies were measured after ingestion of much higher amounts of anthocyanins, from 50 to 320 mg/day (Guo & Ling, 2015). In addition,

dietary habits and choices, geographical situation, purchasing power or age group have a great impact on anthocyanin consumption. As a result, many sections of the population could actually be ingesting insufficient amounts of anthocyanins. As many of these factors are not easy to overcome, the fortification of highly consumed and well-accepted food matrices with anthocyanins represents a good strategy in order to increase or complete anthocyanin consumption to its effective dose.

Grape extracts (GE), a by-product resulting from the juice and wine making process, are very promising ingredients for this purpose. They are natural, inexpensive, can be obtained in considerable quantities and represent a very good source of anthocyanins and other polyphenols (Antoniolli, Fontana, Piccoli, & Bottini, 2015; Katalinić et al., 2010). 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.

However, the structure and composition of the food matrix may either enhance or prevent the release and solubilization of anthocyanins during digestion and hence their bioavailability and effectiveness. Thus, the study of the interactions between the food matrix and anthocyanins is of vital importance during the development of potential effective enriched-foods. The effect of the co-digestion of anthocyanins with different food components, matrices or diets has been proved to affect their bioaccessibility (Ribnicky et al., 2014; Sengul, Surek, & Nilufer-Erdil, 2014; Gordon J. McDougall et al., 2005). The release from liquid or solid food matrices has also been studied but mainly in naturally enriched matrices, such as fruits and juices (Tagliazucchi, Verzelloni, & Conte, 2012; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). However, the inclusion, release and solubilization of anthocyanins into and from non-natural food matrices have been scarcely studied to date.

The objective of the present study was to understand to what extent the inclusion of anthocyanins into dairy and egg matrices could affect their stability after processing and their release and solubility during *in vitro* digestion. For this purpose, a control (a solution of anthocyanins in water in which any food matrix was present), and four matrices enriched with anthocyanins were submitted to *in vitro* digestion. The four food matrices studied were milkshake, custard dessert, omelette and pancake; they were all produced under industrial conditions. Identification and quantification of total and individual anthocyanins were made by mass spectrometry. The effect of processing and oral, gastric and intestinal digestion on total and individual anthocyanins was determined.

## 2. Materials and methods

### 2.1. Chemicals

Type VI-B  $\alpha$ -amylase from porcine pancreas, pepsin from porcine gastric mucosa, porcine bile extract, pancreatin from porcine pancreas 8xUSP, potato starch, 3,5 dinitrosalicylic acid, D(+) maltose monohydrate from potato, bovine blood hemoglobin, N-p-tosyl-L-arginine methyl ester hydrochloride (TAME) and N-benzyol-L-tyrosine ethyl ester (BTEE) were supplied by Sigma Aldrich (St Louis, MO, USA). Malvidin 3-O-glucoside 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. Anthocyanin-rich extract

The anthocyanin-rich extract used to fortify the food matrices was obtained from grape pomace. The extract, registered as Eminol<sup>®</sup>, was obtained by means of a patented extraction system developed by Grupo Matarromera (ES 2 309 032). The extract comes 100% from red grapes (Tempranillo variety, *Vitis vinifera*) harvested from vineyards located in the Ribera de Duero Designation of Origin, in Castilla y León (Spain).

Identification of individual anthocyanins in Eminol<sup>®</sup> was determined by analyzing an aqueous solution of the extract at 40 mg/ml on a Grace/Vydac 201TP C18 column (250 x 4.6 mm i.d., 5  $\mu$ m) connected to an Agilent 1100 HPLC system provided with a photo diode array detector (Agilent technologies, Massy, France). Elution was performed according to Sanza et al. (2004) with slight modifications. The chromatographic conditions were: 30°C; 20  $\mu$ l 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 as 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 for 15 min before the next injection. Absorbance was measured at both 280 and 528 nm. The chromatographic system was fitted to a QSTAR XL mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with an electrospray ionisation source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). The mass spectrometer was operated in positive mode at 5000 V and data collected in full scan mode in the mass range of 400 to 700 m/z. The instrument was calibrated by multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from  $\beta$ -casein,  $\beta$ -CN (193–209). While anthocyanin identification was based on the obtained mass measurements, quantification of single anthocyanins - calculated as malvidin-3-O-glucoside equivalents (M3OGE) - was achieved by measurement of each peak area at 528nm and an external calibration curve.

### 2.3. Enriched food matrices

Two liquid (milkshake and custard dessert) and two solid (pancake and omelette) anthocyanin-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 anthocyanin extract was provided in an independent polyethylene bag and had to be added and mixed with a commercial custard dessert before use. Anthocyanin-enriched pancakes and omelettes were provided frozen in modified atmosphere trays. The control solution was prepared by directly dissolving the anthocyanin-rich extract (provided in an independent polyethylene bag) in water at 40 mg/ml. Compositional details and production flow-charts of the food matrices are presented in Figure 1.

