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Effect of divalent minerals on the bioaccessibility of pure carotenoids and on physical properties of gastro-intestinal fluids

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ABSTRACT

During digestion, high concentrations of divalent minerals (DMs) can lead to insoluble lipid–soap complex formation, hampering carotenoid bioaccessibility. The effect of varying concentrations (0–1000 mg/L) of calcium, magnesium, zinc and sodium (control) on the bioaccessibility of lutein, neoxanthin, lycopene and β-carotene, following *in vitro* gastro-intestinal digestion (GI), was investigated systematically and coupled with physical measurements of the digesta. Addition of DMs significantly decreased ($p < 0.001$) carotenoid bioaccessibility, up to 100% in the case of calcium. Mean half maximal inhibitory concentrations (EC50) for calcium, magnesium and zinc were 270 ± 18, 253 ± 75 and 420 ± 322 mg/L respectively. Increased DM concentrations correlated with decreased viscosity ($r > 0.9$) and decreased carotenoid bioaccessibility. Surface tension of digesta correlated inversely ($p < 0.05$) with the bioaccessibility of carotenoids. This correlation was mineral and carotenoid dependent. Although based on *in vitro* findings, it is plausible that similar interactions occur *in vivo*, with DMs affecting the bioaccessibility and bioavailability of carotenoids and other lipophilic micronutrients and phytochemicals.

1. Introduction

Regular consumption of fruits and vegetables has been commonly associated with the prevention of several chronic diseases (Tapiero, Townsend, & Tew, 2004). Carotenoids are phytochemicals present in a large variety of vegetables and fruits, exerting important biological effects in humans including anti-oxidant, anti-inflammatory and pro-vitamin A activity (Kaulmann & Bohn, 2014; Stahl & Sies, 2005). The association between dietary carotenoid intake and the prevention of chronic diseases such as age-related macular degeneration (Bone et al., 2001), coronary disease (Arab & Steck, 2000), diabetes (Brazionis, Rowley, Itsiopoulos, & O'Dea, 2009) and cancer (Vainio & Rautalahti, 1998) has been the topic of several epidemiological studies. Although results from these studies are not always consistent, evidence for the protective role of carotenoids in the prevention of chronic diseases is increasing (Krinsky & Johnson, 2005).

As humans are not able to synthesize these compounds, they have to be acquired through the diet. However, due to their lipophilic character, bioavailability of carotenoids is low, which has mostly been attributed to their limited bioaccessibility (i.e., the percentage of carotenoids effectively released from the food matrix and available for absorption), varying from 3% to 34% (van Het Hof & West, 2000). In recent years much attention has been given to dietary and host factors influencing carotenoid bioaccessibility (Biehler & Bohn, 2010; Bohn, 2008; Castenmiller & West, 1998; van Het Hof & West, 2000). Some of the factors investigated include the presence of dietary fiber and physical properties such as viscosity (O'Connell, Ryan, O'Sullivan, Aherne-Bruce, & O'Brien, 2008; Riedl & Linseisen, 1999; Verrijssen et al., 2014), type and amount of lipids present in meals (Gleize et al., 2013; Goltz, Campbell, Chitchumroonchokchai, Failla, & Ferruzzi, 2012; Huo, Ferruzzi, Schwartz, & Failla, 2007), concentration of bile acids, as well as gastro-intestinal pH and enzyme variations (Biehler, Kaulmann, Hoffmann, Krause, & Bohn, 2011; Tyssandier, Lyan, & Borel, 2001).

One dietary factor that so far has received little attention is the presence and concentration of divalent minerals (DMs), including trace elements, such as calcium, zinc and magnesium. Previously, we have shown that high concentrations of the DMs calcium, magnesium, iron and zinc impaired the transfer of carotenoids to mixed-micelles to different degrees, thereby reducing micellarization by up to 90% (Biehler, Hoffmann, Krause, & Bohn, 2011),

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presumably via soap formation with fatty acids. Earlier findings have already shown an association between triglyceride digestion and dietary minerals. While Cheng, Morehouse, and Deuel (1949) found that these minerals reduce the digestibility of triglycerides, Tadayyon and Lutwak (1969) conversely reported that a diet rich in poorly absorbable fats considerably decreases the absorption of calcium and magnesium. As carotenoids require the presence of a certain amount of fat (ca. 5–10 g) to be present in a meal (Biehler & Bohn, 2010; Failla, Huo, & Thakkar, 2008) in order to be effectively solubilized and emulsified, DMs could compromise carotenoid bioaccessibility by limiting the presence of available triglycerides and free fatty acids (FFA). DMs may also have the tendency to bind and precipitate bile acids (Baruch, Lichtenberg, Barak, & Nir, 1991; Hofmann & Mysels, 1992), also reducing the bioaccessibility of carotenoids. In turn, the presence of salts may influence surface tension, viscosity and electrostatic properties of the digesta, potentially modifying the bioaccessibility of lipophilic constituents.

Here, we aimed to investigate the effect of several DMs (calcium, magnesium and zinc) at different concentrations, from physiological to dietary supplement range, on the bioaccessibility of the pure carotenes and xanthophylls, including β-carotene, lycopene, lutein and neoxanthin. For this purpose, we employed a previously established *in vitro* gastro-intestinal model (Corte-Real, Richling, Hoffmann, & Bohn, 2014), coupled to bioaccessibility determination and rheological analyses such as viscosity and surface tension of the digesta, to gain insight into the mechanisms of action that can alter carotenoid bioaccessibility.

