BIOAVAILABILITY OF CAROTENOIDS – IMPACT OF HIGH MINERAL CONCENTRATIONS (BIOCAR)

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To my family for their unconditional love and support.

To Jerry, for his loving support and understanding.

"Above all don't fear difficult moments.
The best comes from them"

Rita Levi-Montalcini, Neurobiologist

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AI	Acceptable intake
AUC	Area under the curve
DCM	Dichloromethane
DM	Divalent minerals
DMEM	Dulbecco's modified Eagle's medium
FFA	Free fatty acids
GI	Gastro-intestinal
HCl	Hydrochloric acid
IS	Internal standard
KOH	Potassium hydroxide
MeOH	Methanol
MTBE	Methyl tert-butyl ether
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEFA	Non-essential fatty acids
NTA	Nanoparticle tracking analysis
RDA	Recommended dietary allowances
ROS	Reactive oxygen species
TAG	Triacylglicerides
TRL	Triacylglycerol-rich lipoprotein
UL	Tolerable upper intake levels

ZUSAMMENFASSUNG

Aufgrund ihrer Präsenz in zahlreichen Lebensmitteln und der deshalb stetigen Einnahme nahmen Phytochemikalien schon immer eine wichtige und allgegenwärtige Rolle innerhalb der Humanernährung ein. Karotenoide sind lipophile Phytochemikalien, produziert von sowohl phtotosynthetisch aktiven (Pflanzen und Algen) und inaktiven (Pilzen und Bakterien) Organismen. Im Tierreich, einschließlich des Menschen, kommen Karotenoide nur infolge des Verzehrs von Karotenoiden vor. Die Tatsache, dass diese Moleküle i) sich im menschlichen Körper anreichern, ii) eine Rolle in der Prävention chronischer Krankheiten und für ein gesundes Sehvermögen spielen, iii) vermutlich mit antioxidativen und entzündungshemmenden Prozessen interagieren, iv) und zum Teil als Vitamin A Prekursoren gelten, machen sie für das Gesundheitsmanagement sehr interessant; insbesondere mit Blick auf den sich erhärtenden Zusammenhang des Karotenoid-Status mit chronischen Komplikationen wie Herz-Kreislauferkrankungen, Obesitas, und Diabetes. Allerdings ist das Potential der Karotenoide im menschlichen Körper insbesondere von deren Bioverfügbarkeit (d.h. der Fraktion die von der Nahrung freigesetzt wird und vom Körper für physiologische Prozesse aufgenommen oder gespeichert werden kann) und Faktoren die diese beeinflussen, abhängig, inklusive Nahrungsfaktoren, wie z.B. Spezies der Karotenoide, Nahrungsmittelmatrix, und Faktoren die die Absorption und Biokonversion beeinflussen. Frühere Studien unserers Forschungsteams haben gezeigt, dass der Zusatz von divalenten Mineralstoffen/Spurenelementen, d.h. Calcium, Magnesium, Eisen und Zink, zu *in vitro* verdauten Testmahlzeiten zu einer signifikanten Erniedrigung der Karotenoid-Bioakzessibilität (d.h. die Fraktion, die von der Nahrung freigelassen wird und in gemischte Mizellen eigebaut werden kann) führen kann. Die Erhöhung der Mineralstoffkonzentration von 12.5 auf 25 mM führte etwa zu einer bis zu 90%igen signifikanten Erniedrigung der Bioakzessibilität und der zellulären Aufnahme von Karotenoiden. Auch wenn bis dato bereits über einen Effekt der Mineralstoffe auf Karotenoide gemutmaβt wurde, der in Zusammenhang mit der Bindung von Mineralstoffen und Gallensalzen sowie freier Fettsäuren steht, so wurden diese Effekte bislang nicht weiter systematisch untersucht.

Ziel des Projektes BIOCAR war es deshalb, den Einfluss von divalenten Mineralstoffen auf die Verdauung und Bioverfügbarkeit von Karotenoiden besser zu verstehen. Hierzu wurde das Projekt in eine *in vitro* und eine *in vivo* Kompenente unterteilt. In der *in vitro* Phase untersuchten wir, wie verschiedene Konzentrationen an Calcium, Magnesium, und Zink die Bioakzessibilität von sowohl reinen Karotenoiden als auch Karotenoiden aus verschiedenen Testmahlzeiten (Tomatensaft, Karottensaft, Aprikosennektar, Spinat und Feldsalat) beeinflusst. Zusätzlich wurde der Effekt von Magnesium bei niedrigen Gallensalzkonzentrationen (1 mM) und Pankreatin (100 mg/L) untersucht, um eine Verdauungsinsuffizienz zu simulieren. Die folgenden Parameter wurden für die *in vitro* Experimente miteinbezogen: Die prozentuale Wiederfindung der Karotenoide in der wasserlöslichen Phase nach durchgeführter gastro-intestinaler Verdauung *in vitro* (d.h. Bioakzessibilität), physiko-chemische Eigenschaften des Verdauungsbreis wie spezifische Viskosität und Oberflächenspannung; und schließlich die Mizellengröße und das Zeta-Potential der in Lösung befindlichen Partikel. Abschließend wurde, zwecks Validierung der *in vitro* erhaltenden Resultate, eine Humanstudie durchgeführt: Eine randomisierte, Placebo kontrollierte postprandiale Cross-over-Doppelblindstudie mit 24 männlichen Probanden, um den Einfluss von drei Calcium Dosierungen (0, 500 mg und 1000 mg) auf die Bioverfügbarkeit von Karotenoiden aus einer Spinatmahlzeit als Frühstück zu testen. Für jeden Teilnehmer wurden, im Abstand von je einer Woche, drei volle klinsche Studientage geplant (je zehn Stunden). Die Teilnehmer wurden gebeten, alle Karotenoid-haltigen Lebensmittel während der gesamten Studienphase zu meiden. Die Bioverfügbarkeit der Karotenoide wurde als „Area-Under-the-Curve (AUC)“, also Konzentration über Zeit gemessen, und zwar aus der Triazylglyzerol-reichen Lipoproteinfraktion (TRL), welche neu aufgenommene Karotenoide repräsentiert.

Die Resultate der in vitro Versuche zeigten, dass eine Zugabe von divalenten Mineralstoffen die Bioakzessibilität von sowohl reinen Karotenoiden ($P < 0.001$) als auch von Karotenoiden aus Lebensmitteln signfikant erniedrigten ($P < 0.01$). Das Ausmaβ, in welchem die Bioakzessibilität beeinflusst wurde, hing von der Art des Mineralstoffes und dessen Konzentration ab. Die stärksten Effekte wurden bei Calcium gefolgt von Magnesium und Zink beobachtet. Die Zugabe von Calcium,

insbesondere bei höheren Konzentrationen (500 – 1000 mg/L) verursachten eine Reduktion der Bioakzessibilität von bis zu 100%. Die Zugabe von divalenten Mineralstoffen änderte ebenfalls die physiko-chemischen Eigenschaften des Verdauungsbreis. Die Stärke des Effektes hing allerdings von der Matrix der Testmahlzeit ab. Ein Anstieg in der Konzentration der Kationen korrelierte mit einer verminderten Viskosität ($r > 0.9$) des Verdauungbreis der reinen Karotenoide. Im Falle der Testmahlzeiten wurde eine ähnliche Tendenz beobachtet. Bei Gegenwart von divalenten Mineralstoffen wurde ein signifikanter Abfall der Markroviskosität des Verdauungsbreis detektiert. Dieser war stärker für Nahrungsmatrices mit hohem Pektingehalt, d.h. Aprikose, Tomate und Karotte ($P < 0.05$), verglichen mit Blattgemüse. Die Oberflächenspannung auf der anderen Seite stieg signifikant mit steigender Mineralstoffkonzentration an ($P < 0.05$). Die Effekte auf die Bioakzessibililität und physiko-chemischer Eigenschaften hingen mit Änderungen des Zeta-Potentials der in Lösung befindlichen Partkikel zusammen. Unter Kontrollbedingungen (d.h. ohne Mineralstoffzugabe) waren die Partikel des Verdauungsbreis negativ geladen, mit Ladungen um ca. -30 mV. Mit steigenden Konzentrationen an Calcium und Magnesium reduzierte sich das absolute Zeta-Potential auf beinahe Null, das System wurde folglich instabil. Die Effekte von divalenten Minaralstoffen, insbesondere Magnesium, wurden auch durch die Konzentration an Gallensalzen und – in geringerem Ausmaβ – durch die Konzentration von Bauchspeicheldrüsen-Enzymen beeinflusst. Proben die bei geringerer Konzentration an Gallensalzen (1 mM) verdaut wurden waren anfälliger für den inhibierenden Einfluss von Magnesium auf die Bioakzessibilität von Karotenoiden, verglichen mit jenen die bei 8 mM verdaut wurden ($P < 0.001$). Zusammenfassend lassen die Resultate vermuten, dass divalente Mineralstoffe Gallensalze und andere oberflächenaktive Stoffe binden, die die Löslichkeit von Karotenoiden beeinflussen. Die Beobachtung i) einer abnehmenden Makroviskosität, ii) einer Zunahme der Oberlächenspannung und iii) einer Reduzierung des Zeta-Potentials des Verdauungsbreis, bestätigen das Entfernen von oberflächenaktiven Stoffen aus dem Verdauungssystem, vermutlich durch Präzipitierung als Folge der verringerten Löslichkeit des Komplexes aus Mineralstoffen und oberflächenaktiven Stoffen. Als Folge kam es zu einer Behinderung der Mizellarisierung der Karotenoide, die die beobachtete niedrige Bioakzessibilität erklärt.

Bezüglich der Humanstudie und der drei verschiedenen Calciumdosen zeigte sich kein signifikanter Einfluss einer Supplementierung von 500 oder 1000 mg Calcium (in Karbonat-Form) auf die Bioverfügbarkeit, was gegen die Hypothese und die *in vitro* gewonnenen Befunde spricht. Dem widersprechen auf der anderen Seite Resultate einer kürzlich veröffentlichten Humanstudie, die den Effekt einer Calcium Supplementierung auf die Bioverfügbarkeit von Tomatenpasten-Karotenoiden untersucht. Es kann aufgrund dieser beiden Studienergebnisse vermutet werden, dass der Effekt von Mineralstoffen auf die Bioverfügbarkeit von Karotenoiden, und vermutlich anderer fettlöslicher Nährstoffe, von spezifischen Interaktionen zwischen Supplementform und Löslichkeitskinetik und der Art der Karotenoide sowie der Nahrumgsmatrix abhängt. Weitere Studien die den Zusammenhang von divalenten Mineralstoffen und Karotenoiden sowie anderer Nahrungsbestandteile *in vivo* untersuchen sind angeraten.

SUMMARY

Phytochemicals have always been a significant and ubiquitous element in human nutrition, as they are present in a myriad of food items regularly consumed by humans. Carotenoids are lipophilic phytochemicals, synthesised by both photosynthetic (e.g. plants and algae) and non-photosynthetic organisms (e.g. some fungi and bacteria). However, the animal kingdom, including humans, acquires carotenoids through diet. The fact that these molecules i) accumulate in the human body, ii) have a known role in eye health and in the prevention of eye related diseases, iii) potentially interact with antioxidant and inflammatory related pathways, iv) and that a few are known vitamin A precursors, makes them particularly attractive in health management; especially in the face of an increasing amount of evidence correlating carotenoid status to the incidence of several chronic diseases such as cardiovascular problems, obesity, and diabetes. However, the potential of carotenoids in the body is first and foremost depending on their bioavailability (i.e. fraction of carotenoids that is released from the food matrix and available for uptake and storage in the body), and factors affecting it, including dietary factors such as: species and amount of carotenoids; matrix; and effectors of absorption and bioconversion. Earlier studies from our group found that the addition of divalent cations, i.e. calcium, magnesium, zinc and iron, to test meals digested in an *in vitro* digestion system, significantly affected carotenoid bioaccessibility (i.e. the fraction of carotenoids released from the food matrix and incorporated into mixed micelles) from test meals. Increasing concentrations of minerals from 12.5 to 25 mM led to significant decreases in bioaccessibility and cellular uptake of carotenoids by as high as 90%. Although at the time, it was hypothesised that the reported effects resulted from the binding of divalent cations to bile salts and free fatty acids, forming insoluble mineral bile salts and fatty acid soaps respectively, the effect of divalent cations on carotenoids remained to be investigated in a more systematic manner.

Hence, the aim of this project (BIOCAR) was to better understand how divalent cations act during digestion and modulate carotenoid biovailability. To do so, the project was divided into *in vitro* and *in vivo* experimental components. In the *in vitro* work, we investigated how varying concentrations of calcium, magnesium and zinc affected the bioaccessibility of both pure carotenoids and carotenoids

from different food matrices (tomato juice, carrot juice and apricot nectar, and spinach and field salad). Additionally, the effect of magnesium was also tested at low bile (1 mM) and pancreatic enzyme concentrations (100 mg/L), mimicking an insufficiency scenario. The following parameters were assessed for the *in vitro* experiments: the percentage of carotenoids recovered from the aqueous micellar fraction, following *in vitro* gastro-intestinal digestion (i.e. bioaccessibility); physicochemical properties of the digesta, specifically viscosity and surface tension; and finally the micelle size and the zeta-potential of the particles in solution. Finally, in order to validate, or not, the results obtained *in vitro*, we carried out a randomized and double blinded placebo controlled cross-over postprandial trial (24 male participants), testing the effect of 3 supplementary calcium doses (0 mg, 500 mg and 1000 mg) on the bioavailability of carotenoids from a spinach based meal, given for breakfast. Participants were scheduled to 3 full day (10 h) clinical visits, with one week apart between each one of them. Participants were asked to avoid as much as possible any sources of dietary carotenoids, during the whole trial period. Bioavailability was assessed as the Area-Under-the-Curve (AUC) of time vs. concentration of carotenoids extracted from the plasma triacylglycerol-rich-lipoprotein (TRL) fraction, representing newly absorbed carotenoids.

Bioaccessibility results, from the *in vitro* trials, showed that addition of the divalent cations significantly decreased the bioaccessibility of both pure carotenoids ($P < 0.001$) and those from food matrices ($P < 0.01$). The extent to which bioaccessibility was reduced was dependent on the type of mineral and its concentration. Strongest effects were seen for calcium followed by magnesium and zinc. Addition of calcium, especially at higher concentrations (500 – 1000 mg/L), led to reductions in bioaccessibility as high as 100%. The addition of divalent cations also altered the physico-chemical properties of the digestas. However, the extent of its effect varied according to the type of matrix. An increasing concentration of the cations correlated with decreased viscosity ($r > 0.9$) of the digestas of pure carotenoids. In the case of food matrices, we observed a similar tendency. In the presence of divalent minerals, a significant decrease of the digesta macroviscosity was detected, which was steeper for the food matrices with higher pectin content, i.e. apricot, tomato and carrot juice, than for leafy vegetable matrices. Surface tension, on the other hand, increased significantly ($P < 0.05$) with

increasing concentrations of divalent minerals. The effects on bioaccessibility and physico-chemical properties were accompanied by variations of the zeta-potential of the particles in solution. In control conditions (i.e. no minerals added), the particles in the digesta were negatively charged, with values of approximately of -30 mV. As concentrations of calcium and magnesium increased, the absolute zeta potential decreased, tending towards zero, suggesting that systems became somewhat more instable. The effect of divalent cations, specifically magnesium, was also modulated by the concentration of bile and, to a lesser extent, by pancreatic enzyme concentration. Samples digested with lower concentrations of bile (1 mM) were more susceptible to the inhibitory effects of magnesium on carotenoid bioaccessibility, than those digested with 8 mM ($P < 0.001$). Taken together, results from the *in vitro* trials strongly suggested that divalent cations were able to bind bile salts and other surfactant agents, affecting their solubility. The observed i) decrease in macroviscosity, ii) increase in surface tension, and the iii) reduction of the zeta potential of the digesta, confirm the removal of surfacting agents from the system, most likely due to precipitation as a result of the lower solubility of the mineral-surfactant complexes. As such, micellarization of carotenoids was hindered, explaining the reduced bioaccessibilities.

As for the human trial, the analysis of the AUC of the 3 different calcium doses, showed that there was no significant influence of supplementation with either 500 or 1000 mg of supplemental calcium (in form of carbonate), suggesting that the hypothesis and the *in vitro* results are not supported in such an *in vivo* scenario. However, the results of another recent human trial, investigating the effect of calcium supplementation on the bioavailability of carotenoids from tomato paste, seem to contradict our findings. Hence it can be speculated from these two human trials that the effect of mineral supplements on the bioavailability of carotenoids, and potentially other liposoluble nutrients, may depend on the specific individual interaction of the supplement form and dissolution kinetics and with the type of carotenoid and the food matrix. Further investigations are necessary to understand how divalent cations act during *in vivo* digestion and potentially interact with different nutrients and food constituents.

1. INTRODUCTION

1.1 Outline

For the past two decades there has been an increasing interest in phytochemicals and recognition of their potential benefits in human nutrition and health. Phytochemicals cover a wide range of secondary plant compounds that have not been shown to be essentials for humans, i.e. their absence in the diet does not cause any deficiency symptoms. However, the intake of several dietary phytochemicals has been associated with the prevention of certain chronic conditions, such as cancer (Giovannucci, 2002; Surh, 2003), diabetes type 2 and insulin resistance (Coyne et al., 2005; Ford, Will, Bowman, & Narayan, 1999), and cardiovascular disease (Voutilainen, Nurmi, Mursu, & Rissanen, 2006). Some of these phytochemicals and other food microconstituents are highly lipophilic molecules, i.e. with high octanol-water partition coefficients (log Pc > 8), and tend to readily dissolve in oil. These include, among others, carotenoids, phytosterols, monoterpenes, and triterpenes/triterpenoids, as well as the fat soluble vitamins (A, E, D, and K).

Given their highly lipophilic nature, these food microconstituents tend to share, during digestion, similar crucial steps with the fat soluble vitamins (A, E, D, and K),, including the transfer from the food matrix into a lipid phase, emulsification, inclusion into mixed micelles, diffusion through the mucus to the unstirred water layer, binding to the enterocyte, and cellular uptake (Gropper, Smith, & Groff, 2005). The transfer of lipophilic food microconstituents from the matrix into mixed micelles is especially important and a limiting step in their bioavailability (i.e. the fraction of a nutrient/non-nutrient that is absorbed and available for use and/or storage in body organs). In recent years, dietary aspects influencing the bioavailability of highly lipophilic food microconstituents (HLFMs), particularly carotenoids, have been reviewed comprehensively (Borel, 2003; Hof & West, 2000), and certain studies have highlighted the importance of sufficient dietary lipids (Gleize et al., 2013; Unlu & Bohn, 2005) and a limited amount of co-consumed dietary fibre (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011; Verrijssen et al., 2013) as crucial factors influencing their bioavailability. However,

certainly many more dietary factors remain to be investigated regarding their potential to interact with carotenoid absorption and bioavailability

1.2 Carotenoids: A brief overview

Carotenoids are lipophilic organic pigments synthesised in plants and other microorganisms, with vast physiological functions. Though they colour the world in hues of red, orange and yellow, by absorbing light in the visible range (400-500 nm), their primary function in producing organisms is not pigmentation. In photosynthetic organisms, carotenoids are essential for photosynthesis and photoprotection, while in non-photosynthetic organisms, they take part in mitigating photooxidative damage (Stange & Flores, 2010).

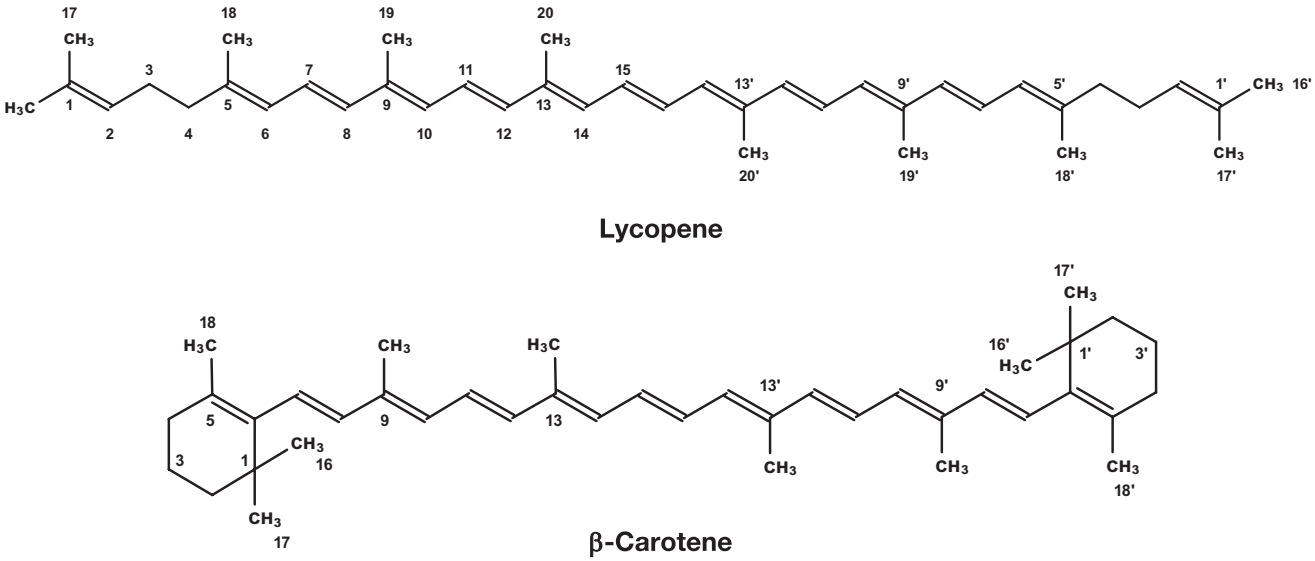

Figure 1 – Basic structure and numbering scheme of lycopene, example of an acyclic carotenoid, and all-trans-β-carotene, as an example of a dicyclic carotenoid.

Until now, more than 700 carotenoids have been isolated from natural organisms, including geometrical and optical isomers (Britton, Liaaen-Jensen, & Pfander, 2004). Despite their structural variety, all carotenoids share the following common features: 1) a polyisoprenoid structure; 2) near symmetry around the central double bond; 3) and a long conjugated chain of double bonds in a central portion of the molecule which gives carotenoids their distinctive molecular shape, chemical activity, and light absorbing properties (Britton, 1995) (Figure 1). It is the existence of the conjugated double

bond system that confers them the ability to absorb energy from other molecules and to act as antioxidant agents (Britton, 1995; Stahl & Sies, 2005, 2012). One of the ways through which carotenoids exert antioxidant functions is by quenching singlet oxygen molecules (Krinsky, 1998). In addition, they also have the ability to interact with free radicals, such as lipid peroxyl radicals (Krinsky, 1998). In order to be an effective antioxidant, carotenoids would have to remove from the system free radicals that are responsible for oxidation reactions, either by forming harmless products with free radicals, or by otherwise disrupting free radical chain reactions (Britton, 1995).

1.3 The relevance of carotenoids in human health

It is partly its antioxidant potential that has sparked the interest in carotenoids in relation to health status and disease prevention. In the human body, reactive oxygen species (ROS) are constantly being produced through normal metabolite activity. The exposure to environmental stressors such as UV radiation, microbes, allergens, CO_2 emissions or cigarette smoke, amplify the generation of ROS (Bouayed & Bohn, 2010), causing oxidative stress and increasing the risk of chronic disease development, including cardiovascular complications, atherosclerosis, diabetes, cancer (Bouayed & Bohn, 2012) and age-related macular degeneration (Hammond, Wooten, & Snodderly, 1996). In this context the antioxidant and biological properties of carotenoids make them appealing as potential players for the prevention of chronic conditions associated with oxidative damage. However, their interaction with gene expression following the binding to transcription factors related to anti-inflammatory and anti-oxidant effects (e.g. NF-kB, Nrf2) has also been highlighted (Kaulmann & Bohn, 2014). The main specific nutrient function of carotenoids, specifically β-carotene, β-cryptoxanthin and α-carotene, is their function as precursors for Vitamin A synthesis. Amongst the more than 700 known carotenoids, around 10% are nutritionally active precursors of this vitamin, though the most relevant in human nutrition are β-carotene, α-carotene and β-cryptoxanthin. Vitamin A is acquired through diet either in the form of provitamin A carotenoids, mainly from fruits and vegetables, or as pre-formed vitamin A in the form of retinyl esters, from animal products, i.e. eggs, meat, fish and dairy (Tang & Russell, 2009).

Despite their antioxidant potential and apparent role in the prevention of chronic diseases, no other specific nutrient function has been identified for carotenoids so far; main reason for which no dietary requirements for carotenoids have been defined. Also, the evidence relative to the antioxidant function of carotenoids in humans, specifically β-carotene, is controversial and failed to meet the definition of a dietary antioxidant as defined on the American Report for Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids (Institute of Medicine (US) & Compounds, 2000) – "A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans." Although reference intakes could be derived from Vitamin A equivalency, there are many host- and dietary-related factors that affect the amount β-carotene effectively converted to Vitamin A (Haskell, 2012), as mentioned below.

1.4 Carotenoid bioavailability

Carotenoid bioavailability can be defined as the percentage of carotenoid that is effectively released from its food matrix and is available for uptake and storage in the human body (Parker, Swanson, You, Edwards, & Huang, 1999; Stahl et al., 2002); and it can be affected by a multitude of factors often described by the mnemonic SLAMENGHI, standing for: S) species of carotenoids; L) linkages at molecular level; A) amount of carotenoid; M) matrix; E) effectors of absorption and bioconversion; N) nutrient status; G) genetics; H) host-related factors; and I) interaction among these variables (Castenmiller & West, 1998). Perhaps the main limiting factors of bioavailability are those related to the release of carotenoids from its food matrix, solubilisation and transfer into mixed micelles – nanoscale sized water-soluble aggregates formed by bile salts and insoluble lipids (i.e. fatty acids, phospholipids and cholesterol), carrying products of lipolysis and other lipophilic food microconstituents, such as carotenoids and liposoluble vitamins (Carey & Small, 1970; Gropper et al., 2005). Also pre-formed vitamin A, as a liposoluble micronutrient and a product of carotenoid metabolism, will be most likely affected by the same factors modulating carotenoid bioavailability.

1.4.1 Gastro-intestinal digestion

Digestion and upper-intestinal uptake of lipophilic food microconstituents and carotenoids occurs in a similar fashion (Figure 2). First of all, they must be released from their food matrices (Borel, 2003; Stahl et al., 2002). In the stomach, under the action of hydrochloric acid and enzymes, carotenoids and vitamin A are partially released from the food matrix into emulsified oil droplets. During small intestinal passage, components of the food matrix and lipid droplets are further degraded through the action of bile and pancreatic enzymes, and the lipolysis products are partitioned into mixed micelles (Failla & Chitchumronchokchai, 2005; Parker, 1996). Carotenoids are then transferred from the oil droplets into mixed micelles, which will diffuse through the unstirred water layer to the intestinal mucus, until the surface of the mucosa of the upper small intestinal tract, where carotenoids can be taken up at the apical surface of the enterocytes (Stahl et al., 2002).

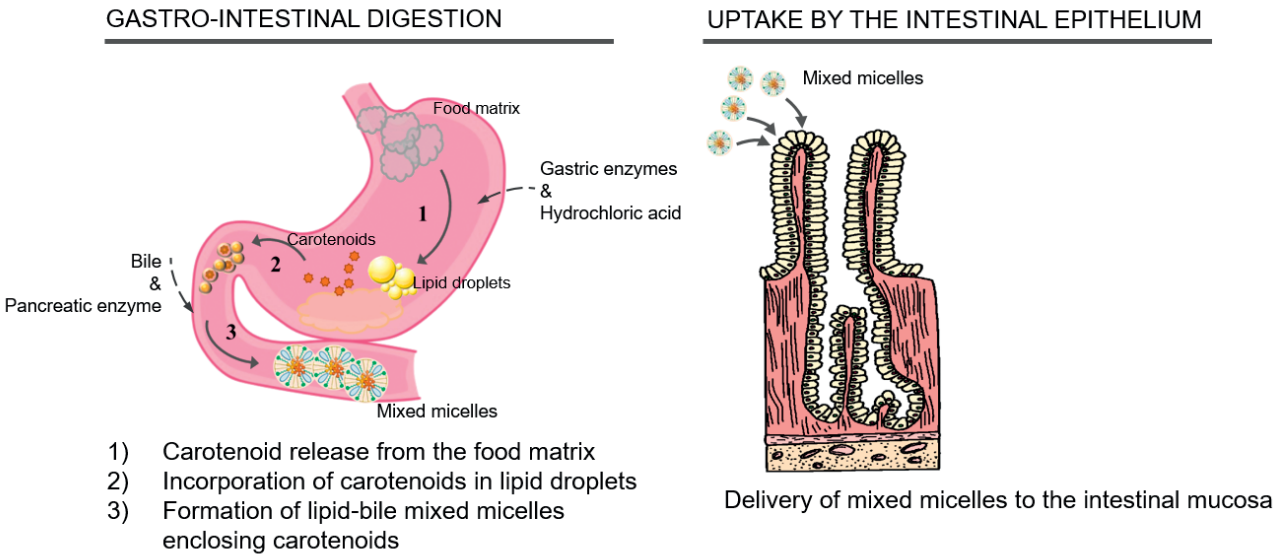

Figure 2 – Simplified illustration of gastro-intestinal digestion and epithelial uptake of dietary carotenoids in humans.

Different dietary factors have the potential to either promote or hamper their transfer of carotenoids into mixed micelles and later uptake, and they are discussed below.

1.4.2 Dietary factors affecting carotenoid bioavailability

Dietary factors can potentially affect carotenoid bioavailability at different stages of the GI digestion. As mentioned previously, the first step into carotenoid bioavailability is their release from the food matrix either mechanically or via enzymatic action (Bohn, 2008). Aspects such as the type of food matrix, food processing techniques, presence of lipids and dietary fibre of carotenoids have been investigated, and are briefly discussed below.

Type of Matrix. In plants, carotenoids are stored in chromoplasts and chloroplasts. While chloroplasts contain membrane-bound carotenoids, in chromoplasts carotenoids are stored in i) lipid droplets (i.e. plastoglobuli), ii) under crystalline form or iii) membrane-bound (Jeffery, Holzenburg, & King, 2012). The different forms of storage will affect the ease of release of carotenoids from its matrix, with those stored in plastoglobuli being the most accessible followed by those in crystalline form and finally membrane-bound (Jeffery, Turner, & King, 2012). Generally, carotenoids tend to be less accessible from food matrices in which the main storage organelles are chloroplast, such as green leafy varieties, and more accessible from food matrices in which carotenoids are stored in chromoplasts, such as pulpous fruits and root vegetables (Schweiggert & Carle, 2016).

Food Processing. Processing techniques such as heating, pureeing or juicing can alter carotenoid bioavailability by breaking down the food matrix, including cell walls, and increasing the release and accessibility of carotenoids. For example, bioavailability of β-carotene, but not of lutein, from minced (6.4%) and liquefied (9.5%) spinach was shown to be significantly higher than the bioavailability from whole spinach leaves (5.1%) (Castenmiller, West, Linssen, van het Hof, & Voragen, 1999). Similarly, the consumption of pureed spinach and carrot, over a 4 week period, resulted in higher plasma concentrations of β-carotene when to compared to the intake of the same amount of β-carotene from the raw vegetables, during the same period of time (Rock et al., 1998). However, food processing methods can also increase carotenoid exposure to oxygen, metals, enzymes, unsaturated lipids, prooxidants/ antioxidants, and light, making them susceptible to degradation (Rodriguez-Amaya, 2001). Heat treatments can lead to the *cis-trans* isomerisation of

carotenoids (Updike & Schwartz, 2003), which will also affect its biovailability since *cis* isomers have been shown to be more bioaccessible and may be taken up better at the cellular level (Ferruzzi, Lumpkin, Schwartz, & Failla, 2006). Hence, heat application can have both favourable and unfavourable consequences regarding carotenoid bioavailability.

Amount and type of lipid. As mentioned previously, carotenoids and vitamin A are liposoluble molecules, and the presence of lipids during digestion is paramount to promote the transfer of carotenoids from the food matrix into mixed micelles. Addition of fat to a meal is hence an important factor that can significantly improve carotenoid bioavailability during GI digestion, both *in vitro* (Biehler, Kaulmann, et al., 2011; Gleize et al., 2013; Kohut, Failla, Watkins, & Ferruzzi, 2007) and *in vivo* (Brown et al., 2004; Goltz, Campbell, Chitchumroonchokchai, Failla, & Ferruzzi, 2012; Unlu & Bohn, 2005). Not only the amount of lipid added, but also its fatty acid profile may influence the transfer of carotenoids into mixed micelles. Oral administration of spinach and saturated fatty acid (SFA) rich butter over a 3 d period led to a higher fasting plasma lutein concentration than spinach and unsaturated FA rich olive or fish oils, in a rat model (Gleize et al., 2013). In contrast, Goltz et al (2012) reported that the addition of unsaturated FA rich canola oil to a salad, led a higher, yet modest ($P = 0.06$), increase of lutein and α-carotene's concentration in the plasma chylomicron fractions of the participants, when compared to the addition of SFA rich butter ($P = 0.08$). The same study also underlined that the amount of fat added to the test meal was more important than the fatty acid profile, and that the addition of 20 g of lipids promoted a higher bioavailability, compared to 3 and 8 g, for all carotenoid species ($P < 0.05$), with the exception of α-carotene ($P = 0.07$) (Gleize et al., 2013).