#### 2.4. *In vitro oro-gastro-intestinal digestion*

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

Before digestion, all of the matrices were prepared/ defrosted and a sufficient quantity of each one was freeze-dried for further anthocyanin 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 omelette, 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 was mixed with 17.5 ml of simulated salivary fluid electrolyte stock solution (SSF), 125  $\mu$ l of 0.3 M  $\text{CaCl}_2$ , 4.875 ml of water and 2.5 ml of  $\alpha$ -amylase solution in SSF (1,500 U/ml), all of them pre-warmed at 37°C. The mix was thoroughly shaken 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  $\mu$ l of 0.3 M  $\text{CaCl}_2$  and a sufficient volume of 1 M HCl to adjust the pH to 3. After the addition of 10 ml of porcine pepsin solution diluted to 20,000 U/ml in SGF and distilled water to a final volume of 100 ml, the mix was thoroughly shaken 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  $\mu$ l of 0.3 M  $\text{CaCl}_2$  and 12.5 ml of 160 mM bile extract solution in SIF. After adjusting the pH to 7 with 1 M 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 removing 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 anthocyanins, the oral and intestinal samples were acidified to pH 2 directly after their digestion. Finally, all digestions were immediately centrifuged at 21,000g and 5°C for 20 min, and the supernatants and pellets collected, freeze-dried and stored until further used for anthocyanin extraction. The composition and preparation of the SSF, SGF and SIF electrolyte stock solutions are detailed in Table 1.

### 2.5. Anthocyanin extraction

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

### 2.6. Anthocyanin identification and quantification

Anthocyanin identification and quantification in the matrices and *in vitro* digestion fractions were performed by RP-HPLC. After the dissolution of the freeze-dried samples in H<sub>2</sub>O/methanol/formic acid (75/11.25/13.75 v/v) and filtration through 0.2 µm cellulose filters (Sartorius ministart RC4 17821), anthocyanins were separated on a Grace/Vydac 201TP C18 column (250 x 4.6 mm i.d., 5 µm) connected to a Waters e2695 separation module provided with a Waters e2489 UV/Visible detector (Waters Inc., Milford, USA) following the chromatographic conditions described in section 2.2. Individual anthocyanins were identified by comparison of the retention times to those of the Eminol® sample identified by mass spectrometry. 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.

### 2.7. Calculations

The proportion of total and individual anthocyanins recovered in the matrices and control solution after manufacturing and/or preparation was calculated as follows:

$$\text{Recovery(\%)} = (\text{Qtt extracted} / \text{Qtt added}) \times 100$$

where  $Q_{tt}$  extracted is the quantity of anthocyanins (mg) extracted after manufacturing and/or preparation and  $Q_{tt}$  added is the quantity of anthocyanins (mg) added to the matrices (based on the quantity of AC present in the Eminol® GE).

The proportion of anthocyanins that were released from the food matrices/control and solubilized into the digestive fluids and the proportion of anthocyanins that remained insoluble during the different phases of digestion were calculated as follows:

$$\text{Soluble (\%)} = (Q_{tt} \text{ supernatant} / Q_{tt} \text{ digested}) \times 100$$

$$\text{Insoluble (\%)} = (Q_{tt} \text{ pellet} / Q_{tt} \text{ digested}) \times 100$$

where  $Q_{tt}$  supernatant is the quantity of anthocyanins (mg) in the supernatant at the end of the corresponding phase of digestion,  $Q_{tt}$  pellet is the quantity of anthocyanins (mg) in the pellet at the end of the corresponding phase of digestion and  $Q_{tt}$  digested is the quantity of anthocyanins (mg) that was submitted to digestion (based on the AC content of the matrices after manufacturing and/or preparation). Finally, total anthocyanin recovery during the steps of digestion was calculated as follows:

$$\text{Total recovery} = \text{Soluble (\%)} + \text{Insoluble (\%)}$$

## 2.8. Statistical assays

The combined effect of manufacturing and the different phases of *in vitro* digestion on anthocyanin recovery and solubility were studied by principal component analysis (PCA). In order to test the reliability of running the PCA on means instead of on the raw data, the absence of a repeatability effect in the measurements of each variable was verified by a one-way ANOVA analysis ( $p > 0.05$ ). PCA was then performed using the Facto-Miner package of the R software (R Core Team, 2013; Le, Josse, & Husson, 2008). The variables were automatically standardized (mean centered and scaled) by the software to give them all the same importance. Recoveries after processing and recoveries in the soluble and insoluble fractions after oral, gastric and intestinal digestion were defined as the active variables. Type of matrix was added as an illustrative factor. PCA transformed the variables into a new set of independent variables called principal components (PCs), which were uncorrelated linear combinations of the original variables which allowed the representation of most of the information in the data by a 2-D graph. The comparison of anthocyanins recoveries after manufacturing and during the different phases of digestion was studied by post one-way ANOVA Tukey's tests at  $\alpha = 0.01$  using the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA).

### 3. Results

#### 3.1. Grape extract composition

Mass spectrometry analyses performed in the control solution identified 12 anthocyanins, all of them 3-O monoglucosides. In decreasing order, the 3-O-glucosides of malvidin, petunidin, peonidin, delphinidin and cyanidin were the most abundant anthocyanins of the GE. In fact, the 3-O-glucosides, including the methylpyranomalvidin, accounted for more than three fourths of the total anthocyanin content (80.8%) of the extract. The remaining anthocyanins were the 3-O-acetylglucosides of malvidin and peonidin, and the 3-O-coumarylglucosides of malvidin, petunidin, delphinidin and peonidin. Identified anthocyanins accounted for 94.4 % of the total anthocyanin content. Although some other minor anthocyanins were detected at 528 nm, they could not be identified. A RP-HPLC chromatogram of the Eminol® GE with the corresponding peak assignments, retention times,  $m/z$  values and relative amounts of the identified anthocyanins is shown in Fig. 2.

