Assessment of *in vitro* Bioaccessibility of Carotenoids and Phenolic Compounds in a Model Milk-mandarine Beverage

Running title: Bioaccessibility of bioactives compounds in a model milk-fruit beverage

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ABSTRACT

Mandarine juice is one of the richest sources of β-cryptoxanthin and flavonoids, which have been positively associated with bone mineral density. Carotenoids are lipophilic isoprenoid compounds with a complex absorption process that can be affected by different factors. In this study we have evaluated the effect of the food matrix on the in vitro bioaccessibility of carotenoids and phenolic compounds in a model milk-mandarine beverage (MMB).

MMBs were formulated with mandarine juice and different dairy products to achieve three fat levels (0.2%, 1.7% and 3.2%) and three calcium levels (120, 310 and 500 mg Ca+2 / 100 ml.). The bioaccessibility was evaluated using a harmonised *in vitro* digestion method. The results showed that the content of milk fat increased the bioaccessibility *in vitro* of phenolic compounds ($p < 0.05$), while a moderate fat level (1.7%) resulted in the highest bioaccessibility for bioactive carotenoids. On the other hand, calcium fortification at the highest level (500 mg Ca^{+2}/100 mL) decreased the bioaccessibility of bioactive carotenoids from 76% to 43% (66% for the major β-cryptoxanthin) compared to the lower calcium fortification level (120 mg Ca^{+2}/100mL). The bioaccessibility of hesperidin, the main flavanone in mandarine juices, was significantly ($p < 0.05$) reduced in the MMB with the highest calcium level.

The bioaccessibility of carotenoids and phenolic compounds is affected by fat and calcium level. When formulating functional beverages, the impact of the formulation should be carefully considered to optimize the bioaccessibility of the bioactive compounds.

Keywords: Beverage formulation; Bioaccessibility; Carotenoids; Calcium fortification; Phenolic compounds

Abbreviations: flavones (FLV), flavanones (FLN), hydroxycinnamic acids (HCA), milk mandarin beverages (MMBs), Rapid Resolution Liquid Chromatography (RRLC), retinol activity equivalent (RAE).

1. Introduction

Consumer awareness of the benefits of a healthy diet is leading the food industry to design new functional foods that satisfy both sensory and health demands. In this sense, the functional beverage market is one of the most active categories in the functional food market and is expected to increase due to healthy lifestyle, disease prevention objectives, and the design of products tailored for the specific needs of the elderly[1,2]. Functional beverages can be formulated by the addition of bioactive ingredients or the removal or reduction of undesirable ingredients, i.e. sugar, fats, to improve stability, bioactivity, and bioavailability [3].

Fruit juices are a good source of bioactive compounds and, among them, orange juice stands out due to its excellent flavour and appealing color [4]. From a nutritional point of view, orange juice is a good source of provitamin A carotenoids and vitamin C, in addition to folate and other bioactive compounds such as flavonoids [5]. A wide variety of carotenoids (more than 100) have been reported in citrus fruits, but violaxanthin and β-cryptoxanthin (β-CRX) both in peel and pulp are the main discriminating factors between the different citrus genotypes [6,7]. In this sense, mandarins are one of the richest sources of β-CRX, one of the six carotenoids found in human blood and tissues [8]. β-CRX is a provitamin A carotenoid, with other relevant health beneficial effects such as antiobesity, antioxidant, anti-inflammatory, and anticancer activities [9]. The role of β-CRX on bone calcification and osteogenesis has been explored *in vitro* and *in vivo* and there is evidence that β-CRX is a suppressor of bone resorption, and has also been associated with increased bone mass, thus decreasing the risk of osteoporosis [10]. Furthermore, daily intake of β-CRX was associated with a lower risk of osteopenia in postmenopausal women [11]. Similarly, the intake of flavonoids has been positively associated with bone mineral density, *in animal* and *cellular-base* studies [12] and also in human studies [13]. Although both milk and mandarine juice are usually consumed in the western diet, some groups of people, such as menopausal women, may benefit from functional beverages which contain a mixed of compounds which are beneficial for bone health. Milk contains more bone-beneficial nutrients, than any food in the adult diet such as protein, Ca, Mg, K, Zn and P per unit energy [14]. However, a high consumption of milk has been associated with augmented concentrations of

oxidative stress, which is one of the risk factors for osteoporosis [15] and of inflammation markers, especially in women, however this effect is attenuated by fruit and vegetable intake [16]. In this sense a good approach to designing a functional drink to improve bone health could be a beverage containing milk or a dairy product and a mandarine juice to provide proteins and minerals and β-CRX and flavonoids, respectively, and the other antioxidant compounds provided by the fruit juice that may counteract the oxidative effect of milk.

An important aspect to consider when designing functional foods is the bioavailability of the bioactive compounds in the matrix of the formulated beverage. For this purpose, *in vitro* gastrointestinal digestion is considered a valuable tool to estimate stability and bioaccessibility of nutrients/phytonutrients from different food matrices, in order to optimize the bioavailability and therefore the nutritional efficacy of any bioactive compound [17,18].

Carotenoids are lipophilic isoprenoid compounds with a complex absorption process that can be affected by different factors, from those related to the food matrix (processing, interaction with other meal components) to factors related to the host (the activity of digestive enzymes, transport efficiency across the enterocyte) [19]. One of the most extensively studied dietary factors is the addition of lipids to the food matrix. The addition of fat / oil increases the bioaccessibility and bioavailability of carotenoids by facilitating the solubilization of carotenoids released from the food matrix during digestion [20,21]. The fat content in dairy products is a potential vehicle to increase carotenoid delivery [22].

There is evidence to suggest that the lipid portion of dairy is a key factor contributing to improved carotenoid bioaccessibility in milk-fruit beverages compared to raw fruit in commercial and model beverages [23,24]. However, the presence of divalent minerals (calcium, present in dairy products) during digestion can drastically reduce their bioaccessibility [25–27]. Few studies have investigated how changes in the food matrix may affect the bioaccessibility of phenolic compounds [28,29], but recent studies also suggest that the bioaccessibility of fruit phenolics are optimize in the presence of a skim-milk matrix [30]

For this reason, this study aims at formulating a model functional beverage for bone health containing mandarine and milk products (milk mandarine beverage, MMB). Different milk-fat

contents and calcium fortifications were used in the model MMB formulations and "*in vitro*" bioaccessibility of carotenoids and phenolic compounds was evaluated to elucidate the influence of these factors (fat content and calcium fortification) on the bioaccessibility of the bioactive compounds.

2. Materials and Methods

2.1. Chemicals

The extraction solvents (methanol, dichloromethane, and acetone) were obtained from Carlo-Erba (Milan, Italy). HPLC grade solvents were acquired from Merck (Darmstadt, Germany). NANO pure Diamond system (Barnsted Inc.) provided the Ultrapure water. Pepsin (porcine gastric mucosa, cat N° P7012, 2000 U/ mL), pancreatin (porcine pancreas, cat. no. P7545, 100 U/mL), bile salt (cat N° P8756, 2.1 g/mL) and other reagents (mineral salts (KCl, NaCl), sodium bicarbonate, monopotassium-phosphate, magnesium chloride hexahydrate) used in the in preparation of stock solutions of simulated digestion fluids (see table 2 of Minekus et al. [31]) were from Sigma-Aldrich (Steinheim, Germany), as well as some of the carotenoids standards (β-carotene, β-CRX, lutein, and zeaxanthin). Other carotenoid standards were isolated from appropriate sources by standard procedures as explained elsewhere [32]. Phenolic compounds were acquired from Sigma-Aldrich (Steinheim, Germany), except didymin from Extrasynthese (Lyon-Nord, France).