Fibre Content. The presence of fibre, oppositely to lipids, has been shown to have a negative effect on carotenoid bioavailability. Physical properties of fibres such as particle size, viscosity, water-binding capacity, gel formation, and bile acid-binding, are likely to result in carotenoids i) not being well released from the fruit and vegetable matrices; ii) being potentially entrapped during upper intestinal digestion and not accessible to cellular uptake; iii) binding to polysaccharides requiring additional enzymatic hydrolysis to be absorbed (Palafox-Carlos et al., 2011). This inhibitory outcome

seems to be valid for both soluble (e.g. pectin, guar and alginate) and insoluble fibres (e.g. wheat bran and cellulose) (Riedl & Linseisen, 1999; Verrijssen et al., 2013).

Type of Carotenoid. Carotenoids can be classified into two groups according to the presence (xanthophylls) or absence (carotenes) of oxygenated functions. The presence of oxygen affects the polarity of the molecule, rendering xanthophylls (e.g. lutein and zeaxanthin) slightly more polar than carotenes (e.g. lycopene and β-carotene), which in turn will influence their bioavailability. In the process of transfer of carotenoids into mixed micelles the apolar, and more hydrophobic, carotenes will presumably localise at the interior of the micelles, while the more polar xanthophylls will tend to distribute at the surface of the micelle, rendering them more accessible for cellular uptake (Borel et al., 1996; Britton, Liaaen-Jensen, & Pfander, 2009).

1.5 Divalent minerals as a potential dietary factor influencing carotenoid bioavailability

One dietary factor that has recently been proposed to affect *in vitro* carotenoid bioaccessibility and cellular uptake is the presence of DM (Biehler, Hoffmann, Krause, & Bohn, 2011), especially at concentrations that are common for dietary supplements, usually at half to full of the Dietary Reference Intakes (DRIs) level recommended by the Food and Nutrition Board of the Institute of Medicine (USA) (2011). For example, Ca^{2+} at intestinal concentrations of 15 mM reduced the *in vitro* availability of β-carotene (Biehler, Hoffmann, Krause, & Bohn, 2011). Very recently, a human trial corroborated potential negative effects on carotenoid absorption, showing that a dietary supplement containing 500 mg Ca^{2+} reduced lycopene absorption from tomato paste by up to 83%, as measured by plasma appearance (Borel et al. 2017). The ability of DM to bind FFA and bile acids, leading to the formation of low solubility soaps (Govers & Van der Meer, 1993) and salts (Hofmann & Mysels, 1992), respectively, may in fact inhibit the transfer of lipophilic food microconstituents into mixed micelles, limiting their bioavailability, as these would then pass on to the colon, where presumably they will not

become available either, or are in part degraded by the microbiota (Goni et al., 2006), and are finally excreted.

1.6 Role of divalent minerals in health and nutrition

Dietary DM include several essential macro-minerals such as Ca^{2+} and Mg^{2+}, and micro-minerals, also termed trace elements, such as iron and Zn^{2+} as the most predominant ones. Due to their participation in many essential functions in the human body, a sufficient intake of these minerals is paramount. While Ca^{2+} is especially of relevance for bone metabolism (Gropper, Smith, & Groff, 2005), Mg^{2+} plays a crucial part in many energy-related metabolic functions, acting as a co-factor for many enzymes (Swaminathan, 2000). Iron is part of haemoglobin and required for the formation of functional erythrocytes, and Zn^{2+} is likewise required for energy-metabolism and constitutes a co-factor for many enzymes (Ross et al., 2012). Despite the current dietary recommendations for macrominerals and trace elements intake, in Europe and the United States (Table I), a significant percentage of the population fails to meet the recommended intake levels. (Cordain et al., 2005; Roman Viñas et al., 2011). For example, it was estimated that in the United States, at least half of the population aged $\geq$ 2, does not meet the country's Recommended Dietary Allowances (RDA) for vitamin B-6, vitamin A, Mg^{2+}, Ca^{2+}, and Zn^{2+} (Cordain et al., 2005). As a solution, due to their dietary importance and convenience, supplements are widely offered on the market and are frequently consumed. For Ca^{2+}, typical dietary supplements contain between 250 and 1200 mg per unit (mostly, a tablet), for Mg^{2+} these are 100 – 600 mg, and for iron and Zn^{2+} up to 65 and 50 mg, respectively. Especially in developed Westernized countries, up to 66% of the population regularly consumes dietary multimineral/multivitamin supplements (Rock, 2007; Skeie et al., 2009), at least in most of these countries. Given their frequent use in today's society, understanding the potential inhibitory effects of DM on lipophilic food microconstituent bioavailability would also be of interest for subjects with digestion malfunctions such as those with pancreatitis (Dutta, Bustin, Russell, & Costa, 1982), and for subjects with already poor absorption of certain micronutrients, such as those with inflammatory bowel disease (Hwang, Ross, & Mahadevan, 2012). Often, these conditions are

associated with deficient bile and pancreatic enzyme secretion, causing steatorrhea and absorption deficiencies of certain nutrients, such as vitamin E, most likely due to the inability to form mixed micelles during the small intestinal passage (Dutta et al., 1982; Stahl et al., 2002). It could be hypothesised that intake of DM as supplementation would worsen the effects of these conditions.

1.7 Effect of Divalent Minerals on Digestion

Dietary minerals are typically released in the stomach, where digestive enzymes (pepsin, gastric lipase) and peristaltic movements work towards the food matrix breakdown, and the rather acidic gastric pH (fasting 1-5-2.0; with food 3-5) (Alminger et al., 2014) aid in releasing the minerals from their bound form such as in organic complexes. Solubilized minerals, present in their free ionised form, can be taken up in the small intestine, both via passive transport and facilitated uptake via e.g. calcitriol-regulated transporters such as for calcium (Gropper et al., 2005, Chapter Macrominerals) though a smaller fraction may also be absorbed by the colon (Roberfroid, 2007; Sellers and Morton, 2014; Gopalsamy et al., 2015). Trace elements (e.g. iron and Zn^{2+}) are principally absorbed in the upper small intestine (duodenum and jejunum), while the macrominerals Ca^{2+} and Mg^{2+} appear to be absorbed throughout the small intestine either by active or passive transport (Gropper et al., 2005), but especially in the latter sections of the small intestine, the ileum. However, release and solubilisation of DM in the stomach does not necessarily ensure absorption in the small intestine. Within neutral to slightly alkaline pH range, as found in the small intestine, DM can complex with bile acids and dietary constituents such as organic acids (Etcheverry, Grusak, & Fleige, 2012), polysaccharides (Debon & Tester, 2001), and fatty acids (Cheng, Morehouse, & Deuel, 1949). For example, it is well comprehended that iron absorption can be negatively influenced by phytic acid (Gupta et al., 2015; Petry et al., 2015) present in many grain cereals, legumes and oilseeds (Schlemmer, Frolich, Prieto, & Grases, 2009), and similar behaviour has been shown for Mg^{2+} (Bohn, Davidsson, Walczyk, & Hurrell, 2004). Other organic acids, such as oxalic acid, present in many leafy vegetables (Holmes & Kennedy, 2000) may also form complexes with minerals, reducing their availability (Bohn et al., 2004).

Table 1 - Population Reference Intakes (PRIs) and Dietary Reference Intakes (DRIs) for calcium, magnesium, iron and zinc

Population Reference Intakes (PRIs), defined by the European Food and Safety Agency (EFSA) [1]

Age (years)	Calcium (mg/d)		Age (years)	Magnesium [a] (mg/d)		Age (years)	LPI[b] (mg/d)	Zinc (mg/d)		Age (years)	Iron (mg/d)	
	Male	Female		Male	Female			Male	Female		Male	Female
18-24	1000	1000	≥ 18	350	300	≥ 18	300	9.4	7.5	> 18	11	16 [c] 11 [d]
≥ 25	950	950					600	11.7	9.3			
							900	14.0	11.0			
							1200	16.3	12.7			

Dietary Reference Intakes, defined by the Food and Nutrition Board from the Institute of Medicine (USA) [2]

Age (years)	Calcium (mg/d)		Age (years)	Magnesium (mg/d)		Age (years)	Zinc (mg/d)		Age (years)	Iron (mg/d)	
	Male	Female		Male	Female		Male	Female		Male	Female
19-50	1000	1000	19-30	400	310	> 18	11	8	19-50	8	18
51-70	1000	1200	> 30	420	320				> 50	8	8
> 70	1200	1200									

d : day; LPI: level of phytate intake

[a] Adequate Intake level – estimated value when a PRI could not be established because the Average Requirement level could not be determined.

[b] Average requirements for dietary zinc necessary to meet physiological requirements were estimated using saturation response modelling, taking into account the inhibitory effect of dietary phytate on zinc absorption.

[c] For pre-menopausal women. [d] For post-menopausal women.

SOURCES: [1] EFSA Summary of Dietary Reference Values – version 3 (August 2017); [2] Dietary Reference Intakes: The Essential Guide to Nutrient Requirements (2006) (http://www.nap.edu/catalog/11537.html)

In general, the strength of complexation has been reported to follow the following ranking: Ca=Zn=Fe> Mg^{2+} (reviewed by Bohn, 2002) due to the stability of the formed complexes.

Perhaps the most relevant complexes formed by the binding of DM, concerning aspects of liposoluble food constituent bioaccessibility, are those formed between cations and bile acids or FFA, given the importance of the latter regarding the formation of lipid-bile mixed micelles. Fatty acids, when released from the diet during digestion of triglycerides and/or phospholipids such as lecithin, have been reported to form insoluble soaps with DM (Vaskonen, 2003). Similar reports have been published demonstrating that DM may react with bile acids, forming poorly soluble complexes (Capurso et al., 1999). In addition, the presence of DM may enhance the binding of bile salts to fatty acids (Pandolf and Clydesdale, 1992). In the following, two main potential pathways of inhibiting mixed micelle formation (i.e. binding with fatty acids and/or bile salts, and their impact on the bioaccessibility of liposoluble constituents), will be evaluated with a focus on carotenoids.

1.7.1 Interaction of divalent minerals with bile-salts during digestion

Bile acids, which consist of a steroid backbone, include primary, secondary, and tertiary bile acids produced in the hepatocytes of the liver from cholesterol (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). The most prominent are cholic acid and chenodeoxycholic acid (Wilde & Chu, 2011), which are conjugated with taurine and glycine in the liver to form glycoconjugates and tauroconjugates, respectively. They are then secreted via the bile into the duodenum, i.e. the uppermost part of the small intestine (Hofmann, 1999), to act as emulsifiers during intestinal digestion. The amount secreted daily is approx. 250 – 1000 mL, of which the bile concentration is around 4-45 mmol/L (Boyer, 2013). The polarity of bile acids has been reported to decrease in the following order: glycine-conjugated < taurine-conjugated < lithocholic acid < deoxycholic acid < chenodeoxycholic acid < cholic acid <ursodeoxycholic acid. Due to their amphiphilic properties they can act as surfactants, aiding in the micellization of liposoluble constituents. If missing, severe malabsorption of lipophilic constituents may occur (Venkat et al., 2014). In subjects without functional bile salt secretion, very low circulating levels of lipophilic vitamins have been reported. For example, Setchell et al. (2013)

reported that in children genetically lacking the possibility for bile salt conjugation, low serum concentrations of especially vitamin D and E were encountered and in part also low vitamin K and A levels, emphasizing the importance of sufficiently high conjugated bile salt concentrations in the gut for optimal micellization and absorption of lipophilic constituents. In children suffering from obstructive jaundice, occurring when the essential flow of bile to the intestine is blocked, the serum concentrations of vitamin E were significantly lower (0.1 mg/mL) compared to children with no history of malabsorption conditions (0.8 mg/mL) (Muller et al., 1974).

Previous *in vitro* trials have shown the dependency of carotenoid bioaccessibility on the concentration of bile during GI digestion (Tyssandier, Lyan, & Borel, 2001), and that low concentration of bile salts compromised the bioaccessibility, i.e. the proportion of a compound available for absorption, of carotenoids from spinach (Biehler, Kaulmann, et al., 2011), highlighting the importance of micellization for especially the more apolar compounds. Similar results were found by Garret et al. (1999) regarding carotenoid bioaccessibility from a mixed salad. Likewise, in a study by Gireesh (2009), uptake of β-carotene into human exfoliated colonic cells increased with increasing bile salt concentration. In a study with gerbils, adding taurocholate at concentrations of 0, 0.5, or 1% to the diet significantly increased hepatic and plasma β-carotene concentrations (Sundaresan et al., 2005). In an *in vitro* trial employing spinach and bile salts, glycodeoxycholate was more effective in micellarizing carotenoids than taurocholate, possibly due to higher polarity of the latter, acting as a less efficient emulsifier (Rich et al., 2003)

As bile acids have a pKa of 4.2-7.3 (Cabral et al., 1986) and are weak acids, they are in part present in the gut in form of a salt. Sodium bile salts have generally a high solubility (Jones, Hofmann, Mysels, & Roda, 1986), and have a lower critical micelle concentration (CMC) (Jones et al., 1986) than their free form, i.e. the concentration threshold above which spontaneous formation of bile salt mixed micelle formation occurs. In contrast, binding of Ca^{2+} ions to bile acids tends to increase the CMC and lower the solubility product of the respective bile salt (Jones et al., 1986), which could lead to bile salt precipitation. However, it is likely that the presence of Na^{+} also lowers the potential negative effect of DM by competition, as bile salts and micelles bind both Ca^{2+} ions as counterions. Such an effect may

explain results from an *in vitro* study by Biehler et al. (2011b), where sodium concentrations of 900 mM in the digesta increased bioaccessibility of spinach originating β-carotene by 60%, though not remarkably influencing xanthophylls.

Addition of DM to *in vitro* digestion models has also negatively affected the surface charge of digested particles, and potentially the stability, of the digestas. The presence of bile confers a negative charge to the system, as a result of repulsive forces created by the surfactant effect of bile salts, which stabilizes the system and prevents aggregation. The higher the concentration of bile salts, the more negative the zeta potential of the emulsion will be (Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998). In the presence of Ca^{2+} ions the zeta potential of the bile salt emulsions decreases, and it has been suggested that this effect is due to the shielding of the surface charge of the emulsion droplets by the Ca^{2+} ions (Wickham et al., 1998). It also has to be considered that factors related to cellular uptake, such as better adhesion to the epithelium, have in general been related to more negatively charged particles (Xu et al., 2013).

Although Ca^{2+} is by far the most investigated divalent mineral both *in vitro* and *in vivo*, other minerals and trace elements also bind bile acids, namely iron, copper and Zn^{2+} (Feroci, Fini, Fazio, & Zuman, 1996). However, *in vivo*, they are normally present at low micromolar concentrations and are hence not likely to significantly affect bile salt solubility.

1.7.2 Interaction of divalent minerals with lipids during digestion

Earlier animal trials had described negative interactions between lipids and DM minerals during GI digestion, resulting in both the non-availability of the mineral and the lipids. In a study by Tadayyoon and Lutwak (1969) on rats receiving either fat-free, 5%, or 25% of either a triolein, tripalmitin or tristearin diet together with Ca^{2+} and Mg^{2+}, it was found that diets with 25% tripalmitin or tristearin reduced Ca^{2+} and Mg^{2+} absorption, while no effect was found for triolein. In another study (Gacs and Barltrop, 1977), the authors investigated whether Ca^{2+} from ^{47}Ca labelled soaps of fatty acids would be bioavailable in rats. It was found that Ca^{2+} absorption was inversely correlated with the chain lengths and level of saturation of the fatty acids, presumably related to the lower solubility of long-

chain and of saturated fatty acid soaps. However, no significant soap formation or reduction in Ca^{2+} absorption was observed when fats were given as triglycerides, unlike the earlier trial by Tadyyoon and Lutwak (1969). It should be pointed out that levels of dietary lipids were quite high in the latter study, and that it is likely that the slow and stepwise release in the small intestine, coupled with parallel ongoing Ca^{2+} absorption in the small intestine, reduced the negative interactions of Ca^{2+} with triglycerides, compared to the FFA from the study of Gacs and Barltrop (1977). Also negative effects regarding lipid digestion and availability were reported in a previous study employing high concentrations of Ca^{2+} (12 g/kg fodder) during a 23 d feeding trial of calves. Fat digestion in calves was inhibited, resulting in 5.6% lower fat digestibility and 90% increased bile acid excretion in the faeces, compared to a low Ca^{2+} control diet (Xu et al., 1998).

Similar studies have also been performed in humans. In an older study (Steggerda and Mitchell, 1951), a diet rich in butter fat did not decrease Ca^{2+} balance in humans, perhaps due to the limited content of butter fat (up to 32% fat content of diet), or to the higher solubility of short chain fatty acids partly present in butter (Souci et al., 2000). In another human study, targeting negative effects on lipids, Ca^{2+} intake from dairy products (800 mg and 350 mg), but not from Ca^{2+} carbonate (850 mg) reduced lipid response in plasma and chylomicrons, used as an indicator of postprandial fat absorption, also suggesting negative interactions with lipid availability and Ca^{2+} intake. It is noteworthy that there was no effect of the Ca^{2+} carbonate supplement, perhaps due to the differences in the chemical form of Ca^{2+} (calcium carbonate has to solubilize in the acid milieu of the stomach first) and altered kinetics of dissolution during digestion (Lorenzen et al., 2007), which may have slowed down potential negative interactions. One pilot study, looking into the effect of supplementation of Ca^{2+} alone (2 g), or in combination with vitamin D, on blood pressure and serum lipids of colorectal adenoma patients, has also looked into plasma carotenoid levels (Chai, Cooney, Franke, & Bostick, 2013). Authors found that daily Ca^{2+} supplementation, either alone or in combination with vitamin D, for a period of 6 months, was able to reduce, though not significantly, serum triglycerides by approximately 30 and 32% ($P = 0.1$), respectively, and plasma carotenoid levels by 14% ($P = 0.07$) and 9% ($P = 0.1$) in groups treated with Ca^{2+} or Ca^{2+}+ vitamin D_3, respectively. It was hypothesized

that these effects were likely due to the formation of Ca^{2+}-lipid complexes in the gut, and that, despite the absence of statistical significance, Ca^{2+} supplementation could indeed lower blood triglycerides and consequently carotenoids on the long term.

In fact, Ca^{2+} supplementation has been used for a few decades as a possible therapeutic means to lower serum circulating lipids. In a number of randomized (n=6) and non-randomized (n=6) human trials, reviewed by Reid (2004), lasting a minimum of 4 d to trials performed over the period of a year, with Ca^{2+} doses ranging from 400 to 2660 mg/d, the majority of these reported significant reductions of the participants' serum triglycerides and also LDL-C levels. While calcium gluconate and citrate were found to be effective, 1 study employing calcium carbonate did not result in any benefits, which may or may not have been due to lower solubility of the carbonate form (Goss, Lemons, Kerstetter, & Bogner, 2007). Some of the studies reviewed by Reid also reported an increase in faecal lipid excretion, supporting the hypothesis that minerals and lipids interact in the gut and limit one another in their absorption. However, the formation of fatty acid soaps with DM and their successive faecal excretion seems to depend on the solubility of the formed soap, which is again related to the digestibility of triglycerides and fatty acid composition. Addition of Ca^{2+} and Mg^{2+} appears to have a particularly negative effect on the solubility of natural fats with high melting points (i.e. > 50ºC) (Cheng et al., 1949) and of long-chain saturated fatty acids (Tadayyon & Lutwak, 1969). Interestingly, it also has to be stated that lipolysis could be enhanced by higher concentrations of Ca^{2+} (Wickham et al., 1998), perhaps also due to the precipitation of lipolysis products, shifting the equilibrium of the reaction to the product side. In support of this proposition, some studies report an increase in lipase activity in the presence of millimolar concentrations of Ca^{2+} (Hu, Li, Decker, & McClements, 2010; Waite, 1985).

1.8 Divalent minerals and lipophilic food microconstituents

Lipophilic food microconstituents, which include liposoluble vitamins (A, E, D and K), carotenoids, other triterpenes such as ursolic acid, phytosterols, and some liposoluble phenols (e.g. curcumin) are assumed to share similar steps during upper GI digestion. Due to their low water solubility, these

compounds have to be emulsified in order to diffuse through the unstirred water layer of the enterocyte for absorption. For this purpose, they are transferred into mixed micelles, emulsified particles containing amphiphilic constituents such as bile salts, monoglycerides, FFA on the outside, and more apolar compounds (cholesterol, carotenoids) in their interior (Parker, 1996; Sy et al., 2012). It could be assumed that any dietary factor that potentially interferes with the ability to form mixed micelles in the gut, including the presence of DM, would also affect food constituents that depend on its formation to be absorbed. However, data on the potential effects of DM/trace elements on the bioavailability of lipophilic food microconstituents, particularly the liposoluble vitamins, is scarce and in need of better elucidation. However, some *in vitro* and *in vivo* studies have looked into the interactions between DM and carotenoids, and the human carotenoid metabolite vitamin A.

1.8.1 Carotenoids, vitamin A and divalent minerals

***In Vitro* Studies.** A few *in vitro* studies have meanwhile investigated interactive effects of various DM, as well as sodium, and carotenoids during simulated GI digestion. The first report mentioning a negative interaction between minerals and carotenoid bioaccessibility emphasized negative effects of Ca^{2+} on β-carotene at concentrations of 5 mM (Biehler et al., 2011b), while sodium enhanced β-carotene bioaccessibility by up to 60% (at 0.9 M vs. controls, i.e. 0.15 M). In a first systematic study by Biehler et al. (2011a), increasing concentrations of iron, Zn^{2+}, Mg^{2+} and Ca^{2+}, added at concentrations of 3.8 (212 mg/L), 3.8 (250 mg/L), 12.5 (300 mg/L) and 7.5 mM (300 mg/L), respectively, resulted in a dose dependent decrease of individual and total carotenoid bioaccessibility (lutein, β-carotene) and Caco-2 cellular uptake of carotenoids from spinach. The half maximal inhibitory concentration (IC50) values for bioaccessibility were reported to be of approximately 7 (390 mg/L), 9 (590 mg/L) and 15 mM (600 mg/L) for Fe, Zn, and Ca, respectively, when compared to physiological saline (0.15 M). The reduction of β-carotene was significantly more pronounced than that of lutein, indicative of a stronger effect of minerals on the more apolar carotenoids, presumably related to their lower aqueous solubility. Thus, negative effects were observed starting from doses equivalent for an intake of approx. 210 mg, 250 mg, 300 mg, and 300 mg for Fe, Zn, Ca, and Mg,

respectively, given their dissolution in approx. 1 L gastrointestinal fluid, i.e. if taken within one meal or supplement. The doses are therefore physiological only for Ca^{2+} and Mg, with RDAs, for 19-30 y old men) of 1000 mg/d and 400 for adults (Institute of Medicine, 2005), respectively, as doses are difficult to achieve even for the latter two minerals via dietary intake without supplements. Dietary supplements for Mg^{2+} range approximately between 50 and 250 mg, and for Ca^{2+} between 250 and 1000 mg, while only up to approximately 50 mg for Zn^{2+} and 65 mg for iron. It was however unclear whether the observed effects were related to decreased bile salt or fatty acid concentration, due to precipitation and/or complexation, or related to other unknown mechanisms such as micelle destabilization. Furthermore, the influence was tested merely on one test meal (spinach). Conversely, positive effects on trace elements absorption have been reported. One study reported that adding pure β-carotene (200 µg or 400 µg, constituting non-physiological doses) to an *in vitro* digestion model significantly improved the bioaccessibility of Zn^{2+} (16.5 – 118.0%) and iron (19.6 – 102.0%) from food grains - rice (*Oryza sativa*), sorghum (*Sorghum vulgare*), green gram (*Phaseolus aureus*) (whole) and chickpea (*Cicer arietinum*) (whole) (Gautam, Platel, & Srinivasan, 2010). Authors also reported that the addition of β-carotene rich vegetables, carrot and amaranth, also enhanced iron, but not Zn^{2+}, bioaccessibility. Although the authors attributed these results to the presence of β-carotene, it is actually difficult to discern whether other food constituents played a role as well. Some of the interactions between carotenoids and DM may be related to factors other than the digestive process. Work from García-Casal and colleagues, for example, has shown that carotenoids, but not vitamin A, were able to improve iron uptake, in the form of NaFe-EDTA and ferrous fumarate, in a Caco-2 cell model (García-Casal & Leets, 2014; García-Casal, Leets, & Layrisse, 2000). Increases in iron absorption were statistically significant ($p < 0.05$) at iron:carotenoid molar ratios: from 1:0.3 to 1:2 iron:β-carotene (García-Casal et al., 2000); at 1:2 and 1:1 for lycopene; from 1:1 to 1:0.13 for lutein, and from 1:1 to 1:0.03 for zeaxanthin (García-Casal & Leets, 2014). However, the exact mechanism behind the effects of carotenoids on iron absorption remained to be elucidated. Though the former studies suggest that carotenoids enhance Fe absorption, it might come at the expense of carotenoid oxidation as reported by Sy et al (2013). The authors, investigating the reactivity of (all-E)-β-carotene

in the presence of heme (0.5–500 mM) and nonheme iron (0.05–25 mM), found that under the acidic conditions (pH = 4) of a simulated gastric digestion, β-carotene (15 μM) was quickly oxidized, while the conversion of Fe^{II} into Fe^{III} was inhibited.

***In Vivo* Studies.** Similar results have also been reported *in vivo* by the same authors. García-Casal and colleagues (2006), tested the effect of adding lycopene (3.6 mg), lutein (1.8 mg) and zeaxanthin (1.8 mg) to a wheat- or corn-based radioactive labelled meal, and iron bioavailability was assessed by measuring the incorporation of radioactive iron into the bloodstream 15 d after the test meals intake. Authors found that iron absorption significantly increased from 8.1% to 22.5%, 15.8%, and 16.5% when 3.6 mg of lycopene, 1.8 mg of lutein, or 1.8 mg of zeaxanthin, respectively, were added to a wheat-based breakfast. Similarly, the same amounts of carotenoids added to a corn-based breakfast led to a significant increase of iron bioavailability from 5.4% to 18.0%, 12.9%, and 11.1%, respectively. Curiously, the investigated carotenoids were also capable of overcoming the coffee-mediated inhibition of iron absorption, which was additionally tested in the human study (García-Casal, 2006). However, it is hard to discriminate whether carotenoids *per se* had an effect on iron absorption, or if the trial results were somehow influenced by the supplement formulation. Thus, despite the *in vitro* an *in vivo* results, the mechanism behind the effect of carotenoids on iron absorption it is yet to be elucidated.

Other studies have reported that iron supplementation is able to improve vitamin A status, while Zn^{2+} supplementation appears to improve carotenoid bioavailability. Kana-Sop et al. (2015) has looked at the influence of iron (20 mg of iron fumarate) and Zn^{2+} (20 mg of zinc sulphate) supplementation (11 days) on the bioavailability of retinol, and provitamin A carotenoid (β-carotene and β-cryptoxanthin) from papaya. Participants were assigned to 1 of the 3 testing groups - 1) iron alone, 2) Zn^{2+} alone, or 3) Zn^{2+}+iron – and followed a free vitamin A and provitamin A carotenoid free diet, from day 6 to day 11 of the trial period. On day 11, participants were given a papaya based test meal for breakfast, and the postprandial concentration of β- and α-carotene, β-cryptoxanthin and retinol on the plasma chylomicron fraction was determined over a period of 7 hours. While postprandial concentration of retinol was significantly higher in the groups taking iron or iron+ Zn^{2+}, compared to Zn^{2+} alone, the

postprandial provitamin A carotenoid concentration in chylomicrons was higher in the groups taking either Zn^{2+} or Zn^{2+}+iron, compared to iron alone. Given these results, the authors proposed that iron supplementation promoted the conversion of the provitamin A carotenoids into retinol, hence the former lower chylomicron appearance when compared to the Zn^{2+} supplement group. However, the study design did not include a placebo group, so it is difficult to evaluate the real influence of Zn^{2+} and iron status/supplementation on the bioavailability of carotenoids and retinol metabolism. It is possible that subjects suffered in part from iron deficiency anemia, characterized by chronic inflammation (Rubin, Ross, Stephensen, Bohn, & Tanumihardjo, 2017), which is known to reduce retinol transport (and thus showing an apparent reduced conversion into retinol).

In another human trial with pregnant women receiving iron (30 mg) and folic acid (0.4 mg), participants were additionally supplemented daily with Zn^{2+} alone (30 mg), β-carotene (4.5 mg) alone, or Zn^{2+}+β-carotene, for their entire pregnancy period. Micronutrient status was assessed 1 and 6 months postpartum. Six months after partum, women that were supplement with Zn^{2+}+ β-carotene showed a significantly higher plasma concentrations of β-carotene compared to the control group (i.e. women taking iron and folic acid only), while no significance was seen when women were supplemented with either Zn^{2+} or β-carotene alone (Dijkhuizen, Wieringa, West, & Muhilal, 2004). While it is not clear why Zn^{2+} supplementation together with β-carotene improved the plasma concentration of the latter, authors suggest that Zn^{2+} could have enhanced β-carotene uptake from the intestinal lumen by, for instance, affecting the excretion of pancreatic enzymes or the composition of bile acids. Studies have also reported that supplementation with Zn^{2+} significantly improved plasma concentration of vitamin A in the participants (Dijkhuizen et al., 2004; Muñoz, Rosado, López, Furr, & Allen, 2000), which could be due to an effect of Zn^{2+} on an increased synthesis of RBP in the liver with a consequent higher production of vitamin A. Indeed, a lack of Zn^{2+} is related to general effects on the human metabolism, slowing down many essential processes (Hambidge, 2000), resulting e.g. in impaired physical growth (Hambidge, 2000), and altered vitamin A metabolism (Smith, 1980). Despite these existing studies, indicating a role for vitamin A on iron status and vice-versa, it has been pointed out by Michelazzo *et al* (2013) that many of the trial designs looking at these interactions, are not without certain flaws

including the following: the absence of a placebo group; lack of randomization; no assessment of the baseline status of either iron or vitamin A; and the lack of assessment of additional markers of anaemia and inflammation in iron and/or vitamin A deficient populations (Michelazzo et al., 2013).

1.9 Goal and objectives of the BIOCAR project

Despite the current knowledge on the interactions between DM and carotenoids, many gaps still exist, particularly concerning more mechanistic aspects related to the action of DM and trace elements during GI digestion on carotenoids, and also their practical relevance regarding concentration-bioaccessibility effects. Prominent questions include:

- Which minerals and trace elements hamper carotenoid bioaccessibility and at what concentrations?
- What are the effects of increasing mineral concentrations on mixed micelle formation, their structure and size?
- How do effects translate into carotenoid micellarization?
- How does it affect micelle and carotenoid uptake?
- Are the effects equal for all species of carotenoids?
- How strong is the effect of various test meals and altered concentrations of bile and digestive enzymes?
- Can the hypothesis be validated *in vivo*?

These are some of the questions that remain to be answered, and where we perceive a gap of information. The goal of the BIOCAR project is thus to investigate the effect of the DM Ca^{2+}, Mg^{2+}, and Zn^{2+} on aspects of bioaccessibility and bioavailability of pure and diet originating carotenoids.

To achieve this goal, the project is divided into *in vitro* and *in vivo* experimental components. During the *in vitro* work we aim to investigate how varying concentrations of Ca^{2+}, Mg^{2+} and Zn^{2+} affect the bioaccessibility of, on one hand, pure carotenoids and, on the other hand, of carotenoids from different

food matrices. To do so we investigate aspects such as: the percentage of carotenoids recovered from the aqueous micellar fraction, following *in vitro* GI digestion; physicochemical properties of the digesta, specifically viscosity and surface tension; and finally the micelle size and the zeta potential of the particles in solution. In order to validate, or not, the results obtained *in vitro*, a randomized double-blind and placebo-controlled human trial is finally carried out to test the of effect of Ca^{2+} supplementation on the bioavailability of carotenoids from a spinach test meal.

Results from this study could be of relevance for several stakeholders, such as consumers of supplements, for subjects having very low intake or status of dietary vitamin A and carotenoids, such as those in some developing countries, and for the food supplement/pharmaceutical industry. Additionally, people suffering from chronic GI disorders such as pancreatitis and inflammatory bowel syndrome could also benefit from the results of this project, considering the potential of DM to increase bile and fatty acid excretion, possibly worsening the aforementioned conditions. This would discourage the combination of high amounts of minerals and carotenoids within the same formulation (e.g. a caplet), and would suggest not taking simultaneously high amounts of minerals and carotenoids in a meal or within supplements. This could have ramifications for the industry to reformulate dietary supplements, as many of these are sold as complex mixtures, and change the view on the biological activity of combination supplements, adding perhaps another hesitation toward high dosing of supplement ingredients. It can even be assumed that the negative effects of formation of insoluble Ca^{2+} soaps with FFA can be extendable to other lipophilic nutrients. From another viewpoint, the question approached by the BIOCAR project represents a novel topic, and the results of this work would be of value to current scientific knowledge.