#### 3.2. PCA analysis

As shown in Fig. 3A, the plane defined by the first two PCs explained almost 92.0% of the variability of the dataset. PC1, which explained 63.7% of the variability, was strongly correlated to the recovery of anthocyanins after manufacturing (0.84) and the recovery of anthocyanins after the oral digestion (0.97 and -0.97 for the soluble and insoluble fractions, respectively) and gastric digestion (0.93 and -0.94 for the soluble and insoluble fractions, respectively). PC2, which explained 28.2% of the variability, was strongly correlated to the recovery of anthocyanins after intestinal digestion (0.95 and 0.88 for the soluble and insoluble fractions, respectively).

As highlighted in Fig. 3B by ellipses and geometrical forms, the distribution of the projected anthocyanins into the 2D plane defined by PCs 1 and 2 could be easily divided into 5 different clusters, each one comprising all of the individual anthocyanins from the same matrix. In pancake and omelette, situated on the left hand-side of the PCA map of individuals by the PC1, the recovery of soluble anthocyanins after the oral and gastric phases of digestion and the recovery of anthocyanins after processing was significantly lower than the overall mean, especially in the omelette. On the contrary, in milkshake and custard dessert (which almost overlap on the right hand-side of the PCA map of individuals), these values were clearly above the overall mean and very close to that of the control solution. Finally, as highlighted by red squares in Fig. 3B, 3-O-coumarylglucosides presented much lower stabilities after the intestinal digestion than the rest of the anthocyanins regardless of the matrix they were included in. In other words, both anthocyanin recovery after processing and anthocyanin bioaccessibility during *in vitro* digestion were highly influenced by the type of matrix in which they were incorporated. In order to better understand and

discuss the results obtained by the PCA, the effect of processing and *in vitro* digestion were analysed separately.

### 3.3. Processing effect

The first part of the study was focused on the evaluation of the food matrix effect on the stability of anthocyanins during the manufacturing and/or preparation processes of the enriched matrices. In order to achieve this goal, total and individual anthocyanin recoveries were calculated in the ready-to-eat products. Results, expressed as recovery percentages, are summarized in Fig 4 and Table 1 of supplementary data.

In custard dessert and milkshake, although total anthocyanin recoveries were very high (96.6 and 91.1% respectively), only that of the custard dessert was not significantly different from that of the control solution. In pancake and omelette, although the times and temperatures applied during manufacturing were almost identical (Fig. 1), total anthocyanin recovery was more than 2-fold higher in pancake than in omelette (74.5% vs.31.4%).

Concerning individual anthocyanins, although custard dessert and milkshake showed higher recoveries than pancake, the three matrices shared an almost identical profile after processing, i.e., except for Del-3CG, which presented the highest recovery in the three matrices, the recoveries of the remaining individual anthocyanins within the same matrix was very similar and almost no significant differences were detected among them. In omelette, recoveries of the 3-O-coumaryl-glucosylated anthocyanins were significantly higher than that of their respective 3-O-glucosides, and of the latter, delphinidin, petunidin and malvidin showed the smallest recoveries. Finally, differences between malvidin and peonidin 3-O-acetylglucosides were also significant, the recovery of malvidin being the highest one.

### 3.4. *In vitro* digestion

#### 3.4.1. General

The second part of the study was focused on the evaluation of the food matrix effect on the release, solubilization and stability of anthocyanins during *in vitro* digestion. In order to achieve this goal, the four egg and dairy anthocyanin-enriched matrices were submitted to *in vitro* digestion and total and individual anthocyanins were quantified in the soluble and insoluble fractions of the oral, gastric and intestinal phases of digestion. Finally, in order to discriminate the food matrix effect from the *in vitro* digestion effect, a control solution (no matrix present) consisting of a water solution of the GE was submitted to *in vitro* digestion and the evolution of anthocyanins was followed throughout digestion. The results, expressed as recovery percentages, are presented in Fig. 5.

### 3.4.2. Control solution

As shown in Fig. 5 and Tables 2 and 3 of the supplementary data, the oral and gastric phases of digestion barely affected the stability of anthocyanins when any food matrix was present (individual anthocyanin recovery yields ranged between 90% and 105%). In terms of solubility, the oral phase of digestion produced the insolubilization of 12.3% of total anthocyanins. This phenomenon, unlike stability, greatly depended on the chemical structure of anthocyanins: while the proportion of glucosylated and acetyl-glucosylated anthocyanins that were recovered in the insoluble fraction was around 10%, coumaryl-glucosylated anthocyanins presented values around 3-fold higher. Among them, Del-3CG and Pet-3CG presented significantly higher values (39% and 35.4%, respectively) than Peo-3CG and Mal-3CG (27.2% and 29.5%, respectively).

On the other hand, the transition from the acid gastric phase to the neutral/basic intestinal phase extensively affected the stability and solubility of anthocyanins: after 2 h of intestinal digestion only 55% of total anthocyanins could be detected: 30% in the soluble fraction and 25% in the insoluble one. Among the different types, the coumaryl-glucosylated anthocyanins were the most affected ones: Del-3CG was not detected and the others, which were only present in the insoluble fraction, did not reach recovery yields over 8%. Among the glucosylated anthocyanins, the nature of the aglycone seemed to have an important effect on their stability: in particular peonidin, malvidin and cyanidin presented significantly higher total recoveries (60.8%, 59.5% and 55.9% respectively) than petunidin and delphinidin (37.9% and 24.5%, respectively). The two acetyl-glucosylated anthocyanins identified in the extract, Peo-3AG and Mal-3AG, presented very similar values to those of their corresponding glucosylated forms (62.3% and 51.4%, respectively).

