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**Negative effects of divalent mineral cations on the bioaccessibility of
carotenoids from plant food matrices and related physical properties of gastro-
intestinal fluids**

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Abstract

Carotenoid intake and tissue levels have been frequently associated with reduced risk of chronic diseases. However, their bioavailability is low and influenced by many dietary related parameters. Divalent mineral cations have been suggested to interfere with carotenoid digestion and to hamper micellarization, a prerequisite for their uptake, via complexation of bile salts and precipitation of fatty acids. In the present investigation, we have evaluated the effects of increasing concentrations of magnesium (0 – 300 mg/L), calcium (0 – 1500 mg/L), zinc (0 – 200 mg/L), and sodium (0 – 1500 mg/L; control monovalent cation), on carotenoid bioaccessibility from frequently consumed food items rich in carotenoids (tomato juice, carrot juice, apricot nectar, spinach and field salad), following simulated gastro-intestinal digestion. In addition, physicochemical parameters of digesta (macroviscosity, surface tension), micelle size, and zeta-potential were evaluated. All divalent minerals (DM) reduced bioaccessibility of total carotenoids ($P<0.01$), as well as of individual carotenoids. Calcium and magnesium led to reductions of up to 100% at the 2 highest concentrations. Curiously, sodium increased ($P<0.01$) carotenoid bioaccessibility of most investigated matrices. The absolute value of the zeta-potential decreased with increasing concentrations of DM, suggesting a decreased stability of the colloidal digesta dispersion. Viscosity decreased, except for apricot nectar samples, while surface tension increased with DM concentration ($P<0.05$). Thus, at physiological ranges, calcium and magnesium could negatively impact carotenoid bioavailability, while for zinc, negative effects were only seen at supplemental concentrations. The potential negative effects of DM on carotenoid bioavailability should be further studied *in vivo*.

Keywords: phytoene; phytofluene; calcium; magnesium; digestion; micellization; surface tension; zeta-potential; bile salts; soap formation.

INTRODUCTION

Carotenoids are secondary metabolites, found across all kingdoms of life. As pigments, absorbing light in the visible spectrum, carotenoids colour many fruits and vegetables in orange, red and yellow^{1,2}. Structurally, they are composed of a C-40 skeleton with a partly conjugated double-bond system that is the key to many of the carotenoids' characteristics. One important property is the ability to absorb excess energy from reactive oxygen species and to quench singlet oxygen, meaning that they can act as anti-oxidants^{1,3,4}. This anti-oxidant potential coupled with anti-inflammatory properties⁵ is of particular interest from a public health perspective for the prevention and delayed progression of several chronic diseases⁶, including eye related degenerative diseases⁷, cardiovascular diseases⁸, diabetes⁹ and prostate cancer^{10,11}. In addition, certain carotenoids, including β -carotene, α -carotene and β -cryptoxanthin, are also sources for vitamin A production by the human body.

Nevertheless, the bioactivity of carotenoids is firstly dependent on their bioavailability, i.e. the fraction of ingested carotenoids that is available for physiological functions and/or storage in organs and tissues¹². The first limiting factor of carotenoid bioavailability is their release from the food matrix and their inclusion in bile acid-lipid mixed micelles^{13,14}, i.e. their bioaccessibility. This step is mainly affected by 1) dietary factors (type and amount of fiber and lipids, form of storage of carotenoids in the matrix, among other)¹⁵⁻²⁰, and 2) host-related physiological factors (enzyme and bile acid concentrations secreted, gastric and intestinal pH, etc.)^{21,22}.

The presence of divalent minerals during gastro-intestinal digestion, particularly calcium and magnesium, has been hypothesized to negatively affect the bioaccessibility of carotenoids^{23,24}. Divalent mineral cations are naturally present in the human gut and are part of our diet. The effects of the interactions between divalent cations and lipids^{25,26}, and divalent cations and bile acids^{27,28} have already been investigated. It has been hypothesized that during digestion, these

cations bind free fatty acids and bile acids to form fatty acid soaps and bile salts of generally low solubility, respectively. Depending on the cation concentration, this could lead to precipitations, removing lipids from the system and ultimately impairing the formation of bile acid-lipid mixed micelles. Previously, we have scrutinized *in vitro* employing simulated gastrointestinal digestion the effects of the presence of divalent minerals, including calcium, magnesium and zinc, on the bioaccessibility of carotenoids alone and from spinach^{23,24}. The concentration-dependent effect of these minerals appeared obvious, with calcium and magnesium reducing significantly, at increasing concentrations, carotenoid fractional bioaccessibility. However, the effect of different food matrices including solid vs. liquid form and thus different complexity on carotenoid bioaccessibility has so far not been studied. Furthermore, we have previously observed an association between decreased viscosity and increased surface tension and carotenoid bioaccessibility²³. Here, we strived to obtain additional insights in this regard from real food matrices, also with respect to micellar stability, as it could be speculated that an altered dynamic of the micellarization process may impinge on micelle formation. The objective of the present study was thus to evaluate the impact of various concentrations of the divalent minerals calcium, magnesium and zinc, present during simulated digestion, on the bioaccessibility of individual carotenoids from different frequently consumed food items rich in carotenoids, and to better understand the mechanism of action of divalent minerals by scrutinizing viscosity and surface tension changes during digestion.