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2. SELECTIVE FACTORS GOVERNING *IN VITRO* β-CAROTENE BIOACCESSIBILITY: NEGATIVE INFLUENCE OF LOW FILTRATION CUTOFFS AND ALTERATIONS BY EMULSIFIERS AND FOOD MATRICES

Preamble

The following chapter has been published in the journal of “Nutrition Research”.

The objective of the experimental work here described was to optimize/define conditions for an *in vitro* digestion protocol to investigate aspects of the bioaccessibility of pure carotenoids

All the experimental work and analysis was carried out at the current Luxembourg Institute of Science and Technology (LIST), past *Centre de Recherche Public Gabriel Lippmann*. Experiments and analysis were performed by myself, and with the help of the group’s laboratory technician, Boris Untereiner.

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Selective factors governing *in vitro* β-carotene bioaccessibility: negative influence of low filtration cutoffs and alterations by emulsifiers and food matrices

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2.1 Abstract

Because of their putative health benefits, the biological fate of carotenoids after digestion has been met with much interest, and ex vivo methods using carotenoid standards to study their digestion and further metabolism have been developed. In the absence of a complex food matrix, that is, when studying isolated carotenoids, protocol conditions of GI digestion models have to be adjusted. In this investigation, we hypothesized that certain selected factors would significantly influence the bioaccessibility of β-carotene *in vitro*. The factors considered included (i) type of lipid matrix employed (milk, cream, or oil), (ii) presence/absence of emulsifiers (e.g. lecithin and taurocholate), (iii) addition of a gastric lipase, and (iv) final filtration (20 or 200 nm) of the digesta. Adding an emulsifier mixture (10 mg lecithin + 50 mg monoolein + 5 mg oleic acid) enhanced β-carotene bioaccessibility 3 times ($P < 0.001$), whereas additional taurocholate and the presence/absence of gastric lipase added before intestinal digestion had no significant effect. β-carotene bioaccessibility was superior with oil than with milk (18.8% ± 0.7% and 6.1% ± 0.7%, respectively; $P = 0.03$), especially after filtration, thus suggesting incomplete micelle formation after addition of milk. Filtration through 20 nm filters reduced carotenoid concentration in the aqueous fraction (from 7.1% ± 0.2% to 5.5% ± 0.2% in samples digested with canola oil, $P < 0.001$), indicating that not all formed micelles compared in size with those normally formed *in vivo*. When studying carotenoid standards during *in vitro* digestion, care should be taken to separate mixed micelles by filtration, and the choice of emulsifier and matrix should be considered.

Keywords: Transfer of carotenoids to mixed micelles; Dietary lipids; *In vitro* digestion; Micellarization; Filtration; Gastric lipase; Emulsifying agents.

2.2 Introduction

Carotenoids are lipophilic organic pigments widespread in nature, produced by photosynthetic (plants and algae) and nonphotosynthetic organisms (bacteria and fungi) (Stange & Flores, 2010). These compounds exhibit antioxidant properties, and some constitute precursors of vitamin A active retinoids, which make them relevant from a dietary and health perspective (Britton et al., 2009). In fact, fruits and vegetables rich in provitamin A carotenoids are often investigated as nutritional agents to fight vitamin A deficiency, especially in underdeveloped countries. In addition, the intake of carotenoids and its concentration in the human body and tissues has been associated with the prevention of chronic diseases such as cardiovascular and eye diseases, type 2 diabetes, and certain types of cancer (Britton et al., 2009).

Yet, bioavailability of carotenoids is relatively low (3%-34%) (Hof & West, 2000) and varying according to the dietary source and cooking procedures (Hof & West, 2000; Stahl et al., 2002). For example, concentration of β-carotene in the human blood is around 0.3 and 1.4 nmol/mL for total carotenoids (Stahl et al., 2002). To be available for absorption and to be bioactive, carotenoids must first be released from the food matrix (Bohn, 2008). During gastric digestion, under the action of hydrochloric acid and enzymes, carotenoids are partially released from the food matrix and incorporated in emulsified oil droplets. In the small intestine, components of the food matrix and lipid droplets are further degraded by pancreatic juices. While bile salts emulsify dietary lipid droplets, the pancreatic lipases further break them down, and the hydrolysed triglycerides, phospholipids, and other lipophilic compounds are partitioned into mixed micelles (Failla & Chitchumronchokchai, 2005; Parker, 1996) with a spherical structure and a diameter of ca. 8 nm (Parker, 1996). The amount of carotenoids that is released from the food matrix and effectively incorporated into mixed micelles, that is, their availability for uptake, is commonly referred to as bioaccessibility. A variety of factors can act on these steps, increasing or decreasing carotenoid bioaccessibility, as for example, the type and amount of dietary fat. It is also known that the efficient emulsification and absorption of carotenoids require a certain amount of fat (5-10 g) to be present in a meal (Failla, Huo, & Thakkar, 2008) and that different

lipid containing matrices translate into different bioaccessibility (Biehler, Kaulmann, et al., 2011; Borel, Tyssandier, & Mekki, 1998).

In vitro models have been used to study the regulation of processes associated with digestion and absorption of carotenoids (Failla, Chitchumroonchokchai, & Ishida, 2008), allowing the study of their bioaccessibility in a relatively inexpensive and technically reproducible but simple way (Failla, Huo, et al., 2008). Thus, these models offer the possibility of easily manipulating biochemical conditions, such as pH, amount and type of dietary fat, bile salts, and enzyme concentrations. This provides useful information on the relative bioaccessibility of carotenoids from different food sources and the effects of different food processing techniques and dietary factors (Failla, Huo, et al., 2008).

Two common and often combined models used to study carotenoid bioavailability *in vitro* are (1) simulated gastric and intestinal digestion followed by (2) Caco-2 cell–based models to investigate carotenoid uptake or transport (Bohn, 2008). One of the challenges when using *in vitro* models is adjusting conditions to those that are physiologically relevant, as the variability of enzymes and bile concentrations and optimal pH in humans is high (Ulleberg et al., 2011). These parameters have been the subject of many debates (Dupont et al., 2011), and models existing up to this day are numerous (Hur, Lim, Decker, & McClements, 2011). Conditions may vary in terms of bile acid and enzyme concentrations, time and pH of the gastric and intestinal phase (Biehler & Bohn, 2010), usage of porcine bile acid extract, or a mix of pure bile acids (Larsson, Cavonius, Alminger, & Undeland, 2012), etc. Some parameters are also governed by practical limitations. For example, although lipid digestion occurs mainly in the duodenum, approximately 10% to 30% of lipolysis takes place in the stomach under the action of a gastric lipase (Huo, Ferruzzi, Schwartz, & Failla, 2007). However, *in vitro* GI models do not usually include gastric lipolysis as human gastric lipases are not commercially available. One possible replacement is the fungal lipase from Rhizopus oryzae (Larsson et al., 2012), but to the best of our knowledge, gastric lipase has never been used in *in vitro* models to study the bioaccessibility of carotenoids. An additional challenge to research of carotenoids embedded in food matrices, such as fruits or vegetables, exists when working with pure carotenoids, which show very low solubility when added in powder form to a dietary matrix, for example, oil, as they have the

tendency to form aggregates. Nevertheless, the addition of pure carotenoids further simplifies the experimental system, allowing the studying of factors that influence the bioaccessibility or bioactivity of individual carotenoid species.

With the aim of setting up an *in vitro* GI digestion model to study the bioaccessibility of pure carotenoids, we investigated various protocol conditions that may influence the transfer of β-carotene into mixed micelles. We focused on different dietary lipid sources (canola oil, cream, and milk), variations in the emulsifying agents (e.g. lecithin, sodium taurocholate), presence of a gastric lipase, and different filtration cutoffs. An improved understanding of using *in vitro* models is important for building a hypothesis for the *in vivo* behaviour and, thus, for human nutrition. Therefore, our hypotheses were that (i) not all lipid sources would yield similar bioaccessibility, (ii) adding gastric lipase may enhance β-carotene bioaccessibility via improving lipid digestion, (iii) the addition of emulsifying agents would enhance β-carotene micellarization, (iv) introducing a filtration cutoff of 20 nm would allow isolating true mixed micelles and prevent overestimating β-carotene bioaccessibility by including carotenoids entrapped in lipid droplets above 20 nm, and (v) filtration of digesta at a 20 nm cutoff before incubation in a Caco-2 cellular model would reduce the toxicity of digesta by removing potentially toxic biliary compounds.

2.3 Materials and methods

2.3.1 Chemicals, minerals and carotenoid standards

Digestive enzymes, that is, pepsin (porcine, ≥ 250 U/mg solid where 1 U produces a change in A280 of 0.001 per minute at pH 2 at 37°C, measured as TCA (trichloracetic acid)-soluble products using hemoglobin as substrate) and pancreatin (porcine), porcine bile extract, fungal lipase from *Rhizopus oryzae* (47 U/mg where 1 U corresponds to the amount of enzyme, which releases 1 µmol fatty acid from triglycerides per minute at pH 7.2 and 37°C), sodium taurocholate, oleic acid (cis-9-octadecenoic acid), lecithin from egg yolk (L-α-phosphatidylcholine, ~60%), and monoolein (1-oleoyl-rac-glycerol) were purchased from Sigma-Aldrich (Bornem, Belgium). Dulbecco's modified Eagle's medium (DMEM + GlutaMAX), phosphate-buffered saline, and heat-inactivated fetal bovine serum were

obtained from GIBCO (N.V. Invitrogen SA, Merelbeke, Belgium). Penicillin/streptopmycin mixture and nonessential amino acids for cell cultures were purchased from Sigma-Aldrich. Hexane, hydrochloric acid, and sodium chloride were acquired from VWR (Leuven, Belgium), and acetone and sodium carbonate, from Merck (Darmstadt, Germany). β-Carotene standard in powder form was purchased from Sigma-Aldrich (purity >95%). Unless otherwise specified, all products were of analytical grade or higher. Used throughout the study, 18 MΩ water was prepared with a purification system from Millipore (Brussels, Belgium).

2.3.2 Dietary lipid sources

Milk (4% fat), cream (18% fat), and canola oil were purchased at local supermarkets in Luxembourg (CACTUS S.A., Esch-sur-Alzette, and DELHAIZE Belval, Esch-sur-Alzette) in the summer of 2013. The same batch of products was used throughout the study, and the material was stored at 4°C. Canola oil has been used previously to aid in the micellarization of carotenoids (Biehler et al., 2012; Failla, Huo, et al., 2008; Huo et al., 2007) and is low in natural occurring carotenoids (Biehler et al., 2012). Milk has likewise been used previously to facilitate β-carotene micellarization (Biehler, Kaulmann, et al., 2011; Granado-Lorencio, Herrero-Barbudo, Olmedilla-Alonso, Blanco-Navarro, & Pérez-Sacristán, 2010; Kohut et al., 2007).

2.3.3 Solubilisation of β-carotene

A standard solution of β-carotene (0.5 mg/mL) was prepared gravimetrically by dissolving β-carotene in chloroform. Concentration of the standard solution was further determined spectrophotometrically at 461 nm (wavelength for maximum absorbance of β-carotene in chloroform), using published extinction coefficients (Britton et al., 2004). Aliquots of the standard solution, equivalent to 30 µg of β-carotene, were pipetted into an amber glass vial, dried under a stream of nitrogen, and stored under argon at −80°C until usage. To promote the resolubilisation of β-carotene and successive formation of mixed micelles during GI digestion, 3 emulsifying agents (monoolein, lecithin, and oleic acid) were added (referred to as emulsifier mix) to the previously dried β-carotene. For this purpose, 5 mg of oleic acid, 50 mg of monoolein, and 10 mg of lecithin were weighed into the amber vial, followed by

the addition of 150 µL of dietary lipid source (milk, cream, or canola oil), and all constituents were sonicated for 10 minutes (Elmasonic Ultrasonic Bath 37 KHz).

2.3.4 Simulation of GI digestion and factors investigated

The *in vitro* digestion protocol was adapted from previous studies (Biehler, Kaulmann, et al., 2011; Garrett et al., 1999) and is described below. The various factors expected to influence carotenoid bioaccessibility investigated are summarized in Part 2, Table 1 and included varying food matrices (milk, cream, or canola oil), presence or absence of emulsifiers (oleic acid, monoolein, lecithin), use of gastric lipase (with, without), the impact of digesta filtration (no filtration, 200 nm, and 20 nm), additional sodium taurocholate before gastric digestion (with or without), and testing the influence of filtration (none, 200 nm, and 20 nm) and dilution of digesta (2, 3, and 4 times) on Caco-2 cell viability as an example of a frequently used cell culture combined with GI digestion.

Part 2, Table 1 - Overview of the effects and conditions varied for studying aspects of β-carotene bioaccessibility in an *in vitro* system.

Effect Studied	Conditions
Dietary lipid source[a] at onset of digestion	▪ Milk, 4% fat (150 µL per digestion) ▪ Cream, 18% fat (150 µL per digestion) ▪ Canola oil (150 µL per digestion)
Presence of emulsifiers[a] at onset of digestion	▪ Without emulsifiers ▪ Lecithin (10 mg) ▪ Emulsifier mix (10 mg lecithin + 50 mg monoolein + 5 mg oleic acid)
Addition of sodium taurocholate at onset of digestion (5 mM)	▪ With addition ▪ Without addition
Addition of fungal lipase to gastric digesta (340 U/mL)	▪ With fungal lipase ▪ Without fungal lipase
Filtration of final derived digesta[a] before extraction	▪ Filtration with 200 nm cutoff filters[b] ▪ Filtration with 20 nm cutoff filters[c] ▪ No filtration of digesta
Effect of dilution of final digesta[a] on cell viability (for cellular uptake studies)[d]	▪ 2× dilution ▪ 3× dilution ▪ 4× dilution
Effect of fitration of final digesta on cell viability (for cellular uptake studies)[d]	▪ Standard 200 nm filtration cutoff ▪ Additional filtration at 20 nm cutoff

Digesta carried out with 30 or 60 µg of β-carotene and dietary lipid source plus mixture of enzymes and bile salts, as described in Methods and materials, brought ot a final volume of 25 mL at small intestinal digestion.

[a] The studied effect was found to be significant at $P < 0.05$ (2 sided)

[b] Filtration with 200 nm Acrodisc Nylon membrane filter

[c] Filtration with a 20 nm Anotop syringe filter

[d] Digestas were diluted in cell media DMEM + GlutaMax

Gastric phase

To simulate the gastric passage, solubilized β-carotene (30 or 60 µg in 150 µL of milk, cream, or canola oil and the emulsifier mix) was added to 7.5 mL of physiological saline, containing or not sodium taurocholate (5 mM), in a 50 mL falcon tube. The sample was sonicated for 20 minutes in an ultrasonic bath and then acidified to pH of 3 with 1 mL of porcine pepsin (40 mg/mL) solution prepared in 0.01 M HCl. The sample was then incubated for 1 hour at 37°C in a shaking water bath (GFL 1083 from VEL Leuven, Belgium) at 100 rpm. The effect of adding a fungal lipase was tested to simulate the action of gastric lipolysis. The solubilized β-carotene was added to 6.5 mL of physiological saline and sonicated for 20 minutes. Samples were acidified to pH of 3 with 1 mL of porcine pepsin (40 mg/mL) solution prepared in 0.01 M HCl and incubated for 30 minutes at 37°C in a shaking water bath. After the 30 minutes of incubation, the pH of the samples was slowly adjusted to 5 with $NaHCO_3$ (0.1 M), and 1 mL of a fungal lipase solution (340 U/mL) that was prepared in physiological saline was added. Samples were then further incubated for an additional 30 minutes at 37°C in a shaking water bath.

Intestinal phase

Porcine pancreatin (4 mg/mL) and porcine bile extract (24 mg/mL) solutions were prepared freshly in $NaHCO_3$ (0.1 M or 0.01 M when fungal lipase was added), and 4.5 mL of the mixture was added to the simulated gastric fluid at the end of the incubation period of the gastric phase. The addition of 4.5 mL of pancreatin and bile solution brought up the pH to 7. The final volume of the samples was adjusted to 25 mL with physiological saline. The incubation time of the intestinal phase was set to 2 hours in a shaking water bath (100 rpm) at 37°C.

2.3.5 Extraction of carotenoids from digesta

Aliquots of 12 mL of digesta were transferred to Beckman Ultra Clear tubes and ultracentrifuged (Beckman Optima C-90U Ultracentrifuge; Beckman Coulter, Palo Alto, CA) in a SW 40-Ti rotor at 164 000g for 35 minutes at 4°C. After ultracentrifugation, 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. A second aliquot of 4 mL was collected from the aqueous phase and filtered first through the same 200 nm Nylon membrane filter. This was followed by a second filtration procedure to eliminate particles with a size above 20 nm (Anotop 20 nm syringe filters; Whatman GmbH, Dassel, Germany).The following extraction procedure was adapted from Biehler et al (Biehler et al., 2012). In short, 2 mL aliquots of the filtered aqueous micellar phases were mixed with 4 mL of hexane/acetone (1:1, v/v), shaken for 1 minute and centrifuged for 2 minutes at 4000g at 4°C. The upper hexane phase was collected into a second 15-mL tube. The extraction procedure was repeated with 4 mL of hexane, followed by a second centrifugation at 4000g. The hexane phases were combined and dried under a stream of nitrogen using a TurboVapLV (Biotage, Eke, Belgium) apparatus. The residue was redissolved in 1 mL of hexane and filtered through a 0.45 µm Acrodisc CR 4 mm syringe filter before spectrophotometric analysis.

2.3.6 Spectrophotometric analysis of β-carotene

Because of the presence of a single carotene (β-carotene) in the aqueous phase, its concentration was determined by spectrophotometry. Maximum absorbance of β-carotene was measured at 450 nm, molar extinction coefficient in hexane was taken from Britton et al. (2004), and then the concentration of β-carotene was calculated by applying the Beer-Lambert formula and taking into account dilution steps, as previously published (Biehler, Mayer, Hoffmann, Krause, & Bohn, 2010). The percentage of β-carotene micellarization was used as the measure for bioaccessibility and is expressed as the percentage of solubilized β-carotene present in the aqueous phase of digesta after *in vitro* GI digestion, compared with the initial amount added to the sample. To minimize potential day-to-day variations between the analyses, normalized bioaccessibility was expressed as the percentage of micellarized β-carotene present in the aqueous phase of the digesta, compared with the respective control condition:

$$Normalized\ bioacessibility = \frac{Micellarization\ of\ sample}{Micellarization\ of\ control\ conditions} \times 100$$

2.3.7 Cell culture and cell viability assay

We hypothesized that particles originating from the GI digestion, such as those rich in enzymes, could be responsible for cellular toxicity effects, as reported in previous studies (Fang et al., 2004; Wachs et al., 2005). Thus, it was tested whether the filtration of the digesta with 20-nm cutoff filters would help reduce the cellular toxicity of the digesta.

The TC-7 subclone (ATCC no. HTB-37) of the Caco-2 parental cell line was originally cloned from a tumor isolated by Jorgen Fogh (Chantret et al., 1994) and was a generous gift from Monique Rousset (Nancy University, France). Cells were routinely maintained in 75 cm^2 plastic flasks (Nunclon, Nunc, Denmark) and grown in DMEM + GlutaMAX supplemented with 1% nonessential amino acids, 20% fetal bovine serum, and 1% penicillin (10 000 U)/streptomycin (10 mg/mL) mixture. Cells were subcultured weekly after reaching 70% to 80% confluence, and the medium was replenished 3 times per week. All flasks and cultured plates were kept in a moist-saturated environment (37°C, 10% CO_2, 90% air) in a CB-210 CO_2 incubator (Binder GmbH, Tuttlingen, Germany).

The resazurine reduction assay was used to test the viability of Caco-2 cells after exposure to simulated digesta. For viability studies, cells were cultured in 6-well plates at densities of 5 × 104 cells/cm^2 and grown in cell media DMEM + GlutaMAX, which was replenished every other day. After 2 weeks of differentiation, Caco-2 cells were exposed for 4 hours to ultracentrifuged digesta obtained as described above and diluted with media DMEM + GlutaMAX by various factors (2, 3, and 4 times), with (20 nm) and without filtration. Control cells were incubated with media only. The resazurine reduction assay itself was performed as described earlier (Kaulmann, Serchi, Renaut, Hoffmann, & Bohn, 2012). Cell viability was expressed as mean cell viability (in percentages) compared with control cells (set at 100%).

2.3.8 Particle size distribution analysis

To study the impact of digestion and filtration on the particle size distribution of the digesta, we performed a nanoparticle tracking analysis (NTA) of the aqueous phase of the ultracentrifuged digesta using the NanoSight NS500 (Malvern, Amesbury, UK). The detection range of particle size was from 10 to 2000 nm. For terms of comparison and to investigate the contribution of enzymes and bile to the particle distribution of the digesta, blank samples were also analysed. The latter was constituted only of digestive fluids, that is, without lipid sources or β-carotene.

2.3.9 Statistical analysis

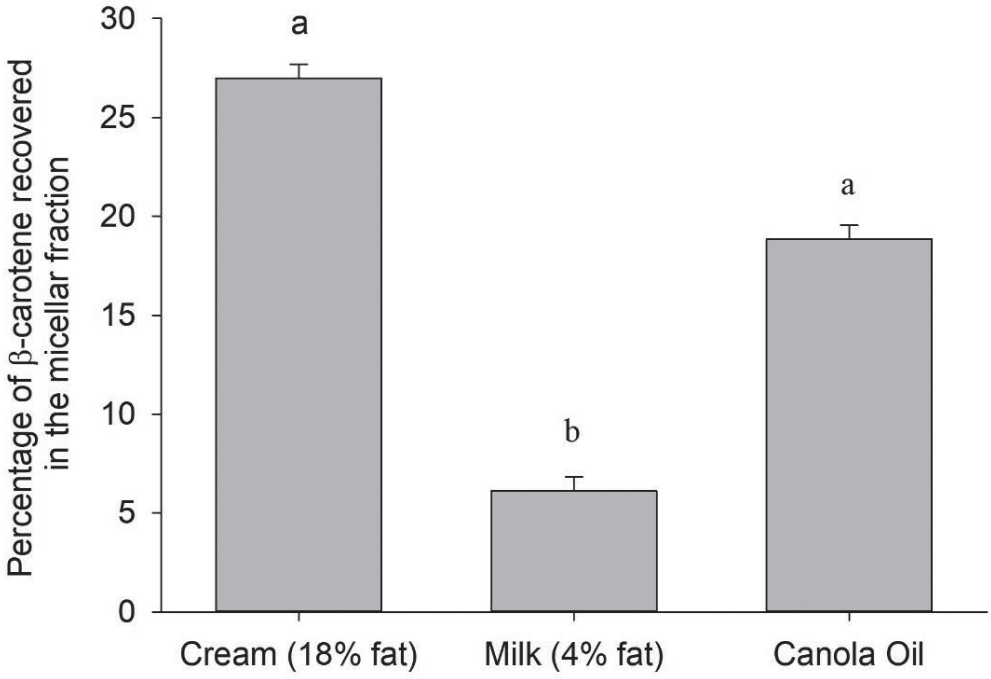
Unless stated otherwise, the statistical analyses were performed in SPSS (IBM, Inc, Chicago, IL) vs 19.0, and all values given in the text are expressed as mean ± SD. All fractional β-carotene micellarization results and cellular viability data were first normalized to a control condition (set to 100% as explained above) to attenuate the variability between independent replicates. The normality of the data distribution was verified by quantile-quantile plots, and the equality of variances was verified by box plots. Data of fractional β-carotene micellarization were log transformed to achieve a normal distribution. A linear mixed model was used to investigative the effects of filtration, emulsifying agents, sodium taurocholate addition, and dietary lipid source on fractional β-carotene micellarization Log-transformed, normalized fractional β-carotene micellarization was taken as the dependent variable and all other investigated factors were selected as fixed factors. $P < 0.05$ (2 sided) was considered significant.

To investigate the effect of filtration and dilution of the digesta (fixed effects) on the cellular viability, a linear mixed model was applied, taking the normalized cellular viability as a dependent variable. A $P < 0.05$ (2 sided) was considered significant. The effect of the addition of fungal lipase on the fractional micellarization of β-carotene was analyzed with a Student unpaired t test, and analysis of the data was performed on SigmaPlot 12.5 (Systat Software, Inc, Erkrath, Germany).

2.4 Results

2.4.1 Effect of different dietary lipid sources on carotenoid bioaccessibility

Samples digested with canola oil, taken as control measurements for statistical comparison, showed an average micellarization (percentages) of 14.3%. Bioaccessibility of β-carotene did not differ significantly between samples digested with canola oil and cream (18% fat), whereas milk resulted in a significantly lower normalized bioaccessibility of β-carotene when compared with canola oil and cream (Part 2, Figure 1). Thus, milk was not considered for the following experiments, and canola oil and cream were used instead



Part 2, Figure 1 - Effect of lipid source on micellarization of β-carotene. The effect of 2 dairy lipid sources (milk 4% fat and cream 18% fat) and 1 vegetal source (canola oil) was tested for their impact on the bioaccessibility of pure β-carotene, determined as the percentage of β-carotene recovered in the aqueous micellar fraction of the ultracentrifuged digesta after *in vitro* GI digestion. Values represent means ± SD of n = 6 replicates. Different superscripts indicate statistical significant differences (P < 0.05).

2.4.2 Impact of the addition of emulsifier, sodium taurocholate, and fungal lipase on the bioaccessibility of β-carotene

The presence of emulsifiers had a clear positive impact on the bioaccessibility of β-carotene ($P < 0.001$; Part 2, Table 2). Following all group-wise comparisons, all 3 conditions studied for this effect were also significantly different among each other ($P < 0.001$). Samples digested with the emulsifier mix showed highest values of β-carotene micellarization, followed by samples where only lecithin was added, and finally samples without the addition of emulsifiers (Part 2, Table 2). The presence of sodium taurocholate before gastric digestion, to promote solubilisation of β-carotene into the emulsifier mix and lipid matrix did not significantly enhance micellarization compared with samples where no sodium taurocholate was added (Part 2, Table 2). Taking into consideration that the presence of sodium taurocholate before the intestinal digestion is not found under physiological conditions and in light of these results, it was decided to exclude the addition of sodium taurocholate from the succeeding protocols.

Furthermore, we have tested herein the effect of the addition of a fungal lipase during gastric digestion on β-carotene micellarization. However, this addition had no significant ($P > 0.05$) impact on the percentage of β-carotene micellarized at the end of *in vitro* digestion (data not shown).

Part 2, Table 2 - Effect of the addition of emulsifiers on the bioaccessibility of pure β-carotene.

Lipid Source	Without emulsifier	Lecithin	Emulsifier Mix*
Canola Oil	4.6 ± 0.2^{a}	9.1 ± 0.2^{b}	13.7 ± 0.2^{c}
Cream (18% fat)	15.9 ± 0.4^{a}	NI	30.1 ± 0.2^{b}

Pure β-carotene was solubilized in the lipid source with or without the addition of emulsifiers (lecithin or emulsifier mix), and the solubilized carotenoid was then subjected to simulated digestion. Values represent the percentage of β-carotene recovered in the micellar fraction of the digesta, at the end of the *in vitro* digestion and are expressed as means ± SD (n = 20 replicates for canola oil, n =18 replicates for cream). Abbreviation: NI, not investigated. Superscripts indicate statistical significance, and values in the same row not sharing the same letters differ significantly from each other ($P < 0.001$).

* Emulsifier mix was composed of 10 mg lecithin + 50 mg monoolein + 5 mg oleic acid

2.4.3 Effect of sequential filtration on β-carotene bioaccessibility

Nanoparticle tracking analysis revealed the distribution of particle sizes and concentration in the aqueous phase of the digesta, following ultracentrifugation (Part 2, Table 3). Using ultracentrifugation, it was possible to exclude particles with sizes above ca. 700 nm. Further filtration of samples, at 200 nm cutoffs, efficiently removed particles with dimensions above that cutoff. However, because of technological limitations, it was not possible to confirm the presence of particles within the range of physiological mixed micelles (5-11 nm of diameter) with this instrument. To verify to which extent, if any, the digestive fluids themselves contributed to the particle distribution in our samples, we performed NTA on blank samples containing only physiological saline, the enzymes, and bile extract solutions. Surprisingly, the digestive fluids appeared to contribute to much of the particle population with sizes between 30 and 130 nm (Part 2, Table 3).

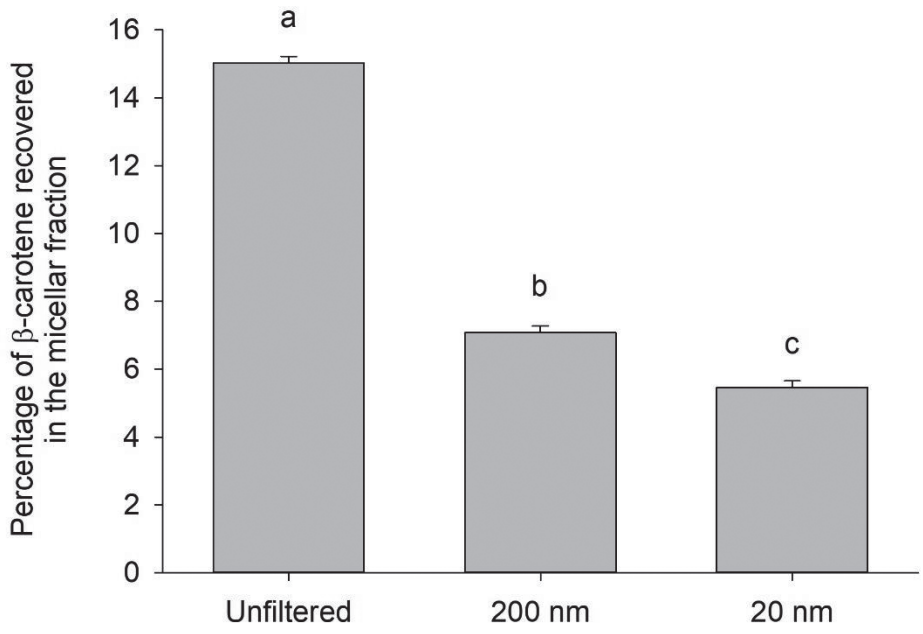
Part 2, Table 3 - Nanoparticle tracking analysis of the aqueous phase of ultracentrifuged digesta after GI digestion with different dietary lipid sources.

Sample		Mean particle diameter (nm)[c]	D10[d]	D50[d]	D90[d]	Concentration (10^8 particles/mL)
Milk 4% fat	Filtered[a]	108 ± 51	45	104	169	155
	Unfiltered	175 ± 106	66	145	343	169
Cream 18% fat	Filtered[a]	104 ± 51	57	91	158	2910
	Unfiltered	113 ± 58	52	98	202	1890
Canola Oil	Filtered[a]	110 ± 45	55	104	170	10 000
	Unfiltered	162 ± 104	68	133	361	11 840
Blank[b]	Unfiltered	91 ± 62	47	76	132	2380

[a] Samples were filtered after ultracentrifugation through a 200 nm filter (nylon membrane syringe filter).
[b] Blank sample contained only simulated digestive fluids with digestive enzymes and bile extract.
[c] Values represent means of particle diameter ± SD (n = 3 replicates) in nm.
[d] D10, D50, and D90 represent the diameter (in nm) of the particles that are the 10th, 50th, and 90th percentiles.

Given the results from NTA, a second filtration using a 20 nm filter was introduced in the protocol to exclude larger particles without compromising true, that is, physiological sized mixed micelles. Filtration trials were performed to evaluate how filtration at different cutoffs affected the estimation of β-carotene bioaccessibility. All 3 different cutoffs evaluated (no filtration, filtration at 200 nm, and filtration at 20 nm) differed significantly (Part 2, Figure 2). According to the filtration trial results, we

estimated that approximately 50% of β-carotene present in the aqueous phase but not incorporated into physiological sized mixed micelles was excluded following filtration through 200 nm cutoff filters. The inclusion of a second filtration step of the digesta, that is, performing a sequential filtration first through a 200 nm pore filter followed by filtration through a 20 nm pore filter, led to a further average exclusion of approximately 28% ($P < 0.0047$) of the β-carotene present in the 200 nm filtered fraction.