### 3.4.3. Anthocyanin-enriched matrices

As shown in Fig.5, the anthocyanin profile obtained during the *in vitro* digestion of the liquid food matrices (custard dessert and milkshake) was very similar to that of the control solution, while during the oral and gastric phases of digestion most anthocyanins remained stable and soluble, a large proportion of them could not be detected and many others were insolubilized during the intestinal phase of digestion. The first main difference that could be observed in comparison to the control solution was that, as a result of a higher insolubilization of anthocyanins during the oral phase of digestion, the proportion of total soluble anthocyanins that were quantified in the liquid matrices was significantly lower (62.7% and 67.4% for the custard dessert and milkshake respectively) than in the control solution (83.4%). The second main difference, and probably more important in terms of bioactivity, was that after the whole *in vitro* digestion process, the proportion of total anthocyanins detected (soluble + insoluble) was significantly higher in custard dessert and

milkshake (63.5% and 76.5%, respectively) than in the control solution (55.2%). In terms of individual anthocyanins, it must be said that in general little difference was observed between the liquid food matrices and the control solution.

On the other side, as can be seen in the lower part of Fig. 5, the *in vitro* digestion of the solid food matrices (omelette and pancake) resulted in a completely different anthocyanin profile to that of the control solution and liquid food matrices. During the oral phase of digestion, most anthocyanins remained insoluble and only a very small proportion of them (28.4% in the pancake and 23.4% in the omelette) were detected in the soluble fraction. Among the different types of anthocyanins, the coumaryl-glucosylated continued to be the most affected. Indeed, their recoveries in the soluble fraction did not exceed values over 9.5%. For the glucosylated and acetyl-glucosylated anthocyanins, these values ranged between 16.5% and 35%. During the subsequent gastric phase, although the proportion of total soluble anthocyanins increased and reached values of around 60% (59.2% and 62.2% in pancake and omelette, respectively), their values continued to be much lower than in the control solution (92.4%), custard dessert (80.2%) or milkshake (89.2%). Once again, the coumaryl-glucosylated ones presented much lower values than the acetyl-glucosylated or glucosylated ones.

Finally, as observed in the liquid food matrices, the presence of the solid food matrices also increased the stability of anthocyanins during the intestinal phase of digestion. In fact, the proportion of total recovered anthocyanins detected after the intestinal digestion of the solid matrices, as well as their distribution between the soluble and insoluble fractions were not significantly different from those of the previous gastric phase of digestion. In other words, at the end of the whole digestion process, almost all of the anthocyanins remained stable (total anthocyanin recovery in pancake and omelette was 111% and 86.5%, respectively) and around half of them remained soluble (62.5% in pancake and 51.5% in omelette). In terms of individual anthocyanins, the presence of the solid food matrices efficiently protected the glucosylated and acetyl-glucosylated anthocyanins, which presented individual recovery values of around 75%-85% in the pancake and 90%-110% in the omelette. As a consequence, the differences among individual anthocyanins previously observed in the control solution and liquid food matrices were much less pronounced and, in many cases, no longer existent. Finally, it must be noted that, on the other hand, the presence of the solid matrices could not protect the coumaryl-glucosylated anthocyanins from intestinal degradation, which could only be detected in the insoluble fractions and generally in relatively low amounts.

#### 4. Discussion

In the present study, four dairy and egg products enriched with anthocyanins were produced on an industrial scale and submitted to *in vitro* digestion to understand to what extent the inclusion of anthocyanins into dairy and egg matrices could affect their stability after processing and their release and solubility during digestion.

##### 4.1. Effect of the food matrix during processing

According to our results, it seems reasonable to state that the quantity of anthocyanins that the different enriched egg and dairy products could actually host after their production was highly determined by the techniques applied during their manufacturing and the physicochemical properties of the food matrices. In custard dessert and milkshake, since any heat treatment was applied during their production, it is very likely that the small losses observed were caused by the interaction of anthocyanins with other food components. These interactions could have produced the formation of insoluble aggregates, the masking of the anthocyanin chromophore or even anthocyanin degradation.

Regarding the heat-treated matrices, although a part of the big difference observed between the pancake and the omelette could have been produced by a lower protection of anthocyanins by the latter during heat treatment, it seems that the development of interactions between anthocyanins and the food components of the omelette was actually the main reason. Some additional analysis carried out to check the stability of anthocyanins after 21 days of storage (data not shown) revealed that the recovery of total anthocyanins in omelette had almost doubled after the storage. Since anthocyanins could not be produced during this 21 day period, the increase on anthocyanin recovery can be only explained by the weakening and/or breaking of previous interactions between anthocyanins and the omelette components. In pancake, on the other hand, anthocyanin recovery after storage did not increase with time. Therefore, in this matrix, the loss of anthocyanins observed after manufacturing seems to be mainly caused by the heat treatment applied during processing, a phenomenon that has been studied in depth before (Patras, Brunton, O'Donnell, & Tiwari, 2010).

The almost identical shape of the profiles obtained after the processing of the pancake (cooked) and the custard dessert and milkshake (non-cooked) shows that neither the nature of the aglycone moiety nor the nature of the glucoside moiety influenced the thermal degradation of anthocyanins during the cooking of the pancake. In other words, the heat treatment applied to the pancakes affected all individual anthocyanins in a similar way, regardless of their chemical differences.

Finally, if we take into consideration that the omelette was cooked under almost identical conditions, it seems reasonable to expect that, if only the heat treatment determined the anthocyanin profile of the omelette, this should have been similar to that of the other 3 matrices. Therefore, it is very likely that the differences detected among individual anthocyanins were caused by their interactions with the omelette components.