2. Materials and methods

2.1. Chemicals, minerals and carotenoid standards

Digestive enzymes, i.e., pepsin (porcine, ⩾250 units/mg solid, measured as TCA-soluble products using hemoglobin as substrate) and pancreatin (porcine, 4× USP specifications of amylase, lipase and protease), porcine bile extract, oleic acid (cis-9-octadecenoic acid), lecithin from egg yolk (L-α-phosphatidylcholine, ~60% TLC) and monoolein (1-oleoyl-rac-glycerol) were purchased from Sigma–Aldrich (Bornem, Belgium). Hexane and hydrochloric acid were from VWR (Leuven, Belgium); acetone, sodium carbonate and sodium chloride from Merck (Darmstadt, Germany). Beta-carotene and lycopene standards were from Sigma–Aldrich (purity > 95%). Neoxanthin and lutein were from CaroteNature GmbH (Ostermundigen, Switzerland). Calcium chloride anhydrous and zinc chloride anhydrous were purchased from VWR while magnesium chloride anhydrous was acquired at Sigma–Aldrich. Unless otherwise specified, all products were of analytical grade or higher. 18 MΩ water was prepared with a purification system from Millipore (Brussels, Belgium) and used throughout the study.

Canola oil was used as a natural and dietary lipid source for the solubilization of pure carotenoids and was purchased at a local supermarket (CACTUS S.A., Windhof, Luxembourg) in summer 2013. Canola oil has been used previously to aid in the micellarization of carotenoids (Biehler et al., 2012; Biehler, Hoffmann, et al., 2011; Biehler, Kaulmann, et al., 2011; Chitchumroonchokchai, Schwartz, & Failla, 2004; Huo et al., 2007) and is low in natural occurring carotenoids.

2.2. Carotenoid standard solutions

Individual standard solutions were prepared gravimetrically by dissolving each pure carotenoid in organic solvent; β-carotene and lycopene were dissolved in chloroform, while lutein and neoxanthin were dissolved in acetone. Concentration of the standard solutions was further determined spectrophotometrically as explained below (2.5). Aliquots of the standard solutions were pipetted into amber glass vials, and stored at −80 °C until usage.

On days of experiments, the concentration of each carotenoid standard solution was determined spectrophotometrically. A volume equivalent to 30 μg of the investigated carotenoid was pipetted into a 50 mL falcon tube and the solvent was evaporated under a stream of nitrogen. To promote the resolubilisation of the carotenoid and successive formation of mixed-micelles during gastro-intestinal digestion, 3 emulsifying agents (referred hereon as emulsifier-mix) were added to the previously dried pure carotenoid, followed by the addition of 150 μL of canola oil. The emulsifier-mix was composed of 100 mg of monoolein, 10 mg of lecithin and 10 mg of oleic acid. The final mixture was then sonicated at 37 kHz (Elmasonic Ultrasonic Bath, Elma, Mägenwil, Switzerland) for 10 min.

2.3. Simulation of gastro-intestinal digestion and factors investigated

The *in vitro* digestion protocol was adapted from Corte-Real et al. (2014) and is described below. The model was used to test the effect of 3 different DMs (calcium, magnesium and zinc) and sodium (used as a control mineral) at 4 different concentrations on the bioaccessibility of 4 different carotenoids (β-carotene, lycopene, lutein and neoxanthin).

Concentrations of divalent minerals and sodium were chosen based on the dietary reference intakes (RDA (recommended dietary allowance) or AI (acceptable intake) if no RDA available) and tolerable upper intake levels (UL) (Food & Nutrition Board, 2011). To determine the concentration of mineral per volume of digesta, we assumed a total volume of 2 L of intestinal fluids during GI digestion. The concentrations for calcium tested were 0, 250, 500 and 1000 mg/L; magnesium 0, 100, 200 and 300 mg/L; zinc 0, 50, 100 and 200 mg/L, and sodium 0, 375, 750 and 1500 mg/L. For the purposes of this study, we have defined as physiological a range of mineral concentrations up to the daily RDA/AI (dissolved in 2 L), and as supplemental concentrations levels above the RDA/AI in 2 L.

To simulate the gastric passage, physiological saline and a standard solution of the investigated mineral were added to the falcon tube containing the previously solubilized carotenoid. Physiological saline and mineral solutions were added in varying volumes, depending on the desired final mineral concentration. The total volume at this step was of 7.5 mL. Samples were then sonicated for 20 min in an ultrasonic bath, followed by the addition of 1 mL of porcine pepsin (40 mg/mL) solution prepared in 0.01 M HCl, bringing the pH to 3. Samples were incubated for 1 h at 37 °C in a shaking water bath (GFL 1083 from VEL®, Leuven, Belgium) at 100 rpm.

At the end of the gastric phase's incubation period, 4.5 mL of freshly prepared solutions of porcine pancreatin (4 mg/mL) and bile (24 mg/mL) in $NaHCO_3$ (0.1 M) were added to the simulated gastric fluid and the pH was brought up to 7. The final volume of the samples was adjusted to 25 mL with physiological saline and samples were incubated for another 2 h in a shaking water bath (100 rpm) at 37 °C.

2.4. Extraction of carotenoids from digesta

Aliquots of 12 mL of digesta were transferred to 15 mL falcon tubes and centrifuged at 4800*g* for 1 h at 4 °C. Following centrifugation, 4 mL were collected from the middle aqueous micellar phase, by means of a syringe and a hypodermic needle. The 4 mL aliquot was then filtered through a 200 nm Nylon membrane filter (Acrodisc® 13 mm Syringe Filters, PALL Life Sciences, Ann Harbor, MI) into a 15 mL falcon tube.