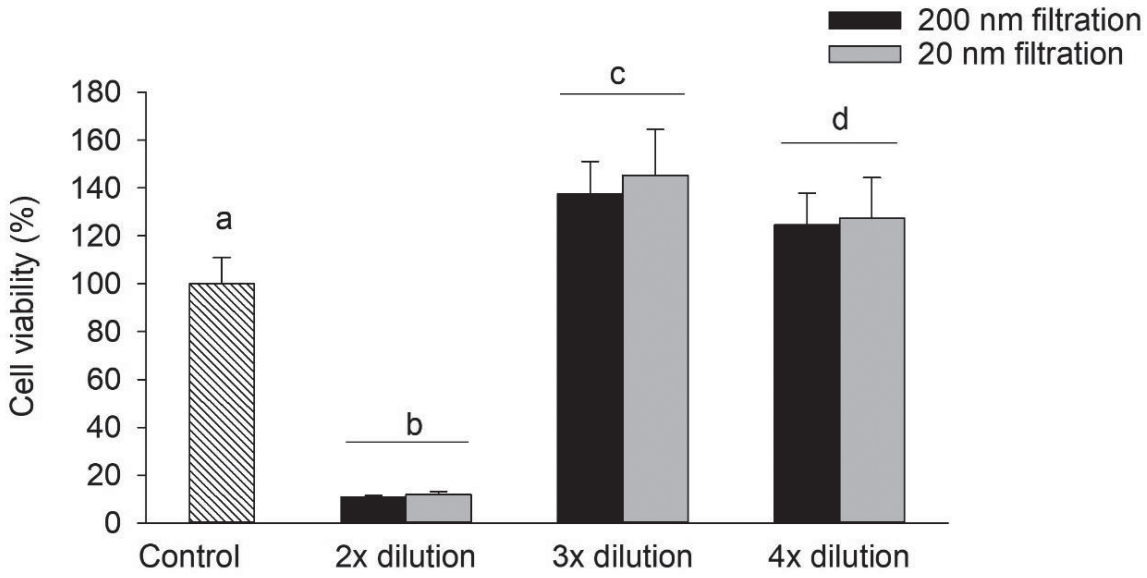


Part 2, Figure 2 - Effect of filtration on β-carotene bioaccessibility. Various filtration cutoffs (no filtration, filtration at 200 nm, and filtration at 20 nm) were tested to estimate the bioaccessibility of pure β-carotene, determined as the percentage of β-carotene recovered in the aqueous micellar fraction of the digesta. After *in vitro* GI digestion, samples were ultracentrifuged, and aliquots of the aqueous phase were taken for filtration trials, and the bioaccessibility of β-carotene was compared across the different filtration cutoffs. Values represent means ± SD of n = 20 replicates. Bars not sharing the same superscript are significantly different ($P < 0.05$).

2.4.4 Toxicity of digesta expressed as cellular viability

No significant differences were found for the cellular viability of Caco-2 cells exposed to filtered vs. unfiltered digesta across dilution factors (Part 2, Figure 3). However, there were significant statistical differences in cellular viability between cells incubated with different dilution factors of digesta (Part 2, Figure 3). A 2-fold dilution factor appears to be still toxic for the cells. When cells were incubated

for 4 hours with the digesta diluted only 2-fold, they showed a complete loss of adherence to the well plates, although diluting the digesta by 3-fold and 4-fold did not appear to negatively affect the integrity of the Caco-2 cells. Results showed significant increases in cellular viability, compared with control conditions (Part 2, Figure 3). However, this result does not possibly indicate improved viability. Higher fluorescence readings may reflect instead a mitochondrial stress caused by the bile salts or other digesta ingredients. In this perspective, a 3-fold dilution of the digesta appeared to be significantly ($P < 0.014$) more “stressful” than a 4-fold dilution.



Part 2, Figure 3 - Effect of filtration of the digesta on the cellular viability (assessed by the resazurine assay) of Caco-2 differentiated cells at different dilutions. Caco-2 cells were incubated with ultracentrifuged digesta for a period of 4 hours. To test the effect of dilution factor and filtration, the digesta were diluted with cell media DMEM + GlutaMAX (2-, 3-, and 4-fold), and for each dilution, samples were either filtered through a 200-nm Acrodisc Nylon membrane syringe filter (standard procedure) or subjected to an additional filtration with a 20 nm Anotop syringe filter. Control cells were incubated with cell media only. Fluorescence readings were normalized to the control condition. Values represent means ± SD from n = 4 (for the 2-fold dilution), n = 7 (for the 3-fold and 4-fold dilutions), and n = 11 (for the control) samples. The filtration of the digesta had no significant effect on the cellular viability. Groups represented by different letters were significantly different ($P < 0.05$).

2.5 Discussion

To study the bioaccessibility of lipophilic compounds *in vitro*, optimization of digestion parameters toward those resembling physiological conditions, including the formation of mixed micelles, is paramount. It has been highlighted that *in vitro* methods to assess aspects of carotenoid bioavailability often do vary in a variety of parameters (Biehler & Bohn, 2010), and up to now, there exists no standardized *in vitro* GI protocol to study aspects of carotenoid bioavailability.

In an attempt to optimize an *in vitro* digestion protocol to study pure β-carotene micellarization and factors that may affect it, we investigated several conditions used in simulated GI digestion protocols that were deemed crucial for the observed results. This included effects that, to our knowledge, have never been reported in protocols used to study carotenoid bioaccessibility, that is, the application of filtration at a 20 nm particle cutoff to ensure isolation of pure mixed micelles, and the effect of a fungal lipase at gastric digestion to mimic gastric lipolysis.

One of the many dietary factors affecting carotenoid bioaccessibility is the type of fat present in the meal, that is, triglyceride profile such as the chain length and degree of saturation of fatty acids (Borel et al., 1996; Gleize et al., 2013; Hof & West, 2000). In this study, we compared 3 different dietary lipid sources, 1 vegetal (canola oil) and 2 dairy sources (4% fat milk and 18% fat cream). Although cream (18% fat) and canola oil led to higher micellarization values, 4% fat milk yielded the lowest (6.1% ± 0.7%) bioaccessibility, representing ca. 68% less than with canola oil, and 77% less than with cream. It is possible that the amount of milk added to the samples (150 µL) did not contain enough fat to promote the formation of mixed micelles. In the case of β-carotene, it is shown to be better incorporated into chylomicrons in the presence of long-chain fatty acids (Borel et al., 1996), and shorter fatty acids may compromise incorporation also into mixed micelles before absorption. The degree of saturation of fatty acids has also been shown to affect carotenoid bioaccessibility, and the absorption of β-carotene seems to be favoured in the presence of monounsaturated long-chain fatty acids, such as oleic acid (C18:1), vs. polyunsaturated and unsaturated fatty acids (Goltz et al., 2012; Hollander & Ruble, 1978). This suggests that canola oil, which is richer in long-chain fatty acids and

is made up of ca. 62% of monounsaturated fatty acids (Przybylski, Mag, Eskin, & McDonald, 2005), may be a more suitable matrix compared with dairy sources that contain high proportions of medium-chain fatty acids (Bauer, Jakob, & Mosenthin, 2005).

In addition to dietary lipids, the emulsifiers lecithin and monoolein have been previously used in systems to study factors impacting carotenoid bioaccessibility (Biehler et al., 2012) and cellular uptake (Kaulmann et al., 2012; Xu et al., 1999). Phospholipids, such as lecithin, take part in the formation of mixed micelles, are essential for the solubilisation of lipophilic compounds, and have been used to stabilize biological emulsions containing pure carotenoids (Borel et al., 1996) and to enhance the micellarization of β-carotene and lutein in spinach (Rich, Bailey, et al., 2003). In the present investigation, adding a mixture of emulsifiers (lecithin, monoolein, and oleic acid) before digestion had a clear positive effect on the micellarization of pure β-carotene, which increased by ca. 300% in samples digested with canola oil and by ca. 200% in samples digested with cream.

In some studies, investigating bioaccessibility of purified carotenoids, sodium taurocholate has been used as a surfactant to induce the formation of artificial micelles embedding carotenoids, and to foster emulsification (Biehler et al., 2012; Chitchumroonchokchai, Schwartz, & Failla, 2004; Kaulmann et al., 2012; Sy et al., 2012; Xu et al., 1999). Here, it was tested whether the nonphysiological addition of sodium taurocholate (5 mM) before GI digestion would enhance β-carotene emulsification. It was found that adding sodium taurocholate did not significantly alter β-carotene micellarization, thus suggesting that the action of bile salts and pancreatin during small intestinal digestion was sufficient and more important for the formation of mixed micelles.

At the end of GI digestion, the fraction of β-carotene that is not transferred into mixed micelles is typically removed along with undigested lipids, as these separate from the aqueous phase during ultracentrifugation. Thus, it is possible that the extent of fat digestion represents a limiting factor for β-carotene bioaccessibility. Although the digestion and breakdown of dietary lipids occur mainly in the duodenum, a fraction of lipids (10%-30%) may undergo lipolysis in the stomach under the action of the human gastric lipase and release fatty acids, monoglycerides, and diglycerides (Bauer et al., 2005; Pafumi et al., 2002), vital for the formation of mixed micelles. Human gastric lipase attacks primarily

the sn-3 position of triacylglycerols in short- and medium-chain fatty acid linkages, rather than long-chain fatty acids, generating FFA and a mixture of diacylglycerols and monoacylglycerols (Bauer et al., 2005). A possible replacement for the human enzyme is the commercially available lipase from the fungus *R oryzae*, which has a 1,3-regiospecificity mainly in medium- and long-chain fatty acids (Oba & Witholt, 1994; Ray, Nagy, Smith, Bhaggan, & Stapley, 2013). Under the present conditions, the addition of the fungal lipase had no significant impact on the amount of β-carotene recovered at the end of GI digestion. This could be due to differences in the pH range at which the enzymes are stable and the pH optima of the fungal lipase. Although the gastric human lipase is stable at ca. pH 1.5 to 8, lipolysis only occurs at pH 3 to 6.5 (Christophe & DeVriese, 2000). In the case of the free *Rhizopus oryzae* lipase, this enzyme is active between pH 3 and 11; however, its activity decreases substantially at a pH less than 5 and a pH greater than 9, while reaching a maximum activity around pH 8 (Ghamgui, Miled, Karra-chaâbouni, & Gargouri, 2007; Ray et al., 2013). During gastric digestion, samples were incubated for 30 minutes with fungal lipase at pH 5, at which the residual activity of the lipase is around 30% (Ghamgui et al., 2007; Kharrat, Ali, Marzouk, Gargouri, & Karra-Châabouni, 2011). The short time of incubation and the reduced activity of the enzyme at pH 5 might have limited its action on canola oil lipolysis. For the moment, no truly suitable replacement for human gastric lipase is available for research purposes.

The filtration of digested samples with filters of a 200 nm cutoff is a common practice to remove insolubilized carotenoids and to reduce microbial contamination. However, mixed micelles have been reported to possess a diameter of approximately 5 to11 nm (Elsayed & Cevc, 2011; Sy et al., 2012). Thus, the filtration of samples with 200 nm filters could introduce the risk of overestimating the emulsification of β-carotene in true mixed micelles. The filtration of samples at different cutoffs (unfiltered, 200 nm, or 20 nm) showed that with every filtration, there was a significant decrease in estimated β-carotene bioaccessibility. These results indicate an overestimation of β-carotene bioaccessibility of 175% when not using any filter and 30% when using the 200 nm filters. This was in comparison to results with 20 nm filters, where only physiologically resembling mixed micelles or other possible vehicles able to solubilize carotenoids (eg, vesicles, proteins) (Reboul & Borel, 2011)

would pass. To our knowledge, filtration of samples at 20 nm has never been applied in studies of carotenoid bioaccessibility. Nevertheless, it could be recommended as a measure to prevent the possible overestimation of bioaccessibility.

The cellular toxicity of bile salts and acids has been previously reported in studies using liver cell models (Fang et al., 2004; Palmeira & Rolo, 2004; Payne et al., 2005), as they mediated changes in the mitochondrial membrane potential and respiration induced mitochondrial permeability transition, causing membrane damage, and the production of reactive oxygen species (ROS) that cause oxidative stress (Fang et al., 2004; Schulz et al., 2013). In the NTA results, it was seen that digestion of blank samples alone that contain only bile extract and enzymes contributed largely to the particle population in our digesta, with a mean particle size of 90 nm. Filtration of samples at 20 nm was thought to constitute a simple and cost-effective solution to exclude these particles from our digesta. However, filtering digesta at a 20 nm cutoff had no significant effect on reducing its toxicity and on ensuring cellular viability of Caco-2 cells, compared with a standard 200 nm filtration. Dilution of the digesta by 3 or 4 times seemed to be the best option to reduce digesta-related toxicity, although the increase in fluorescence intensity following incubation with the digesta (3 and 4-fold dilution) may have indicated mitochondrial stress. In the presence of higher amounts of ROS, the reduction of resazurin to resafurin would also be higher, hence emitting higher fluorescence compared with cells under control conditions (medium only).

Thus, several adaptations may be considered for studying the bioaccessibility of isolated carotenoids in *in vitro* systems, such as for mechanistic investigations. Firstly, the source of lipids should be considered, with dairy sources perhaps constituting a source fostering emulsification but not true micelle formation. The use of emulsifiers is recommended, although the addition of further sodium taurocholate before digestion is not physiological and may only have small effects. While gastric lipolysis is a process occurring *in vivo*, the addition of a gastric lipase in an *in vitro* system may have limited effects on β-carotene emulsification, although this deserves further investigation. An important consideration is sample filtration to remove small lipid droplets above 20 nm. Finally, when coupling *in vitro* digestion to Caco-2 based studies, sufficient dilution with media should be carried out to reduce

the potential toxicity of the digesta. Despite advantages of *in vitro* models to study GI digestion and aspects of carotenoid bioavailability such as simplicity, the model still has its limitations. It is a static, nondynamic model with pre-set concentrations and pH, not allowing for adaptive changes of pH, enzymes, and bile salts concentrations as *in vivo*. Further, the specific conditions were optimized for pure β-carotene and may not represent ideal conditions for other carotenoids, such as xanthophylls, or carotenoids embedded in more complex matrices. The obtained results highlight merely some potential factors that could impact bioaccessibility of carotenoids in digestive models, thus emphasizing the need for more standardized models and improved physiological conditions.

In conclusion, as hypothesized, β-carotene bioaccessibility significantly differed across sources of dietary lipids. In addition, adding certain emulsifiers (lecithin, monoolein, and oleic acid) significantly enhanced β-carotene bioaccessibility. Contrary to expectations, fungal lipase addition showed no significant improvement of β-carotene micellarization. It appears likely that the conditions used, or the choice of fungal lipase, may have compromised our results; thus, further research is warranted for examining the role of human lipase for digesting lipophilic micronutrients. Finally, introducing different filtration cutoffs indicated an overestimation of carotenoid bioaccessibility when no filtration or filtration with large filter pores is performed, which may partially explain differences in bioaccessibility findings across different laboratories. However, filtration of the digesta did not reduce cellular toxicity in a coupled Caco-2 cellular model.

2.6 Acknowledgments

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3. EFFECT OF DIVALENT MINERALS ON THE BIOACCESSIBILITY OF PURE CAROTENOIDS AND ON PHYSICAL PROPERTIES OF GASTRO-INTESTINAL FLUIDS

Preamble

The following chapter has been published in the journal of “Food Chemistry”.

The following chapter describes the first step, after optimization of an *in vitro* GI digestion protocol, towards the study of the DM on the bioavailability of carotenoids. The objective of the experimental work here described was to test the effect of DM on the bioaccessibility of pure individual carotenoids, during gastrointestinal digestion, and on the physico-chemical properties of the simulated digestive fluids, namely viscosity and surface tension.

All the experimental work and analysis were carried out at the current Luxembourg Institute of Science and Technology (LIST), past *Centre de Recherche Public Gabriel Lippmann*. Bioaccessibility assays and analysis were performed by myself and Master student Mohammed Iddir, and with the help of the group’s laboratory technician, Boris Untereiner. The assays and data analysis for viscosity and surface tension analysis were performed by the group’s member Dr. Christos Soukoulis.

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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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3.1 Abstract

During digestion, high concentrations of divalent minerals (DM) can lead to insoluble lipid–soap complex formation, hampering carotenoid bioaccessibility. The effect of varying concentrations (0–1000 mg/L) of Ca^{2+}, Mg^{2+}, Zn^{2+} and sodium (control) on the bioaccessibility of lutein, neoxanthin, lycopene and β-carotene, following in vitro 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 Ca^{2+}. Mean half maximal inhibitory concentrations (EC50) for Ca^{2+}, Mg^{2+} and Zn^{2+} 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.

Keywords: Xanthophylls; Carotenes; Calcium; Magnesium; Zinc; Micellarization; Digestion; Solubility.

3.2 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% (Hof & West, 2000). In recent years much attention has been given to dietary and host factors influencing carotenoid bioaccessibility (Biehler & Bohn, 2010; Castenmiller & West, 1998; 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 et al., 2012; Huo et al., 2007), concentration of bile acids, as well as gastro-intestinal pH and enzyme variations (Biehler, Kaulmann, et al., 2011; Tyssandier et al., 2001).

One dietary factor that so far has received little attention is the presence and concentration of DMs, including trace elements, such as Ca^{2+}, Zn^{2+} and Mg^{2+}. Previously, we have shown that high concentrations of the DMs Ca^{2+}, Mg^{2+}, iron and Zn^{2+} impaired the transfer of carotenoids to mixed micelles to different degrees, thereby reducing micellarization by up to 90% (Biehler, Hoffmann, et al.,

2011), 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 Ca^{2+} and Mg^{2+}. 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, et al., 2008) in order to be effectively solubilized and emulsified, DMs could compromise carotenoid bioaccessibility by limiting the presence of available triglycerides and FFA. DMs may also have the tendency to bind and precipitate bile acids (Baruch, Lichtenberg, Barak, & Nir, 1991), 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 (Ca^{2+}, Mg^{2+} and Zn^{2+}) 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* GI 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.

3.3 Materials and methods

3.3.1 Chemicals, minerals and carotenoid standards

Digestive enzymes, i.e., pepsin (porcine, P250 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-a-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 solubilisation 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 et al., 2004; Huo et al., 2007).) and is low in natural occurring carotenoids.

3.3.2 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 (3.3.5). 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 re-solubilisation of the carotenoid and successive formation of mixed micelles during GI 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.

3.3.3 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 (Ca^{2+}, Mg^{2+} and Zn^{2+}) 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 or AI (acceptable intake) if no RDA available) and tolerable upper intake levels (UL) (Food and 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 Ca^{2+} tested were 0, 250, 500 and 1000 mg/L; Mg^{2+} 0, 100, 200 and 300 mg/L; Zn^{2+} 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.

3.3.4 Extraction of carotenoids from digesta

Aliquots of 12 mL of digesta were transferred to 15 mL falcon tubes and centrifuged at 4800g 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.

The extraction procedure was adapted from Biehler, Hoffmann, *et al*. (2011) and Biehler, Kaulmann, *et al*. (2011). In short, 2 mL aliquots of the filtered micellar phases were mixed with 4 mL of hexane:acetone (1:1, v/v), shaken for 1 min and centrifuged for 2 min at 4000g at 4 °C. The hexane phase was collected into a second 15 mL tube. The extraction procedure was repeated with 4 mL of hexane, followed by a second centrifugation at 4000g. The hexane phases were combined and dried under a stream of nitrogen. The residue was re-dissolved in 1 mL of hexane, acetone or chloroform, depending on the investigated carotenoid, and filtered through a 0.45 µm Acrodisc® CR 4 mm syringe filter (Pall, Hoeegarden, Belgium) prior to spectrophotometric analysis.

3.3.5 Spectrophotometric analysis of carotenoids

Absorbance was measured between 350 nm and 600 nm. The concentration of each individual carotenoid was calculated based on the Beer–Lambert law and the formula was taken from Britton, Liaaen-Jensen, and Pfander (2004):

(1) $$C = \frac{ABS_{max}}{A^{1\%} \times d} \times 10$$

where C = concentration of the solution (mg/mL), Abs_{max} = maximum absorbance of the carotenoid, $A^{1\%}$ = mass extinction coefficient of the carotenoid in a particular solvent (g^{-1} L cm^{-1}) and d = length of the cuvette (cm). Quantification was based on the extinction coefficient of: β-carotene in hexane ($A^{1\%}$ = 2590), lutein in acetone ($A^{1\%}$ = 2340), neoxanthin in acetone ($A^{1\%}$ = 2240) (Britton et al., 2004) and lycopene in chloroform ($A^{1\%}$ = 2810) (Naviglio et al., 2008).

Percentage of carotenoid micellarization was used as a measure for bioaccessibility, and is expressed as the percentage of solubilized carotenoid present in the aqueous phase of digesta after *in vitro* GI digestion, compared to the initial amount added to the sample.

3.3.6 Rheological measurements and surface tension of the digesta

Steady state shear flow measurements of micellar fractions of digesta samples (middle phase of digesta centrifuged at 4800g for 1 h at 4 °C) were carried out in an Anton-Paar rheometer (MCR 302, WESP, Graz, Austria), using a double gap concentric cylinder geometry (DG 26.7). All measurements were performed at 25 ± 0.03 °C. Steady state shear flow measurements 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}) were carried out. Shear stress (τ) – shear rate data ($\dot{\gamma}$) data were fitted according to the Ostwald–de Waale model:

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

where: τ_0 = the yield stress (Pa), K = consistency coefficient (mPa * s^{-n}) and n = rheological behaviour index (dimensionless). The air–water interfacial properties of digesta samples, pre-conditioned at 25 ± 0.1 °C, were determined via the weight-drop method as previously described by Permprasert and Devahastin (2005). Surface tension of digesta samples were calculated as follows:

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

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

3.3.7 Statistical analysis

In order to minimize potential day-to-day variations between the experiments, we have used the normalized bioaccessibility for statistical analysis, expressed as the percentage of micellarized β-carotene present in the aqueous phase of the digesta, compared to the respective daily control condition (no mineral added).

(4) $$Normalized\ bioacessibility = \frac{Micellarization\ of\ sample}{Micellarization\ of\ control\ conditions} \times 100$$

The normality of data distribution was verified by quantile–quantile (Q–Q) plots and equality of variance was verified by box plots. A linear mixed model was applied to study the effect of 4 minerals (Ca^{2+}, Mg^{2+}, Zn^{2+} and sodium) at 4 concentrations on the bioaccessibility of 4 individual carotenoids (β-carotene, lutein, neoxanthin and lycopene). Normalized bioaccessibility was the dependent variable; the type of mineral and carotenoid were set as fixed factors while mineral concentration was nested within mineral. Following significant F-values, Bonferroni post hoc tests were conducted for all pairwise comparisons. A P-value below 0.05 (2-sided) was considered as statistically significant. Linear correlation between the bioaccessibility and physical (macroviscosity and surface tension) data was verified via Pearson correlation coefficients. All statistical analyses were performed with SPSS (IBM, Inc., Chicago, IL) vs. 19.0. All values in the text are expressed as mean ± SD.

3.3.8 Calculation of half maximal effect concentration (EC50)

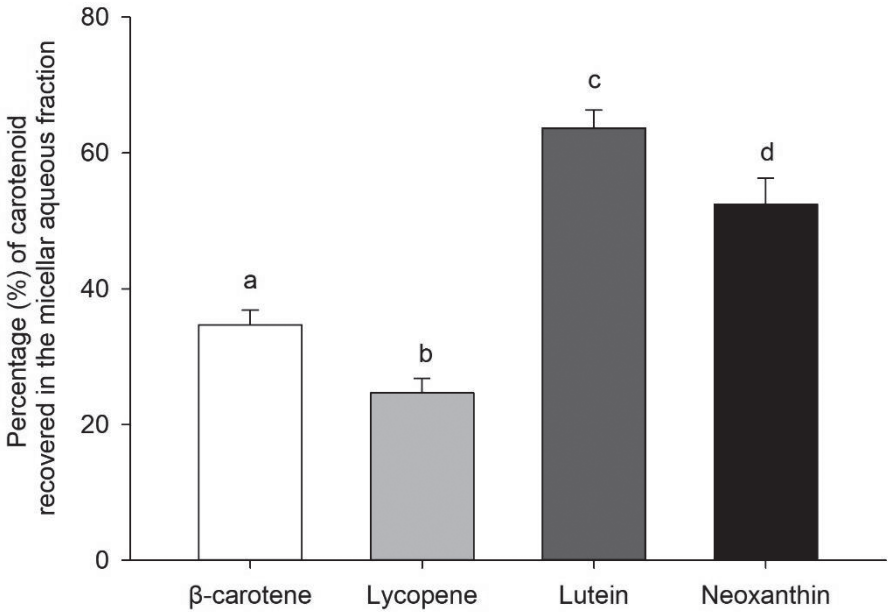
The half-maximal effect concentrations (EC50) of Ca^{2+}, Mg^{2+} and Zn^{2+} were calculated for the parameters bioaccessibility, surface tension and apparent viscosity, in the case of each individual carotenoid. The EC50 values were determined by applying the Hill model on the REGTOX macro for Excel. REGTOX is freely distributed and is available from the internet (http://www.normalesup.org/~vindimian/fr_index.html).

3.4 Results

3.4.1 Bioaccessibility of carotenoids following in vitro GI digestion

Under control conditions (no addition of DMs), micellarization of the pure carotenoids following GI digestion differed significantly between the different species of carotenoids ($P < 0.001$) (Part 3, Figure 1). Xanthophylls (lutein and neoxanthin) were more bioaccessible than carotenes (lycopene and β-carotene).

The addition of DMs to the simulated GI digestion significantly affected the bioaccessibility of pure carotenoids in a concentration-dependent manner ($P < 0.001$). The addition of DMs also led to the formation of insoluble complexes that precipitated and accumulated at the bottom of the falcon tubes. The aqueous fraction appeared clearer and less turbid than the respective control conditions.



Part 3, Figure 1 - Overall bioaccessibility of carotenoids under control conditions (i.e., no additional mineral added to the system), at the end of *in vitro* GI digestion. Values represent mean ± SD of all the control conditions in the experimental design (n = 24 for β-carotene, n = 20 for lycopene, n = 23 for lutein and n = 21 for neoxanthin). Different superscripts indicate statistical significant differences between carotenoids ($P < 0.001$).

Sodium, employed as a control mineral, had a positive effect on the micellarization of β-carotene, lutein and neoxanthin when compared to the respective control conditions. This effect depended on the concentration of sodium ($P < 0.001$) (Part 3, Figure 2C); for 750 and 1500 mg Na/L, there was a significant improvement of carotenoids recovered in the micellar aqueous fraction at the end of the GI digestion. The addition of Ca^{2+} ions significantly ($P < 0.001$) decreased the micellarization of all tested carotenoids, and this effect depended on the concentration of Ca^{2+}. From the analysis of the EC50 curves, an average concentration of 270 mg/L was able to inhibit the micellarization of the pure carotenoids by 50% (Part 3, Table 1). At 500 mg/L the micellarization of most carotenoids was reduced to zero (Part 3, Figure 2A). Similarly to Ca^{2+}, the addition of Mg^{2+} significantly decreased carotenoid micellarization during *in vitro* GI digestion in a concentration dependent manner

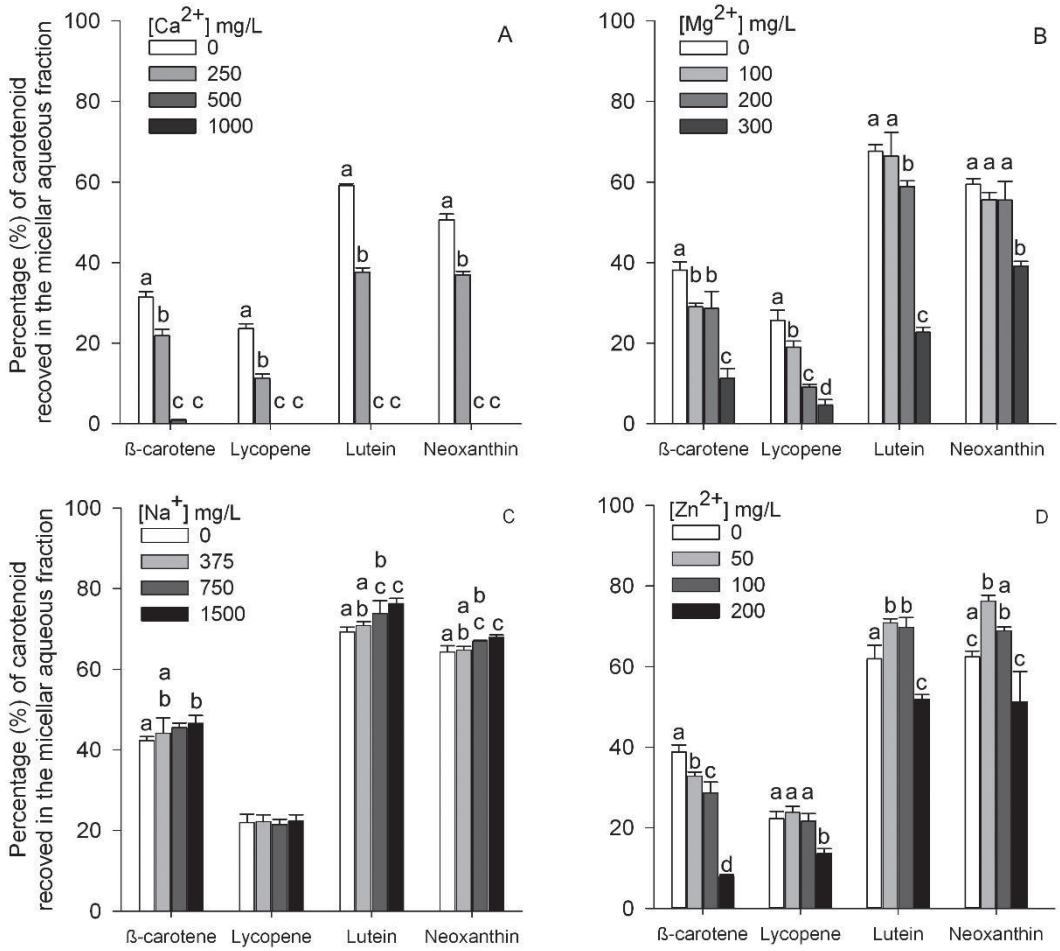
($P < 0.001$). On average, a concentration of 252 mg Mg^{2+}/L was necessary to bring down the micellarization of carotenoids to 50% (Part 2, Table 1).

Part 3, Table 1 - Half maximal effect concentration (EC50) values of calcium, magnesium and zinc for the bioaccessibility of carotenoids, surface tension, and apparent viscosity of digestive fluids. Values represent the concentration (mg/L) of divalent minerals at which bioaccessibility and surface tension is decreased and viscosity increased by 50%. EC50 values were determined applying the Hill model on RegTox macro for Excel.

	Ca^{2+}	Mg^{2+}	Zn^{2+}
Bioaccessibility			
β-Carotene	290	260	170
Lycopene	250	150	230
Lutein	260	270	880
Neoxanthin	280	330	400
Surface tension			
β-Carotene	386	134	254
Lycopene	346	166	58
Lutein	nd	266	115
Neoxanthin	99	216	196
Apparent viscosity			
β-Carotene	255	102	110
Lycopene	194	211	105
Lutein	nd	244	421
Neoxanthin	228	343	105

Nd: not determined

Contrary to Mg^{2+} and Ca^{2+}, Zn^{2+} did not significantly ($P > 0.05$) affect the micellarization of carotenoids within a physiological range of concentrations (i.e., ≤ daily RDA) (data not shown). Significant effects of Zn^{2+} on the micellarization of carotenoids were only seen for concentrations above the physiological level (Part 3, Figure 2D, $P < 0.001$).



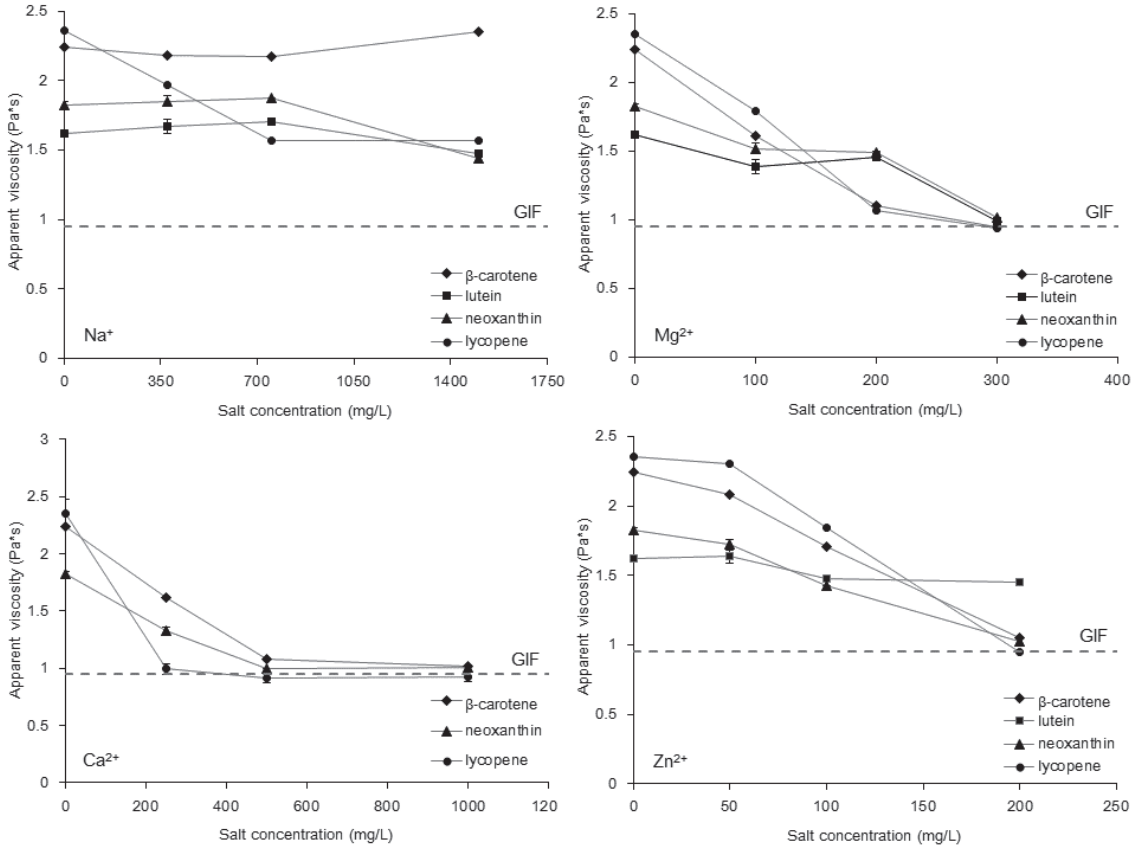
Part 3, Figure 2 - Effect of divalent minerals, calcium (A), magnesium (B), zinc (D) and control mineral sodium (C) on the bioaccessibility of carotenoids. Carotenes (β-carotene and lycopene), and xanthophylls (lutein and neoxanthin) were digested in the presence of varying concentrations of DMs and sodium: [Ca^{2+}] from 0 to 1000 mg/mL; [Mg^{2+}] from 0 to 300 mg/L; [Zn^{2+}] from 0 to 200 mg/mL and [Na+] from 0 to 1500 mg/L. The bioaccessibility is represented as the percentage of carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, when compared to the amount of carotenoid added to the system at the beginning of digestion. Values are mean ± SD of n = 4 for lutein, neoxanthin and β-carotene and n = 3 for lycopene. Bars not sharing the same superscript differ significantly from each other (P < 0.05) – no effect on lycopene was seen for sodium (graph c).