Calcium (Mastical, Takeda Farmacéutica, Spain) was obtained from a pharmacy as calcium carbonate.

2.2. Milk Mandarine Beverages (MMBs)

A commercial fresh squeezed pasteurized mandarine juice not from concentrate and ultra-high temperature milk products (including cream milk, whole milk, semi-skimmed milk, and skimmed milk for the same brand) were acquired from a local supermarket (Spain). The model MMBs were prepared in the laboratory by mixing mandarine juice and dairy products as described below using a domestic blender (MOULINEX, 400 W) for 2 min at high speed.

i) Milk Mandarine Beverages. Initially three model MMBs were prepared by mixing 50% v/v of mandarine juices and % 50 v/v of different milk products (added as whole, semi-skimmed or skimmed milk or cream) to obtain three different fat levels as shown in **Table 1**. The bioaccessibility of carotenoids was assessed in MMB1/MMB2/MMB3 (as decribed below) and the MMB with the highest bioaccessibility value for β-CRX (MMB2) was selected for the calcium fortification assay.

ii) Fortified Milk Mandarine Beverages. For the calcium fortification assay, three new MMBs were prepared by fortification of MMB2 with calcium at three levels: 120, 310 and 500 mg Ca+2/100 ml. These levels were selected based on the range of calcium found in commercial milk or yoghurt (aprox. 120-125 mg/100 mL), and the calcium levels of commercial beverages fortified with this mineral (between 300 and 500 mg Ca+2 / 100 mL).

2.3. Physicochemical parameters

The six MMB models were characterized for fat content using the Gerber method [33]. Proteins were anlyzed by the Kjeldahl´s method [34]. Calcium was quantified by ICP-OES (Spectro Spectroblue, Metrohm AG, Herisau, Switzerland). The samples were digested by microwave oven (Milestone UltraWAVE, Metrohm AG, Herisau, Switzerland). The calibration curve was prepared in the range of 0.5 ppm to 10 ppm, and the sample was diluted 10 times.

The formulation and final composition of the MMBs are shown in **Table 1**. The pH values were 4.58± 0.01 for MMBs. All MMBs were prepared as previously described and just before analysis. No further treatments were performed since MMBs were not intended for human consumption studies. All analyzes were performed in triplicate.

2.4. In vitro digestion method

An international consensus methodology explained elsewhere [31] was used for *in vitro* simulated gastrointestinal digestion. Briefly, 5 ml of each beverage sample was submitted to the *in vitro* digestion process using the enzyme concentration, time, pH, incubation temperature, and simulation of gastric and duodenal fluids detailed in Minekus et al. [31] adapted by Stinco et al. [24]

Once the digestion process was completed, the samples were centrifuged at 3900 × g for 20 minutes at 4 ºC [35] using an Allegra X-12R centrifuge (Beckman Coulter, USA). The supernatant

was filtered through a 0.22-µm nylon membrane (Millipore Iberica S.A., Madrid, Spain). The bioaccessible fraction was flushed with nitrogen and stored at -20 ° C in an atmosphere of nitrogen until analysis.

2.5. Bioactive Compound Analysis

2.5.1. Phenolic compounds

The undigested beverage and digesta were centrifuged at 18000 g for 15 min at 4 ° C and subsequently filtered through a 0.22-µm pore size membrane filter. The samples were analyzed by Rapid Resolution Liquid Chromatography (RRLC) by direct injection on an Agilent 1260 System equipped with a diode array detector and a quaternary pump. UV spectra were recorded from 200 to 770 nm and the chromatograms were monitored at 280 and 320 nm. Separation was carried out on a C18 analytical column (Kinetex Biphenyl 2.6 µm, 50 × 4.6 mm, Phenomenex; Torrance, CA, USA) coupled with a Security Guard (ULTRA UHPLC Biphenyl filter, Phenomenex; Torrance, CA, USA). The mobile phase was: solvent A, 0.1% formic acid in water and solvent B, acetonitrile and the gradient elution (min/% of A): 0 /100,5/95, 20/50; 22/100, 25/100. The flow rate was set at 1.5 ml / min, the column temperature was kept at 25 ° C, and the injection volume was 0.5 µL. All samples were analyzed in triplicate.

The identification of the individual phenolic compounds was carried out by comparing their retention times and spectroscopic characteristic, within the range 200–770 nm, with those of appropriate standards. Other phenolics, for which there are no commercially available standards, were identified based on their retention times and spectral features, as compared with those reported in the literature. These compounds have been assayed by assuming that their molar absorptivity is the same as that of the corresponding free standard molecule. For quantification, linear calibration curves of external standards were used (320 nm for hydroxycinnamic acids (HCA) and flavones (FLV) and 280 nm for flavanones (FLN)). The results were expressed in mg/L of MMB, as mean ± standard deviation. The total phenolic compounds were calculated as the sum of individual compounds.

The method was validated: linearity, limits of quantification and detection (LOQ and LOD), and precision (repeatability and reproducibility) were calculated. The validating parameters of each

calibration curve are described in **Supplementary Table 1**. Excellent linearity was observed for all phenolic compounds ($R^2 \geq 0.999$) tested range, except Apigenin ($R^2 \geq 0.994$). The LOD and LOQ for all compounds were in the range of 0.01 to 0.02 mg / L and 0.01 at 0.06 mg/L, respectively. Repeatability and reproducibility were evaluated by the relative standard deviation (RSD) and a good precision for the RRLC was obtained.

2.5.2. Carotenoids

2.5.2.1. Extraction

The extraction and saponification of the undigested samples and micellar fractions were carried out according to the method described by Stinco et al. [36]. The dry extracts were redissolved in 1 mL of dichloromethane and saponification was performed with 1 mL of methanolic KOH (30% w/v) under dim light at room temperature. After 1 h, the samples were washed with 5% NaCl and water. The obtained coloured extract was concentrated to dryness in a rotary evaporator at a temperature below 30 ° C and dissolved in 50 µL of ethyl acetate before injection into the RRLC system. The samples were analyzed in triplicate.

2.5.2.2. RRLC analysis

RRLC analyzes were carried out according to the validated method described by Stinco et al. [37]. The identification of carotenoids was made by comparison of their chromatographic and UV/vis spectroscopic characteristics with those of the standards. This includes the maximum absorption wavelength (λmax) and the shape of the spectrum (fine structure considering %III/II and Q ratio) as well as retention time and chromatographic conditions. The carotenoid content of the beverages was achieved by external calibration with the corresponding standards. Total carotenoid content was assessed as the sum of the content of individual pigments. The analyzes were performed in triplicate.

The vitamin A activity of the beverages was expressed in terms of retinol activity equivalents (RAE) [38] using the following formula:

$$RAE = \frac{\mu g\ \beta\ carotene}{12} + \frac{\mu g\ \beta\ cryptoxanthin + \mu g\ \alpha\ carotene}{24} \quad (1)$$

2.6. Bioaccessibility of Bioactive Compounds

The bioaccessibility in percentage for each bioactive compound (carotenoid and phenolic compound) was calculated as follows:

$$\%\ Bioaccessibility_{biactive\ compound} = \frac{[BC]_{digesta}}{[BC]_{beverage}} x\ 100 \quad (2)$$

Where: $BC_{digesta}$ corresponds to carotenoid concentration in micellar fraction (mg/L) or phenolic compound concentration (mg/L) in digesta after *in vitro* digestion, while $BC_{beverage}$ corresponds to each bioactive compound in the model MMB before digestion.

2.7. Statistical analysis.

Results are presented as mean and standard deviation of independent determinations. Statistical analyzes were performed with Statistica v.8.0 software. Means were compared using variance analyzes (ANOVA) and Tukey´s test ($p < 0.05$). The results of the experiments were compared according to one factor (fat level or calcium content) and the discussion below was performed according to each of them separated.