#### *4.2. Effect of the food matrix during in vitro digestion.*

Since 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 phases) 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 should contain the bioaccessible anthocyanins, e.g. those anthocyanins that, once released from the matrices and solubilized into the digestive fluids, would be available for absorption into the systemic circulation after transport through the digestive tract. The insoluble fraction, on the other hand, should include all of the non-bioaccessible anthocyanins, e.g. those anthocyanins that are not released from the matrix and those that, once released, precipitate during digestion. These anthocyanins, not available for absorption, would reach the successive compartment of digestion, or the large intestine at the end of the intestinal phase. Finally, the non-detected anthocyanins would correspond to anthocyanins degraded during digestion or associated with other food components and therefore undetectable.

The results obtained after the digestion of the control solution, which were in line with those of other authors, showed that most anthocyanins remained stable and soluble during the oral and gastric steps of digestion. By contrast many of them were extensively modified or insolubilized during the intestinal step of digestion.

Despite the neutral pH environment, the oral step of digestion is known to be propitious for anthocyanin destruction (Kay et al., 2009), and the degradation of anthocyanins during this step of digestion was almost insignificant. Since this anthocyanin degradation at neutral and basic pH has been described as a time-dependent process, it is very likely that the very short duration of the oral phase (only 2 min) was not long enough to affect the stability of anthocyanins. During the gastric phase, the transition of anthocyanins from the very unstable quinoidal-base form (the most abundant form of anthocyanins at pH 7) to the very stable flavylium cation form (the most abundant form at pH 3)(Mazza & Brouillard, 1987) yielded total recovery of practically unaltered anthocyanins. The high stability of anthocyanins during gastric digestion is a well-documented phenomenon (Tagliacucchi et al., 2010; Liang et al., 2012; McDougall et al., 2005)

The decrease of total anthocyanin content during intestinal digestion has already been described (McDougall et al., 2005; Tagliacruzchi et al., 2012; Podsędek et al., 2014). It can mainly be attributed to the high instability of anthocyanins at physiological pH, rather than to the effect of the digestive enzymes (Bermúdez-Soto et al., 2007; Tagliacruzchi et al., 2010). The change from acidic to neutral/basic conditions produces the transformation of the flavylum cation to the chalcone pseudobase, which can be further broken down into hydroxybenzoic acids and aldehydes, such as protocatechuic acid or phloroglucinaldehyde (Kay et al., 2009). Although all anthocyanins were affected, the presence of the coumaryl group resulted in a much higher degradation. This phenomenon, according to our knowledge, has been never described before.

In terms of solubility, since no proline-rich proteins were added to simulate the presence of salivary proteins, the insolubilization of anthocyanins observed could be explained by the formation of insoluble anthocyanin- $\alpha$  amylase aggregates. The decrease of insoluble rates during the subsequent gastric phase suggests that these aggregates were scarcely resolubilized in these conditions. The capacity and different specificity of amylase to form insoluble aggregates with anthocyanins and other polyphenols has already been reported (Xiao et al., 2013; Akkarachiyasit et al., 2010).

As has been described in the results section, the inclusion of the anthocyanins into the dairy and egg-based matrices, had a very different effect on the stability and solubility of anthocyanins during digestion. In fact the, two very different behaviours could be observed.

In the liquid food matrices the presence of the matrices did not greatly impact the stability and solubility of anthocyanins, which presented a very similar behaviour to that of the control solution. If we take into consideration that custard dessert and milkshake were completely solubilized into the digestive fluids and that, in consequence, all of the anthocyanins should be soluble from the beginning of the digestion, all of the observed differences with respect to the control solution can only be explained by the interactions between the food components of the matrices and anthocyanins, i.e. by the composition of the matrix. According to the obtained results it can be stated that the main components of these matrices: milk, sugar, maltodextrin of modified starch, did not seem to greatly affect the solubility and stability of anthocyanins.

In pancake and omelette, the release, solubilization and stability of anthocyanins during digestion appeared to be controlled, not only by the composition of the matrices, but also by the solid structure of their matrices. As a result, the anthocyanin profile obtained after the digestion of these matrices was very different to that of the control solution and the liquid food matrices.

During the oral phase of digestion, although the simulation of the mastication and the addition of the simulated salivary fluid transformed the solid matrices into a dense liquid oral bolus, the high proportion of anthocyanins that were recovered in the insoluble fraction seems to indicate that, at this early stage of digestion, a large proportion of anthocyanins remained entrapped within the solid structure of pancake and omelette. In addition, the significantly higher proportion of insoluble coumaryl-glucosylated anthocyanins in comparison to the glucosylated and acetyl-glucosylated ones could indicate that a proportion of the anthocyanins that had been released from the solid matrices were further insolubilized by the formation of insoluble aggregates with the  $\alpha$ -amylase.

During the gastric phase of digestion, the progressive degradation of the solid matrices by the proteolytic action of the pepsin, combined with the high stability of anthocyanins at low pH, produced the observed decrease in the proportion of insoluble anthocyanins and the corresponding increase in the proportion of soluble anthocyanins.