EXPERIMENTAL

Chemicals and standards

Digestive enzymes, i.e., pepsin (porcine, ≥ 250 units/mg solid, measured as trichloroacetic acid-soluble products using hemoglobin as substrate) and pancreatin (porcine, 4x US Pharmacopeia

specifications of amylase, lipase and protease), and porcine bile extract were purchased from Sigma–Aldrich (Bornem, Belgium). The solvents hexane, diethyl ether, methanol (MeOH), acetonitrile (ACN), and dichloromethane (DCM) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), and all solvents were from Rotisol® HPLC grade. Acetone was purchased from VWR (Leuven, Belgium), and methyl tert-butyl ether (MTBE) from Sigma–Aldrich (Bornem, Belgium). Potassium hydroxide (KOH), sodium hydroxide (NaOH), hydrochloric acid (HCl), calcium chloride anhydrous and zinc chloride anhydrous were purchased from VWR (Leuven, Belgium). Sodium bicarbonate and sodium chloride were purchased from Merck (Darmstadt, Germany), while magnesium chloride anhydrous was acquired at Sigma–Aldrich, and ammonium acetate from VEL® (Leuven, Belgium). Lycopene, β -carotene and β -apo-8'-carotenal standards were acquired from Sigma–Aldrich; violaxanthin, neoxanthin, lutein, phytoene and phytofluene standards were purchased from CaroteNature GmbH (Ostermundigen, Switzerland). Unless otherwise specified, all products were of analytical grade or higher. 18 M Ω water was prepared with a purification system from Millipore (Brussels, Belgium) and used throughout the study.

Food matrices and test meals

Five food items were investigated: Eden Organic 100% Carrot Juice (other ingredients: lemon juice); Granini Apricot Nectar from concentrate (minimum fruit content: 40%) (other ingredients: sugar, concentrate from lemon juice, ascorbic acid); Rauch 100% Tomato Juice from concentrate (other ingredients: 0.3% salt, citric acid); frozen spinach leaves (*Spinachia oleracea*) from CORA supermarket (Foetz, Luxembourg); and fresh field salad (*Valerianella locusta*) from Delhaize (Belval, Luxembourg) supermarket. Matrices were chosen based on their carotenoid profile, and in order to cover the range of several of the most common carotenoids present in human diet. Fruit juices were aliquoted into 50 mL amber conical

propylene tubes, frozen in liquid nitrogen and stored at -80°C until the day of the experiments. Spinach aliquots were prepared as follows: i) spinach was left to defreeze; ii) the excess water was drained and the leaves were pat dry; iii) spinach was homogenized stepwise in a Grindomix GM 200 (Retsch, Aartselaar, Belgium) at 3, 4 and 5 x 1000 rpm (5 seconds each); iv) the homogenized spinach was weighted and aliquoted into polypropylene sample containers, flushed with argon and stored at -80°C. Aliquots of field salad were prepared in a similar fashion (step iii and iv).

To promote the solubilization of dietary carotenoids and micelle formation during gastrointestinal digestion, we have chosen to add coffee creamer (10% fat) to the tested matrices, as a natural source of triglycerides and phospholipids, which are essential for the formation of mixed bile-lipid micelles. Similar dairy products have been used previously in our lab in order to foster carotenoid bioaccessibility^{24,29,30}. INEX coffee creamer (10% fat) was purchased at a local supermarket (Delhaize).

Simulation of gastro-intestinal digestion and factors investigated

To investigate the effect of divalent minerals (calcium, magnesium and zinc), we tested the following concentrations: calcium 0, 250, 500 and 1000 mg/L; magnesium 0, 100, 200 and 300 mg/L; zinc 0, 12.5, 25, 50, 100 and 200 mg/L; and sodium 0, 375, 750 and 1500 mg/L, which was used as a control monovalent cation. Concentrations were chosen based on the dietary reference intakes (RDA (recommended dietary allowance), or AI (acceptable intake) when no RDA was available) and tolerable upper intake levels (UL)³¹. To determine the concentration of the mineral per volume of digesta, we assumed a total volume of 2 L of intestinal fluids during gastrointestinal (GI) digestion. For the purposes of this study, we have defined as physiological a range of cation concentrations up to the daily RDA/AI (dissolved in 2 L), and as supplemental concentrations above the RDA/AI per 2L. Standard solutions of divalent

minerals were prepared with a physiological saline solution. The *in vitro* gastro-intestinal digestion protocol was adapted from Biehler *et al.* (2011)²² and is described below.

Gastric phase. The previously prepared frozen aliquots of the food matrices were thawed, and 4 g of matrix were weighed into 50 mL amber Falcon tubes. To improve carotenoid bioaccessibility, 2 mL of coffee creamer (10% fat) were added to each test meal^{24,29}. To simulate the gastric passage, varying volumes of physiological saline and of a standard solution of the investigated mineral were added to equal final volumes of the test meal. The volumes of the physiological saline and of the mineral solution were determined based on the desired cation concentration at the intestinal phase. Test meal was acidified by adding 2 mL of pepsin (40 mg/mL in HCl 0.01 M), and the pH was adjusted for each test meal at the beginning of the gastric phase to a pH of 3. The tubes were sealed with Parafilm®, and incubated during 1h at 37°C, in a shaking water bath (GFL 1083 from VEL®, Leuven, Belgium) with reciprocating motion at 100 strokes per minute.

Intestinal Phase. At the end of the incubation period, a volume of 9 mL of solution of porcine bile extract (24 mg/mL) and pancreatin (4 mg/mL) in NaHCO₃ (0.1 M) was added to the simulated digestive fluids, and the pH was adjusted to 7. The final volume of the samples was brought to 50 mL with physiological saline. The tubes were once again sealed with Parafilm and to simulate the intestinal passage, samples were incubated for 2 h in a shaking water bath (100 rpm) at 37°C. At the end of the gastro-intestinal digestion, aliquots of 12 mL were transferred into 15 mL Falcon tubes and centrifuged (Thermo Scientific Heraeus Multifuge X3R) at 4700g for 1 h, at 4 °C. After centrifugation, a 6 mL aliquot was collected from the middle aqueous fraction with a syringe and hypodermic needle, and filtered through a 0.2 µm

nylon membrane filter (Acrodisc® 13mm Syringe Filters, PALL Life Sciences, Ann Harbor, MI) into a new 15mL Falcon tube. Four mL were then taken for extraction of carotenoids.