3.Results and Discussion

3.1. Effect of fat content on the bioaccessibility of phenolic compounds and carotenoids

3.1.1. Phenolic Compounds

The 13 identified phenolic compounds are shown in **Table 2**. They can be classified into three major categories: 7 hydroxycinnamic acids (HCA): ferulic acid (2 derivatives), caffeic acid (2 derivatives), p-coumaric acid (2 derivatives) and sinapic acid derivative; 2 flavones (FLV): vicenin-2 and a derivative of luteolin; and 4 flavanones (FLN): (naringin derivative, naritrutin, hesperidin, and dydimin. Vicenin-2 (apigenin 6,8-C-diglucoside) was identified as the predominant flavone, while hesperidin (hesperetin-7-O-rutinoside) and narirutin (naringenin-7-O-rutinoside) were the main flavanone in the commercial mandarine juice.

Table 2 illustrates the total and individual phenolic content in the model MMBs and in the bioaccessible phenolic content. There were no significant differences in the individual and total phenolic composition of the different MMBs as they were all formulated with the same commercial mandarine juice.

However, the phenolic content in digesta, was significantly affected ($p < 0.05$) by the fat content. Specifically, beverages with the lowest fat level (MMB1 and MMB2) showed significantly lower ($p < 0.05$) contents of HCA (*p*-coumaric, caffeic, ferulic, and sinapic acids), while MMB3 showed the highest. Similarly, flavones, as well as flavanones and total phenols, were affected by fat level, except for the narigin derivative. These results suggest that the fat content above 1.7% positively ($p < 0.05$) affects the amount of phenolic compounds in digesta ($p < 0.05$).

The bioaccessibility (as % BIO) of the individual phenolic compounds was calculated as the relationship between the mean level of each compound in the MMBs and the digesta (**Table 2**). The results are shown in **Figure 1.** The bioaccessibility of HCA varied from 12.8 to 19%; of the four types of HCA, ferulic acid had the highest percentage (20-30%), followed by sinapic acid (18.5-26%), caffeic acid (9-13.4%) and finally p-coumaric acid (8.6-14%), respectively. A similar trend was reported by Rodríguez-Roque et al. [39], who found that the bioaccessibility of HCAs during the *in vitro* digestion of a milk-fruit beverage varied from 12% for caffeic and 11% for p-coumaric acid to 14% for ferulic acid. Similarly, the percentage of flavones transferred to the digesta ranged from 12 to 18.5%. The bioaccessibility of FLN bioaccessibility was higher than that of HCA, between 42 and 65 %, being the highest bioaccessibility for hesperidin (54.8-88%) followed by didymin (27-68%), narirutin (23-31%) and the derivative of naringin (18.6-22%). These results are similar to those reported in the literature [28,39,40]. In addition, in orange juices, Aschoff et al. [40] reported 91-94% of bioaccessibility for hesperidin and narirutin after *in vitro* digestion and Rodríguez-Roque et al. [28] reported 87 and 97 % for hesperidin and naringenin respectively. The lower bioaccessibility values obtained in this study could be related to the composition of the beverage, which was 75% fruit juice and 17.5% milk since phenolic compounds can bind to milk protein and carbohydrates which affects their determination in heterogeneous matrices [28].

An ANOVA analysis was carried out to evaluate the effect of fat content on bioaccessibility of phenolics. As shown in **Figure 1,** the beverage with the highest fat content (MMB3) showed the highest bioaccessibility for flavanones, flavones, HCA and total phenols ($p < 0.05$). Ortega et al.

271 [41] also reported an improvement in the digestibility of some phenolic compounds in cocoa
272 samples related to fat content. The protective effect of lipids on bioaccessible phenolic
273 compounds has already been described by different authors. Jakobek [42] reported that lipids can
274 interact and 'capture' polyphenols protecting them during digestion, due to better micellization
275 that allows better stability of polyphenols during their passage through the gastrointestinal tract.
276 During intestinal digestion bile salts and biliary phosphatidylcholine (PC) emulsify lipids and
277 break them into micelles under the actions of lipase before they are absorbed. Phenol-lipid
278 interactions due hydrophobic interactions results in incorporation of phenolic compounds into
279 the lipid phase of the micelles which prevents the degradation [43].Taking into account the overall
280 results, which are consistent with previous studies discussed above, it could be inferred that the
281 milk fat content above 1.8% positively affects the bioccessibility of all the phenolic compounds
282 analyzed [44].

283 3.1.2. Carotenoids

284 Eleven carotenoids were identified (**Table 2**), which can be classified into two major categories:
285 xanthophylls (antheranxanthin isomers, mutatoxanthin epimer A and B; lutein, zeaxanthin,
286 zeinoxanthin and β-CRX) and carotenes (α- and β- carotene; phytoene and phytofluene). β-CRX
287 was the most abundant carotenoid in mandarine juice and therefore in formulated MMBs,
288 accounting for 45-55% of the total carotenoids content. **Table 2** shows the carotenoid content
289 and vitamin A activity expressed as equivalents of retinol activity (RAE) in the model MMBs
290 and the bioaccessible carotenoid content in the micellar fractions. No statistically significant
291 differences were found in individual and total carotenoids among the three formulated MMBs
292 (MMB1, MMB2 and MMB3) were found.

293 After in vitro digestion, total carotenoids decreased significantly decreased ($p < 0.05$) in the
294 micellar fraction from 74% to 88 % as shown in **Table 2**. Similarly, retinol activity equivalents
295 (RAE) decreased by 4 to 10 times in the micellar fractions. The epoxycarotenoids carotenoids
296 (violaxanthin, antheraxanthin, luteoxanthin) that are also incorporated into micelles are also
297 shown in **Table 2**, however, they are not found in human plasma and their functions remain
298 unknown as well as their relevance in nutrition [45].

The content of bioactive carotenoids in the micellar fraction of the MMBs was affected by the fat content ($p < 0.05$). Only lutein and zeaxanthin (referred as macular carotenoids) remained unchanged regardless of the fat level in the MMBs. Provitamin A and colorless carotenoids were significantly higher ($p < 0.05$) in the micellar fraction of the MMB2, with moderate fat level, compared to the micellar fractions of the other two MMBs, as reflected by the total carotenoid content and RAE. The amount of fat in the diet needed for the optimal incorporation of carotenoids released into mixed micelles in the intestine is a controversial fact [46]. Several studies suggest that a minimum amount of fat is required for carotenoid absorption [47], which depends on the structure of the individual carotenoid and the matrix [48]. Hedren et al. [49] reported that the addition of 20% oil per gram to freeze-dried carrot matter resulted in a significant increase in the bioaccessibility of β-carotene, however increasing the amount of added oil over 60% did not.

Figure 1 shows the bioaccessibility of individual bioactive carotenoids in model MMBs. Among the provitamin A carotenoids, β-carotene showed the highest bioaccessibility (16-47.5%), followed by α-carotene (22-35%), and finally β-CRX (9-23%), respectively. Estévez- Santiago et al. [50], also reported the lowest bioaccessibilityfor β-CRX in mandarine and loquat. For macular carotenoids, bioaccessibilities ranged from 9 to 17% and for colorless carotenoids, ranged from 19 to 37% for phytoene and from 22 to 40% for phytofluene.

According to the fat content, the MMB with moderate fat level (1.7%) showed the highest bioaccessibility for bioactive carotenoids. These results are consistent with others previously published results, Da costa et al. [51] reported of an increase in carotenoid bioaccessibility linked to increased fat in milk-fruit beverages formulations (from 1.5-1.8 % fat). Similarly, Rodriguez-Roque et al. [21] reported that the bioaccessibility of total carotenoids decreased from 0.63%, 0.28% to 0%, respectively, with the decrease in fat content in beverages formulated with 75% of a blended fruit juice and 17.5% of milk, soy milk or distilled water and 7.5% of sugar, respectively.

