Food Chemistry 239 (2018) 751–759

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Magnesium affects spinach carotenoid bioaccessibility *in vitro* depending on intestinal bile and pancreatic enzyme concentrations

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ARTICLE INFO

Article history:
Received 11 May 2017
Received in revised form 27 June 2017
Accepted 28 June 2017
Available online 29 June 2017

Keywords:
Lutein
Carotenes
Divalent minerals
Calcium
Bioavailability
Digestion
Lipids
Micelle formation

ABSTRACT

Magnesium may reduce carotenoid bioavailability by forming insoluble complexes with bile salts/fatty acids, inhibiting micelle formation. Here, we investigated whether altering bile/pancreatin concentration influenced potential negative effects of magnesium on carotenoid bioaccessibility. Spinach (4 g) was digested *in vitro* with added magnesium (0, 200, 400 mg/L) and canola oil/coffee creamer, at varying bile extract (1 or 8 mM) and pancreatin (100 or 990 mg/L) concentrations. Bioaccessibility was determined for β-carotene, lutein, and total carotenoids via HPLC. Additionally, lipolysis, particle size, and zeta potential of the micellar fractions were investigated. Increasing magnesium concentrations negatively affected carotenoid bioaccessibility ($p < 0.001$), lipolysis, particle size and zeta potential. The impact of magnesium on carotenoid bioaccessibility was modulated mainly by bile concentration, with samples digested with 1 mM of bile being more susceptible to inhibitory effects of magnesium than those digested with 8 mM ($p < 0.001$). Thus, magnesium was found to potentially interfere with carotenoid bioaccessibility at various physiologically plausible conditions.

1. Introduction

Carotenoids are lipophilic C30- and C40-based secondary plant compounds, recognized mainly for their anti-oxidant potential. Several carotenoids, such as β-carotene and β-cryptoxanthin, can also act as vitamin A precursors, and lutein and zeaxanthin have been suggested to protect against age-related macular degeneration (Lima, Rosen, & Farah, 2016; Scripsema, Hu, & Rosen, 2015). Though their mechanism of action has not been completely elucidated, direct anti-oxidant effects (Krinsky & Yeum, 2003), and modulation of the expression of genes involved in inflammatory and anti-oxidant pathways (Kaulmann & Bohn, 2014), likely play a role.

Carotenoid absorption efficiency is rather low and variable. It depends on both dietary (Desmarchelier & Borel, 2017) and host-related factors (Bohn et al., 2017). The food matrix is considered to be a key factor, as, in order to be absorbed, carotenoids first need to be extracted from their food matrix and incorporated into mixed micelles. This process of transferring carotenoids from the food matrix into lipid globules already starts in the stomach. By the time carotenoids reach the small intestine, bile acids and pancreatic lipase are released into the duodenum, stimulated by dietary fat. While pancreatic enzymes break down lipids, bile, acting as a surfactant, facilitates the formation of smaller sized mixed bile-lipid micelles that enclose carotenoids (Britton, Liaaen-Jensen, & Pfander, 2009).

Recently, it has been hypothesized (Biehler, Hoffmann, Krause, & Bohn, 2011) that dietary divalent cations, such as calcium and magnesium could negatively affect carotenoid absorption, by preventing the formation of mixed-micelles as a result of fatty acid (Atteh & Leeson, 1985; Boyd, Crum, & Lyman, 1932) and bile salt precipitation (Gu, Hofmann, Ton-Nu, Schteingart, & Mysels, 1992; Hofmann & Mysels, 1992). This has been shown *in vitro* for a variety of divalent cations and trace elements, following simulated digestion experiments with isolated carotenoids (Corte-Real et al., 2016) and carotenoids released from regular food matrices, such as spinach or carrot juice (Corte-Real, Bertucci et al., 2017). The concentrations required to observe these negative effects were equivalent to approximately half the recommended dietary allowance (RDA) for calcium and magnesium. However, recent human trials investigating the effect of calcium have found contradictory

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http://dx.doi.org/10.1016/j.foodchem.2017.06.147
0308-8146/

results. While in a study employing tomato paste, 500 mg of calcium decreased post-prandial plasma lycopene concentration (Borel et al., 2016), another trial employing spinach and calcium doses of 500 and 1000 mg did not find any significant changes in carotenoid concentrations in plasma triacylglycerol-rich lipoprotein (TRL) fractions (Corte-Real, Guignard et al., 2017).

While thus the findings remain controversial, even less information is available on the potential influence of dietary magnesium, the second most abundant dietary cation after calcium (RDA: 1000 mg/d), with an RDA of 400 mg/d (Institute of Medicine, 2005). *In vivo*, the interaction between magnesium and other divalent cations and carotenoids is likely to be influenced by varying concentrations of bile and pancreatic enzymes, reflecting a more adaptive system compared to the *in vitro* static situations employed previously (Alminger et al., 2014; Biehler & Bohn, 2010). Magnesium also has the ability to bind triglycerides and form soaps of different solubility, depending on the degree of fatty acid chain saturation. Magnesium soaps formed from saturated fatty acids showed lower solubility than those from unsaturated fatty acids (Bohn, 2008), which could lead to precipitation and fat excretion (Tadayyon & Lutwak, 1969).