Extraction of carotenoids

Extraction from food matrices. Aliquots of 4 g of food material were weighted into 50 mL falcon tubes. Spinach and field salad were wetted with 5 mL of methanol, and 1 mL of 30% aqueous KOH was added for saponification of chlorophylls. Samples were then vortexed, sonicated at 37 kHz for 10 min in an ultrasonic bath (Elmasonic Ultrasonic Bath, Elma, Mägenwil, Switzerland), and further incubated in the dark for 20 min (still with KOH) at room temperature. Samples were then centrifuged for 5 min at 1300g at 4°C. The supernatant was collected into a second 50 mL Falcon tube. The following extraction steps were similar for both green leafy matrices and fruits juices. Matrices were extracted once with 9 mL hexane:acetone (1:1), vortexed, sonicated for 5 min, and centrifuged (5 min, 1300g, 4°C). The supernatants were collected into a second 50 mL Falcon tube, while for saponified samples the supernatant was combined with the methanol phase. Extraction was repeated once with 9 mL of hexane and a second time with 9 mL hexane plus 4 mL of saturated NaCl. Supernatants were combined in the second tube. For the extraction of the fruit juice matrices, only half of the above mentioned volumes were used. All matrices were extracted once more with 4 mL of diethyl ether, vortexed, sonicated and centrifuged, and the supernatant was combined with the previously collected organic phases. To promote phase separation from water residues, samples were spun down at 1300g, for 1 min, at 4°C. When water was present, the organic phase was transferred into a third tube, and the total volume was written down prior to collecting a 10 mL aliquot for evaporation. The aliquots of the combined extracts were dried under a stream of nitrogen using a TurboVapLV (Biotage, Eke, Belgium) apparatus, for 45 min at 25°C. Dried extracts were re-dissolved in 5 to 7 mL of MTBE:MeOH (3:7), filtered through a 0.2 µm PVDF syringe filter, and the filter was rinsed with 1 mL of MTBE:MeOH (3:7).

Extraction from the bioaccessible fraction. A 4 mL aliquot of the bioaccessible fraction was extracted with 6 mL of hexane:acetone (2:1) – in the case of green leafy matrices an additional 1 mL of aqueous KOH (30%) was added for saponification - vortexed and centrifuged for 2 min at 4000g, at 4°C. The supernatant was transferred to a new 15 mL falcon tube. The bioaccessible fraction was re-extracted once with 5 mL of hexane, and a second time with 5 mL of diethyl ether. The combined and homogenized organic phases were centrifuged, during 2 min at 4000g, to separate the organic phase from any water carried over during extraction. The totality of the organic phase was transferred into a new tube, and evaporated under a stream of nitrogen on a TurboVap for 45 min at 25°C. The dried carotenoid extracts were spiked with an appropriate amount of internal standard (IS), β -apo-8'-carotenal, of known concentration (10 μ g/mL) to obtain a final concentration of 1 μ g/mL in the sample, and re-dissolved in 500 μ L of MTBE:MeOH (3:7). Samples were filtered through a 0.2 μ m PVDF syringe filter (PALL Life Sciences, Ann Arbor, MI, USA), into an HPLC amber vial. The filter was then rinsed with 100 μ L of MTBE:MeOH, to reduce possible losses of carotenoids to the filter membrane, which was combined with the previous filtered sample, to a total volume of 600 μ L.

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HPLC analysis

Carotenoids were separated on an Agilent 1260 Infinity Preparative HPLC instrument (Agilent Technologies, De Kleetlaan Belgium) by gradient elution with (A) water:MeOH (60:40) with 30 mM of ammonium acetate, and (B) ACN:DCM (85:15), passing through an Accucore™ C30 column (2.6 μ m particle size, 100 mm length, 3 mm diameter, from Thermo Fisher Scientific) at 30°C, 10 μ L injection volume. Elution gradient was as follows: 0 min, 48% B; 4 min, 48% B; 5 min, 52% B; 11 min, 52% B; 13 min, 75% B; 18 min, 90% B; 35 min, 90% B; 36min, 42% B. Carotenoids were detected with a UV/VIS photodiode array detector, and identified according to their retention times and spectral data, based on the comparison to the

corresponding individual standard. All peaks were integrated manually at 286 nm (phytoene), 350 nm (phytofluene), 440 nm (neoxanthin and violaxanthin), 450 nm (lutein and α -carotene), 455 nm (β -carotene, β -cryptoxanthin and IS), and at 470 nm (lycopene), according to each carotenoid's absorption maxima. Quantification was done using the internal standard method³².