Surprisingly, in this study, we have included a highest fat content (3.2%) that did not show higher levels of bioaccessibility. This striking result could be explained considering that the

micellization process is affected by other factors, such as the complexity of the food matrix (fat and protein content), and the fat solubility of individual carotenoids. Furthermore, in milk-fruit beverages, there is a debate whether the enhancement of carotenoids bioaccessibility is more related to the role of milk proteins in the micellization process than to the role of milk fat [51]. The interaction of carotenoids with milk proteins increases the solubility of carotenoids in aqueous medium. Factors that may modulate the binding between milk protein and carotenoids are related to both the protein nature and the carotenoid structure and also environmental conditions, such as pH and temperature [52,53]. In this case, the protein content in the formulated beverages was not statistically different (**Table 1**) however the profile of casein/whey proteins may vary between the formulations due to the different milk derivatives used (whole milk, skim milk, cream). The main binding mechanism between carotenoids and proteins is hydrophobic interactions and casein has a higher hydrophobicity than whey proteins (β-Lactoglobulin and α-Lactalbumin) [54].

3.2. Effect of calcium fortification on the bioaccessibility of phenolic compounds and carotenoids

3.2.1. Phenolic Compounds

Table 3 shows the individual phenolic compounds identified and quantified in the different MMBs formulated before and after in vitro digestion. MMBs did not differ in the content of individual phenolic compounds, total phenolics, HCA; FLV and FLN. Similarly, no significant differences ($p < 0.05$) in phenolic compounds in the digesta were found for the different calcium levels tested.

Figures 2A and B summarize the bioaccessibility of individual HCA, and total HCA, FLV, FLN, and phenolic compounds in the model MMBs. The bioaccessibility of phenolic compounds was affected by the calcium content, except for the total FLN that was significantly ($p < 0.05$) lower in the drink with the highest calcium level (MMB6). The effect of phenolics intake on mineral bioavailability has been a subject of interest and several studies have reported that some polyphenols (phenolic acids and flavonoids) decrease the assimilation of several

355 minerals and trace elements, including iron, zinc, and copper, most likely due to chelation [55].
356 However, to the best of the authors' knowledge, no study has previously evaluated the effect of
357 calcium content on the bioavailability of phenolic compounds. The negative effect of minerals
358 on polyphenol absorption could be inferred from the study by Matsumoto et al. [56]. These
359 researchers reported an improvement in anthocyanin levels (up to approx. 15-fold) in plasma
360 and urine after supplementing the diet of animals and subjects with blackcurrant anthocyanin in
361 the presence of phytic acid (1% solutions) compared to the same supplement without phytic
362 acid. Divalent minerals are strongly chelated by phytic acid, and this may prevent the formation
363 of mineral-polyphenol complexes. However, the duration of the gastrointestinal passage
364 increased with phytic acid, which could have altered the absorption kinetics.
365
366 3.2.2. Carotenoids
367 **Table 3** summarizes the carotenoid content and RAE in the different beverages formulated with
368 three levels of calcium content before and after the digestion process. The MMB4, MMB5, and
369 MMB6 were all equal in terms of individual and total carotenoid content. On the contrary, in the
370 micellar fraction, the bioactive carotenoid content was inversely related to calcium fortification.
371 In other words, the macular, provitamin A and colorless carotenoids and therefore the total
372 carotenoid content and RAE were higher in the micellar fraction of the beverage with the lowest
373 calcium level (MMB4).
374 **Figures 2C** and **D** show the bioaccessibility (as a percentage) of the bioactive carotenoids in the
375 MMBs with different calcium levels. As it can be observed, bioaccessibility decreased
376 significantly with increasing calcium content. Thus, β-CRX bioaccessibility was reduced by
377 66% in MMB6 compared to MMB4 (the one with the lowest total calcium content), (see **Table**
378 **1**), while other bioactive carotenoids decreased its bioaccessibility from 76% (in ZEA) to 43%
379 (in BCAR). These results are consistent with previous studies reporting the effect of calcium
380 concentration in digesta on micellarization and bioaccessibility of carotenoids [25,26,57].
381 Biehler et al. [57] reported an inhibition of micelle formation (>40% on average) with a calcium
382 content greater than 13.8 mM presumably due to the generation of insoluble soaps with fatty

acids and bile salts, which is in accordance with the decrease in bioaccessibility observed in MMB5 to MMB6 (21.9 to 33. 1 mM). Likewise, Corte Real et al. [25–27] reported a negative effect of calcium in vitro on the bioaccessibility of different carotenoids in different matrices and this effect depended on calcium concentration. Although they observed an increase in bioaccessibility for samples with calcium concentrations up to 250 mg/l of digest, bioaccessibility was negatively affected over this value. Similarly, we observed a significant ($p < 0.05$) change in the bioaccessibility related to calcium concentrations in the digesta, so it was 26% for MMB2 (142 mg/L in digesta) (**Figure 1D**) and 40% for MMB4 (426 mg Ca+2 /L in digesta) (Figure 2). However, for calcium concentrations in digesta above this value (426 mg Ca+2 /L), bioaccessibility was reduced ($p < 0.05$) to 30% and 12% in MMB5 and MMB6 (875.4 and 1325 mg Ca+2 /L respectively). This fact points out that there is a critical calcium concentration for optimal bioaccessibility of carotenoids (426 mg/L of calcium in the digesta, in this case) while levels over or under this value may negatively affect it.

However, when it comes to in vivo studies, contradictory results have been published. Borel et al. [58] in a randomized crossover study with 10 subjects who consumed 19 mg of lycopene (tomato paste), reported that 500 mg of dissolved calcium was able to reduce the bioavailability of lycopene by 83%. In contrast, Corte-Real et al. [59] reported that high calcium supplementation at physiological concentrations (500-1000 mg) in a spinach-based meal did not significantly affect the concentration of any carotenoid in plasma triacylglycerol-lipoprotein fraction (TRL), thus the bioavailability. These contradictory results could be related to the variability of factors such as type of carotenoid, matrix, calcium kinetic, and the endpoints selected.

4. Conclusions

The results obtained in this study suggest that when functional beverages containing carotenoids or flavonoids are formulated, the impact of the formulation should be carefully considered to optimize the bioaccessibility of the bioactive compounds. The proposed functional beverage for bone health should be formulated with mandarine juice to have the highest content of β-CRX and also whole milk which provides vitamin D and enough fat to optimize the bioaccessibility

411 of carotenoids and flavonoids and fortified with < 120 mg of Ca+2/ 100 ml of beverages.
412 However more human studies are needed to understand the critical factors affecting the
413 bioaccessibility of carotenoids and flavonoids.
414

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542 **Figure Captions**

543 **Figure 1.** Bioaccessibility in percentage (BIO %) of phenolic compounds (A: Individual HCA,
544 B: Total HCA, Flavones, Flavanones and Phenols) and bioactive carotenoids (C: Individual
545 bioactive carotenoids, D: total carotenoids and retinol activity equivalent RAE) in the model
546 milk mandarine beverages (MMBs) formulated with different fat contents (**Table 1**).
547
548 **Figure 2.** Bioaccessibility in percentage (BIO %) of phenolic compounds (A: Individual HCA,
549 B: Total HCA, Flavones, Flavanones and Phenols) and bioactive carotenoids (C: Individual
550 bioactive carotenoids, D: total carotenoids and retinol activity equivalent RAE) in the the model
551 milk mandarine beverages (MMBs) formulated with different calcium fortification **(Table1).**