Finally, the protection of anthocyanins during the intestinal phase of digestion could be explained by the progressive release of anthocyanins from the solid matrices. As explained above, since the degradation of anthocyanins at neutral and basic pH is not instantaneous but rather a time-dependent process, the progressive release of the anthocyanins from the solid matrices would decrease the contact time of anthocyanins with the intestinal fluids and in consequence their degradation. Another possibility is that the liberation of free fatty acids from the intestinal digestion of the pancake and omelette fats could have acidified the pH of the intestinal media and therefore increased the stability of anthocyanins. This hypothesis could also explain why in milkshake and custard dessert, liquids matrices containing considerably smaller amounts of fat than the omelette and pancake, the degradation of anthocyanins was significantly higher. In them, not only would anthocyanins have stayed in contact with a more basic intestinal environment than in the solid food matrices, but also for a longer period of time

## 5. Conclusion

Anthocyanin recovery after processing largely varied among the different matrices, mainly due to the treatments applied and the interactions developed with other food components. The digestion of the control solution showed that most anthocyanins of the extract were quite stable and soluble during the oral and gastric phases of digestion but were highly degraded and insoluble during the intestinal phase, especially the coumaryl-glucosylated ones. In the liquid enriched matrices, the release, solubilization and stability of anthocyanins during digestion was very similar to that of the control solution and seemed to be governed, apart from the digestion effect itself, by the composition of the matrices. In the solid matrices, the composition, and to a larger extent the

structure, of the matrices produces the progressive release of anthocyanins during digestion and, in consequence, the obtainment of a very different anthocyanin profile. In all matrices, in any case, the presence of the food matrices protected anthocyanins against intestinal digestion. At the end of the whole digestive process the four enriched matrices, especially the solid ones, delivered significantly higher proportions of anthocyanins than the control solution.

Although the *in vitro* bioaccessibility essays performed in our study do not permit assessment of the potential health benefits of the different matrices and more relevant essays should be performed, the results here obtained shows that: 1- the inclusion of anthocyanins into food matrices could be an effective way to protect them against intestinal degradation, and 2 – the incorporation of anthocyanins into matrices with different compositions and structures could represent an interesting and effective method to control the delivery of anthocyanins within the different compartments of the digestive tract.

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#### Figure captions

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

**Fig. 2.** RP-HPLC chromatogram of an Eminol sample at T=0 with the corresponding peak assignments, abbreviations, retention times, mass spectral data and relative amount of the identified anthocyanins.

**Fig. 3.** (A) Projection of variables onto the plane defined by the first two principal components (PCs) of principal component analysis (PCA). The coordinates of each variable are the correlation coefficients with the two first PCs: the closer the arrow to the circle, the better the representation of

the variable. The smaller the angle between the direction of two variables, the higher the correlation between them. (B) PCA map of individual anthocyanins projected on the 2D plane defined by PC1 and PC2. The type of matrix into which anthocyanins were included is indicated by different geometrical forms and grouped by ellipses: control solution (empty diamonds), milkshake (full squares), custard dessert (empty stars), pancake (empty triangles) and omelette (full circles). Additionally, 3-O-coumarylglucosylated anthocyanins of each matrix are displayed in red and grouped into rectangles. Individual anthocyanins are numbered following Figure 2 assignments. Total anthocyanin values are represented by an asterisk (\*)

**Fig. 4.** Total and individual anthocyanin recoveries in the control solution and anthocyanin-enriched food matrices after manufacturing and/or preparation. Data are means  $\pm$  SD (n=3). Individual anthocyanins are named following Figure 2 abbreviations. For total anthocyanin recovery, <sup>1</sup> denotes significant difference at  $p < 0.01$  (t-test) with respect to control's total recovery. Recoveries of individual anthocyanins within the same matrix without common letter superscripts denotes significant difference at  $p < 0.01$  after one-way ANOVA and Tukey' test.

**Fig. 5.** Total and individual anthocyanin recovery in the control solution and anthocyanin-enriched food matrices during *in-vitro* digestion. The proportion of soluble and insoluble anthocyanins recovered during the oral (Or), gastric (Gs) and intestinal (In) steps of digestion are represented by white and grey bars, respectively. Data are means  $\pm$  SD (n=3). Individual anthocyanins are named following Figure 2 assignments.

#### Table captions

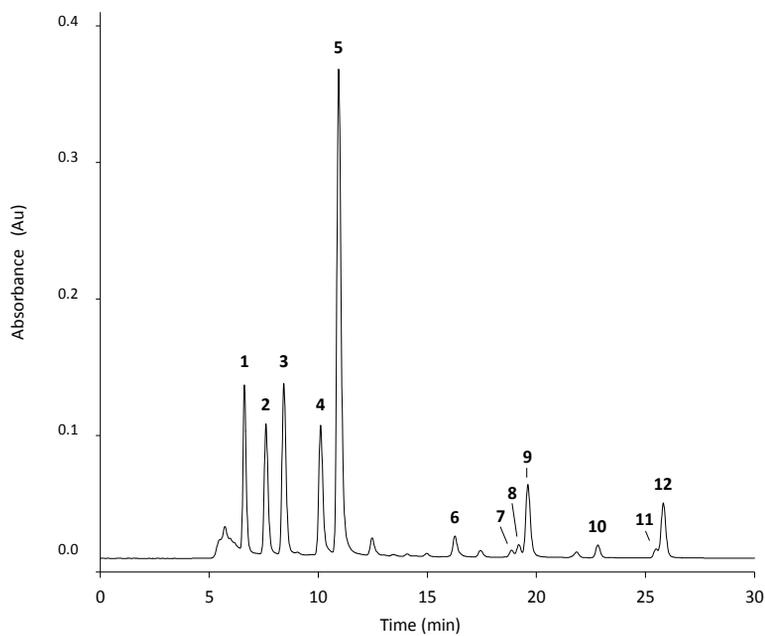
**Table 1.** Composition and preparation of the simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) electrolyte stock solutions. The volumes indicated were calculated to obtain 400ml of each simulated stock solution with distilled water (1.25x concentrate). The further addition of enzymes, bile salts,  $\text{Ca}^{2+}$  solution etc. and water resulted in the correct electrolyte concentration in the final digestion mixture.  $\text{CaCl}_2 \cdot (\text{H}_2\text{O})_2$  was not added to the electrolyte stock solutions as precipitation may occur. Instead, it was added to the final mixture of simulated digestion fluid and food matrices a. Source: Minekus et al. (2014).