Surface tension and macroviscosity analysis

To investigate if the addition of divalent minerals affected the physico-chemical characteristics of the digesta, we measured the surface tension and macroviscosity of the aqueous micellar fractions of the digesta. Macroviscosity was determined by measuring the steady state shear flow of the samples in an Anton-Paar rheometer (MCR 302, WESP, Graz, Austria), using a double gap concentric cylinder geometry (DG 26.7). Measurements were carried out by applying an upward–downward ramp shear stress range from 0.1 to 200 s⁻¹, with a 60 s maintenance shear rate step (at 200⁻¹). Shear stress (τ) – shear rate data ($\dot{\gamma}$) data were fitted according to the Ostwald–de Waele model:

$$(1) \quad \tau = K\dot{\gamma}^n$$

where: τ_0 = the yield stress (Pa), K = consistency coefficient (mPa * s⁻ⁿ) and n = rheological behavior index (dimensionless). All measurements were performed at 25 $\pm$ 0.03 °C. Surface tension of digesta samples, pre-conditioned at 25 $\pm$ 0.1 °C, were determined via the weight-drop method as previously described³³ by Permprasert & Devahastin (2005). The air–water interfacial properties of digesta were calculated as follows³⁴:

$$(2) \quad \sigma_{digesta} = \frac{m_{digesta}}{m_{H2O}} \times \sigma_{H2O}$$

where σ_{H2O} = 71.99 dyn/cm is the surface tension of pure water (Pallas & Harrison, 1990).

Micelle size and zeta potential analysis

An aliquot of the aqueous micellar fraction was taken for the analysis of the micelle size and zeta potential (assumed to modulate the stability of the particles in solution). Aliquots were filtered beforehand, through a 0.2 µm syringe filter. The intensity-weighted mean hydrodynamic radius and zeta-potential were determined by dynamic light scattering, and Laser Doppler Micro-electrophoresis respectively. Measurements were done at room temperature with a Zetasizer Nano Zs (Malvern Instruments, Malvern, UK).

Statistical analysis

Unless described otherwise, all values are given as mean ± SD. Normality of distribution and equality of variance of the data were tested by normality plots and box plots, respectively. If required, log-transformation was conducted. All bioaccessibility data was normalised to a respective control which was run for every set of analyses. Bioaccessibility itself was calculated as the amount recovered in the final micellar phase compared to the original matrix content. A general linear model was developed to test the effect of minerals and their concentration on bioaccessibility, with concentration, matrix, type of mineral and carotenoid (total carotenoids for comparison across matrices, individual carotenoids to compare within-matrix effects) as fixed factors, and fractional bioaccessibility as the dependent (observed) parameter. A P-value below 0.05 (2-sided) was considered statistically significant. Following significant Fisher F-values, Bonferroni's post hoc-tests were conducted.

RESULTS

Carotenoid profile of the food matrices

In terms of total carotenoid content (µg/g wet weight of matrix), frozen spinach contained the highest amount, followed by tomato juice, carrot juice, field salad, and finally, apricot nectar (Table I).

In the green leafy matrices (spinach and field salad), the xanthophylls (lutein, violaxanthin and neoxanthin) were the major carotenoids, representing 94% and 88% of the determined total carotenoid content, respectively, which is rather high compared to earlier studies³⁵, due to the rather low β -carotene content found in the samples. In the case of the carrot and tomato juices and apricot nectar, carotenes (β - and α -carotene, and lycopene) were the major carotenoids, with only a very small contribution from lutein. Also, all the three latter matrices were rich in the colourless carotenoids phytoene and phytofluene, which were not detected in green leafy varieties. The contribution of phytofluene and phytoene to the total quantified carotenoid content was 34% in carrot juice, 46% in tomato juice and 83% in apricot nectar.

Bioaccessibility of total carotenoids across matrices

The bioaccessibility of total carotenoids was significantly different ($p < 0.001$) between most matrices, except for apricot nectar and tomato juice. Bioaccessibility of total carotenoids was higher from the juices than it was from the green leafy varieties (Figure 1) and followed the order: apricot nectar (21.4%) > tomato juice (20.1%) > carrot juice (17.1%) > field salad (9.7%) > spinach (5.2%).

Another aspect we observed was the remarkable bioaccessibility of phytofluene and phytoene of 18.5 – 46.9% and 21.1 – 47.6%, respectively (Supplementary Table I), and their large contribution to average total carotenoid bioaccessibility in the juices and nectar (Fig. 1). This was especially evident in the case of tomato juice, where phytofluene and phytoene together represented 91.5% of the total bioaccessible fraction, while in the original matrix they made up 46.2% of the total carotenoid content.

Bioaccessibility of β -carotene: effect of divalent minerals across different matrices

Beta-carotene was the only carotenoid detectable in all of the 5 investigated matrices, thus it served as a reference carotenoid to compare the effect of the minerals across all tested matrices.

Bioaccessibility of β -carotene was significantly affected ($p < 0.001$) by the divalent minerals in a concentration-dependent fashion (Figure 2). Addition of magnesium led to a significant decrease of its bioaccessibility in all matrices. The addition of 200 mg/L magnesium to the test meals reduced the bioaccessibility of β -carotene by more than half (compared to the control), while at 300 mg of magnesium, no more β -carotene was found in the micellar fraction (except for spinach). Similarly, a higher concentration of calcium, i.e. 1000 mg/L, also reduced the bioaccessibility of β -carotene up to 100% for all tested matrices. Interestingly, the addition of sodium had an overall significant positive effect on the bioaccessibility, especially in the case of β -carotene from the juices and apricot nectar, while for the green leafy varieties the effect was less pronounced (field salad) or inexistent (spinach). Regarding zinc, its presence significantly improved β -carotene bioaccessibility from field salad and spinach for all concentrations tested, and in carrot juice for a concentration range between 12.5 and 50 mg/L. On the other hand, at higher concentrations (≥ 100 mg/L), the presence of zinc significantly decreased β -carotene bioaccessibility from apricot nectar and tomato juice (up to 43%), and up to 100% in carrot juice.