Highlights

- Milk-madarine beverages contain bioaccessible bioactive compounds
- Fat and calcium level differently affect bioaccessibility of bioactive compounds
- Milk-fat content is a limiting factor in the bioaccessibility of carotenoids
- Bioaccessibility of phenolics is positively affected the by fat- content
- Calcium fortification negatively affect bioaccessibility of bioactives

1 **Table 1**. Model milk mandarin beverages (MMBs) formulation and composition

	MMBs					
	MMB1	**MMB2**	**MMB3**	**MMB4**	**MMB5**	**MMB6**
Ingredients %						
Mandarin juice	50	50	50	50	50	50
Whole milk	0	50	40	50	50	50
Semi-skimmed milk	15	0	4	0	0	0
Skimmed milk	35	0	0	0	0	0
Cream milk	0	0	6	0	0	0
Calcium added (mg/100mL)	0	0	0	120.0	310.0	500.0
Composition						
Fat (g/100g)	0.2 ±0.1	1.7±0.1	3.2±0.1	1.7±0.1		
Protein (g/100g)	1.6±0.1	1.6±0.1	1.5±0.16	1.7±0.1		
Calcium (mg/100mL) in MMB	56.8±0.3			170.4±0.5	350.2±0.8	530.0 ± 1.2
Calcium (mg/L) calculated in the digested	142.0			425.9	875.4	1325.0

2

Table 2. Summary of the mean phenolic compounds and carotenoids (mg/L and Retinol Activity Equivalents (RAE μg/100mL)) in the model milk mandarin beverages (MMBs) and in the digesta (phenolic compounds)/micellar fraction (carotenoids).

		Non-digested			Digesta /Micellar fraction		
Bioactive compounds		**MMB1**	**MMB2**	**MMB3**	**MMB1**	**MMB2**	**MMB3**
Phenolic Compounds	caffeic acid-d_1	1.50±0.07a	1.44±0.04a	1.50±0.06a	0.15±0.01A	0.19±0.01B	0.27±0.02C
	caffeic acid-d_2	1.54±0.13a	1.52±0.05a	1.59±0.06a	0.12±0.02A	0.09±0.01B	0.14±0.01A
	ferulic acid-d_1	1.20±0.03a	1.26±0.04a	1.28±0.01a	0.24±0.02A	0.24±0.01A	0.36±0.02B
	sinapic acid-d	0.91±0.01a	0.92±0.01a	0.93±0.01a	0.19±0.03A	0.1700.01A	0.24±0.02B
	ferulic acid-d_2	1.22±0.02a	1.21±0.02a	1.23±0.01a	0.25±0.05A	0.27±0.04A	0.40±0.03B
	p-coumaric acid-d_1	2.77±0.04a	2.70±0.11a	2.80±0.03a	0.22±0.05A	0.24±0.03A	0.36±0.03B
	p-coumaric acid dimer	0.25±0.03a	0.26±0.02a	0.24±0.01a	0.04±0.04A	0.04±0.01A	0.06±0.01B
	∑HCA	9.40±0.24a	9.30±0.19a	9.58±0.17a	1.21±0.18A	1.23±0.09A	1.83±0.13B
	vicenin-2	4.73±0.06a	4.74±0.11a	4.79±0.04a	0.46±0.05A	0.47±0.02A	0.69±0.05B
	luteolin -d	2.02±0.15a	1.99±0.09a	1.71±0.15a	0.37±0.05A	0.37±0.04A	0.51±0.03B
	∑FLV	6.75±0.13a	6.73±0.14a	6.50±0.19a	0.84±0.10A	0.84±0.06A	1.20±0.08B
	naringin-d	3.24±0.07a	3.05±0.04a	3.16±0.05a	0.71±0.09A	0.56±0.09A	0.62±0.10A
	narirutin	3.59±0.27a	3.40±0.05a	3.38±0.11a	0.82±0.10A	0.85±0.07A	1.06±0.04B
	hesperidin	11.31±0.16a	10.90±0.27a	10.83±0.46a	6.20±0.53A	7.21±0.70A	9.55±0.46B
	didymin	0.62±0.08a	0.64±0.12a	0.52±0.05a	0.17±0.02A	0.17±0.01A	0.35±0.02B
	∑FLN	18.76±0.29a	17.99±0.30a	17.890±0.56a	7.91±0.39A	8.79±0.71A	11.58±0.42B
	∑Total Phenols	34.92±0.3a	34.02±0.49a	33.966±0.86a	9.95±0.26A	10.86±0.62A	14.61±0.56B
Carotenoid Compounds	MUT epimer A	0.20±0.01a	0.19±0.02a	0.17±0.01a	0.02±0.01A	0.03±0.01A	0.03±0.01A
	LUT	0.21±0.01a	0.20±0.02a	0.18±0.01a	0.02±0.01A	0.03±0.01A	0.03±0.01A
	MUT epimer B	0.37±0.04a	0.35±0.06a	0.31±0.01a	0.04±0.01A	0.07±0.02	0.04±0.01A
	ZEA	0.16±0.02a	0.12±0.03ab	0.09±0.01b	0.02±0.01A	0.02±0.01A	0.01±0.01A
	(9*Z*)- or (9´ *Z*)-ANT	0.43±0.05a	0.41±0.07a	0.41±0.05a	0.03±0.01A	0.12±0.01B	0.06±0.01C
	ZEINO	0.08±0.01a	0.08±0.01a	0.09±0.01a	0.01±0.01A	0.01±0.01B	0.02±0.01C
	BCR	3.01±0.04a	2.71±0.30a	2.94±0.08a	0.29±0.01A	0.62±0.11B	0.34±0.04A
	ACAR	0.04±0.01a	0.04±0.01a	0.04±0.01a	0.01±0.01A	0.01±0.01B	0.01±0.01B
	BCAR	0.18±0.01a	0.17±0.01a	0.21±0.01b	0.03±0.01A	0.08±0.01B	0.05±0.01C
	PT	0.39±0.01a	0.40±0.01a	0.39±0.02a	0.11±0.02A	0.16±0.01B	0.09±0.01A
	PF	0.17±0.01a	0.18±0.01a	0.17±0.01a	0.03±0.01A	0.07±0.01B	0.04±0.01A
	Total Carotenoids	5.35±0.18a	4.93±0.51a	5.05±0.09a	0.64±0.07A	1.27±0.18B	0.72±0.06A
	RAE*	14.16±0.16a	12.91±1.23a	14.16±0.36a	1.48±0.17A	3.33±0.53B	1.89±0.19A