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Constituent	Stock conc.		SSF		SGF		SIF	
			pH 7		pH 3		pH 7	
	g/l	mol/l	Vol. of stock ml	Conc. In SSF mmol/l	Vol. of stock ml	Conc. In SGF mmol/l	Vol. of stock ml	Conc. In SIF mmol/l
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
$\text{KH}_2\text{PO}_4$	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$\text{NaHCO}_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$\text{MgCl}_2(\text{H}_2\text{O})_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(\text{NH}_4)\text{CO}_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
<b>For pH adjustment.</b>								
	mol/l		ml	mmol/l	ml	mmol/l	ml	mmol/l
NaOH	1		-	-	-	-	-	-
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
<b><math>\text{CaCl}_2</math> is not added to the simulated digestion fluids, see details in legend.</b>								
	g/l	mol/l	mmol/l		mmol/l		mmol/l	
$\text{CaCl}_2(\text{H}_2\text{O})_2$	44.1	0.3	1.5 (0.75*)		0.15 (0.075*)		0.6 (0.3*)	

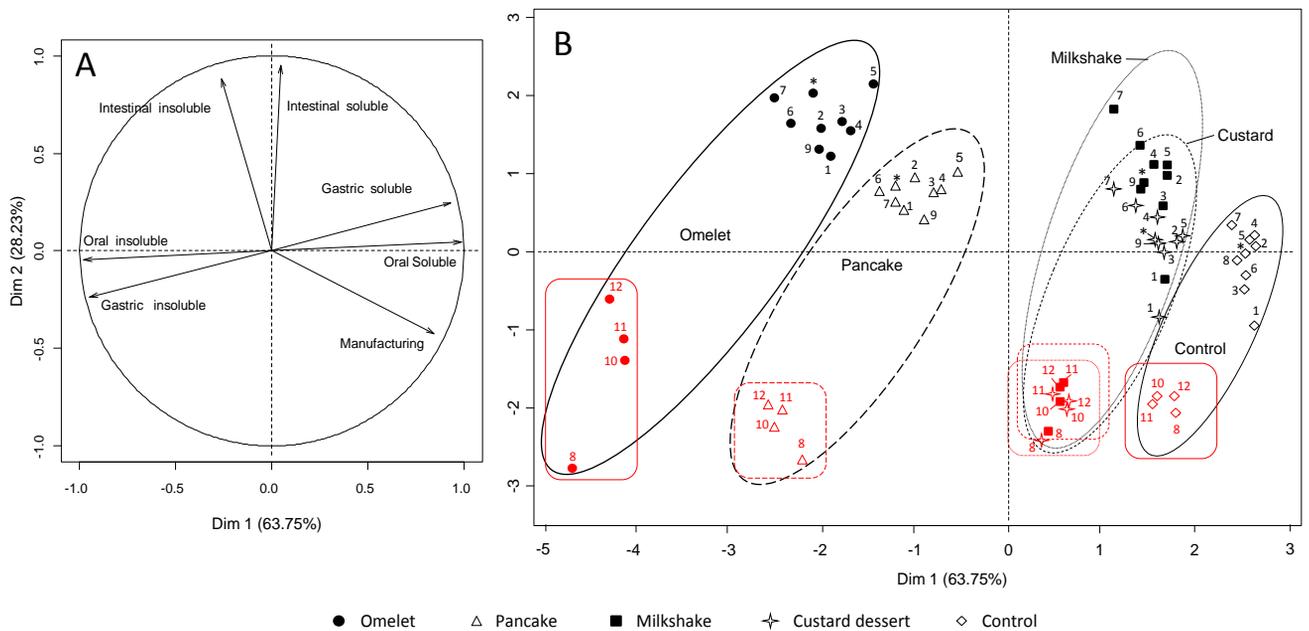
<sup>a</sup> \* In brackets is the corresponding  $\text{Ca}^{2+}$  concentration in the final digestion mixture.