In the case of xanthophylls, concentrations of Zn^{2+} of 50 and 100 mg/L improved slightly, yet significantly, the micellarization of lutein and neoxanthin. For β-carotene, the latter concentrations decreased micellarization, while no significant effect was seen in the case of lycopene. Negative effects of Zn^{2+} on the bioaccessibility were observed, for all carotenoids, when mineral concentrations were above 200 mg Zn^{2+}/L. The concentration of Zn^{2+} required to reduce carotenoid micellarization

by 50% (EC50) varied greatly according to the carotenoid, from 170 mg/L, for β-carotene up to 880 mg/L for lutein (Part 2, Table 1).

3.4.2 Effect of minerals on the surface tension and viscosity of digesta

In order to provide insight on the physical phenomena occurring throughout digestion, in the absence and presence of DMs and sodium, the digesta were subjected to viscosimetric and surface tension analyses. Based on the acquired rheological spectra (data not shown), in the absence of DMs and sodium, the digesta exhibited a Newtonian behavior, suggesting an isotropic micellar solution (Hofmann & Mysels, 1992).



Part 3, Figure 3 - Effect of divalent minerals on the macroviscosity of the digesta following gastro-intestinal digestion. GIF represents the level of the gastrointestinal digesta without any added mineral. Each point represents the mean of 3 independent digesta.

Upon adding DMs, the digesta underwent precipitation, increasing proportionally to the mineral concentration in the bulk aqueous phase. As expected, the addition of DMs was associated with a significantly reduced ($P < 0.001$) macroviscosity of the micellar digesta fractions (Part 3, Figure 3).

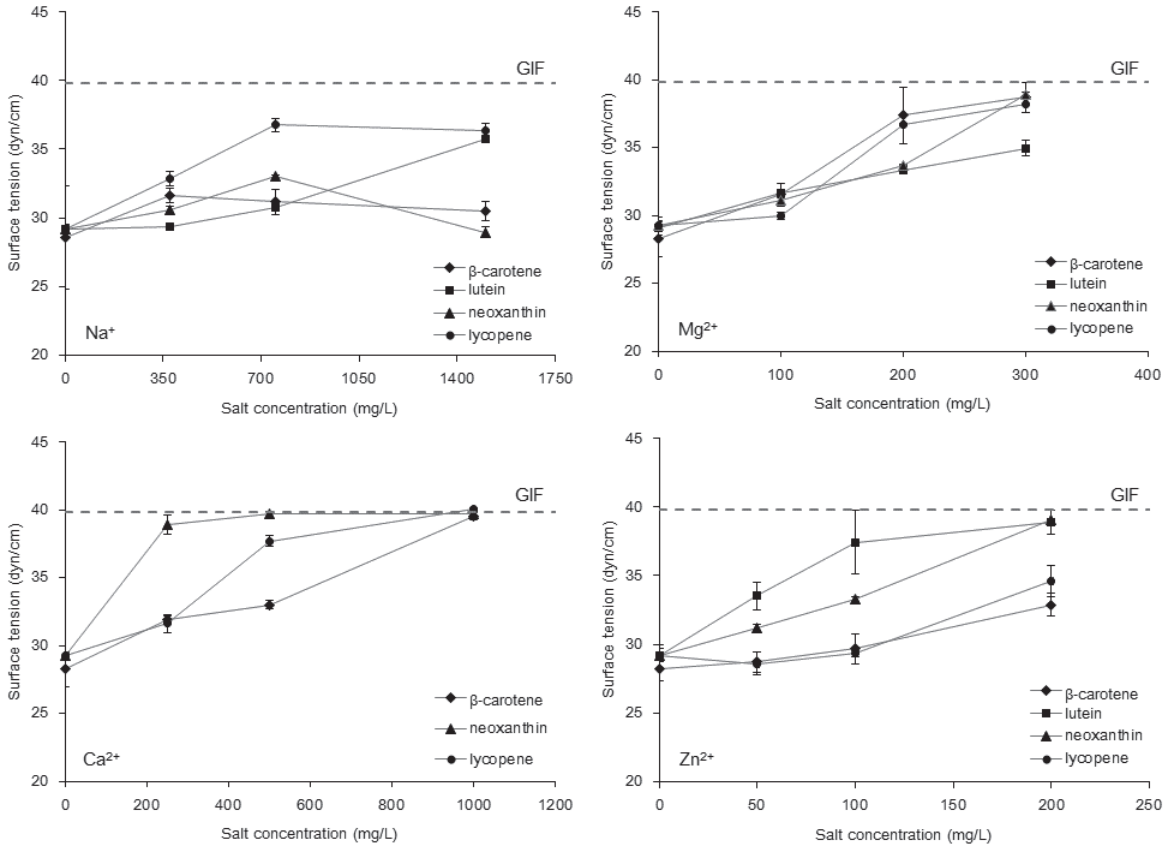
In contrast, digesta containing sodium did not undergo significant macroviscosity changes except for lutein, lycopene and neoxanthin containing digesta samples supplied with 1500 mg/L. Notwithstanding the non-significant differences in the macroviscosity between the digesta samples that were detected (ANOVA mean macroviscosity values ranged from 1.364 to 1.538 Pa * s), the responsiveness of the digesta systems to DM concentrations followed the order $Zn^{2+} > Ca^{2+} > Mg^{2+}$ (Part 3, Table 1). Lutein containing digesta tended to exert generally lower macroviscosity, though differences attained were not significant compared to the other carotenoids.

Part 3, Table 2 - Linear Pearson correlation coefficients depicting the dependence of carotenoid bioaccessibility on the surface tension and apparent viscosity of gastro-intestinal digested samples at 25 °C, in dependency of the addition of various minerals.

		Surface tension	Viscosity
Ca^{2+}	Global correlation[a]	-0.630**	0.730**
	β-Carotene	-0.829*	0.978*
	Lutein	nd	nd
	Neoxanthin	-0.787^{NS}	0.944*
	Lycopene	-0.948*	0.902*
Mg^{2+}	Global correlation[a]	-0.606**	0.339^{NS}
	β-Carotene	-0.997**	-0.972*
	Lutein	-0.814*	0.936*
	Neoxanthin	-0.955*	0.965*
	Lycopene	-0.972*	-0.992**
Na^{+}	Global correlation[a]	-0.396^{NS}	-0.365^{NS}
	β-Carotene	0.698^{NS}	0.291^{NS}
	Lutein	0.911*	-0.569^{NS}
	Neoxanthin	0.157^{NS}	-0.681^{NS}
	Lycopene	-0.235^{NS}	0.189^{NS}
Zn^{2+}	Global correlation[a]	0.297^{NS}	-0.150^{NS}
	β-Carotene	0.158^{NS}	0.900*
	Lutein	-0.275^{NS}	0.472^{NS}
	Neoxanthin	-0.683^{NS}	0.691^{NS}
	Lycopene	-0.997**	0.963*

NS = non-significant, nd = not detected/measured.
[a] Global correlation represents the dependence of the pooled bioaccessibility values, taking into account all 4 carotenoids and 4 different concentrations for a given DM, on the surface tension and apparent viscosity of the digesta. * represents $P < 0.05$. ** represents $P < 0.01$.

The surface tension of the digesta (Part 3, Figure 4) was significantly influenced by the type ($P < 0.001$) and the amount ($P < 0.001$) of minerals (Part 2, Table 2). Specifically, the presence of divalent ions was associated with higher surface tension values (ranging from 33.8 to 34.4 dyn/cm) compared to sodium ions (30.3 dyn/cm). However, similarly as to macroviscosity, all DMs impacted equally the inter- facial properties of the digesta. Based on the calculated EC50 values, surface tension responsiveness on DM type was higher for Ca^{2+} (277 mg/L) followed by Mg^{2+} (196 mg/L) and Zn^{2+} (156 mg/ L), corroborating the observations for macroviscosity (Part 3, Figure 3).



Part 3, Figure 4 - Effect of divalent minerals on the surface tension of the digesta following GI digestion. GIF represents the level of the gastrointestinal digesta without any added mineral. Each point represents the mean of 3 independent digesta.

The correlation coefficients between bioaccessibility, digesta macroviscosity and surface tension are illustrated in Part 2, Table 2. Linear correlations between physical properties and bioaccessibility were rather weak when the entire (global) carotenoid dataset was subjected to analysis. Nevertheless, when the bioaccessibility and physical properties were inspected based on carotenoid class, i.e.,

carotenes (β-carotene, lycopene) vs. xanthophylls (neoxanthin, lutein), a strong correlation between carotene bioaccessibility and digesta macroviscosity (positive) and surface tension (negative) was observed. In addition, the strongest dependency of bioaccessibility on the physical properties of the digesta was the case of Ca^{2+} and Mg^{2+}, while in the presence of Zn^{2+}, only lycopene bioaccessibility was significantly associated with the physical profile of the digesta.

3.5 Discussion

In this study, we investigated the effect of three DMs (Ca^{2+}, Mg^{2+} and Zn^{2+}) at physiological to dietary supplement concentration range, on the bioaccessibility of pure carotenoids, two xanthophylls (neoxanthin and lutein) and two carotenes (β-carotene and lycopene). These carotenoids are predominant in the diet of most people, and are also among the most investigated. Beta-carotene, lycopene and lutein are also commonly found in supplements, often together with other macro- and micro-nutrients such as Ca^{2+}, Mg^{2+}, or Zn^{2+}. Previously, we reported that the presence of Ca^{2+}, iron and Zn^{2+}, during simulated *in vitro* digestion of spinach, inhibited the micellarization and cellular uptake of total carotenoids including β-carotene and lutein, and that these effects were less pronounced for Mg^{2+} (Biehler, Hoffmann, et al., 2011; Biehler, Kaulmann, et al., 2011). However, the effects of DMs have never been studied systematically and with individual carotenoids, and the novelty of the present study rests in demonstrating the potential detrimental effects of minerals on individual, already solubilized carotenoids, and in showing effects on selected related physical parameters of the digesta.

The addition of DMs and sodium to the GI digestive fluids affected significantly, either positive or negative, carotenoid micellarization. These effects depended on the concentration of the mineral and the type of carotenoid. We further observed the presence of a precipitate that was evident with increasing concentrations of DMs. Under physiological conditions, bile salts are able to aggregate with phospholipids and glycerides to form mixed micelles (Mukhopadhyay & Maitra, 2004), in which carotenoids are solubilized and become available for cellular uptake (Stahl et al., 2002). The capacity of bile salts to form micelles depends, among other, on the critical micelle concentration (CMC) of the

bile acids (the concentration threshold above which the spontaneous formation of bile salt micelle formation is taking place), temperature and pH (Baruch et al., 1991; Carey & Small, 1969; Feroci et al., 1996).

The presence of Ca^{2+} and Mg^{2+} resulted in a significant concentration-dependent decrease of carotenoid bioaccessibility, even at concentrations representing 50% of the daily RDA (i.e., 250 mg Ca/L and 100 mg Mg/L). Two possible reactions may mainly contribute to this observation, namely the precipitation of FFA following the formation of insoluble soaps, or, alternatively, the precipitation of (conjugated) bile salts, for which a mineral-concentration dependent precipitation was reported earlier (Jones et al., 1986). The precipitation of conjugated bile salts would dampen the formation of mixed micelles, whereas the binding of FFA to DMs would hinder the transport of carotenoids from the lipid droplets to the formed mixed micelles. It was therefore postulated that, in the presence of sufficiently high concentrations of DMs, carotenoids remain unavailable for uptake, either solubilized in undigested lipid droplets or occluded in the precipitates.

In the case of Ca^{2+} at 500 mg/L, almost no carotenoids were recovered in the micellar fraction after digestion. Jones et al. (1986) found that increasing Ca^{2+} concentration from 0 to ca. 400 mg/L led to the precipitation of insoluble soaps, possibly due to the low solubility of Ca^{2+} products, increasing the CMC of the bile conjugates and resulting in precipitation above a certain threshold for Ca^{2+} concentration.

Despite the impact of DMs on the bioaccessibility, the addition of low amounts of Ca^{2+} (250 mg/L) and Mg^{2+} (100 mg/L) did not significantly modify the macroviscosity and surface tension of the digesta, except for neoxanthin, implying that carotenoid bioaccessibility reduction is rather driven by their occlusion in the soap precipitants formed via the interaction of lipolysis products with the existing DMs. At moderate to high concentrations of Ca^{2+} and Mg^{2+}, a synergistic action between the formation of insoluble soaps and insoluble conjugated bile salts may take place, leading to carotenoid and surface active lipophilic compound depletion of the digesta, suppressing drastically their uptake from the mixed micelles.

In the case of Zn^{2+}, physiological concentrations of this mineral (i.e., up to the RDA) did not significantly affect the micellarization of the pure carotenoids. The effect of Zn^{2+} was only significant at concentrations above 50 mg/L, which can be considered as being in the supplement range, as this concentration is already above the reference UL. In a study on chicks, testing the effects of excess Zn^{2+} on tissue levels of α-tocopherol, it was found that excess Zn^{2+} depressed the absorption of α-tocopherol (Lü & Combs, 1988), another liposoluble micronutrient requiring micellarization prior to cellular uptake, though rather other mechanism such as pancreas perturbations and lack of lipase were suggested in this study. In the present work, sodium was taken as a negative control.

Contrary to what was expected, additional Na^{+} ions improved slightly, though significantly, carotenoid micellarization, especially at higher concentrations (1500 mg/L), except for the more apolar lycopene. This could be attributed to the ability of sodium to lower the CMC of bile salts (Jones et al., 1986). Jones and colleagues found that the presence of Na^{+} counterions, provided mainly in the form of physiological saline (~0.154 M) and $NaHCO_3$ counteracted the effect of the Ca^{2+} ions. Sodium appears to exert a modulating role on the existing colloidal state of the digesta, i.e., the presence of micellar isotropic solution vs. precipitation, and at low to moderate Ca^{2+} and Mg^{2+} concentrations during digestion, the formation of mixed micelles is barely interrupted by bile salt precipitation, probably due to the ability of sodium to increase bile salt solubility.

The macroviscosity of the control digesta showed a fairly isotropic character, suggesting that conjugated salts are existing either in the dilute or the micellar form state (Hofmann & Mysels, 1992). A further increase of sodium was associated with a slightly decreased macroviscosity and increased surface tension, possibly indicative of a mild precipitation of bile salts due to their adversely impacted water solubility. However, as no significant correlation between bioaccessibility and macro-viscosity or surface tension was observed for sodium, the formation of mixed micelles was not considerably hindered. In summary, the experimental results show that at physiological concentrations, Ca^{2+} and Mg^{2+} ions had the ability to significantly reduce carotenoid bioaccessibility. On the other hand, sodium ions had a slight but significant positive effect on carotenoid bioaccessibility. Despite the negative effect of high DM concentration on the bioaccessibility of carotenoids, the nature of the formed

precipitate remains to be clarified, specifically the contribution of bile salts and fatty acids to its composition, and the fate of the carotenoids that were not micellarized. It should also be kept in mind that the human GI system is a far more complex and dynamic system. The constant secretion of bile and enzymes could counteract some of the effects observed *in vitro*, e.g., avoiding some of the precipitation encountered in simulated systems (Hofmann & Mysels, 1992). In addition, under real physiological conditions, i.e., *in vivo*, it is possible that DMs themselves may be complexed by other molecules, such as phosphates from animal products, or phytic acid/oxalic acid from plant matrices, attenuating the negative impact that DMs may have on carotenoid bioaccessibility. A possible comparable negative effect of high DM concentrations on other liposoluble micronutrients and phytochemicals should also be considered. Confirmation of these results *in vivo* is still in need.

3.6 Conflict of interest

The authors declare no conflict of interests.

3.7 Acknowledgments

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4. NEGATIVE EFFECTS OF DIVALENT MINERAL CATIONS ON THE BIOACCESSIBILITY OF CAROTENOIDS FROM PLANT FOOD MATRICES AND RELATED PHYSICAL PROPERTIES OF GASTRO-INTESTINAL FLUIDS

Preamble

The following chapter has been published in the journal of “Food and Function”.

The experimental work described in the subsequent chapter derives from the work performed in the previous chapter. Given the results obtained for the effect of divalent minerals on the bioaccessibility of pure isolated carotenoids, the next step was to investigate similar experimental conditions on more complex carotenoid-containing matrices, such as those from fruits and vegetables. Hence, the objective was to test the effect of DM on the bioaccessibility, during GI digestion, and cellular uptake of dietary carotenoids from different food matrices, and to assess changes in physico-chemical properties of the simulated gastrointestinal fluids.

Bioaccessibility assays, cellular trials, HPLC analysis, viscosity and surface tension measurements were carried out at the current Luxembourg Institute of Science and Technology (LIST), past *Centre de Recherche Public Gabriel Lippmann*, with the help of Master student Marie Bertucci, laboratory technician Boris Untereiner, and Dr. Christos Soukoulis. Micelle size and zeta potential measurements were performed in collaboration with Dr. Charles Desmarchelier and Dr. Patrick Borel, at the *Nutrition Obésité et Risque Thrombotique* (NORT) Unit from the *Institut National de la Santé et de la Recherche Médicale* (INSERM), France.

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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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4.1 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 Mg^{2+} (0 – 300 mg/L), Ca^{2+} (0 – 1500 mg/L), Zn^{2+} (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 Mg^{2+} led to reductions of up to 100% at the 2 highest concentrations. Curiously, sodium increased ($P < 0.01$) carotenoid bioaccessiblity 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, Ca^{2+} and Mg^{2+} could negatively impact carotenoid bioavailability, while for Zn^{2+}, 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.

4.2 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 (Fraser & Bramley, 2004; Stange & Flores, 2010). 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(Britton, 1995; Demmig-Adams, Gilmore, & Adams III, 1996; Stange & Flores, 2010). This anti-oxidant potential coupled with anti-inflammatory properties (Kaulmann & Bohn, 2014) is of particular interest from a public health perspective for the prevention and delayed progression of several chronic diseases (Krinsky & Johnson, 2005), including eye related degenerative diseases (Scripsema, Hu, & Rosen, 2015), cardiovascular diseases (Voutilainen et al., 2006), diabetes (Brazionis et al., 2009) and prostate cancer (Kucuk et al., 2002; Sonn, Aronson, & Litwin, 2005). 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 tissues (Castenmiller & West, 1998). The first limiting factor of carotenoid bioavailability is their release from the food matrix and their inclusion in bile acid-lipid mixed micelles (Hof & West, 2000; Stahl et al., 2002), i.e. their bioaccessibility. This step is mainly affected by 1) dietary factors (type

and amount of fibre and lipids, form of storage of carotenoids in the matrix, among other) (Goltz et al., 2012; Huo et al., 2007; Jeffery, Holzenburg, et al., 2012; Jeffery, Turner, et al., 2012; O'Connell et al., 2008; Palafox-Carlos et al., 2011), and 2) host-related physiological factors (enzyme and bile acid concentrations secreted, gastric and intestinal pH, etc.) (Biehler, Kaulmann, et al., 2011; Tyssandier et al., 2001).

The presence of DM during GI digestion, particularly Ca^{2+} and Mg^{2+}, has been hypothesized to negatively affect the bioaccessibility of carotenoids (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2016). Divalent mineral cations are naturally present in the human gut and are part of our diet. The effects of the interactions between DM and lipids (Gacs & Barltrop, 1977; Tadayyon & Lutwak, 1969), and DM and bile acids (Baruch et al., 1991; Jones et al., 1986) 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 GI digestion, the effects of the presence of DM, including Ca^{2+}, Mg^{2+} and Zn^{2+}, on the bioaccessibility of carotenoids alone and from spinach (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2016). The concentration-dependent effect of these minerals appeared obvious, with Ca^{2+} and Mg^{2+} 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 bioaccessibility (Corte-Real et al., 2016).

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 Ca^{2+}, Mg^{2+} and Zn^{2+}, 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.

4.3 Experimental

4.3.1 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 Mg^{2+} 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 MΩ water was prepared with a purification system from Millipore (Brussels, Belgium) and used throughout the study.

4.3.2 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 solubilisation of dietary carotenoids and micelle formation during GI 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 (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2014; Kaulmann, André, Schneider, Hoffmann, & Bohn, 2016). INEX coffee creamer (10% fat) was purchased at a local supermarket (Delhaize).

4.3.3 Simulation of gastro-intestinal digestion and factors investigated

To investigate the effect of divalent minerals (Ca^{2+}, Mg^{2+} and Zn^{2+}), we tested the following concentrations: Ca^{2+} 0, 250, 500 and 1000 mg/L; Mg^{2+} 0, 100, 200 and 300 mg/L; Zn^{2+} 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, or AI when no RDA was available, UL (Food and Nutrition Board, 2011). To determine the concentration of the mineral per volume of digesta, we assumed a total volume of 2 L of intestinal fluids during 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 DM were prepared with a physiological saline solution. The *in vitro* GI digestion protocol was adapted from Biehler et al. (2011) 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 (Biehler, Hoffmann, et al., 2011; Kaulmann et al., 2016). 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 (spm).

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 spm) at 37°C. At the end of the GI 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® 13 mm Syringe Filters, PALL Life Sciences, Ann Harbor, MI) into a new 15 mL Falcon tube. Four mL were then taken for extraction of carotenoids.

4.3.4 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 MeOH, 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 MeOH 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 µg/mL) to obtain a final concentration of 1 µg/mL in the sample, and re-dissolved in 500 µL of MTBE:MeOH (3:7). Samples were filtered through a 0.2 µm PVDF syringe filter (PALL Life Sciences, Ann Arbor, MI, USA), into an HPLC amber vial. The filter was then rinsed with 100 µL 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 µL.

4.3.5 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 µm particle size, 100 mm length, 3 mm diameter, from Thermo Fisher Scientific) at 30°C, 10 µL 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 method (Rome & Mcintyre, 2012).

4.3.6 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) $$\tau = K\dot{\gamma}^{n}$$

where: τ_0 = the yield stress (Pa), K = consistency coefficient (mPa * s^{-n}) and n = rheological behaviour index (dimensionless). All measurements were performed at 25 ± 0.03 °C. Surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, were determined via the weight-drop method as previously described by Permprasert & Devahastin (2005). The air–water interfacial properties of digesta were calculated as follows (Pallas & Harrison, 1990):

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

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

4.3.7 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).

4.3.8 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.

4.4 Results

4.4.1 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 (Part 4, Table 1).

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 studies (Biehler et al., 2012), 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.

Part 4, Table 1 - Carotenoid content (µg/g food wet weight) for each of the tested food matrices.

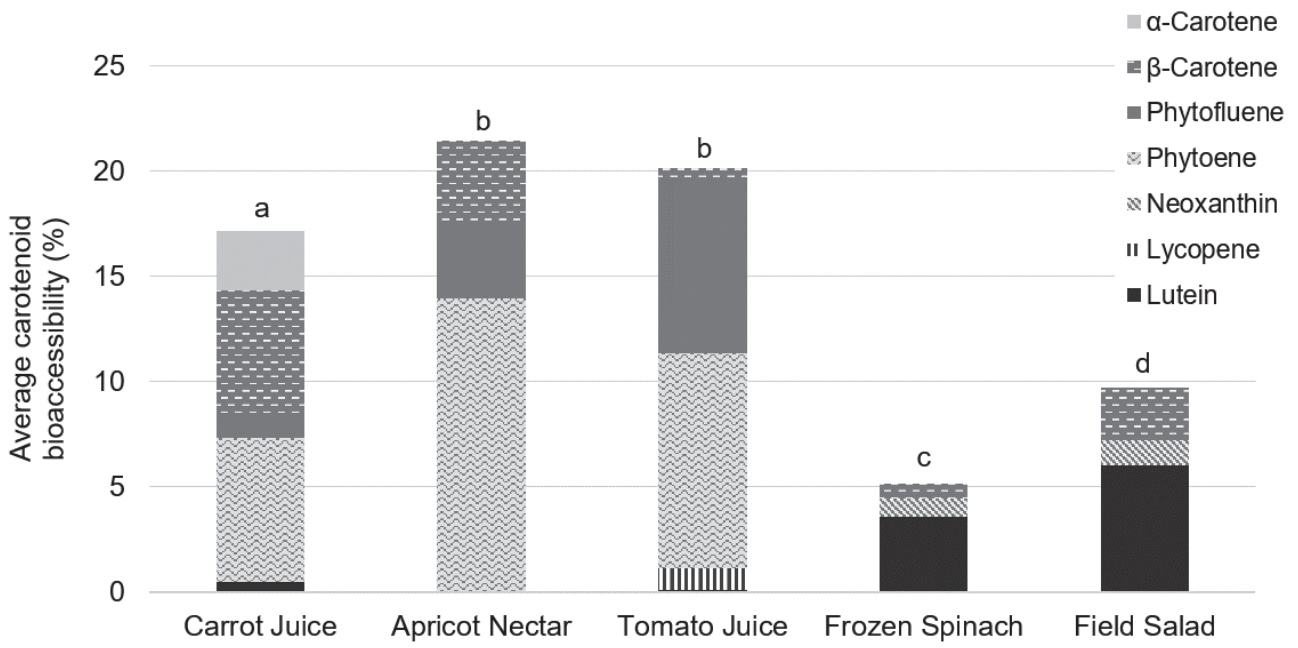
Food matrix	Latin name	α-Car	β-Car	Lyc	Lut	Vio	Neo	PTE	PTF	Total
Apricot nectar	*Prunus armeniaca*	nd	2.87	nd	nd	nd	nd	11.27	2.79	16.87
Tomato juice	*Solanum lycopersicum*	nd	2.98	85.04	0.31	nd	nd	65.09	8.20	158.6
Carrot juice	*Daucus carota*	20.52	66.67	nd	1.24	nd	nd	40.44	4.33	133.2
Frozen spinach	*Spinacia oleracea*	nd	10.54	nd	100.1	64.80	16.26	nd	nd	191.7
Field Salad	*Valerianella locusta*	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.

4.4.2 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 (Part 4, 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 1), and their large contribution to average total carotenoid bioaccessibility in the juices and nectar (Part 4, Figure 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.



Part 4, 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.

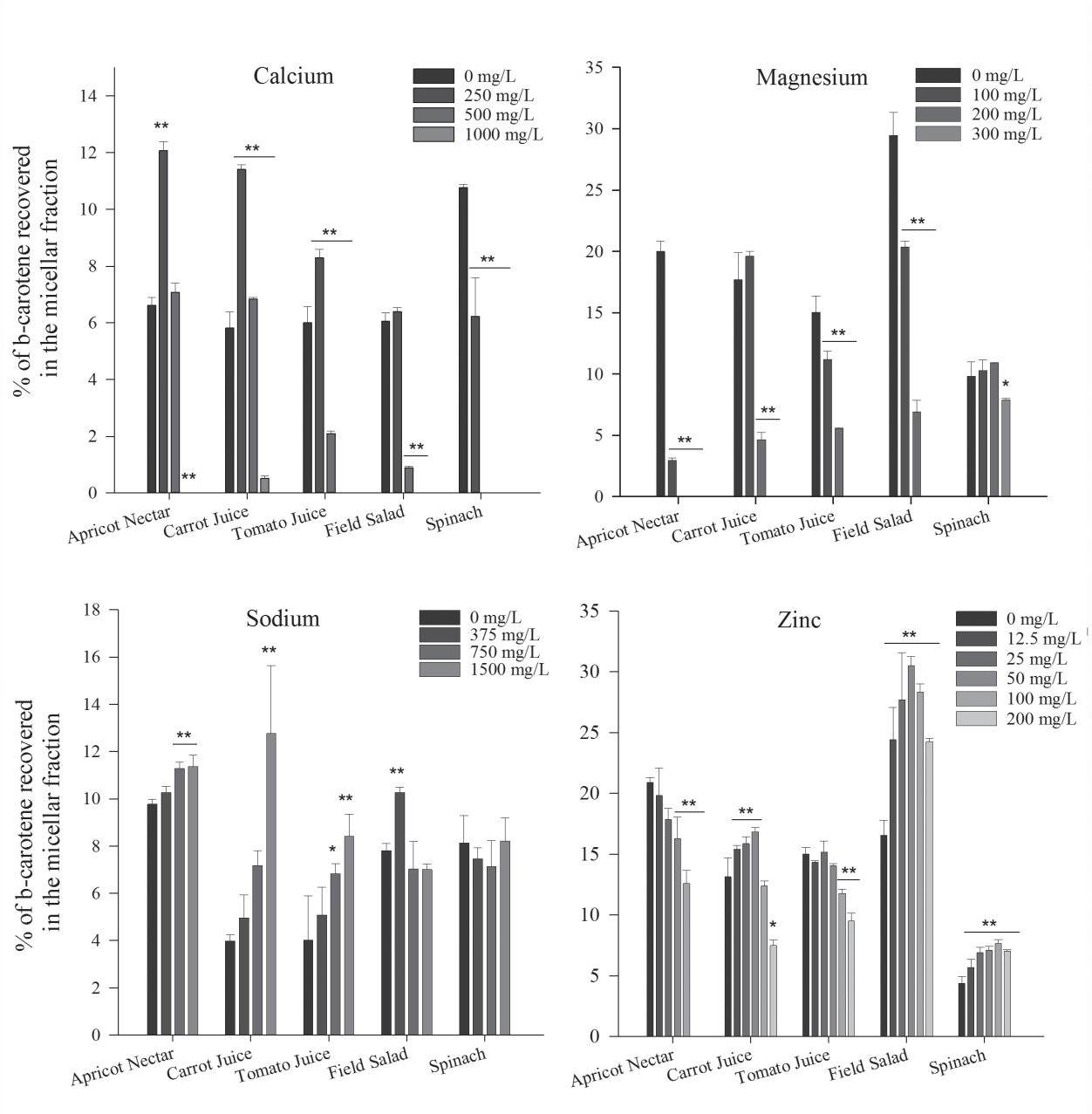
4.4.3 Bioaccessibility of β-carotene: effect of divalent minerals across different matrices

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 DM in a concentration-dependent fashion (Part 4, Figure 2). Addition of Mg^{2+} led to a significant decrease of its bioaccessibility in all matrices. The addition of 200 mg/L Mg^{2+} to the test meals reduced the bioaccessibility of β-carotene by more than half (compared to the control), while at 300 mg of Mg^{2+}, no more β-carotene was found in the micellar fraction (except for spinach). Similarly, a higher concentration of Ca^{2+}, 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 Zn^{2+}, 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 Zn^{2+} significantly decreased β-carotene bioaccessibility from apricot nectar and tomato juice (up to 43%), and up to 100% in carrot juice.