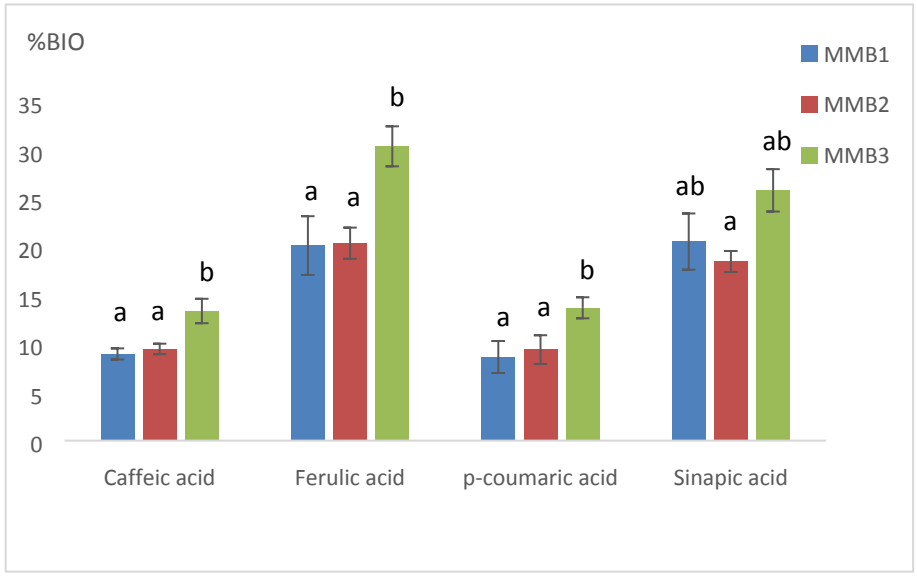
*: Retinol activity equivalent: calculated as RAE (μg/100 mL) = (μg of β-carotene)/12 + (μg β-cryptoxanthin + μg α-carotene)/24. Different lower case letters in the same row show significant differences ($p<0.05$) among MMBs. Different capital letters in the same row indicate significant differences ($p<0.05$) among digesta/micellar fraction of each sample. FLV = (flavones), FLN = (flavanones), HCA = (hydroxycinnamic acids), ANT = (antheraxanthin), MUT= mutatoxanthin, LUT= (lutein), ZEA = (zeaxanthin), ZEI = (zeinoxanthin), BCR = (β-cryptoxanthin), ACAR= (α-carotene), BCAR= (β-carotene), PT= (phytoene), PF= (phytofluene).

Table 3. Summary of the mean phenolic compounds and carotenoids (mg/L and Retinol Activity Equivalents (RAE μg/100mL)) in the model milk mandarin beverages (MMBs) and in the digesta (phenolic compounds)/micellar fraction (carotenoids).

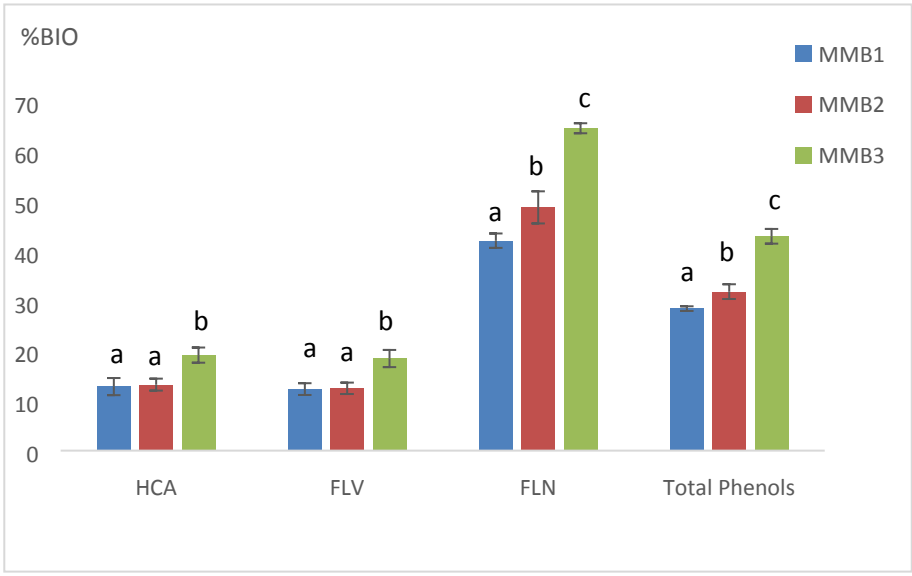
		Non-digested			Digesta /Micellar fraction		
Bioactive compounds		**MMB4**	**MMB5**	**MMB6**	**MMB4**	**MMB5**	**MM**
Phenolic Compounds	caffeic acid-d_1	1.81±0.16a	1.80±0.07a	1.85±0.13a	0.20±0.01A	0.22±0.01A	0.24±0.03A
	caffeic acid-d_2	2.02±0.13a	1.99±0.04a	1.95±0.08a	0.10±0.01A	0.12±0.02A	0.14±0.02A
	ferulic acid-d_1	1.48±0.08a	1.45±0.03a	1.46±0.02a	0.25±0.02A	0.27±0.04A	0.26±0.03A
	sinapic acid-d	1.13±0.07a	1.15±0.01a	1.16±0.01a	0.19±0.02A	0.21±0.04A	0.23±0.02A
	ferulic acid-d_2	1.29±0.06a	1.24±0.02a	1.24±0.01a	0.28±0.02A	0.36±0.06A	0.35±0.05A
	p-coumaric acid-d_1	3.10±0.13a	3.04±0.02a	3.01±0.05a	0.25±0.02A	0.32±0.05A	0.31±0.04A
	p-coumaric acid dimer	0.32±0.02a	0.33±0.01a	0.34±0.02a	0.04±0.01A	0.04±0.01A	0.05±0.01A
	∑HCA	11.15±0.05a	11.00±0.16a	11.00±0.18a	1.32±0.10A	1.53±0.23A	1.57±0.19A
	vicenin-2	4.09±0.21a	3.94±0.08a	3.98±0.21a	0.49±0.05A	0.57±0.10A	0.55±0.07A
	luteolin -d	2.67±0.19a	2.60±0.07a	2.64±0.10a	0.38±0.04A	0.46±0.06A	0.44±0.06A
	∑FLV	6.77±036a	6.54±0.14a	6.62±0.29a	0.86±0.08A	1.03±0.15A	0.99±0.12A
	naringin-d	3.65±0.20a	3.53±0.07a	3.23±0.54a	0.55±0.04A	0.44±0.03A	0.43±0.10A
	narirutin	3.46±0.21a	3.24±0.10a	3.21±0.06a	0.88±0.04A	1.00±0.09A	0.96±0.23A
	hesperidin	12.58±0.71a	12.83±0.64a	13.41±0.39a	7.87±0.47A	7.70±0.65A	7.04±0.42A
	didymin	0.61±0.01a	0.57±11a	0.60±0.05a	0.22±0.01A	0.33±0.05B	0.19±0.02A
	∑FLN	20.29±1.10a	20.17±0.91a	20.45±0.46a	9.53±0.51A	9.47±0.78A	8.61±0.32A
	∑Total Phenols	38.21±2.09a	37.71±1.15a	38.07±0.67a	11.71±0.68A	12.03±1.15A	11.17±0.42A
Carotenoid Compounds	MUT epimer A	0.37±0.02a	0.34±0.04a	0.35±0.01a	0.10±0.01A	0.07±0.01AB	0.04±0.01B
	LUT	0.08±0.01a	0.08±0.01a	0.08±0.01a	0.02±0.01A	0.01±0.01A	0.01±0.01C
	MUT epimer B	0.18±0.02a	0.20±0.03a	0.17±0.01a	0.06±0.01A	0.04±0.01B	0.02±0.01B
	ZEA	0.12±0.01a	0.12±0.01a	0.13±0.01a	0.03±0.01A	0.02±0.01AB	0.02±0.01B
	(9*Z*)- or (9´ *Z*)-ANT	0.18±0.03a	0.18±0.01a	0.18±0.02a	0.11±0.01A	0.06±0.02B	0.03±0.01B
	ZEINO	0.06±0.01d	0.07±0.01a	0.07±0.01a	0.02±0.01A	0.01±0.01B	0.01±0.01C
	BCR	1.35±0.10a	1.36±0.13a	1.37±0.08a	0.54±0.02A	0.41±0.03B	0.18±0.01C
	ACAR	0.07±0.01a	0.07±0.01a	0.07±0.01a	0.02±0.01A	0.02±0.01B	0.01±0.01C
	BCAR	0.20±0.01a	0.20±0.01a	0.21±0.02a	0.09±0.01A	0.06±0.01B	0.02±0.01C
	PT	0.20±0.01a	0.20±0.01a	0.20±0.01a	0.13±0.01A	0.12±0.01A	0.06±0.01B
	PF	0.09±0.01a	0.09±0.01a	0.10±0.01a	0.08±0.01A	0.07±0.01A	0.03±0.01B
	Total Carotenoids	2.99±0.08a	2.99±0.26a	3.00±0.06a	1.22±0.03A	0.92±0.08B	0.44±0.03C
	RAE*	7.56±0.39a	7.62±0.58a	7.74±0.16a	3.06±0.11A	2.27±0.07B	0.98±0.10C