Comparison of the bioaccessibility of carotenes, xanthophylls, and colourless carotenoids

Differences between carotenes and xanthophylls. The relative variations of the bioaccessibility of the carotene β -carotene, as a function of varying concentrations of the investigated minerals, were compared to those of the xanthophylls lutein and neoxanthin, from either spinach or field salad (Figure 3, supplementary Table X for bioaccessibility values). The statistical interaction “matrix * mineral” had a significant ($P < 0.001$) effect on the bioaccessibility of carotenoids.

Carotenoids from spinach were overall less affected by the addition of minerals, than those from field salad. Sodium and zinc had no statistically significant effect on the bioaccessibility of xanthophylls measured in spinach test meals, while β -carotene bioaccessibility increased significantly in the presence of zinc (> 12.5 mg/L, Figure 2). As for field salad, zinc improved significantly ($P < 0.001$) the bioaccessibility of both lutein, neoxanthin and β -carotene, by up to 39%, 31%, and 63%, respectively.

Addition of sodium to the field salad test meals led to a significant ($P < 0.05$) increase in lutein and β -carotene bioaccessibility, by ca. 4% and 31%, respectively, at a concentration of 350 mg/L. At highest concentrations (750 mg/L and 1500 mg/L), bioaccessibility of xanthophylls, but not that of β -carotene, was significantly reduced (up to 35%).

Also for magnesium, the bioaccessibility of carotenoids from spinach appeared less strongly affected than from field salad. The significant effects were seen > 300 mg/L for spinach samples and > 100 mg/L in field salad samples. Addition of calcium at a concentration of 250 mg/L improved significantly the amount of lutein (ca. 13%) and neoxanthin (ca. 24%) recovered in the micellar fraction from field salad test meals, followed by a significant ($P < 0.001$) reduction (> 500 mg/L).

Differences between lycopene and phytoene and phytofluene. Despite their similar molecular structure, the bioaccessibility of lycopene was significantly lower ($P < 0.001$) than that of phytoene and phytofluene, from tomato juice samples. Under control conditions (i.e. no additional mineral), the average bioaccessibility of lycopene from tomato juice was of $2.9 \pm 1.8\%$, compared to $49.0 \pm 14.5\%$ and $53.6 \pm 24.4\%$ for phytoene and phytofluene, respectively.

Concerning the response of these carotenoids to the added minerals (Figure 4), the patterns of bioaccessibility variations between these three carotenoids were similar in the presence of

additional sodium and magnesium. Adding magnesium at >100 mg/L to the tomato juice digesta decreased ($P<0.001$) bioaccessibility of all three carotenoids, and at 300 mg/L the bioaccessibility dropped close to zero. Sodium, at 750 mg/L significantly ($P<0.001$) enhanced the bioaccessibility of phytoene and phytofluene; at 1500 mg/L the bioaccessibility of all three carotenoids significantly improved by 87% for lycopene, 118% for phytoene and 148% for phytofluene. Adding 250 mg/L of calcium increased the recovery of phytoene and phytofluene in the micellar fraction by 18% and 22%, while it had no significant effect on lycopene. Similarly to other carotenoids, 500 mg/L calcium reduced the bioaccessibility by more than 50%, and at 1000 mg/L no more carotenoids were detectable in the micellar fraction. Zinc, at concentrations <50 mg/L had no effect on the bioaccessibility of lycopene, phytoene and phytofluene, while higher concentrations reduced lycopene and phytofluene bioaccessibility (Figure 4).

Effect of divalent minerals on the physico-chemical properties of the digesta

Effect of mineral addition on the zeta-potential of the digesta. To verify the presence of mixed micelles in the micellar fraction of digesta and to evaluate the stability of the particles in solution, we attempted to measure micelle size as well as the zeta potential. However, due to time and cost reasons, measurements were performed only for spinach and carrot juice samples. Unfortunately, micelle size results were not informative. As measurements were not done *in situ* and had to be shipped, micellar fractions had to be previously frozen and thawed on the day of the analysis. The temperature changes might have caused particle size variations, making it impossible to accurately measure the micelle size distribution of the micellar fraction of the digesta.

The average zeta-potential value of the micellar fraction of the spinach and carrot juice digesta, under control conditions (i.e. no added mineral, $C = 0$ mg/L) was of -30.9 ± 2.4 mV and -30.6

± 9.6 mV, respectively. Variations of the zeta potential of the micellar fractions, depended on the type of mineral and concentration (Figure 5 and 6). While zinc and sodium did not cause visible variations in the zeta-potential, a more obvious variation was seen for calcium and magnesium. As the concentrations of the divalent minerals increased, the zeta-potential tended towards zero (Fig 5 and Fig 6), especially in the case of calcium and magnesium, suggesting that the colloid became unstable. Lowest zeta-potential values (-2.53 mV) were observed for spinach samples digested with calcium (at 1000 mg/L).

Effect of divalent minerals on the macroviscosity and surface tension of the digesta. In general, the digesta were prevalently characterised by a Newtonian fluid behaviour, with digesta from tomato, spinach and field salad exerting the lowest macroviscosities (0.97-1.02 mPa·s). Significantly ($P<0.01$) higher macroviscosity values were recorded in the case of carrot juice (ca. 1.08 mPa·s) and apricot nectar (2.16 mPa·s). It should be noted that in the latter case, the digesta exhibited a rather pseudoplastic (shear thinning) behaviour, suggesting the presence of a loosely structured/thickened bulk phase. Regarding sodium addition, the digesta macroviscosities exerted a slight increase (up to 4%), as a result of its buffering role to the biopolymers' hydrodynamic radii. In the presence of divalent minerals, a significant decrease of the digesta macroviscosity was detected. This was steeper for the high pectin content systems i.e. apricot, tomato and carrot juice. Contrary to calcium containing digesta, the apricot nectar – magnesium or zinc digesta exerted a distinct macroviscosity inflection point at low mineral concentrations, followed by a steep decrease upon further mineral content increase. In the case of leafy vegetable matrices, macroviscosity was also adversely affected by the presence of divalent minerals, but to a much lesser extent.