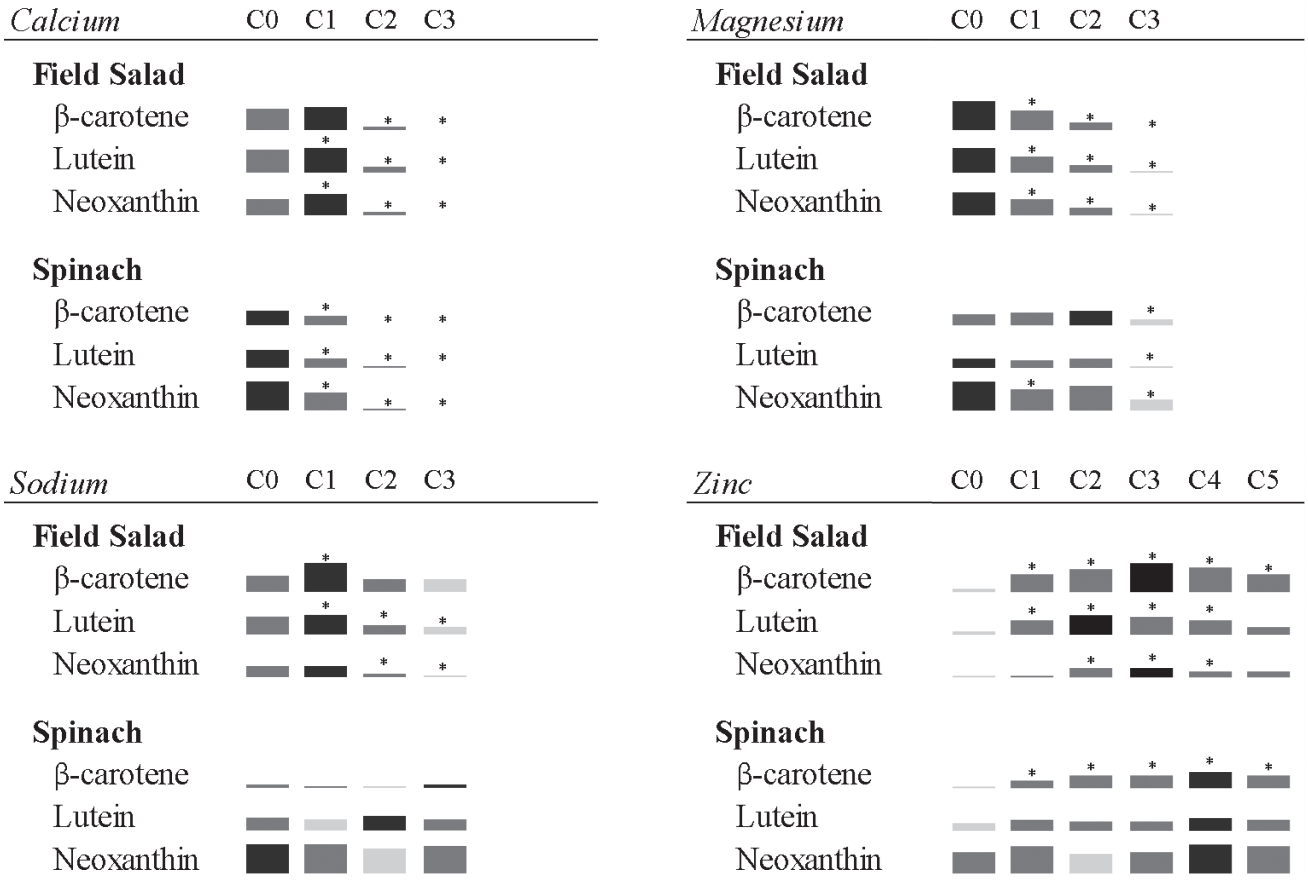
4.4.4 Comparison of the bioaccessibility of carotenes, xanthophylls, and colourless carotenoids

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 (Part 4, Figure 3, Supplementary Table 1 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 Zn^{2+} 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 Zn^{2+} (> 12.5 mg/L, Part 4, Figure 2). As for field salad, Zn^{2+} improved significantly ($P < 0.001$) the bioaccessibility of both lutein, neoxanthin and β-carotene, by up to 39%, 31%, and 63%, respectively.



Part 4, Figure 2 - Effect of divalent minerals 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 minerals (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 ± 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.

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%).

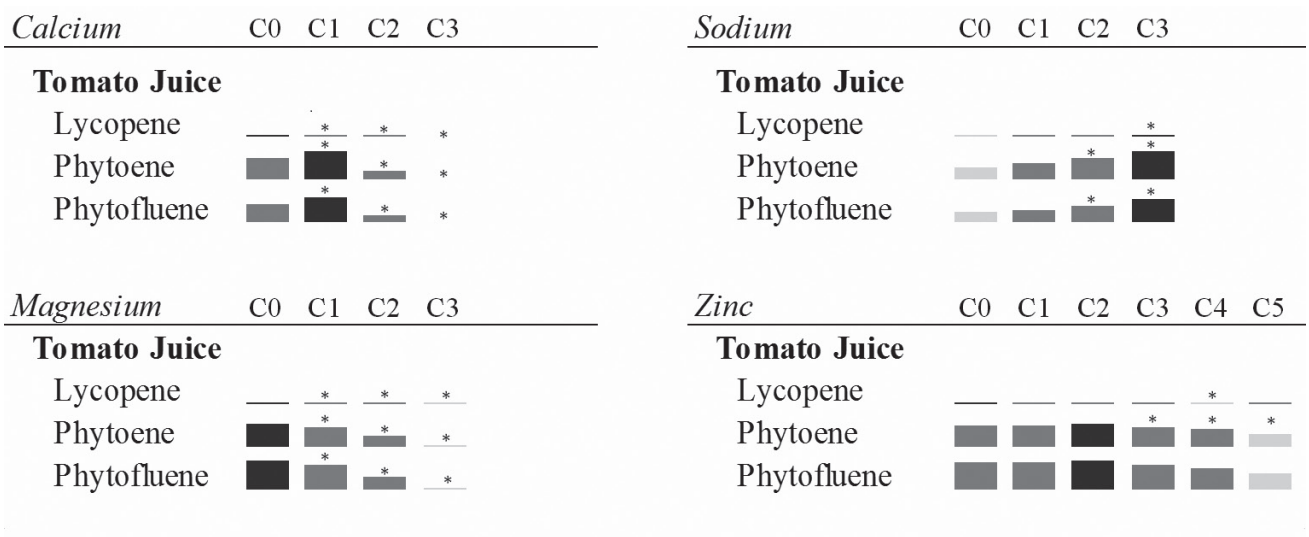


Part 4, Figure 3 - Graphical representation of trends in bioaccessibility variation of carotenoids from spinach and field salad, as effected by cationic minerals at different concentration ranges. Bars in dark and light grey represent the highest and lowest points, respectively, of the measured bioaccessibility. 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, i.e. C0).

Also for Mg^{2+}, 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 Ca^{2+} 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±1.8%, compared to 49.0±14.5% and 53.6±24.4% for phytoene and phytofluene, respectively.

Concerning the response of these carotenoids to the added minerals (Part 4, Figure 4), the patterns of bioaccessibility variations between these three carotenoids were similar in the presence of additional sodium and Mg^{2+}. Adding Mg^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 phytoene, while higher concentrations reduced lycopene and phytofluene bioaccessibility (Part 4, Figure 4).

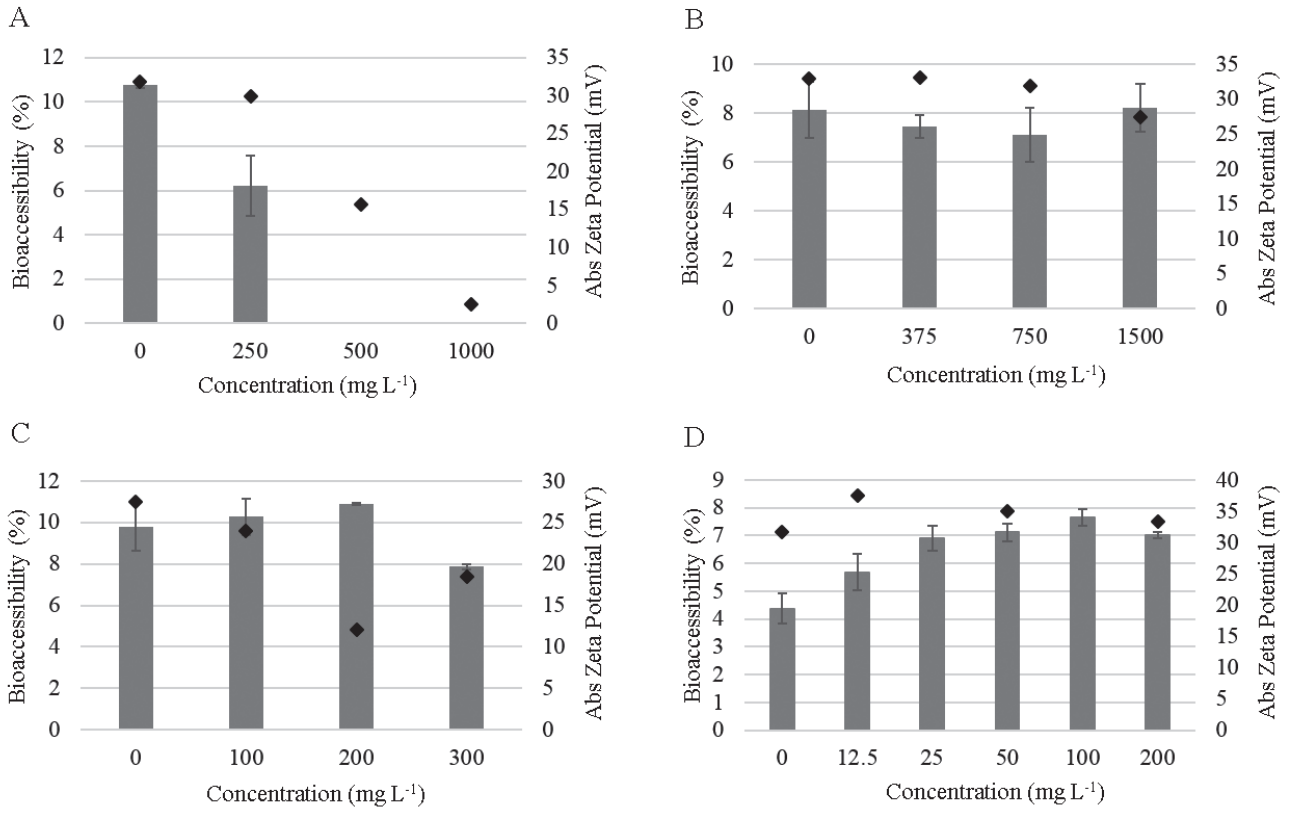


Part 4, Figure 4 - Graphical representation of the trends in bioaccessibility variation of lycopene and the colourless carotenoids, phytoene and phytofluene, from tomato juice, as an effect of cations at different concentration ranges. Bars in dark and light grey represent the highest and lowest points, 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, i.e C0).

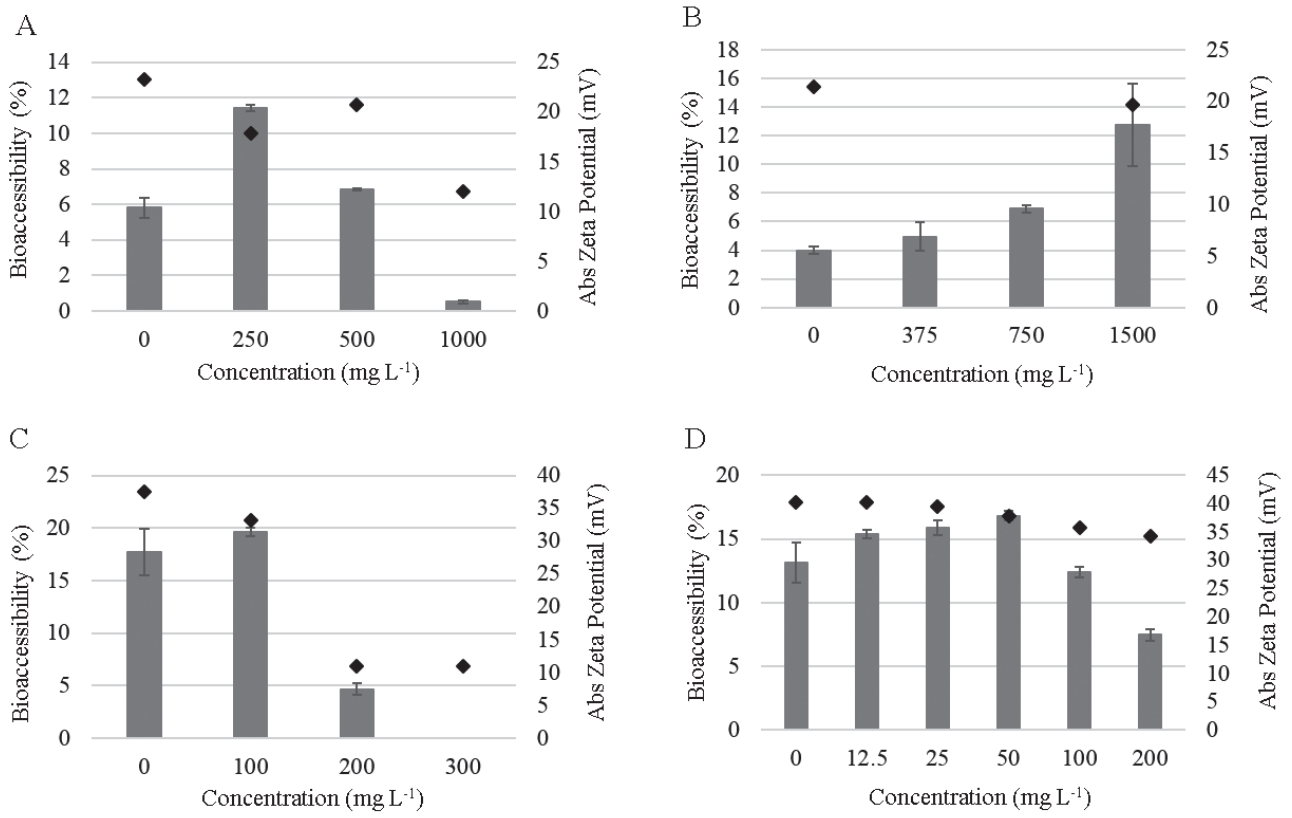
4.4.5 Effect of divalent minerals on the physico-chemical properties of the digesta

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 (Part 4, Figure 5 and Part 4, Figure 6). While Zn^{2+} and sodium did not cause visible variations in the zeta potential, a more obvious variation was seen for Ca^{2+} and Mg^{2+}. As the concentrations of the divalent minerals increased, the zeta potential tended towards zero (Part 4, Figure 5 andPart 4, Figure 6), especially in the case of Ca^{2+} and Mg^{2+}, suggesting that the colloid became unstable. Lowest zeta potential values (-2.53 mV) were observed for spinach samples digested with Ca^{2+} (at 1000 mg/L).



Part 4, 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 ◆ represent the zeta potential. A: Calcium; B: Sodium; C: Magnesium; D: Zinc. Error bars represent standard deviation of n = 4 replicates.

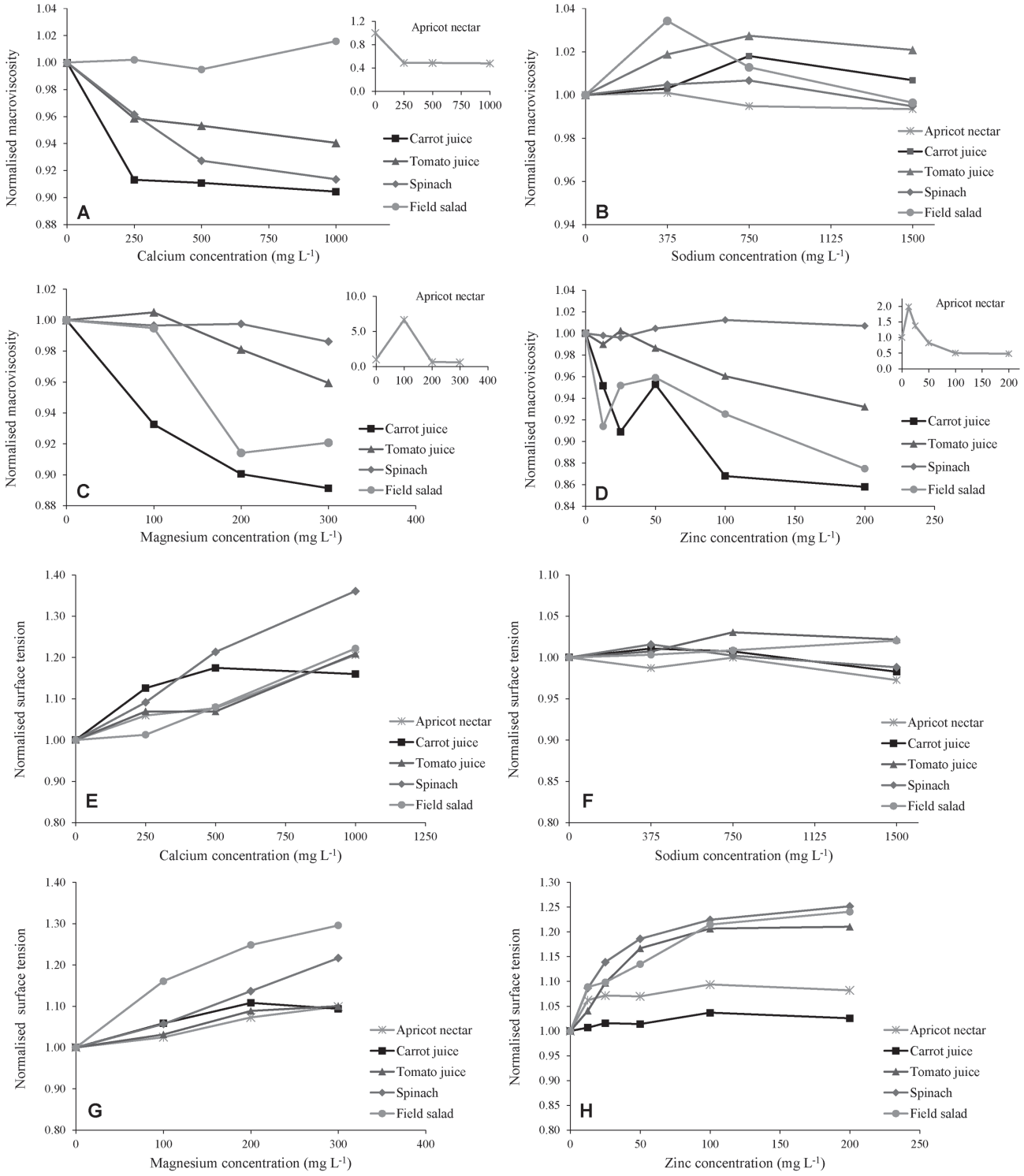


Part 4, Figure 6 - Average carrot juice-borne β-carotene bioaccessibility (%), and absolute zeta- potential (mV) of carrot juice aqueous micellar fraction, after digestion with different cations at varying concentrations. Bars represent bioaccessibility, while markers ◆ represent the zeta-potential.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

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.



Part 4, Figure 7 - Normalised macroviscosity and surface tension values of the aqueous micellar fractions of different food matrices after digestion with varying cationic mineral concentrations.

This was steeper for the high pectin content systems i.e. apricot, tomato and carrot juice. Contrary to Ca^{2+} containing digesta, the apricot nectar – Mg^{2+} or Zn^{2+} 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 (Part 4, Figure 7 E-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.

4.5 Discussion

In the present study, we investigated the interaction of divalent minerals with carotenoids from various food matrices during simulated GI digestion. Previously, we found that the presence of Ca^{2+} and Mg^{2+}, 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 digesta (Corte-Real et al., 2016). The different food matrices chosen in the present study represented regularly consumed fruits and vegetables, providing the most common dietary carotenoids (Biehler et al., 2012) : 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 tomato (Biehler et al., 2012). Carotenoid bioaccessibility was reduced significantly in all matrices by the highest concentrations of Mg^{2+} and Ca^{2+} studied, to almost zero. This is similar

to what has been reported earlier by Biehler et al (2011), investigating micellarization of spinach-borne carotenoids digested with Ca^{2+}, Mg^{2+} 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 Ca^{2+} and Mg^{2+} generally resulted in reduced bioaccessibility of carotenoids following digestion. However, at lower concentrations (250 mg/L Ca^{2+}, 100 mg/L Mg^{2+}), 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 DM, but this remains speculative.

The negative effects of the DM 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 matrix (Corte-Real et al., 2016). 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 DM to unconjugated bile acids (Hofmann & Mysels, 1992) and FFA, forming bile salts of poor solubility and insoluble fatty acids soaps (Appleton, Owen, Wheeler, Challacombe, & Williamson, 1991; Lorenzen et al., 2007; Tadayyon & Lutwak, 1969), 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 FFA, 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 formation (Jones et al., 1986)

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 DM 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. FFA) 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 displaced (Wilde & Chu, 2011). 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 DM also have the ability to cross-link proteins (Etcheverry et al., 2012) and soluble fibres (Debon & Tester, 2001; Grant, Morris, Rees, Smith, & Thom, 1973), 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 neoxanthin (Britton et al., 2009), 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 (Corte-Real et al., 2016; Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012; Sy et al., 2012), 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 tissues (Britton et al., 2009) - chromoplasts (for carrot and tomato) vs. chloroplast

pigment-protein complexes (spinach and field salad) (Jeffery, Turner, et al., 2012; Ljubesic, Wrischer, & Devide, 1991; Rich, Faulks, Wickham, & Fillery-Travis, 2003), and other matrix constituents such as dietary fiber (Bohn, 2008). Furthermore, food processing (Hof & West, 2000) and different methods of disruption of the cell matrix also impact the release of carotenoids. Juicing of carrots has been shown to improve the bioaccessibility of carotenoids from the food matrix (Rich, Faulks, et al., 2003) compared to raw carrots, as a result of cell rupture, making carotenoids more accessible.

Phytoene and phytofluene were present in the tested juices and apricot nectar, where they contributed at least to a third to the total carotenoid content. Despite their high contribution to total carotenoid content of many different fruits and vegetables (Biehler et al., 2012), their appearance in blood plasma (Meléndez-Martínez, Mapelli-Brahm, Benítez-González, & Stinco, 2015), and reported bioactive aspects such as anti-oxidant activity (Engelmann, Clinton, & Erdman Jr, 2011), not much information is available on bioavailability aspects, including bioaccessibility. In the present investigation, phytoene and phytofluene fractional bioaccessibilities were surprisingly high, between 27% and 70%. Bioaccessibility nonetheless depended on the type of matrix. Carrot juice showed a significantly lower bioaccessibility of these colourless carotenoids than tomato juice and apricot nectar, while apricot nectar digestion resulted in significantly higher phytoene and phytofluene bioaccessibility, compared to the other two matrices. These results are in line with those found in a previous study (Mapelli-Brahm, Corte-Real, Meléndez-Martínez, & Bohn, 2017). Despite being similar to lycopene in its molecular structure, very different bioaccessibilities were encountered. Meléndez-Martínez et al. (2015) suggested that the number of conjugated double bonds (cDB) influences the shape of the molecule. Its high number in lycopene (11 cDB) would prevent molecule folding, while phytoene (3 cDB) and phytofluene (5 cDB) bonds are able to rotate more freely, assuming less rigid shapes. This could facilitate the incorporation of the colourless carotenoids into mixed micelles, explaining their higher bioaccessibility. Similarly, another study reported higher bioaccessibility of phytoene, from different food matrices, compared to β-carotene and even lutein, including in carrots and tomatoes (Jeffery, Turner, et al., 2012). Contrarily, phytoene bioaccessibility was comparable to that of

β-carotene from orange pulp and juice, perhaps due to the low amount of β-carotene present in those matrices, facilitating its solublity (Rodrigo, Cilla, Barbera, & Zacarias, 2015).

Regarding physicochemical properties, the higher macroviscosity of the apricot juice was likely due to the high content of pectin and/or due to higher total solids (> 14% w/w) and sugar content (> 13% w/w), promoting viscosity via decreasing the hydrodynamic free volume between the biopolymer molecules, triggering hydrophobic interaction or hydrogen bonding of side chain segments. In case of leafy vegetable matrices, their very low viscosimetric responsiveness is likely to be attributed to their higher content in insoluble fibre, compared to juices (Souci, Fachmann, & Kraut, 2000). Regardless of the food matrix, it is assumed that the observed macroviscosity reduction with increasing divalent mineral concentration was mainly induced by the precipitation of bile salt matter, depleting total solids. The rheological behaviour of the digesta were diverse, with apricot nectar – Mg^{2+} or Zn^{2+} binary systems undergoing structure conformational changes as suggested by the observed rheological behaviour transitions, e.g. pseudoplastic to Newtonian. The inflection point for apricot digesta, containing Mg^{2+} or Zn^{2+}, is also likely explained by its pectin content. It is well established that pectins (depending on their methyl ester content) may undergo electrostatic interactions (via the egg-box structure conformation) with DM, exerting a selective chemical affinity (for polygalacturonic segments) following the order: $Ca^{2+} > Zn^{2+} > Mg^{2+}$ (Bassett, Håti, Melø, Stokke, & Sikorski, 2016). This implies that electrostatic bridging of Ca^{2+} with pectins can be triggered at much lower concentrations compared to the other two cations. This would explain the absence of the inflection point in the case of apricot nectar – Ca^{2+} digesta samples. Upon increasing the cation concentration, an antagonistic ion binding action between bile salts and polygalacturonic segment occurs, with the latter being disfavoured, inducing a strong precipitation. As for surface tension, it is hypothesised that the bile salt binding capacity antagonism between DM and pectins played a buffering role on aggregative depletion phenomena. The amount of soluble pectin in the bulk phase appeared to be well corroborating the average depletion of surfactants i.e. 9% (Mg^{2+}) to 16% (Ca^{2+})

for carrot juice and apricot nectar and 20% (Mg^{2+}) to 26% (Ca^{2+}) for tomato juice, spinach and field salad.

4.6 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 DM on bioaccessibility appear as a result of a complex interaction between matrix, type of carotenoids and minerals and their concentration. Interestingly, concentrations of DM, much below the equivalent to the RDA for Ca^{2+} and Mg^{2+}, 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 DM doses. Further investigations *in vivo*, possibly also including other liposoluble dietary constituents are warranted to attest these observations.

4.7 Acknowledgements

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4.9 Supplementary Material

The following section of supplementary material compiles experimental and analytical results that were not included in the printed version of the scientific article, compiled in Part 4 of this mnuscript, but are nonetheless deemed important for the reader.

The succeeding material is presented:

- **Appendix 1**: Average bioaccessibility of carotenoids from different food matrices digested with divalent minerals at varying concentrations.
- **Appendix 2**: Cellular uptake trials results on the effect of DM on carotenoid *in vitro* bioavailability, conducted during the Master Thesis project of Marie Bertucci.

Appendix 1: Average bioaccessibility of carotenoids from different food matrices digested with divalent minerals at varying concentrations

Supplementary Table 1 - Average bioaccessibility (%)[+] of carotenoids from different food matrices digested with divalent minerals at varying concentrations*. Results represent mean ± SD (n =4).

Calcium (mg/L)	0		250		500		1000	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tomato Juice								
β-Carotene	6.00	0.57	8.23	0.30	2.09	0.10	0.00	0.00
Lutein	71.61	1.15	50.41	2.96	18.19	1.09	0.00	0.00
Lycopene	1.47	0.03	1.17	0.06	0.22	0.03	0.00	0.00
Phytoene	47.56	3.36	56.14	3.95	16.05	0.82	0.00	0.00
Phytofluene	40.23	2.36	49.09	2.60	13.47	0.91	0.00	0.00
Carrot Juice								
β-Carotene	5.81	.58	11.41	0.16	6.83	0.06	0.051	0.09
α-Carotene	14.64	3.05	31.33	0.40	23.33	0.39	1.57	0.26
Lutein	82.72	3.90	82.95	1.90	83.45	1.81	11.00	0.37
Phytoene	21.06	5.27	57.76	2.12	52.46	1.77	0.00	0.00
Phytofluene	18.51	4.45	49.79	1.01	50.44	1.12	0.00	0.00
Apricot Nectar								
β-Carotene	6.62	0.28	12.07	0.31	7.08	0.33	0.00	0.00
Phytoene	46.77	4.70	63.87	2.57	38.36	2.46	0.00	0.00
Phytofluene	46.86	1.54	63.92	2.03	37.34	0.65	0.00	0.00
Spinach								
β-Carotene	10.76	0.12	6.22	1.37	0.00	0.00	0.00	0.00
Lutein	13.92	0.21	6.94	1.13	0.07	0.00	0.00	0.00
Neoxanthin	21.38	0.39	13.79	0.68	0.17	0.01	0.00	0.00
Field Salad								
β-Carotene	6.05	0.30	6.40	0.14	0.89	0.06	0.00	0.00
Lutein	6.36	0.10	7.17	0.27	1.38	0.07	0.00	0.00
Neoxanthin	4.65	0.28	5.83	0.21	1.23	0.06	0.00	0.00

Magnesium (mg/L)	0		100		200		300	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tomato Juice								
β-Carotene	15.03	1.35	11.17	0.68	5.56	0.05	0.00	0.00
Lutein	92.50	6.15	69.50	0.98	37.68	3.99	0.00	0.00
Lycopene	5.09	0.54	3.40	0.14	1.13	0.02	0.00	0.00
Phytoene	59.06	4.02	46.56	1.24	24.29	2.44	0.94	0.21
Phytofluene	73.05	6.32	57.55	1.08	27.64	1.93	2.16	0.06
Carrot Juice								
β-Carotene	17.69	2.21	19.62	0.40	4.66	0.56	0.00	0.00
α-Carotene	26.43	3.52	32.46	0.65	9.99	1.85	0.00	0.00
Lutein	60.71	3.12	60.57	1.71	24.42	0.21	0.00	0.00
Phytoene	27.06	3.99	35.70	1.19	13.45	0.54	0.00	0.00
Phytofluene	39.44	2.85	51.88	0.28	16.57	1.12	0.00	0.00
Apricot Nectar								
β-Carotene	20.00	0.84	2.95	0.20	0.00	0.00	0.00	0.00
Phytoene	46.94	2.99	3.64	4.21	0.00	0.00	0.00	0.00
Phytofluene	54.44	4.04	0.00	0.00	0.00	0.00	0.00	0.00

Spinach								
β-Carotene	9.81	1.16	10.28	0.89	10.88	0.06	7.87	0.13
Lutein	9.19	0.62	8.24	0.78	9.13	0.03	5.93	0.08
Neoxanthin	16.05	1.13	13.63	1.25	14.90	0.14	9.70	0.91
Field Salad								
β-Carotene	29.43	1.90	20.37	0.46	6.90	0.95	0.00	0.00
Lutein	26.16	1.89	16.61	0.43	6.84	1.05	0.88	0.06
Neoxanthin	23.93	1.18	15.85	0.68	7.15	0.83	1.41	0.08

Zinc (mg/L)	**0**		**12.5**		**25**		**50**		**100**		**200**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tomato Juice												
β-Carotene	15.00	0.55	14.32	0.13	15.19	0.91	14.05	0.18	11.77	0.37	9.51	0.65
Lutein	95.42	3.52	89.28	0.76	92.09	0.25	85.97	2.51	69.99	1.56	58.90	1.26
Lycopene	4.16	0.33	3.90	0.33	3.84	0.20	3.43	0.18	2.71	0.14	3.13	1.03
Phytoene	62.09	2.41	59.16	0.39	65.00	1.54	57.79	0.74	49.23	0.56	38.38	1.65
Phytofluene	77.74	8.67	78.55	0.60	80.90	3.38	72.88	1.82	62.60	0.95	47.42	0.80
Carrot Juice												
β-Carotene	13.13	1.55	15.41	0.32	15.88	0.57	16.83	0.34	12.39	0.41	7.48	0.44
α-Carotene	23.70	2.34	28.63	2.63	29.50	1.03	31.10	0.59	22.79	0.92	15.87	1.49
Lutein	88.41	5.42	81.62	2.70	80.97	0.18	76.02	0.86	68.40	0.62	54.74	2.51
Phytoene	31.23	2.55	36.23	3.94	36.81	2.10	38.12	2.31	28.76	1.18	22.74	1.62
Phytofluene	52.50	4.63	59.42	5.53	62.47	1.15	64.99	1.83	45.43	1.40	35.33	1.53
Apricot Nectar												
β-Carotene	20.93	0.37	19.82	2.26	17.84	0.93	16.28	1.78	12.59	1.06	0.00	0.00
Phytoene	22.65	1.32	20.47	1.64	18.40	2.26	15.95	0.85	10.99	2.71	0.00	0.00
Phytofluene	12.55	0.72	11.42	0.90	10.23	1.21	8.90	0.44	5.47	0.78	0.00	0.00
Spinach												
β-Carotene	4.37	0.55	5.68	0.66	6.90	0.45	7.11	0.32	7.66	0.30	7.02	0.11
Lutein	5.98	1.05	6.76	0.59	6.19	0.27	6.14	0.83	7.17	0.10	6.60	0.45
Neoxanthin	8.84	1.17	10.20	0.92	8.76	0.54	9.10	1.37	10.52	0.56	10.12	0.69
Field Salad												
β-Carotene	16.58	1.20	24.42	2.66	27.71	3.89	30.53	0.74	28.36	0.67	24.27	0.28
Lutein	16.35	0.71	22.36	1.72	25.86	3.20	24.06	0.77	22.53	0.92	18.90	0.28
Neoxanthin	14.73	1.98	15.66	0.81	19.69	1.88	19.92	0.42	18.05	0.18	17.32	0.09

Sodium (mg/L)	**0**		**375**		**750**		**1500**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tomato Juice								
β-Carotene	4.03	1.86	5.07	1.17	6.83	0.42	8.42	0.93
Lutein	58.99	0.96	56.46	3.27	61.63	1.95	70.14	7.94
Lycopene	1.07	0.59	1.58	0.38	1.82	0.08	2.00	0.41
Phytoene	28.96	11.10	37.42	8.22	49.37	3.08	63.40	8.50
Phytofluene	23.19	9.64	29.20	5.90	38.79	3.79	57.60	4.44
Carrot Juice								
β-Carotene	3.98	0.26	4.96	0.97	6.89	0.27	12.76	2.87
α-Carotene	8.61	0.57	10.86	2.09	16.15	1.39	28.98	6.51
Lutein	74.96	0.36	77.33	6.55	89.00	9.29	92.93	3.02
Phytoene	12.50	0.77	15.51	2.14	22.78	1.66	41.84	3.55
Phytofluene	8.01	0.84	9.94	1.40	15.01	0.79	26.10	5.22
Apricot Nectar								
β-Carotene	9.77	0.21	10.27	0.26	11.27	0.28	11.36	0.49

Phytoene	67.25	0.76	74.33	2.17	84.96	2.18	88.36	3.81
Phytofluene	65.08	2.66	66.77	1.52	81.30	1.37	80.70	6.34
Spinach								
β-Carotene	8.13	1.16	7.46	0.46	7.13	1.11	8.22	0.96
Lutein	11.37	0.27	10.47	0.82	12.03	1.50	10.87	0.68
Neoxanthin	16.98	0.53	16.82	0.79	15.69	0.51	16.14	0.46
Field Salad								
β-Carotene	7.81	0.30	10.27	0.21	7.02	1.17	7.00	0.23
Lutein	8.09	0.05	8.43	0.13	6.25	0.23	5.68	0.09
Neoxanthin	6.55	0.13	6.67	0.33	5.16	0.36	4.28	0.11

*Concentrations given in final digestion stage (small intestine)
+Bioaccessibility expressed as percentage of originally present carotenoids before digestion

Appendix 2: Cellular uptake trials results on the effect of DM on carotenoid in vitro bioavailability, conducted during the Master Thesis project of Marie Bertucci.