*: Retinol activity equivalent: calculated as RAE (μg/100 mL) = (μg of β-carotene)/12 + (μg β-cryptoxanthin + μg α-carotene)/24. Different lower case letters in the same row show significant differences ($p<0.05$) among MMBs. Different capital letters in the same row indicate significant differences ($p<0.05$) among digesta/micellar fraction of each sample. FLV = (flavones), FLN = (flavanones), HCA = (hydroxycinnamic acids), ANT = (antheraxanthin), MUT= mutatoxanthin, LUT= (lutein), ZEA = (zeaxanthin), ZEI = (zeinoxanthin), BCR = (β-cryptoxanthin), ACAR= (α-carotene), BCAR= (β-carotene), PT= (phytoene), PF= (phytofluene).

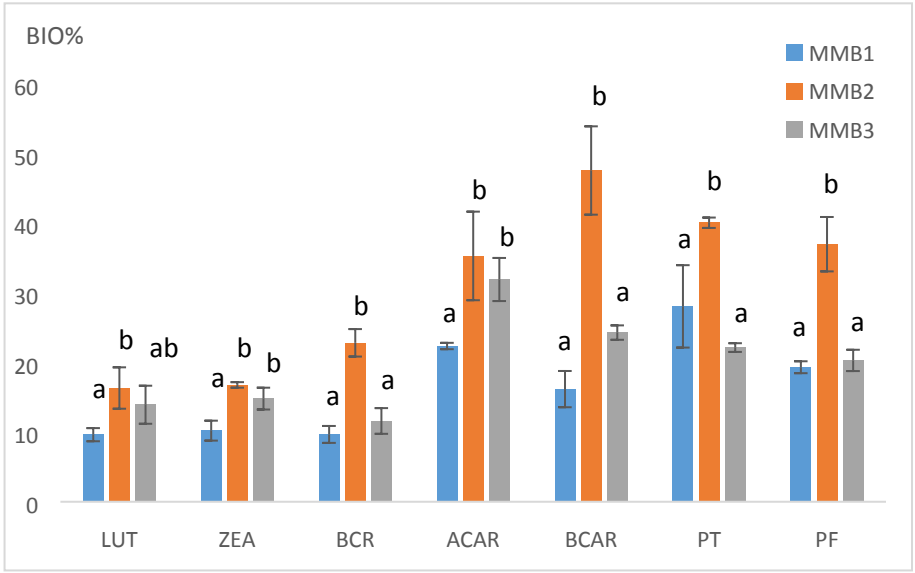
Figure 1.