As for surface tension (Fig. 7e-h), the cationic mineral species present in the intestinal chymes, and to a lesser extent, the type of the food matrix, substantially impacted the air-water

interfacial properties. Surface tension for control digesta systems ranged from ca. 36.2 (for apricot nectar and carrot juice) to 37.5 dyn/cm (for tomato, spinach and field salad) showing a moderate negative ($r=-0.74$, $P<0.05$) correlation to the macroviscosity data. In the presence of the divalent cationic species, surface tension increased by 5 to 30%, depending on the digested food matrix, with field salad and spinach based digesta exerting the highest depletion in surface active compounds. Apricot nectar and carrot juice obtained digesta experienced a rather moderate increase of surface tension.

DISCUSSION

In the present study, we investigated the interaction of divalent minerals with carotenoids from various food matrices during simulated gastro-intestinal digestion. Previously, we found that the presence of calcium and magnesium, at varying concentrations, had the ability to significantly decrease the amount of isolated carotenoids (in absence of a food matrix), present in the micellar fraction of the digesta²³. The different food matrices chosen in the present study represented regularly consumed fruits and vegetables, providing the most common dietary carotenoids³⁵: β -carotene and α -carotene, lycopene, and lutein. Two additional regularly consumed carotenoids investigated were the colourless carotenoids phytoene and phytofluene. Although they are less commonly reported, they are present at relatively high concentrations in a broad spectrum of fruits and vegetables, including apricot, carrot and tomato³⁵. Carotenoid bioaccessibility was reduced significantly in all matrices by the highest concentrations of magnesium and calcium studied, to almost zero. This is similar to what has been reported earlier by Biehler et al (2011)²⁴, investigating micellarization of spinach-borne carotenoids digested with calcium, magnesium and iron. However, the influence of the matrix was apparent when evaluating the effects of divalent minerals on the bioaccessibility of carotenoids. Responses to divalent minerals appeared to differ between green leafy matrices

and the juices. Increasing concentrations of calcium and magnesium generally resulted in reduced bioaccessibility of carotenoids following digestion. However, at lower concentrations (250 mg/L calcium, 100 mg/L magnesium), the bioaccessibility of carotenes, specifically β -carotene, was enhanced in juices and nectar, though neither in spinach nor field salad. Similarly, though employed as a control monovalent ion, sodium improved bioaccessibility in juices and apricot nectar, especially at higher concentrations (≥ 750 mg/L), while lutein and neoxanthin bioaccessibility were significantly ($P < 0.05$) reduced in field salad. It is possible that the more rigid cellular matrix of solid constituents, with carotenoids not yet present in lipid droplets at earlier phases of digestion, is less prone to interactions with divalent cations, but this remains speculative.

The negative effects of the divalent cations at higher concentrations were correlated with increased surface tension, suggesting the depletion of surfactants (bile salts) from the system, similar as observed earlier for carotenoids in systems without food matrix²³. The fact that macroviscosity in parallel decreased, towards values similar to that of pure water, also suggested removal of soluble compounds from the system by flocculation, as observed also visually. Depletion of carotenoids is likely to occur as a consequence of the binding of divalent cations to unconjugated bile acids³⁶ and free fatty acids, forming bile salts of poor solubility and insoluble fatty acids soaps^{25,37,38}, resulting in precipitation. This precipitation was visible at the end of the digestion, when compared to control conditions (no minerals added). Without the presence of bile acids, or free fatty acids, stimulating the formation of mixed-micelles, bioaccessibility of carotenoids is expected to be drastically reduced. Similarly, sodium can also interact with bile acids, resulting in higher solubility of the bile salts, and lower critical micelle concentrations, promoting mixed-micelle formation²⁸ and thus bioaccessibility of carotenoids, explaining the higher recovery from the aqueous micellar fraction of the digesta with added sodium.

The observed positive effects of divalent minerals at lower concentrations on bioaccessibility are more challenging to explain, but could include lipolysis. Lipolysis occurs via the adsorption of pancreatic lipase to the surface of lipid droplets. As lipid digestion proceeds and the products of lipolysis (e.g. free fatty acids) build up at the interface, lipase activity is inhibited. In order for lipolysis to proceed, fatty acids and other molecules adsorbed to the surface of lipids (e.g. proteins and soluble fibres) need to be displaced³⁹. This would occur with increasing cation concentrations, precipitating the products, allowing for continuous lipase activity, aiding in the transferral of lipid droplets to mixed micelles and fostering carotenoid release from lipid droplets. Calcium and other divalent cations also have the ability to cross-link proteins⁴⁰ and soluble fibres^{41,42}, perhaps removing some factors that could negatively affect carotenoid bioaccessibility.

Concerning differences in carotenoid bioaccessibility between matrices, spinach contained the highest amount of total carotenoids, followed by tomato juice, carrot juice, field salad, and finally apricot nectar. Nevertheless, all tested matrices had a unique carotenoid profile, and apart from β -carotene, no other carotenoid was commonly measured across all the five matrices. The investigated spinach and field salad had similar carotenoid composition as previously reported for other green leafy vegetables, containing β -carotene, lutein, violaxanthin and neoxanthin⁴³, with 90% of total carotenoids constituted by xanthophylls (lutein, violaxanthin and neoxanthin). In contrast, xanthophylls accounted for less than 1% of the total carotenoid content determined for the juices and apricot nectar.