Background

Cellular uptakes trials were carried to study in order to access the potential effects of varying concentrations of DM on the *in vitro* bioavailability of dietary carotenoids. Due to time management reasons, only two food matrices and two minerals were investigated. Spinach leaves were chosen as a representation of a green leafy matrix, and also as the *in vitro* counterpart to the *in vivo* bioavailability studies (Part 5). Carrot juice was therefore chosen to represent a differently processed food matrix, in this liquid. Calcium was chosen as the main DM to be investigated in the *in vitro* trials, as it was also chosen for the subsequent *in vivo* bioavailability studies (Part 5). Sodium was hence taken as a control monovalent cation.

Experimental

The TC-7 subclone of the Caco-2 cell line (donation from Dr. M. Rousset, Nancy University, France) was seeded at a density of 5x104 cells/m2 in Falcon® 6-well plates two weeks before exposure to the digested matrices, to allow cells to reach confluency and differentiate into an enterocyte-like phenotype. Cellular toxicity assays were performed to find the better dilution ratio for the digestas, in order to prevent bile salt induced cellular toxicity. Results showed that after 4 hours of incubation the ratio of 1:5 allowed the least dilution with lower toxicity, as measured by cell viability employing the Alamar blue test (Rampersad, 2012). On the day of exposure, cells were incubated with 3 ml of a pooled digesta (n = 4 replicates) and DMEM+GlutaMax® (1:5, v:v). Each condition was tested in 4 replicates. Digestas where concentration of added mineral was 0 mg/L was considered as the positive control, and cells incubated with DMEM+GlutaMax® alone were taken as a negative control.

Caco-2 cells were incubated for 4 hours, at 37°C and 10% CO2, which is the time needed to simulate physiological intestinal passage. After the incubation periods, the wells were washed once with 2 ml of cold PBS, including 2 mg/ml of bile extract to remove potential carotenoids attached to the cell membrane, and a second time with 2 ml of pure cold PBS. To detach cells from the bottom of the

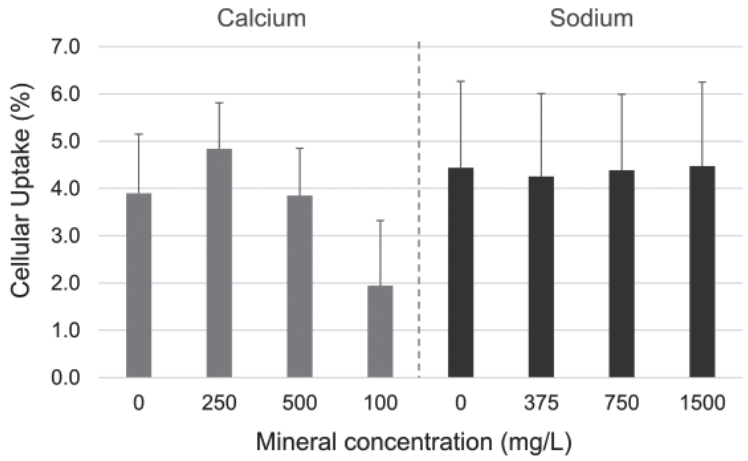
wells, 0.7 mL of trypsin was added to each well, and left to act for 5 min (at 10% CO2 at 37°C). Trypsin was then inactivated by the addition of 1.5 ml of DMEM + GlutaMax®, and cells were re-suspended and collected in 50 mL tubes. Replicates were pooled and centrifuged at 300 g for 5 min. Following centrifugation, the supernatant was discarded and cells were re-suspended in ice cold water (2 ml for each treatment and 4 ml for each control) and incubated in the dark on ice for 20 min to lyse cells by osmotic stress. In order to facilitate the rupture of the cell membrane, tubes were sonicated for 4 minutes, after the incubation period, and vortexed (40s). Cell exposure assays were carried out in 3 biological replicates (corresponding to 3 independent days).

For carotenoid extraction from the Caco-2 cells, 4 ml of hexane-acetone (1:1, v:v) were added to the cellular lysate and tubes were sonicated (2 min) and vortexed. To achieve phase separation, tubes were centrifuged at 4000 g at 4°C for 2 min. The hexane phase was transferred into a new 15 ml tube. A second extraction was carried out with 4 ml of hexane and a third one with 3 ml of diethyl ether. Organic phases were combined in the 15 ml tubes and dried under a stream of nitrogen for 45 min at 25°C. Dried extracts were flushed with Argon and stored at -80°C until analysis.

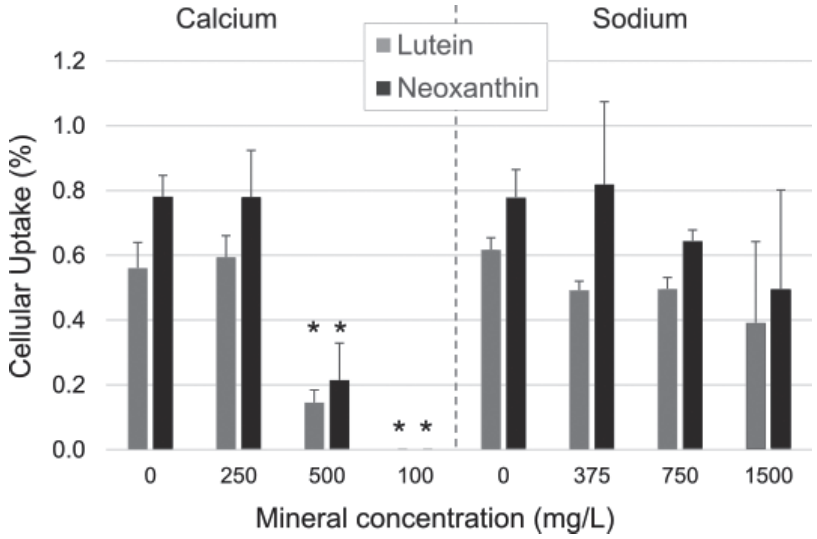
Results

Cellular uptake of carotenoids from carrot juice was low (Supplementary Figure 1), below 5%, and only lutein was detected. There was no effect of neither the type ($P = 0.06$) nor of the mineral concentration ($P = 0.52$).

Cellular uptake of carotenoids was lower than that of carrot juice, below 1%. Both neoxanthin and lutein were significantly ($P < 0.001$) affected by the concentration of Ca^{2+}, specifically at concentrations ≥ 500 mg/L (**Supplementary Figure 2**).



Supplementary Figure 1 – Average Caco-2 cellular uptake of lutein from carrot juice after digestion with varying concentrations of calcium and sodium. Error bars represent standard deviation from 3 replicates. No statically significant (P > 0.05) effect was found for the effect of the mineral concentration on the cellular uptake of lutein.



Supplementary Figure 2 - Average Caco-2 cellular uptake of lutein and neoxanthin from spinach leaves after digestion with varying concentrations of calcium and sodium. Error bars represent standard deviation from 3 replicates. * represents a statically significant (P < 0.05) effect of the mineral concentration on the uptake of carotenoid when compared to the control condition (i.e. concentration is 0 mg/L).

5. NO INFLUENCE OF SUPPLEMENTAL DIETARY CALCIUM INTAKE ON THE BIOAVAILABILITY OF SPINACH CAROTENOIDS IN HUMANS

Preamble

The following chapter has been published in the “British Journal of Nutrition”.

The results from the *in vitro* trials have showed that divalent minerals, at concentrations that could be equivalent to those in dietary supplements, had a negative effect on the bioaccessibility of carotenoids, both pure and from dietary sources, and on the cellular uptake of dietary carotenoids. Nevertheless, these results remained to be validated *in vivo*. Hence the objective of the work described in this chapter was to perform a human trial that would allow testing the hypothesis of the project *in vivo* and validating, or not, the results obtained in the *in vitro* trials.

The planning and execution of the human trial was the product of the collaboration between the Clinical and Epidemiological Investigation Center (CIEC) of the Luxembourg Institute of Health (former CRP Santé), and the Luxembourg Institute of Science and Technology (former CRP Gabriel Lippmann). Clinical visits and sample collection were performed at the premises of the CIEC, with the help of trained research nurses. Blood biochemistry and hematology analysis were done by *Laboratoires Reunis*, a private laboratory for clinical analysis. Extraction, and HPLC analysis of carotenoids were done by myself with the help of the laboratory technician Boris Untereiner.

Full Reference:

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No influence of supplemental dietary calcium intake on the bioavailability of spinach carotenoids in humans

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5.1 Abstract

Dietary carotenoid intake, especially from fruits and vegetables, has been associated with a reduced incidence of several chronic diseases. However, its bioavailability can vary, depending on the food matrix and host factors. Recently, it has been suggested that divalent minerals negatively impinge on carotenoid bioavailability by reducing bile salt and non-esterified fatty-acid levels in the gut, which normally aid in emulsifying carotenoids. The aim of the present study was to investigate whether supplemental Ca^{2+} would negatively influence carotenoid absorption in humans. A total of twenty-five healthy, non-obese men (age: 20–46 years, BMI < 30 kg/m2) were recruited for this postprandial, randomised, crossover, double-blinded trial. Following a randomised block design, each participant received (after 2-week washout periods), on three occasions separated by 1 week, 270 g of spinach-based meals (8·61 (SD 1·08) mg carotenoids/100 g fresh weight), supplemented with 0, 500 or 1000 mg of Ca^{2+} (as calcium carbonate), with each participant acting as his or her own control. Blood samples were collected at regular postprandial intervals for up to 10 h following test meal intake, and standardised lunches were served. TAG-rich lipoprotein fractions were separated and carotenoid concentrations determined. AUC for meals without supplemented Ca^{2+} were 22·72 (SEM 2·78) nmol × h/l (lutein), 0·19 (SEM 3·90) nmol × h/l (β-carotene) and 2·80 (SEM 1·75) nmol × h/l (β-cryptoxanthin). No significant influence of supplementation with either 500 or 1000 mg of supplemental Ca^{2+} was found. In conclusion, Ca^{2+} – the most abundant divalent mineral in the diet –

given at high but physiological concentrations, does not appear to have repercussions on the bioavailability of carotenoids from a spinach-based meal.

5.2 Introduction

Carotenoids are natural pigments that can be produced by plants, bacteria and certain fungi, but not by humans. Their dietary intake, especially in the form of fruits and vegetables, as well as circulating tissue levels have been associated in epidemiological studies with not only the reduced incidence of several chronic diseases including cardiometabolic complications (Hamer & Chida, 2007; Osganian et al., 2003) and some types of cancer (Key et al., 2015; Li & Xu, 2014), but also with all-cause mortality (Buijsse et al., 2005), though their contribution to vitamin A intake, especially in developing countries and for vegetarians, is also important (Faber & Laubscher, 2008; D. Weber & Grune, 2012). Furthermore, low carotenoid intake has been associated with age-related macular degeneration (Liu et al., 2015; Wang et al., 2014), the major cause of blindness in the elderly.

The exact mechanism by which carotenoids act on the host is unknown, though direct antioxidant properties (Krinsky & Johnson, 2005) and anti-inflammatory as well as antiapoptotic effects produced by acting on gene transcription (Kaulmann & Bohn, 2014) may play a role. However, the bioavailability of carotenoids is extremely variable, depending on carotenoid type, dietary (i.e. matrix-related) factors (Bohn et al., 2015; Borel, 2003) and host factors (Bohn, 2016; Borel, 2012). For instance, a diet rich in lipids and low in dietary fibre has been proposed to increase the bioavailability of carotenoids (Bohn, 2008; Unlu & Bohn, 2005), mainly by improving bioaccessibility – that is, the fraction of carotenoids that are available for cellular uptake in the small intestine (Biehler & Bohn, 2010) – as both conditions increase the fraction of emulsifiable carotenoids, a prerequisite for their absorption.

Recently, it has been suggested that dietary DM may negatively impinge on carotenoid bioavailability, as they could bind to bile salts and non-essential fatty acids (NEFA) in the gut, resulting in their precipitation and unavailability for emulsification. This emulsification is crucial in order to allow for mixed micelle formation and assure solubilisation of the lipophilic carotenoids. However, so far, this negative effect has only been demonstrated *in vitro*, following simulated gastrointestinal digestion

(Corte-Real et al., 2016), and also following carotenoid cellular uptake into Caco-2 cells, a frequently employed model of the small intestinal lining (Biehler, Hoffmann, et al., 2011). In addition, concentrations required to reduce carotenoid bioaccessibility and cellular uptake have been shown to be quite high, possibly not reachable by trace elements, not even in supplement form. However, a high but physiological intake of Ca^{2+} and Mg^{2+}, such as those obtained via supplements, at concentrations reflecting approximately 33 and 100 % of the RDA (1000 mg for Ca^{2+} and 400 mg for Magnesium for adult males), respectively (Institute of Medicine, 2005), have been shown to reduce micellarization and cellular uptake by over 60 % (Biehler, Hoffmann, et al., 2011). The observed reductions in carotenoid bioaccessibility were further well associated with increased surface tension and reduced viscosity *in vitro*, also suggesting precipitation of emulsifying compounds as the likely mechanism (Corte-Real et al., 2016). However, as the *in vitro* methods that have been employed thus far represent rather static models – that is, not accounting for potential dynamic adaptations of the human body with respect to enzyme and bile salt secretions – such findings are rather suggestive and do not constitute definite proof.

In the present human study, we aimed to prove or disprove the hypothesis that dietary DM, and specifically Ca, are able to hamper the emulsification and thus the bio- availability of carotenoids from a vegetable matrix. For this purpose, Ca^{2+} was given in the form of supplements together with a spinach meal, and the TAG-rich lipoprotein (TRL) fraction, representing newly absorbed carotenoids, was measured post-prandially over time.

5.3 Methods

5.3.1 Chemicals

Unless otherwise stated, all chemicals used were of analytical or superior quality and were procured from Sigma-Aldrich. Carotenoid standards were from Sigma-Aldrich (β-carotene, lycopene, internal standard trans-β-apo-8'-carotenal) and Carotenature (lutein, β-cryptoxanthin, violaxanthin, neoxanthin, phytofluene). Only 18 MΩ (Millipore) water was used.

Calcium capsules and the placebo were produced by a private company, Econophar SPRL and contained either 250 mg of elemental Ca^{2+} (as carbonate) or 560 mg of mannitol, respectively. Formulation of Ca^{2+} capsules followed that of a commercially available Ca^{2+} supplement, Steocar 250 mg gelatine capsules from Nycomed.

5.3.2 Participants

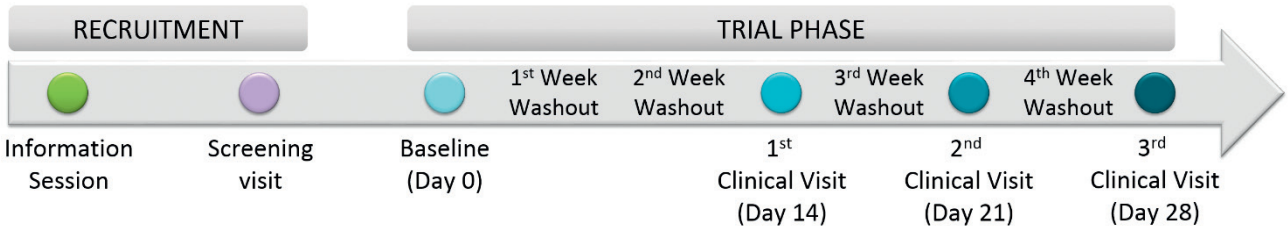
A total of twenty-five male participants were recruited for this study. For this purpose, flyers were distributed at various places in Luxembourg and an advertisement was also issued via email and word-of-mouth. Inclusion criteria were: being male, being between 18 and 50 years of age, living independently in Luxembourg, being healthy and not being obese (BMI < 30 kg/m^2). Exclusion criteria were having any chronic diseases, use of antibiotics during the past 6 months, regularly taking supplements, being vegetarian/vegan, being obese (BMI > 30 kg/m^2), consuming more than 2 units of alcohol/d regularly, engaging in regular physical exercise for >150 min/d, having a haematocrit value <35 % or a Hb level <12 g/100 mL, having fasting blood glucose concentrations >120 mg/dL, being hyperlipidaemic (having both total cholesterol and TAG concentrations >200 mg/dL), having any food allergies, being a current smoker or having a recent smoking history (past 2 years) or typically consuming >five portions of fruits/vegetables per day. Subjects with any history of malabsorption or surgery in the digestive tract were also excluded. The study was approved by the National Research Ethics Committee (CNER, www.cner.lu) of Luxembourg (Protocol 201305/05), and was conducted according to the Declaration of Helsinki. The study was carried out at the Clinical Investigation and Epidemiological Center (CIEC) of the Luxembourg Institute of Health.

5.3.3 Study design

For recruitment, all subjects were informed in detail about the study design as well as its risks and benefits, and those willing to participate signed a written informed consent form. At a first eligibility screening (Part 5, Figure 1), subjects were asked to fill out a ‘Health and Lifestyle’ questionnaire, and their height, weight, body fat (percentage by impedance method, Touch II balance; Ozeri) and waist

and hip circumferences were measured. Subjects were considered eligible if they did not meet any of the exclusion criteria, and were scheduled for a second screening. A fasting blood sample was collected for haematology and lipid-profile analysis (TAG, total cholesterol, LDL-cholesterol and HDL-cholesterol). Subjects also donated a urine spot-sample to screen for any physiological abnormalities and to assure eligibility to participate in the trial. For this purpose, GAK Combi Screen Plus 9 strips were obtained from Analytikon, allowing for testing of glucose, ketones, ascorbic acid, pH, leucocytes, nitrite, proteins, blood and bilirubin in a urine spot-sample. Subjects considered eligible at this stage were enrolled for the trial phase, and will be referred to as participants henceforth.

The trial phase had a total duration of 4 weeks, which included an initial 2 weeks of washout (i.e. participants were asked to restrict themselves from consuming carotenoid-containing foods as much as possible) and three clinical visits that were 1 week apart, during which participants were asked to continue the washout diet. A randomised block design with all six possible combinations of Ca^{2+} intake sequences was employed (high, medium–low v. medium–high–low, etc., with four participants per block). Randomisation was achieved by lots, carried out by the statistical group of the Department. Both participants and all personnel included in carrying out the trial were blinded. The study, from recruitment to finalisation, was carried out between July 2012 and July 2015.



Part 5, Figure 1 -Design of the clinical trial.

At the start of the trial phase, participants visited the CIEC premises to give a 12 h fasting blood sample for assessment of glucose, blood lipids, Ca^{2+} and plasma carotenoid levels at the beginning of the washout phase. During the following 2 weeks, participants were asked to follow a diet low in carotenoids. For this purpose, participants received a list with foods to avoid and a list with foods

allowed. This included not only frequently consumed food items rich in carotenoids, such as green leafy vegetables, bell peppers, tropical fruits and some local fruits such as plums and apricots as well as tomato and tomato products but also certain animal products such as egg yolk and salmon. The list of allowed foods was to ensure suggesting alternatives, including onions, apples, pears and other food items typically low in carotenoids (<approximately 0·5 mg/100 g). In addition, participants received a booklet and were asked to enter major meals on a daily basis. Following the washout phase, participants arrived for their first clinical visit. After an overnight fast (12 h), participants reported to the general clinical research facility between 06.30 and 07.00 hours. Upon arrival, the participant's weight and body fat (%) were measured and a catheter was placed into the arm of the participant by a trained nurse. A first blood sample (20 ml) was obtained and investigated for plasma carotenoids, TRL carotenoids, blood lipids, blood glucose and Ca^{2+}. For this purpose, separate Vacuette® (Greiner Bio-One GmbH) type tubes were employed. For carotenoid analyses, 10 ml Vacuette K-EDTA tubes were used.

Participants were then given the test meal, which had to be consumed within 30 min. Following this, successive blood samples (20 mL) were collected postprandially at 2, 3, 4, 5, 6, 8 and 10 h. At noon, a standardised lunch was served. Following the 10 h clinical stay, a complimentary snack was offered – that is, the participants were free to choose from various sandwiches. During the entire clinical visit, no own foods or beverages were allowed. Participants stayed under medical supervision on the premises of the CIEC postprandially, for 10 h.

Following this clinical visit, each participant maintained an additional washout time of 1 week, before visiting the CIEC premises for the second full clinical day, and a similar procedure was followed for a third visit. In total, each participant had three full stays at the CIEC premises, receiving either the meal with 500 or 1000 mg of elemental Ca^{2+}, or the Ca^{2+}-free placebo. Thus, the intervention differed only in the amount of Ca^{2+} supplemented. Doses were chosen so as to reflect approximately the half and full RDA of Ca^{2+}, being 500 and 1000 mg (for healthy males/females aged 19–50 years), respectively. All other meals and beverages served during the clinical days were identical.

5.3.4 Test meals

Each participant received for breakfast one glass of water (300 ml) low in minerals (total mineral content <40·0 mg/l, with a Ca^{2+} content of 1·0 (SD 0·3) mg/l), 270 g of microwave-heated spinach to which 18 ml of rapeseed oil was added in order to foster carotenoid bioavailability, and 40 g toasted (white wheat) bread served with 20 g cream cheese (17·5 % fat). Spinach was chosen despite its content of dietary fibre and oxalic acid, as it is a frequently consumed vegetable rich in carotenoids (Souci et al., 2000), and as it was the focus of several earlier *in vitro* trials (Biehler, Hoffmann, et al., 2011; Biehler, Kaulmann, et al., 2011; Corte-Real, Bertucci, et al., 2017) with which we wished to compare our results. Nutrient composition was estimated from information given on the labels, and, if not available, by employing a local database as well (Souci et al., 2000). Each test meal contained approximately 1255 kJ (300 kcal) energy, 18 g fat, 21 g carbohydrates, 13 g proteins, 8 g dietary fibre and 390 mg Ca. Together with the meal, four capsules containing the placebo, 500 or 1000 mg of elemental Ca^{2+} were given, which were swallowed with the water, after the spinach portion had been consumed. Thus, the three test meals differed only in their total Ca^{2+} content, which was approximately 390 mg for the placebo meal, 890 mg for the 500 mg Ca^{2+} dose and 1390 mg for the 1000 mg Ca^{2+} dose. Calcium was given in the form of carbonate, which is among the most used Ca^{2+} supplements. The total carotenoid content in the morning test meal was approximately 23 mg (Part 5, Table 1). Considering the content of provitamin A carotenoids, it can be estimated that this portion delivered approximately 10 % of the daily vitamin A requirements (RDA) (Food and Nutrition Board, 2011). For lunch, each participant received 300 mL of water with low mineral content, one banana, one toasted sandwich (white wheat bread, approximately 60 g) with approximately 60 g turkey and butter (10 g) and one portion of Greek yogurt (2·5% fat level, 140 g), containing a total of 2188 kJ (523 kcal) energy, 22 g protein, 24 g fat, 52 g carbohydrates and 4 g of fibre. The Ca^{2+} content was approximately 207 mg. Water with low mineral content (Ca^{2+} content approximately 1 mg/L) was allowed between meals upon request.

Food items were purchased at local supermarkets (CORA SA or CACTUS SA). Spinach was purchased in frozen form from the same batch and stored in the freezer at − 20°C until the day before serving. Aliquots of spinach were kept for later analysis of carotenoid content.

Part 5, Table 1 - Average content of the individual carotenoids in the spinach meal (mean values and standard deviations; n = 23)

	Content per 100 g (mg/100 g)		
Carotenoids	**Mean**	**SD**	**Content in test meal**
Lutein	5.59	0.69	15.10
β-Carotene	0.38	0.34	1.02
Neoxanthin	0.72	0.11	1.94
Violaxanthin	1.87	0.40	5.05
Sum	8.61	1.08	23.25

5.3.5 Sample processing

Samples were collected according to the study design (see above), and were either processed *in house* (carotenoids) or by an external commercial laboratory (accredited according to ISO 15189) for glucose, Ca^{2+}, lipids and haematology (*Laboratoires Réunis*). This external laboratory was accredited in 2006 and undergoes periodic surveillance by an accreditation committee from Luxembourg. Different kinds of Vacuette® tubes were employed depending on the type of analysis to be performed: Vacuette K-EDTA tubes were used for carotenoid and haematology analysis; Vacuette Na-Fluoride tubes were used for glucose analysis; and Vacuette Serum Gel tubes were used for the analysis of lipids. The latter were centrifuged on site, according to laboratory guidelines. In short, the tubes were left standing (upright position) for 30 min to allow clotting. The tubes were then centrifuged for 10 min at 1670 g, at 20°C, and maintained at room temperature until pickup. Quantitative determination of TAG was carried out using the Roche/Hitachi Cobas C analyser following the manufacturer's instructions (Roche Diagnostics).

All blood samples collected for later carotenoid determination were left standing for 5 min and spun down for 6 min at 20°C in a Sigma 2-16KC centrifuge (©Thermo Fisher Scientific Inc.) at 2060 g. Following separation of the blood plasma, the TRL fraction rich in chylomicrons was separated

similarly as described earlier (Bohn et al., 2011; Unlu, Bohn, Francis, Clinton, & Schwartz, 2007). In short, 2·9 ml of plasma was overlaid with 1·4 ml of a 1·006 g/L NaCl solution in a 4 mL propylene conical™ Quick-Seal® (Beckman Coulter) tube, and TRL fractions were separated at 170 000 g for 1 h at 4°C in a Beckman Optima™ C-90U Ultracentrifuge (Beckman Coulter) with a SW 40-Ti titanium swinging-bucket rotor. The lower plasma phase was removed by puncturing the vials, and the upper TRL fraction (0·5 mL) was separated and brought up to 1·5 ml using 0·9 % NaCl, frozen in N_2 and stored at −80°C. Likewise, plasma samples were stored at −80°C for carotenoid analyses.

5.3.6 Extraction of carotenoids

Carotenoids were extracted from spinach samples as described earlier (Corte-Real, Bertucci, et al., 2017). Blood plasma and plasma TRL fraction extraction protocols were adapted from Unlu et al. (2007). In short, after the addition of 3.25 ml ethanol containing 0.1 % butylated hydro-xytoluene (BHT) to approximately 1.5 ml of TRL fraction or to 1.0 mL of plasma, fractions were left to stand on ice for 5 min and were spun down at 4°C for 2 min, at 600 g. The supernatant was collected into a new tube and the precipitates were re-extracted with 3 mL of diethyl ether–hexane (1:2, v/v) containing 0.02 % BHT. Samples were centrifuged at 1250 g for 2 min at 4°C. Supernatants were combined, to which 2 mL of saturated NaCl wa added. Samples were extracted once with 4 mL of hexane + BHT (0.02 %), and a second time with 3 mL diethyl ether–hexane (1:2, v/v). Extracts were spun down for 1 min at 1250 g to remove any water remnants, transferred into new tubes and evaporated to dryness under a stream of N in a TurboVap LV (Biotage) apparatus. Tubes were then flushed with Ar, and samples were stored at −80°C until analysis.

5.3.7 Carotenoid analysis

Before measurements, samples were reconstituted with methanol–methyl-tert-butyl-ether (7:3) in volumes of 600 µL (plasma samples), 100 µL (TRL fraction) or 6 mL (spinach extracts). During this procedure, an aliquot of internal standard (trans-β-apo-8'-carotenal) was also added, to obtain a final concentration of 1 µg/mL (spinach and plasma extracts) or 0.1 µg/mL (TRL extracts). Carotenoid

analyses were carried out using liquid chromatography as reported previously (Corte-Real, Bertucci, et al., 2017). In brief, separation was achieved using an Agilent 1260 Infinity U-HPLC instrument, in combination with gradient elution. Eluents were: (A) water–MeOH (60:40) containing 30 mM of ammonium acetate, and (B) acetonitrile–dichloromethane (85:15). 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; 36 min, 42 % B. An Accucore C30 column (2.6 µm particle size, 2.1 mm i.D.100 mm length; Thermo Fisher Scientific Inc.) at 28 °C was used for separation of carotenoids. The injection volume was 10 µL. Carotenoids were detected using a coupled UV/vis photodiode array detector and identified according to their retention times and spectral data, based on comparison with the corresponding individual standard. All peaks were integrated manually at 350 nm (phytofluene), 440 nm (neoxanthin and violaxanthin), 450 nm (lutein and α-carotene), 455 nm (β-carotene, β-cryptoxanthin and internal standard) and at 470 nm (lycopene), according to each carotenoid's absorption maxima. Quantification was achieved using the internal standard method, with trans-β-apo-8'-carotenal used as the internal standard.

5.3.8 AUC and C_{max}

The postprandial AUC of time v. concentration of the respective carotenoids extracted from TRL fractions was determined on the basis of seven postprandial time points plus the baseline (fasting state, before test meal intake). Concentrations of carotenoids were determined using the internal standard method. The AUC was then determined from baseline-corrected (each concentration was subtracted from its baseline value) values using the trapezoidal method. Individual C_{max} values reflect the highest carotenoid concentrations measured in the TRL fraction of one participant on one clinical day, irrespective of the time point, and these were used to calculate the average C_{max}.

5.3.9 Chemicals, minerals and carotenoid standards

Unless otherwise stated, all data are reported as means and standard deviations. Normality and equality of variance of AUC data reflecting bioavailability were tested using normality plots and box

plots, respectively. For the sample size, it was estimated that, even following several drop-outs, twelve to fifteen participants would provide sufficient statistical strength to observe a difference in carotenoid absorption. For example, a difference in carotenoid absorption of approximately 5 % (e.g. 10 v. 15 %) could be resolved in a pairwise design with a relative standard variation of fractional (%) absorption of approximately 50 % for an α of 0.05 and a power of 80 % (http://www.quantitativeskills.com/sisa/calculations/samsize.htm). With twenty-three participants, an absorption of 3.5 % (11 v. 14.5 %) could be detected with an α of 0.05 and a power of 80 %. For comparing the effect of Ca^{2+} dosing, a generalised linear univariate model was employed for each carotenoid and TG, with baseline-corrected AUC as the observed dependent variable, and carotenoid (lutein, β-carotene, β-cryptoxanthin), participant, sequence of intervention (visits 1, 2 or 3) and supplemental- Ca^{2+} dose (0, 500, 1000 mg) as the independent, fixed factors. TRL baseline concentration (time 0 h on each test day, i.e. before test meal intake) of each respective carotenoid was also included as a covariate. Following significant Fisher's F tests, all pairwise comparisons were carried out using Fisher's protected LSD (least significance difference) test. Values with $P < 0{\cdot}05$ (two-sided) were regarded as statistically significant.

5.4 Results

5.4.1 Drop-out and arbitrary effects, description of participants

A total of twenty-five healthy and free-living male participants, between 20 and 46 years of age, were enrolled in the study. The BMI of the participants ranged from 16.9 to 29.5 (average 24.8 (SD 2.8) kg/m^2), and the measured body fat (%) was between 2.0 and 22.1 (average 13.3 (SD 4.9) %). Total cholesterol and TAG concentrations ranged from 118 to 305 (average 179.7 (SD 44.8) mg/dL) and from 36 to 207 (average 81.0 (SD 41.4) mg/dL), respectively (Part 5, Table 2). Despite some elevated cholesterol (n = 6) and TAG values (n = 1) observed, the participants were still admitted into the study under the advice of a medical doctor. No participant had both TAG and total cholesterol concentrations >200 mg/dL.