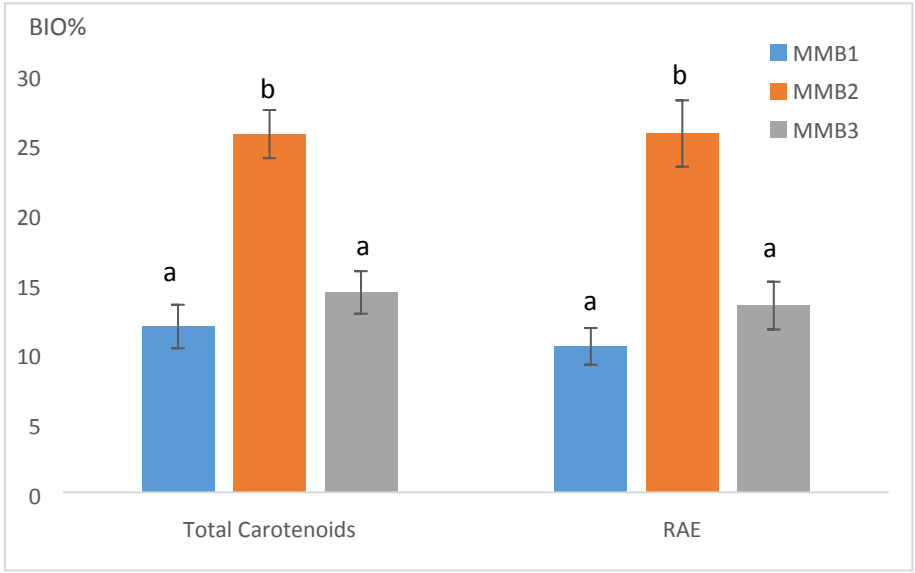
A



B



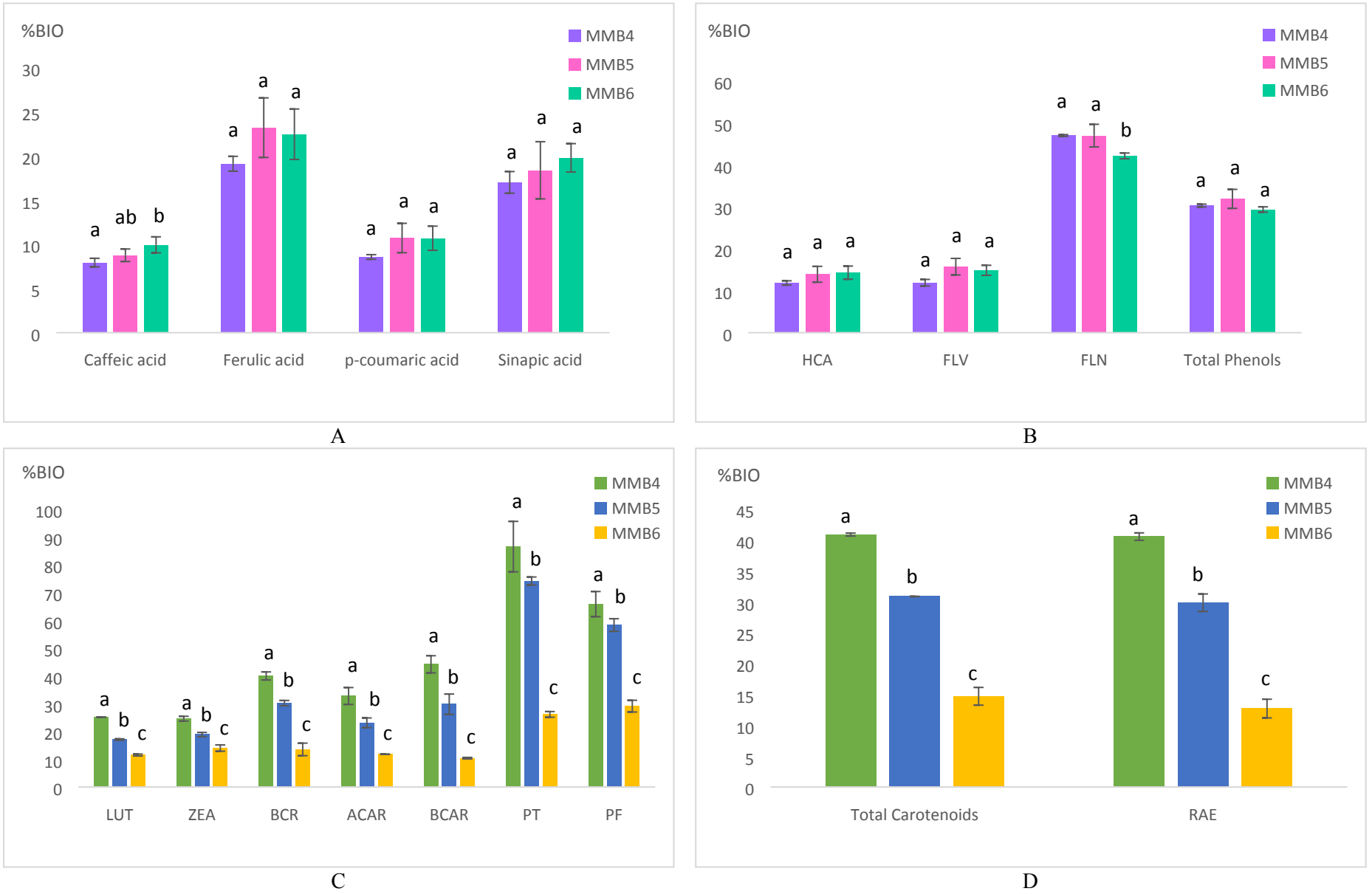
C



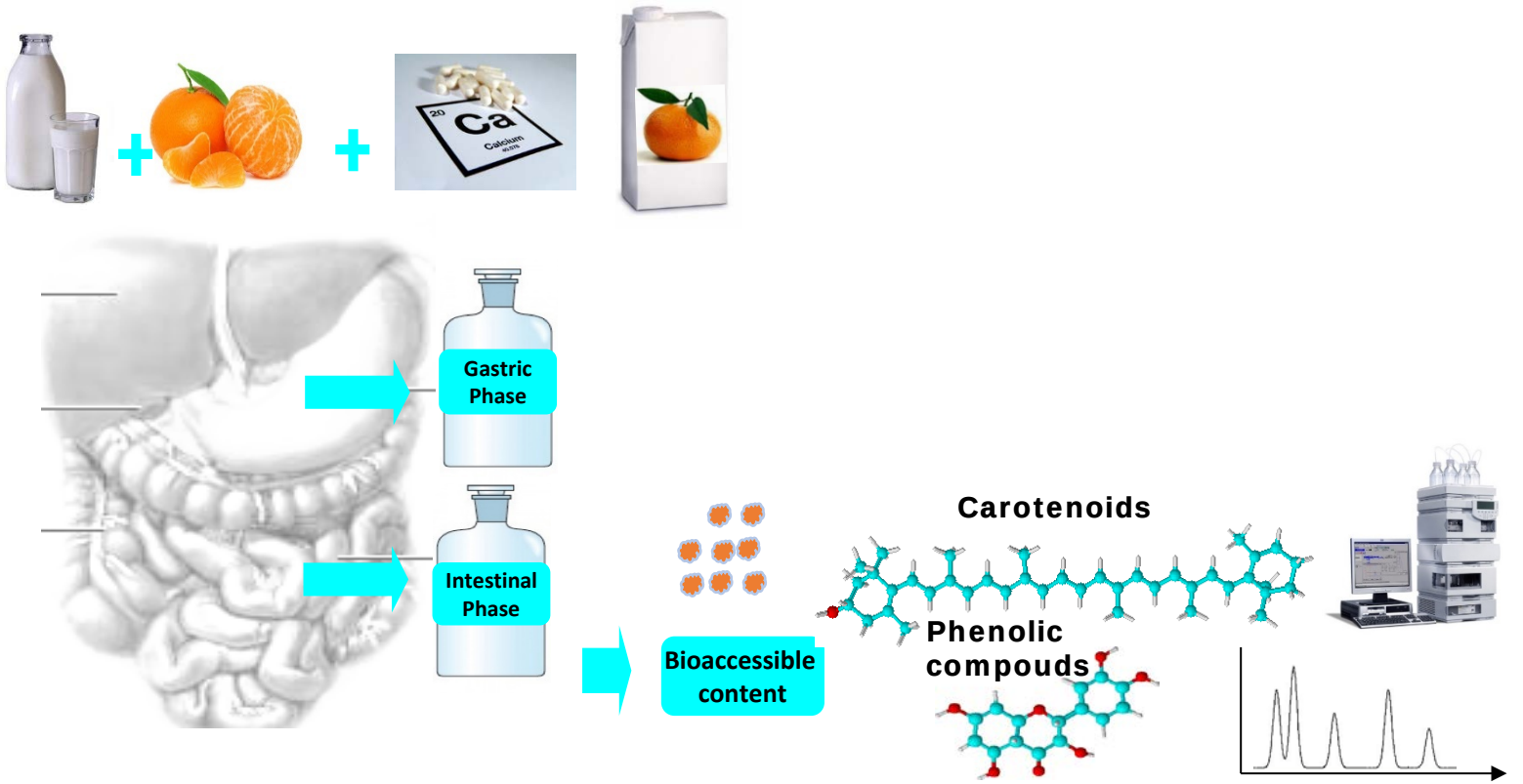
D

a,b,c Different letter indicate statistically significant differences ($p < 0.05$) among bioaccessibility (BIO %) of each compound among formulations. FLV = flavones, FLN = flavanones, HCA = hydroxycinnamic acids, LUT= (lutein), ZEA = (zeaxanthin), BCR = (β-cryptoxanthin), ACAR= (α-carotene), BCAR= (β-carotene), PT= (phytoene), PF= (phytofluene).

Figure 2.



a,b,c Different letter indicate statistically significant differences ($p < 0.05$) among bioaccesibility (BIO %) of each compound among formulations. FLV = (flavones), FLN = (flavanones), HCA = (hdroxycinnamic acids), LUT= (lutein), ZEA = (zeaxanthin), BCR = (β-cryptoxanthin), ACAR= (α-carotene), BCAR= (β-carotene), PT= (phytoene), PF= (phytofluene).


20
Ca
Calcium
Gastric Phase
Intestinal Phase
Bioaccessible content
Carotenoids
Phenolic compouds

Supplementary Materials: Table S1: Linearity, limits of quantification and detection (LOQ and LOD), and precision (repeatability and reproducibility) for the polyphenols analytical method.

	Retention Time (min)	Compound	Wavelength (nm)	Regression equation	R^2 [a]	LOD [b] (µg)	LOQ [c] (µg)	Intra-day (n=3)	Inter-day (n=18)
Phenolic Compounds									
HCA	7.821	*Caffeic acid*	320	y = 3141.53x +2.11	*0.9987*	0.001	0.001	1.77	3.16
	9.179	*p-coumaric acid*	320	y = 4062.04x +3.66	*0.9999*	0.001	0.001	1.31	1.80
	9.882	*Ferulic acid*	320	y = 3687.28x +2.04	*0.9999*	0.001	0.001	1.34	1.49
	10.048	*Sinapic acid*	320	y = 3160.65x + 8.156	*0.9999*	0.001	0.001	1.37	1.58
FLV	14.417	*Apigenin*	320	y = 1772.74x + 2.50	*0.9939*	0.002	0.006	2.22	3.54
FLN	11.411	*Naringenin*	*280*	y = 2154.57x +1.28	*0.9995*	0.001	0.001	1.28	2.54
	11.617	*Hesperidin*	280	y = 860.73x + 0.80	*0.9992*	0.002	0.006	0.52	2.47
	11.892	*Naringin*	280	y = 807.71x +0.29	*0.9995*	0.001	0.002	0.43	1.08
	13.430	*Dydimin*	280	y = 1100.79x +0.463	*0.9997*	0.001	0.001	0.98	1.25

Values are expressed as means ± standard deviation; R^2 [a]: coefficient of determination, **LOD[b]:** limit of detection; **LOQ[c]:** limit of quantification. FLV = flavones, FLN = flavanones, HCA = hydroxycinnamic acids