Despite the fact that spinach and field salad contained mainly xanthophylls, reported as being more bioaccessible than carotenes^{23,44,45}, their average total carotenoid bioaccessibility was significantly lower than that of the juices and nectar. This could have been the result of different storage form of carotenoids in plant tissues⁴³ - chromoplasts (for carrot and tomato) vs. chloroplast pigment-protein complexes (spinach and field salad)^{19,46,47} and other matrix

474 constituents such as dietary fiber⁴⁸. Furthermore, food processing¹³ and different methods of
475 disruption of the cell matrix also impact the release of carotenoids. Juicing of carrots has been
476 shown to improve the bioaccessibility of carotenoids from the food matrix⁴⁶ compared to raw
477 carrots, as a result of cell rupture, making carotenoids more accessible.

478 Phytoene and phytofluene were present in the tested juices and apricot nectar, where they
479 contributed at least to a third to the total carotenoid content. Despite their high contribution to
480 total carotenoid content of many different fruits and vegetables³⁵, their appearance in blood
481 plasma⁴⁹, and reported bioactive aspects such as anti-oxidant activity⁵⁰, not much information
482 is available on bioavailability aspects, including bioaccessibility. In the present investigation,
483 phytoene and phytofluene fractional bioaccessibilities were surprisingly high, between 27%
484 and 70%. Bioaccessibility nonetheless depended on the type of matrix. Carrot juice showed a
485 significantly lower bioaccessibility of these colourless carotenoids than tomato juice and
486 apricot nectar, while apricot nectar digestion resulted in significantly higher phytoene and
487 phytofluene bioaccessibility, compared to the other two matrices. These results are in line with
488 those found in a previous study⁵¹. Despite being similar to lycopene in its molecular structure,
489 very different bioaccessibilities were encountered. Meléndez-Martínez *et al.* (2015) suggested
490 that the number of conjugated double bonds (cDB) influences the shape of the molecule. Its
491 high number in lycopene (11 cDB) would prevent molecule folding, while phytoene (3 cDB)
492 and phytofluene (5 cDB) bonds are able to rotate more freely, assuming less rigid shapes. This
493 could facilitate the incorporation of the colourless carotenoids into mixed micelles, explaining
494 their higher bioaccessibility. Similarly, another study reported higher bioaccessibility of
495 phytoene, from different food matrices, compared to β -carotene and even lutein, including in
496 carrots and tomatoes¹⁹. Contrarily, phytoene bioaccessibility was comparable to that of β -
497 carotene from orange pulp and juice, perhaps due to the low amount of β -carotene present in
498 those matrices, facilitating its solubility⁵².

499 Regarding physicochemical properties, the higher macroviscosity of the apricot juice was
500 likely due to the high content of pectin and/or due to higher total solids (>14% w/w) and sugar
501 content (>13% w/w), promoting viscosity via decreasing the hydrodynamic free volume
502 between the biopolymer molecules, triggering hydrophobic interaction or hydrogen bonding of
503 side chain segments. In case of leafy vegetable matrices, their very low viscosimetric
504 responsiveness is likely to be attributed to their higher content in insoluble fibre, compared to
505 juices⁵³. Regardless of the food matrix, it is assumed that the observed macroviscosity
506 reduction with increasing divalent mineral concentration was mainly induced by the
507 precipitation of bile salt matter, depleting total solids. The rheological behaviour of the digesta
508 were diverse, with apricot nectar – magnesium or zinc binary systems undergoing structure
509 conformational changes as suggested by the observed rheological behaviour transitions, e.g.
510 pseudoplastic to Newtonian. The inflection point for apricot digesta containing magnesium or
511 zinc is also likely explained by its pectin content. It is well established that pectins (depending
512 on their methyl ester content) may undergo electrostatic interactions (via the egg-box structure
513 conformation) with divalent cations, exerting a selective chemical affinity (for
514 polygalacturonic segments) following the order: calcium > zinc > magnesium⁵⁴. This implies
515 that electrostatic bridging of calcium with pectins can be triggered at much lower
516 concentrations compared to the other two cations. This would explain the absence of the
517 inflection point in the case of apricot nectar – calcium digesta samples. Upon increasing the
518 cation concentration, an antagonistic ion binding action between bile salts and polygalacturonic
519 segment occurs, with the latter being disfavoured, inducing a strong precipitation. As for
520 surface tension, it is hypothesised that the bile salt binding capacity antagonism between
521 divalent cations and pectins played a buffering role on aggregative depletion phenomena. The
522 amount of soluble pectin in the bulk phase appeared to be well corroborating the average

depletion of surfactants i.e. 9% (magnesium) to 16% (calcium) for carrot juice and apricot nectar and 20% (magnesium) to 26% (calcium) for tomato juice, spinach and field salad.

CONCLUSIONS

Bioaccessibility of different carotenoids, from different matrices, was affected negatively by divalent mineral cations, and this was mirrored by decreased macroviscosity, increased surface tension of the digesta, as well as by the decrease of the absolute zeta-potential of the mixed micelles. Interestingly, the colourless carotenoids phytoene and phytofluene, displayed a bioaccessibility higher than those obtained for other carotenes. However, the outcomes of the influence of divalent cations on bioaccessibility appear as a result of a complex interaction between matrix, type of carotenoids and minerals and their concentration. Interestingly, concentrations of divalent minerals, much below the equivalent to the RDA for calcium and magnesium, appeared to improve bioaccessibility of carotenoids, likely by acting synergistically with bile acids, possibly promoting lipolysis. The results presented could be relevant regarding the intake and dosage of food supplements, specifically when combining carotenoids with high divalent mineral doses. Further investigations in vivo, possibly also including other liposoluble dietary constituents are warranted to attest these observations.