In the present trial, we investigated whether altering factors required for the micellarization of carotenoids (i.e. type and source of lipids, and bile and pancreatic enzyme concentrations) would influence the effect of magnesium on the bioaccessibility of carotenoids, as determined by fractional carotenoid bioaccessibility, following *in vitro* gastro-intestinal digestion. Frozen spinach leaves were chosen as a model matrix due to their high carotenoid content (>5 mg/100 g), the presence of both xanthophylls (lutein, neoxanthin and violaxanthin) and carotenes (β- and α-carotene), and their previous use as a model vegetable (Biehler, Hoffmann et al., 2011; Castenmiller, West, Linssen, van het Hof, & Voragen, 1999; Corte-Real, Bertucci et al., 2017; Rock et al., 1998). As the interactions are expected to affect the extent of lipolysis and the micelle size and stability of the colloidal system, these parameters were also assessed.

2. Materials and methods

2.1. Chemicals and standards

Pepsin (porcine, ≥250 units/mg solid, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as substrate), pancreatin (porcine, 4X USP specifications), and porcine bile extract were purchased from Sigma-Aldrich (Bornem, Belgium). Methanol (MeOH), hexane and hydrochloric acid were from VWR (Leuven, Belgium); acetone, sodium carbonate and sodium chloride from Merck (Darmstadt, Germany); methyl *tert*-butyl ether (MTBE) was purchased from Sigma-Aldrich; acetonitrile (ACN) and dichloromethane (DCM) were obtained from Carl Roth (Karlsruhe, Germany). Beta-carotene and trans-β-apo-8′-carotenal standards were from Sigma-Aldrich (purity >95%), while neoxanthin and lutein were from CaroteNature GmbH (Ostermundigen, Switzerland). Calcium chloride anhydrous was purchased from VWR and magnesium chloride anhydrous from 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. As dietary lipid sources for the solubilisation of pure carotenoids, canola oil (purchased at a local supermarket CACTUS S.A., Windhof, Luxembourg) and INEX coffee creamer (10% fat, Delhaize supermarket Esch-sur-Alzette, Luxembourg) were used. Both were previously employed to aid in the micellarization of carotenoids (Biehler, Kaulmann, Hoffmann, Krause, & Bohn, 2011; Corte-Real, Richling, Hoffmann, & Bohn, 2014; Corte-Real et al., 2016). The carotenoid containing food matrix, spinach (*Spinachia oleracea*), was purchased in frozen raw form (Cora supermarket, Foetz, Luxembourg).

2.2. Simulation of gastro-intestinal digestion and factors investigated

The *in vitro* digestion protocol was described earlier (Corte-Real et al., 2014). The model was used to test the effect of magnesium (at 0, 200 and 400 mg/L) on spinach carotenoid bioaccessibility, as a function of bile extract (1 and 8 mM) and pancreatin (100 and 990 mg/L) concentrations, expressed as concentrations after small intestinal digestion. Concentrations of enzymes and bile were chosen to represent plausible physiological ranges (Alminger et al., 2014). Concentrations of minerals were those representing earlier observed inhibitory concentrations (Corte-Real et al., 2016) and the upper tolerable limit (UL) (Institute of Medicine, 2005). In addition, the influence of lipid source from either canola oil or coffee creamer (10% fat) was studied. These were chosen to represent lipids easily soluble (coffee creamer) with the bulk of the food and a typical oil dressing, and also, as an earlier investigation suggested, different behavior on carotenoid bioaccessibility (Corte-Real et al., 2014).

Spinach aliquots were left to unfreeze, the excess water was drained, and the leaves were patted dry. Spinach was then further homogenized by maceration in a mortar, using liquid nitrogen. The homogenized spinach was weighed and aliquoted into polypropylene sample containers, flushed with argon and stored at -80 °C. Test meals for digestion contained 4 g (wet weight) spinach plus either 0.3 mL of canola oil or 2 mL of coffee creamer (10% fat) (approximately 250 mg of fat, i.e. 6% of the test meal). A solution of magnesium chloride was added to test meals, to achieve targeted concentrations, and digestion proceeded with a gastric phase carried out at pH 3, for 1 h at 37 °C, followed by an intestinal phase at pH 7, for 2 h at 37 °C. The final volume of digesta was 50 mL in physiological saline. These were then centrifuged briefly (5 min, 2000*g*) and either processed further directly (bioaccessibility) or snap frozen at −80 °C for further investigation (micelle size, zeta potential and lipolysis).

2.3. Separation of bioaccessible fraction and bioaccessibility calculation

Aliquots of digesta (12 mL) were transferred to 15 mL falcon tubes and centrifuged at 4800*g* for 1 h at 4 °C. Following centrifugation, 6 mL were collected from the middle aqueous micellar phase, by means of a syringe and a hypodermic needle. The 6 mL aliquot was then filtered through a 0.2 μm Nylon membrane filter (Acrodisc® 13 mm Syringe Filters, PALL Life Sciences, Ann Harbor, MI) into a 15 mL falcon tube. Carotenoid bioaccessibility was calculated as the percentage of carotenoids recovered in the final micellar phase compared to the original matrix's carotenoid content.

2.4. Extractions

Carotenoid extraction procedures have been described previously (Corte-Real, Bertucci et al., 2017). In short, aliquots of 4 g of spinach were wetted with 5 mL of MeOH, and 1 mL of 30% aqueous KOH for saponification of chlorophylls, vortexed, sonicated, and incubated in the dark for 20 min. Following centrifugation, supernatants were collected and spinach was re-extracted once with 9 mL hexane:acetone (1:1), and re-extracted with 9 mL hexane plus 4 mL of saturated NaCl and again with 4 mL of diethyl ether. Aliquots of the combined organic phases were dried under a stream of nitrogen using a TurboVapLV (Biotage, Eke, Belgium) apparatus (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 polyvinylidene