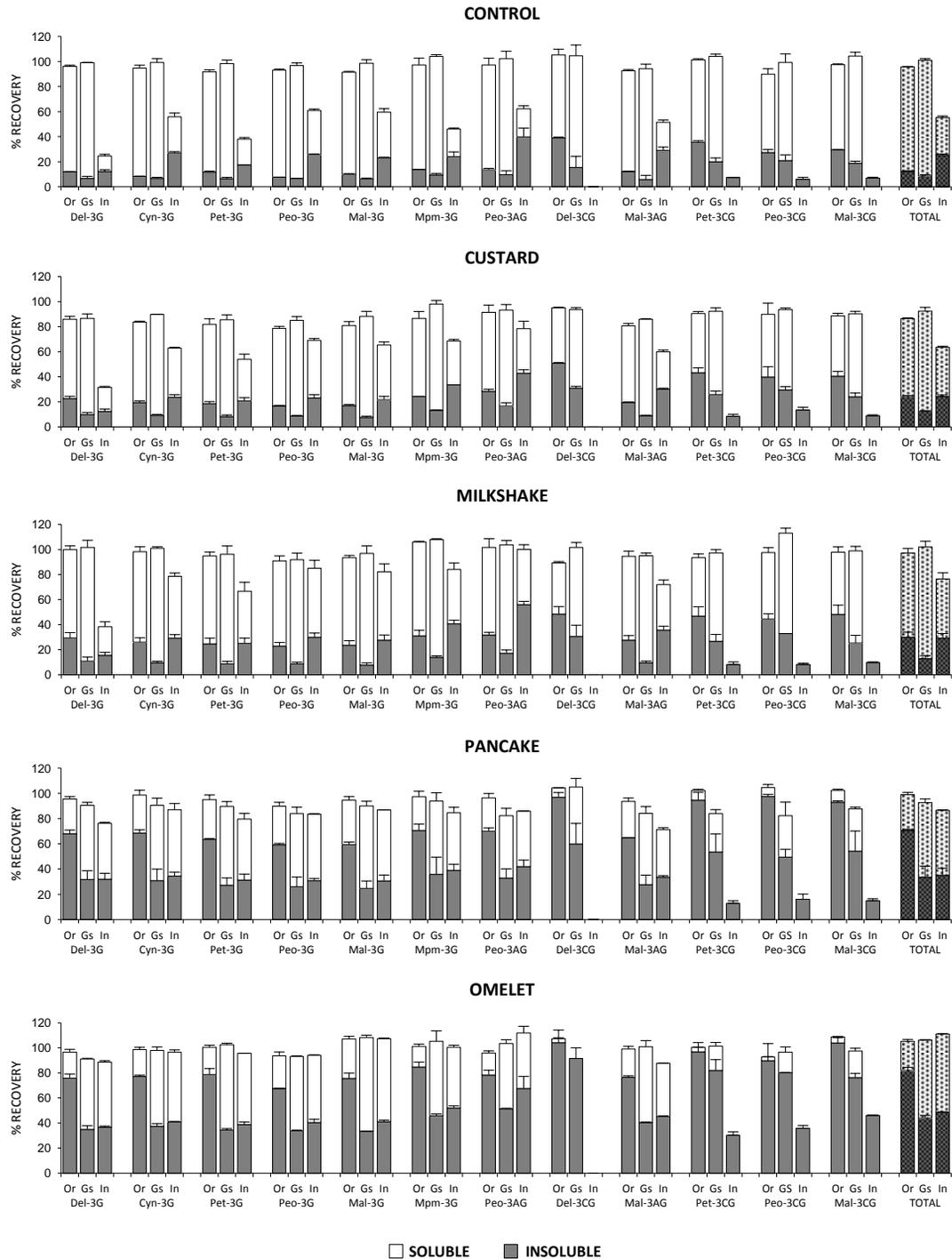


Peak	Anthocyanin	Abbreviation	RT	m/z (+ve)	Relative amount
1	Delphinidin 3-O-glucoside	Del-3G	6.45	465.0	10.05
2	Cyanidin 3-O-glucoside	Cyn-3G	7.49	449.0	9.52
3	Petunidin 3-O-glucoside	Pet-3G	8.34	479.0	12.39
4	Peonidin 3-O-glucoside	Peo-3G	10.07	463.0	10.24
5	Malvidin 3-O-glucoside	Mal-3G	10.93	493.0	36.54
6	Methylpyranomalvidin 3-O-glucoside	Mpm-3G	16.25	531.0	2.02
7	Peonidin 3-O-acetylglucoside	Peo-3AG	18.84	505.1	0.65
8	Delphinidin 3-O-coumarylglucoside	Del-3CG	19.18	611.1	1.04
9	Malvidin 3-O-acetylglucoside	Mal-3AG	19.59	535.1	5.83
10	Petunidin 3-O-coumarylglucoside	Pet-3CG	22.79	625.1	1.10
11	Peonidin 3-O-coumarylglucoside	Peo-3CG	25.50	609.1	0.69
12	Malvidin 3-O-coumarylglucoside	Mal-3CG	25.79	639.1	4.37

**Fig. 2.** RP-HPLC chromatogram of an Eminol sample at T=0 with the corresponding peak assignments, abbreviations, retention times, mass spectral data and relative amount of the identified anthocyanins.



**Fig. 3.** (A) Projection of variables onto the plane defined by the first two principal components (PCs) of principal component analysis (PCA). The coordinates of each variable are the correlation coefficients with the two first PCs: the closer the arrow to the circle, the better the representation of the variable. The smallest the angle between the direction of two variables, the highest the correlation between them. (B) PCA map of individual anthocyanins projected on the 2D plane defined by PC1 and PC2. The type of matrix in which anthocyanins were included into is indicated by different geometrical forms and grouped by ellipses: control solution (empty diamonds), milkshake (full squares), custard dessert (empty stars), pancake (empty triangles) and omelet (full circles). Additionally, 3-O-coumaroylglucosylated anthocyanins of each matrix are displayed in red and grouped into rectangles. Individual anthocyanins are numbered following figure 2 assignments. Total anthocyanin values are represented by an asterisk (\*)



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**Title: The food matrix affects the anthocyanin profile of fortified egg and dairy matrices during processing and in vitro digestion**

#### **Highlights**

- AC content in matrices: controlled by food-AC interactions and treatments applied
- Stability and solubilization of AC during digestion greatly varied between matrices
- The previous variations are controlled by the food matrix composition & structure
- Food matrices protect most anthocyanins from degradation during intestinal phase