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Table I – Carotenoid content (µg/g food wet weight) for each of the tested food matrices.

Food matrix	Latin name	α-Car	β-Car	Lyc	Lut	Vio	Neo	PTE	PTF	Total
Apricot nectar	<i>Prunus armeniaca</i>	nd	2.87	nd	nd	nd	nd	11.27	2.79	16.87
Tomato juice	<i>Solanum lycopersicum</i>	nd	2.98	85.04	0.31	nd	nd	65.09	8.20	158.6
Carrot juice	<i>Daucus carota</i>	20.52	66.67	nd	1.24	nd	nd	40.44	4.33	133.2
Frozen spinach	<i>Spinacia oleracea</i>	nd	10.54	nd	100.1	64.80	16.26	nd	nd	191.7
Field Salad	<i>Valerianella locusta</i>	nd	14.79	nd	69.80	30.92	15.06	nd	nd	130.6
Coffee creamer (10% fat)		nd	21.55	nd	nd	nd	nd	nd	nd	21.55

α-Car: α-Carotene; β-Car: β-Carotene; Lyc: Lycopene; Lut: Lutein; Vio: Violaxanthin; Neo: Neoxanthin; PTE: Phytoene; PTF: Phytofluene, nd: not detectable. All forms represent the more abundant carotenoids isomers (i.e. all-trans). Values are means of duplicate analysis.

FIGURE HEADINGS

Figure 1. Average total carotenoid bioaccessibility (%) between matrices. Each bar represents the relative contribution of the individual carotenoids, detected in each matrix, to the percentage of total carotenoids recovered in the bioaccessible fraction of the digesta, relative to the total carotenoid amount present in the undigested test meal. Different letters represent statistically significant differences ($P < 0.001$). Total number of replicates was 16.

Figure 2. Effect of divalent cations and sodium on the bioaccessibility of β -carotene across different food matrices. Food matrices were digested in the presence of varying concentrations (mg/L) of 3 different divalent cations (calcium, zinc and magnesium) and of the monovalent cation (sodium). Bioaccessibility values are represented as the percentage of β -carotene recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the amount of β -carotene present in the original matrix. Values represent mean $\pm$ SD of $n = 4$. Bars signalled with either * ($P < 0.05$) or ** ($P < 0.01$) were statistically different from the control condition, i.e. [added mineral] = 0 mg/L.

Figure 3. Graphical representation of trends in bioaccessibility variation of carotenoids from spinach and field salad, as effected by cationic minerals at different concentration ranges. Bars in dark and light grey represent the highest and lowest points, respectively, of the measured bioaccessibility. Concentration range is depicted as C0 to C5, corresponding to the following: Calcium C0 = 0mg/L; C1 = 250mg/L; C2 = 500 mg/L; C3 = 1000 mg/L. Sodium C0 = 0 mg/L; C1 = 375 mg/L; C2 = 750 mg/L; C3 = 1500 mg/L. Magnesium C0 = 0 mg/L; C1 = 100 mg/L; C2 = 200 mg/L; C3 = 300 mg/L. Zinc C0 = 0 mg/L; C1 = 12.5 mg/L; C2 = 25 mg/L; C3 = 50 mg/L; C4 = 100 mg/L; C5 = 200 mg/L. Symbol * above bar indicates statistical significance ($P < 0.05$), compared to controls (no mineral added).

Figure 4. Graphical representation of the trends in bioaccessibility variation of lycopene and the colourless carotenoids, phytoene and phytofluene, from tomato juice, as an effect of cations at different concentration ranges. Bars in dark and light grey represent the highest and lowest points, respectively, of the measured bioaccessibility. Concentration range is depicted as C0 to C5, which correspond to the following values: Calcium C0 = 0mg/L; C1 = 250mg/L; C2 = 500 mg/L; C3 = 1000 mg/L. Sodium C0 = 0 mg/L; C1 = 375 mg/L; C2 = 750 mg/L; C3 = 1500 mg/L. Magnesium C0 = 0 mg/L; C1 = 100 mg/L; C2 = 200 mg/L; C3 = 300 mg/L. Zinc C0 = 0 mg/L; C1 = 12.5 mg/L; C2 = 25 mg/L; C3 = 50 mg/L; C4 = 100 mg/L; C5 = 200 mg/L. Symbol * above bar indicates statistical significance ($P < 0.05$), compared to control (no mineral added).

Figure 5. Average spinach-borne β -carotene bioaccessibility (%), and absolute zeta-potential (mV) of spinach aqueous micellar fraction, after digestion with different cations at varying concentrations. Bars represent bioaccessibility, while markers $\blacklozenge$ represent the zeta potential. A: Calcium; B: Sodium; C: Magnesium; D: Zinc. Error bars represent standard deviation of $n = 4$ replicates.

Figure 6. Average carrot juice-borne β -carotene bioaccessibility (%), and absolute zeta-potential (mV) of carrot juice aqueous micellar fraction, after digestion with different cations at varying concentrations. Bars represent bioaccessibility, while markers $\blacklozenge$ represent the zeta-potential. A: Calcium; B: Sodium; C: Magnesium; D: Zinc. Error bars represent standard deviation of $n = 4$ replicates.

Figure 7. Normalised macroviscosity and surface tension values of the aqueous micellar fractions of different food matrices after digestion with varying cationic mineral concentrations.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7