Part 5, Table 2 - Anthropometric characteristics and fasting blood biochemistry from twenty-five male participants, at the time of recruitment (Mean values and standard deviations)

	Mean	SD	Min	Max
Anthropometric Measures				
Age	30	8	20	46
Height (cm)	176.9	5.3	167.0	185.0
Weight (Kg)	77.5	10.0	52.3	97.7
BMI (kg/m^2)	24.8	2.8	16.9	29.5
Body fat (%)*	13.3	4.9	2.0	22.1
Waist (cm)	85.9	8.4	66.0	102.0
Hip (cm)	98.7	5.7	82.0	108.0
Waist/Hip	0.87	0.07	0.70	1.00
Blood biochemistry profile§				
Glucose (mg/dL)	85.4	12.0	53.0	109.0
Cholesterol (mg/dL)	179.7	44.8	118.0	305.0
Cholesterol-HDL (mg/dL)	56.5	13.0	36.0	90.0
Non-HDL-Cholesterol (mg/dL)	122.0	40.3	71.0	215.0
Cholesterol-LDL (mg/dL)	104.8	39.4	57.0	203.0
Triglycerides (mg/dL)	81.0	41.4	36.0	207.0
Calcium (mmol/L)	2.3	0.1	2.1	2.5

*Measured via impedance method.
§ Fasting blood levels
SD: standard deviation; BMI: body mass index; min: minimum; max: maximum

Serum carotenoid profile and concentrations were also investigated. The following carotenoids were identified in the fasting plasma samples of the participants: β-carotene (0.45 (SD 0.37) μm); lutein (0.13 (SD 0.08) μm) α-carotene together with 9-cis-β-carotene (0.07 (SD 0.07) μm); β-cryptoxanthin (0.10 (SD 0.08) μm); lycopene (0.51 (SD 0.36) μm); and phytofluene (0.18 (SD 0.13) μm) (Part 5, Table 3). Calcium levels in plasma at the time of the first screening were 2.35 (SD 0.10) mmol/L (i.e. 94.2 (SD 4.0) mg/L), and within the reference levels provided, and thus did not vary widely.

Among the enrolled participants, one dropped out for personal reasons, after the first clinical visit. For one other participant, the number of pills packed during production were two instead of four, meaning that the participant was given a placebo dose once and a 500 mg dose of Ca2+ supplement twice. Finally, the results from the first participant enrolled in the study were excluded from further plasma TRL analysis, as samples from this participant were used for method-optimisation purposes.

In summary, twenty-four participants completed the trial, and data were analysed for twenty-three of the twenty-five participants.

Part 5, Table 3 - Blood plasma carotenoid concentrations of the participants at the time of recruitment (n = 25). (Mean values and standard deviations)

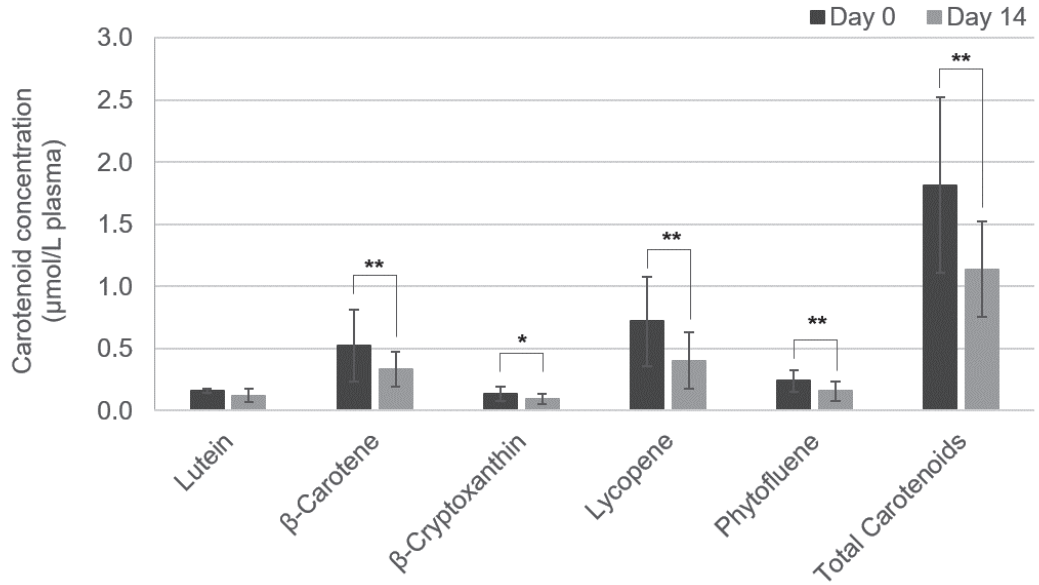
	Mean	SD	Max	Min
Lutein (µM)	0.13	0.08	0.34	0.03
β-Carotene (µM)	0.45	0.37	1.46	0.00
*α-Carotene (µM)**	0.07	0.07	0.28	0.00
β-Cryptoxanthin (µM)	0.10	0.08	0.27	0.00
Lycopene (µM)	0.51	0.36	1.08	0.00
Phytofluene (µM)	0.18	0.13	0.39	0.00
*Total carotenoids*** *(µM)*	1.43	0.89	3.40	0.05

SD: standard deviation. Min: minimum; Max: maximum
* α-carotene represents concentration of both α-carotene and 9-cis-β-carotene
**sum of all indiviudal carotenoids listed above

All participants consumed the totality of the three test meals that were given, plus the supplement pills, without any observed adverse effect. Nevertheless, some participants had more difficulty than others in finishing their spinach dose, requiring up to approximately 20 min, which introduced some variability in the time-span of the test meal intake.

5.4.2 Washout phase

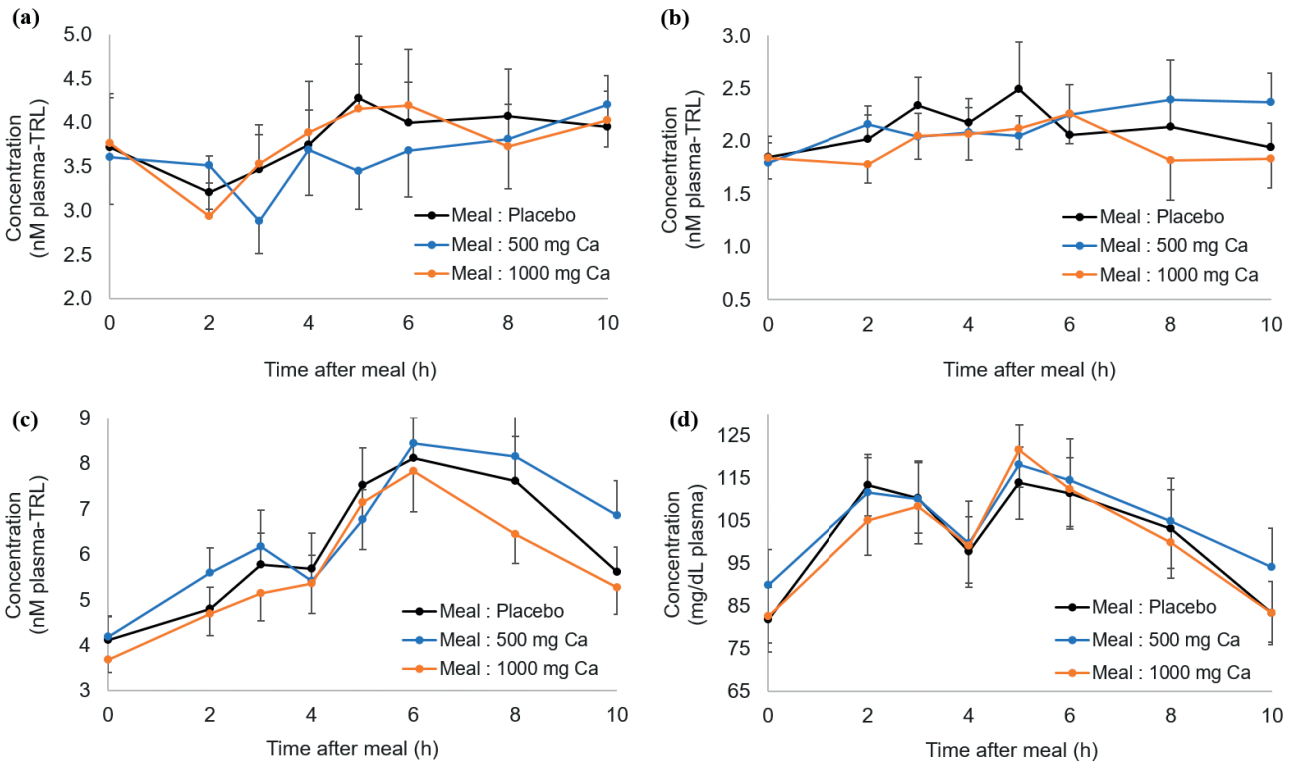
Participants were asked to avoid, as far as possible and during the entire trial phase, carotenoid-containing food items. After 2 weeks of initial washout, the concentration of total serum carotenoids decreased significantly ($P < 0{\cdot}01$), on average by approximately 38 % (Part 5, Figure 2), with an approximately 30 % decrease for both lutein and β-carotene (twenty-four participants). However, we did observe that for some participants, there was an increase in the plasma circulation of some of the individual carotenoids (data not shown).



Part 5, Figure 2 - Effect of 2 weeks of washout on blood plasma carotenoids. Fasting plasma carotenoid concentrations were assessed at day 0 (■) of the trial phase, and participants were asked to restrict themselves to a washout diet (i.e. avoiding, as far as possible, the consumption of carotenoid-containing foods) for a period of 2 weeks. Fasting plasma carotenoid concentrations were assessed once more on the day of their first clinical visit (day 14, ■), and values were compared to evaluate the effect of washout on the reduction of plasma carotenoid concentrations. Values are means (n = 23) and standard deviations represented by vertical bars. Total carotenoids include: lutein, β-cryptoxanthin, β-carotene, α-carotene, + 9-cis-β-carotene, lycopene and phytofluene. Statistical significant difference between the two measures of each carotenoid: * P < 0·05, ** P < 0·01.

5.4.3 Influence of calcium supplements on the AUC of serum TAG and carotenoids in the plasma TAG-rich plasma lipoprotein fraction

Contrary to what was expected, supplementation with either 500 or 1000 mg of Ca^{2+} did not significantly affect the absorption of carotenoids from a spinach-based meal (Part 5, Figure 3). Although the values for the AUC for test meals supplemented with 1000 mg of elemental Ca^{2+} were generally inferior to those of the other two groups, differences were not statistically different and variance was high (Part 4, Table 4). A statistically significant influence was found for participant ($P < 0.01$) and the sequence of visit ($P = 0.012$). For TAG, a biphasic curve pattern was found, with peaks at approximately 2 and 6 h postprandially (after lunch intake).



Part 5, Figure 3 - Effect of different calcium doses on the postprandial plasma TAG-rich lipoprotein (TRL) concentration of carotenoids from a spinach-based meal. Changes in plasma TRL carotenoids of β-carotene (a), β-cryptoxanthin (b), lutein (c) and serum TAG (d) were assessed over a 10-h period after the intake of a test meal together with one of three doses of the calcium supplement (placebo, 500 or 1000 mg of elemental calcium). Plasma TRL concentrations (nM) and plasma TAG concentrations (mg/dl) are expressed as mean values (n = 23) with their standard errors. —●— Meal: placebo; —●— meal: 500 mg Ca; —●— meal: 1000 mg Ca. AUC were compared for statistically significant differences as outlined in the 'Statistical Analyses' section. No statistically significant differences were found.

Including additional covariates such as BMI, body fat and fasting TAG and cholesterol concentrations into the statistical model did not change the absence of an effect of Ca^{2+} on carotenoid absorption. Likewise, no effects were found when analysing data separately for the participants with (n = 6) and without elevated cholesterol levels (n =17).

Part 4, Table 4 - AUC results and maximum concentrations (Cmax) for lutein, β-carotene and β-cryptoxanthin in the plasma TAG-rich lipoprotein (TRL) fractions, and for serum TAG, following the consumption of a spinach-rich test meal with either 0, 500 or 1000 mg supplemental calcium (n = 23). (Mean values with their standard errors; mean values and standard deviations)

	Placebo group*	500 mg Ca^{2+}	1000 mg Ca^{2+}
Area Under the Curve (AUC)≠			
Lutein (nmol.h/L)	22.72 ± 5.44	24.29 ± 5.31	21.38 ± 5.41
β-Carotene (nmol.h/L)	0.19 ± 3.90	0.80 ± 2.77	-0.77 ± 3.07
β-Cryptoxanthin (nmol.h/L)	2.80 ± 1.75	3.71 ± 1.54	1.20 ± 1.60
Total carotenoids (nmol.h/L)§	25.71 ± 9.58	28.81 ± 7.72	21.81 ± 8.40
Triglycerides (mg.h/dL)	210.41 ± 50.40	163.26 ± 54.98	193.81 ± 36.92
Triglycerides (mg.h/dL)	210.41 ± 50.40	163.26 ± 54.98	193.81 ± 36.92
Baseline TRL concentration‡			
Lutein (nmol.h/L)	4.09 ± 0.53	4.20 ± 0.44	3.68 ± 0.29
β-Carotene (nmol.h/L)	3.78 ± 0.56	3.55 ± 0.54	3.76 ± 0.56
β-Cryptoxanthin (nmol.h/L)	1.85 0.20	1.79 ± 0.19	1.84 ± 0.22
Total carotenoids (nmol.h/L)§	9.72 ± 1.08	9.58 ± 0.80	9.37 ± 0.89
Triglycerides (mg.h/dL)	81.47 ± 7.64	90.22 ± 8.24	82.48 ± 6.23
C_{max}			
Lutein (nmol/L)	9.60 ± 4.60	10.34 ± 6.36	9.04 ± 4.90
β-Carotene (nmol/L)	5.70 ± 3.57	5.20 ± 3.05	5.79 ± 3.46
β-Cryptoxanthin (nmol/L)	3.28 ± 2.10	3.15 ± 1.76	2.94 ± 1.59
Triglycerides (mg/dL)	131.61 ± 40.23	133.91 ± 46.64	133.43 ± 40.24

≠ Baseline (fasting) subtracted values, i.e. concentration at time zero before test meal intake was subtracted from all following concentrations. AUC results represent mean ± SEM. C_{max} values represent mean ± SD.

*Values were compared following generalized univariate linear models for each carotenoid and TGs, with AUC as the observed dependent variable, and carotenoid (lutein, β-carotene, β-cryptoxanthin and total carootenoids), participant, sequence of intervention (visit 1, 2 or 3) and calcium supplemental dose (0, 500, 1000 mg) as the independent, fixed factors. No significant effects were found.

§Sum of lutein, β-carotene and β-cryptoxanthin.

5.5 Discussion

Previously, we investigated the effect of different doses of the macrominerals Ca^{2+} and Mg^{2+} and divalent trace elements (iron and Zn^{2+}), on the bioaccessibility of both pure carotenoid standards and carotenoids from different food matrices, including spinach, showing that Ca^{2+} had a strong negative effect on the micellarization of carotenoids (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2016;

Corte-Real, Bertucci, et al., 2017). As carotenoids are liposoluble, and as their intestinal uptake is ultimately related to the formation of lipid–bile mixed micelles, we hypothesised that supplemental dietary Ca^{2+} would bind to fatty acids released from the lipids present in the test meal, and to the bile salts, hindering the formation of mixed micelles and thus the delivery of carotenoids to the enterocytes, and also occluding carotenoids in the precipitates of fatty acid soaps, which would eventually be excreted.

Although this hypothesis was previously observed *in vitro*, it remained to be confirmed *in vivo*, as it is possible that in a dynamic and biologically complex scenario of gastrointestinal digestion, the body might compensate for the precipitation-related effects of Ca^{2+} seen *in vitro* – for example, by up-regulating bile salt and pancreatic enzyme secretion. By supplementing a spinach-based test meal with different high, but still physiologically plausible, supplemental doses of Ca^{2+}, we expected to note differences in the serum concentration of TAG and of carotenoids from the plasma TRL fraction (representing newly absorbed carotenoids) between meals supplemented with placebo (no additional Ca^{2+}) and those supplemented with either 500 or 1000 mg of Ca^{2+}. However, statistical analysis of the carotenoid and TAG AUC of different meal groups showed no statistically significant differences between them. Interestingly, a very recent study investigating the effect of supplemental dietary Ca^{2+} on the bioavailability of lycopene from tomato paste has reported a decrease of 83 % in lycopene bioavailability after supplementation with 500 mg of elemental Ca^{2+} as carbonate (Borel et al., 2016), based on postprandial plasma samples measured over 7 h. Thus, despite not finding any significant effects of Ca^{2+} on the bioavailability of spinach-borne carotenoids, our original hypothesis is not to be completely discarded.

Different factors could have played a role in the different outcomes of these two studies, including differences in test meal composition (spinach v. tomato paste, with respect to richness in insoluble fibre), the profile of the individual carotenoids in the test meal (being more apolar for lycopene) and the investigated target compartment (TRL v. plasma). For example, bioavailability of carotenoids has been shown to improve after processing of the food matrix. Plasma concentrations of lutein and β-carotene have been reported to be significantly higher from the intake of spinach leaves that were

liquefied/puréed, compared with minced or whole-leaf spinach (Castenmiller et al., 1999; Rock et al., 1998). Another study reports a higher bioavailability of total and all-trans-lycopene from tomato paste compared with fresh tomatoes (Gartner, Stahl, & Sies, 1997). In the present study, spinach meals given to participants were composed of whole leaves, which may have introduced issues of low bioaccessibility in the study design. In previous *in vitro* trials, where we compared bioaccessibility from different food matrices, total carotenoids from green leafy varieties (spinach and field salad) had markedly lower bioaccessibilities than those from liquid matrices (carrot or tomato juice) (Corte-Real, Bertucci, et al., 2017). Also, *in vivo* trials have shown that the bioavailability of carotenoids from spinach is inferior to that from other dietary sources (Chung, Rasmussen, & Johnson, 2004; Tang, Qin, Dolnikowski, Russell, & Grusak, 2005), possibly related to their storage in pigment–protein complexes, which renders carotenoids less accessible than those stored in chromoplasts (as it is the case for tomato) (Jeffery, Holzenburg, et al., 2012). Considering the amount of individual carotenoids estimated to be present in the spinach test meal (Part 4, Table 4), and their low bioaccessibility, and that the percentage of carotenoids effectively taken up by the intestinal lumen is lower than its actual bioaccessibility, the resulting low bioavailability may have impeded finding significant effects of the Ca^{2+} supplementation, despite the relatively large number of participants and the crossover design. When comparing, for example, the AUC from the present study with that of other trials with various carotenoid-containing food sources, the observed values are relatively low (Bohn et al., 2011; Unlu & Bohn, 2005), though they are comparable with another recent trial with spinach (Eriksen, Luu, Dragsted, & Arrigoni, 2016).

However, this does not explain the lack of a significant effect of Ca^{2+} supplementation on the TAG AUC. Calcium ions can bind both bile acids and NEFA to form bile salts and fatty acid soaps of low solubility, respectively, which tend to complex and precipitate (Gacs & Barltrop, 1977; Hofmann & Mysels, 1992). Previous trials carried out in animals (Gacs & Barltrop, 1977) and humans (Chai et al., 2013) have reported an increase in faecal fat excretion when meals were supplemented with Ca^{2+}. Furthermore, in a recent meta-analysis of randomised controlled trials testing the effects of dairy products and dietary supplements on faecal fat excretion, results indicated that increasing the intake

of dietary Ca^{2+} to 1241 mg/d impaired the absorption of dietary fat and increased faecal fat excretion (Christensen et al., 2009). In fact, according to the EFSA (European Food Safety Authority) Scientific Opinion on dietary reference values for Ca^{2+}, dairy products remain the main dietary sources of Ca^{2+} in Europe, and the average intake in adults (>18 years) is between 690 and 1122 mg/d (European Food Safety Authority, 2015).

In the present study, calcium carbonate supplements were provided in the form of gelatine capsules, and participants were asked to take them at the end of the meal with 300 ml of water. By comparison, in the study by Borel et al. (2016), calcium carbonate supplementation was provided in the form of effervescent pills dissolved in water, and taken together with the meal. Thus, the different form of the Ca^{2+} supplement and the test meals (liquid v. solid and fibre rich) could have resulted in different mixing patterns during digestion. Also, pectins in the tomato paste could have formed a gel with the added Ca^{2+} (Soukoulis & Bohn, 2018). In addition, spinach contains oxalic acid (0·97 g/100 g) (Haytowitz & Matthews, 1984), which may have chelated Ca^{2+} to some degree, though surely not entirely (molar ratio of Ca^{2+} : oxalic acid approximately 1.6 for the meal highest in Ca^{2+}). Finally, it cannot be completely excluded that the different type of participants included in the present study, compared with those in the study by Borel et al. (2016), contributed to the different observations as follows: elderly participants included in the present study may tend to have lower plasma carotenoid levels; likewise, their BMI was somewhat higher; and participants had, in part, either elevated cholesterol or TAG; and these factors are known contributors to interindividual plasma carotenoid variability (Bohn et al., 2017).

Finally, other aspects to consider are the physiological and genetic variability between participants. Although individual differences in enzymatic activity and concentration of bile acids during gastrointestinal digestion (Ulleberg et al., 2011) could influence the degree of bioaccessibility of carotenoids from one participant to the other, genetic variability could predispose participants to a higher or lower uptake efficiency of carotenoids in the enterocyte and downstream transport into the bloodstream via the chylomicrons (Bohn et al., 2017; Borel, 2012a). On the other hand, as the intraindividual variability has been estimated to be approximately half of the interindividual variability,

week-to-week variations within one participant could be substantial (Bohn et al., 2017). Analysis of the serum and plasma samples for fasting levels of TAG and carotenoids, respectively, on each clinical visit day, showed that values varied from week to week, without any specific trend and for inexplicable reasons, especially for the circulating carotenoids. Although participants were asked to avoid foods containing carotenoids before the first clinical visit, in order to lower the plasma carotenoid concentration, it should be noted that 2 weeks may not be sufficient to completely clear circulating carotenoids in blood plasma fractions, as the body stores dietary carotenoids in different organs and tissues: for example, the liver and adipose tissues (Bohn et al., 2017). However, at the time of the first clinical visit, the concentration of total circulating plasma carotenoids had dropped on average by 38%, and longer washout periods would have been ethically questionable because of the difficulty in excluding coloured fruits and vegetables from the daily diet for a prolonged period of time.

Although this study had its strengths, including a double blinded, randomised, placebo-controlled trial design in which each participant was his or her own control, a high protocol adherence by he participants and a randomised block design where all six possible combinations of Ca^{2+} intake were realised (high, medium–low v. medium–high–low, etc.), it also had its limitations. Only the effect of high doses (i.e. 50 or 100 % of RDA) of Ca^{2+} was tested for short-term treatments, and only spinach was chosen as a representative carotenoid leafy vegetable. Also, the intake of dietary Ca^{2+} was not assessed, which may have potentially enhanced the variance of responses, as it was suggested that low Ca^{2+} consumers tend to utilise more transcellular absorption pathways, as opposed to participants with a higher intake, in whom paracellular pathways possibly play a role, and thus kinetics of Ca^{2+} disappearance from the gut could differ (Bronner, 2003), which may have influenced carotenoid interactions. Faecal TAG excretion was not considered in the experimental design to reduce the burden on the participant.

In conclusion, despite the negative effects of DM on the *in vitro* bioaccessibility of carotenoids reported earlier, we did not find a statistically significant effect of Ca^{2+} supplementation on the bioavailability of individual carotenoids from a spinach-based meal. Nevertheless, a recent human trial with another carotenoid source indicated that, under certain conditions, DM may in fact negatively affect carotenoid

uptake. It can be speculated from these human trials that the effect of mineral supplements on the bioavailability of carotenoids, and potentially other liposoluble nutrients, may depend on the interaction of the supplement form with the type of carotenoid and the food matrix. Further investigations are necessary to fully understand the complex interactions taking place during gastrointestinal digestion between DM and carotenoids.

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T. B. and L. H. planned and designed the study. J. C.-R. coordinated and carried out the major part of the human trial. C. G. organised the carotenoid analyses. K. B. and B. W. conducted the blood biochemistry and haematology analyses. E. R. supervised the PhD student (J. C.-R.) and aided in the planning and writing-up of the study. The manuscript was written by J. C.-R. and T. B., with additional input from all other authors. The authors declare that there are no conflicts of interest.

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6. MAGNESIUM AFFECTS SPINACH CAROTENOID BIOACCESSIBILITY *IN VITRO* DEPENDING ON INTESTINAL BILE AND PANCREATIC ENZYME CONCENTRATIONS

Preamble

The following chapter has been published in the journal “Food Chemistry”.

The previous *in vitro* and *in vivo* experiments were designed to test the effect of divalent minerals under conditions that would either mimic (*in vitro* experiments) or address (*in vivo* human trial) physiological conditions found in healthy humans. However certain chronic conditions related to the gastro-intestinal tract, namely biliary and pancreatic insufficiency, may potentiate the negative effect of divalent minerals on carotenoid absorption. Hence, the goal of the following work package was to assess the importance of bile and pancreatin on the possible mechanism of action of divalent minerals, specifically Mg^{2+}, by testing the former at low concentrations, somewhat mimicking an insufficiency scenario.

In vitro digestions, carotenoid extraction and HPLC analysis were carried out at the current Luxembourg Institute of Science and Technology (LIST), past *Centre de Recherche Public Gabriel Lippmann*, with the help of laboratory technician Boris Untereiner. Micelle size and zeta potential measurements were performed in collaboration with Dr. Charles Desmarchelier and Dr. Patrick Borel at the *Nutrition Obésité et Risque Thrombotique* (NORT) Unit from the *Institut National de la Santé et de la Recherche Médicale* (INSERM), France.

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

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6.1 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 Mg^{2+} on carotenoid bioaccessibility. Spinach (4 g) was digested *in vitro* with added Mg^{2+} (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 Mg^{2+} concentrations negatively affected carotenoid bioaccessibility ($P < 0.001$), lipolysis, particle size and zeta potential. The impact of Mg^{2+} on carotenoid bioaccessibility was modulated mainly by bile concentration, with samples digested with1 mM of bile being more susceptible to inhibitory effects of Mg^{2+} than those digested with 8 mM ($P < 0.001$). Thus, Mg^{2+} was found to potentially interfere with carotenoid bioaccessibility at various physiologically plausible conditions

Keyowrds: Lutein; Carotenes; Divalent minerals; Calcium; Bioavailability; Digestion; Lipids; Micelle formation.

6.2 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 et al., 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 et al., 2009).

Recently, it has been hypothesized (Biehler, Hoffmann, et al., 2011) that dietary DM, such as Ca^{2+} and Mg^{2+} 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 DM 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 Ca^{2+} and Mg^{2+}. However, recent human trials investigating the effect of Ca^{2+} have found

contradictory results. While in a study employing tomato paste, 500 mg of Ca^{2+} decreased post-prandial plasma lycopene concentration (Borel et al., 2016), another trial employing spinach and Ca^{2+} 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 Mg^{2+}, the second most abundant dietary cation after Ca^{2+} (RDA: 1000 mg/d), with an RDA of 400 mg/d (Institute of Medicine, 2005). *In vivo*, the interaction between Mg^{2+} and other DM 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 Mg^{2+} on the bioaccessibility of carotenoids, as determined by fractional carotenoid bioaccessibility, following *in vitro* GI 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 et al., 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.

6.3 Materials and methods

6.3.1 Chemicals and standards

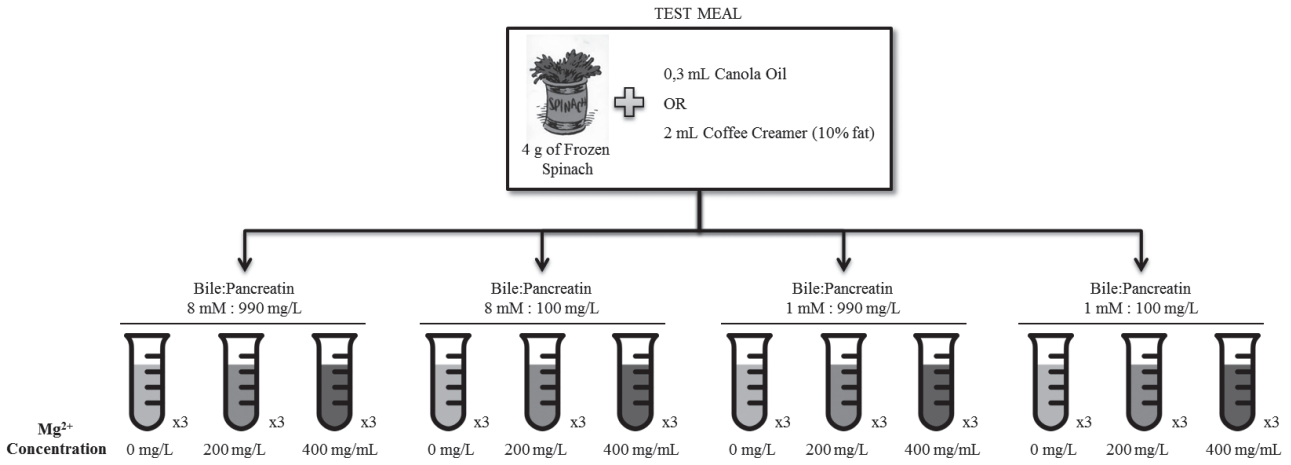
Pepsin (porcine, 250 units/mg solid, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as substrate), pancreatin (porcine, 4X USP specifications), and porcine bile extract were purchased from Sigma-Aldrich (Bornem, Belgium). Methanol (MeOH), hexane and hydrochloric acid were from 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-80-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 Mg^{2+} 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 super- market Esch-sur-Alzette, Luxembourg) were used. Both were previously employed to aid in the micellarization of carotenoids (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2016, 2014). The carotenoid containing food matrix, spinach (*Spinachia oleracea*), was purchased in frozen raw form (Cora supermarket, Foetz, Luxembourg).

6.3.2 Simulation of gastro-intestinal digestion and factors investigated

The *in vitro* digestion protocol was described earlier (Corte-Real et al., 2014), in Part 2. The model was used to test the effect of Mg^{2+} (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), in Part 3, 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 behaviour 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, 2000g) and either processed further directly (bioaccessibility) or snap frozen at 80 °C for further investigation (micelle size, zeta potential and lipolysis).



Part 6, Figure 1 - Schematic diagram of the study design

6.3.3 Separation of bioaccessible fraction and bioaccessibility calculation

Aliquots of digesta (12 mL) were transferred to 15 mL falcon tubes and centrifuged at 4800g 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.

6.3.4 Extractions

Carotenoid extraction procedures have been described previously (Corte-Real, Bertucci, et al., 2017), in Part 4. 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 fluoride (PVDF) syringe filter (PALL Life Sciences, Ann Arbor, MI, USA), and the filter was rinsed in 100 mL of MTBE:MeOH (3:7). For digesta, 4 mL aliquots of the bioaccessible fraction, obtained after centrifugation, were extracted with 6 mL of hexane:acetone (2:1) with an additional 1 mL of aqueous KOH (30%) for saponification, vortexed and centrifuged (2 min, 4000g,4 °C). The supernatant was transferred to a new tube and the bioaccessible fraction re-extracted once with 5 mL of hexane and a second time with 5 mL of diethyl ether. The combined organic phases were evaporated under a stream of nitrogen in a TurboVap (45 min, 25 °C). The dried carotenoid extracts were spiked with an appropriate amount of internal standard (trans-β-apo-80-carotenal at 10 mg/mL), to obtain a final concentration of 1 mg/mL per sample, and were re-dissolved in 500 mL of MTBE:MeOH (3:7). Samples were filtered through a 0.2 µm PVDF syringe filter, into HPLC amber vials. The filter was

then rinsed with 100 mL 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 mL

6.3.5 HPLC Analysis

Carotenoids were separated on an Agilent 1260 Infinity U-HPLC instrument (Agilent Technologies, De Kleetlaan Belgium S.A./N.V.). A gradient elution with (A) water:MeOH (60:40) with 30 mM of ammonium acetate, and (B) ACN:DCM (85:15), passing through an AccucoreTM C30 column (2.6 µm particle size, 100 mm length, 3 mm diameter, from Thermo Fisher Scientific Inc.) was applied at 28 °C, with 10 mL injection volumes. Elution gradient was: 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; 36 min, 42% B. Carotenoids were detected by a coupled UV/VIS photodiode array detector, and identified according to their retention times and spectral data, compared to the corresponding external standards. All peaks were integrated manually at 440 nm (neoxanthin and violaxanthin), 450 nm (lutein and α-carotene), 455 nm (β-carotene and trans-β-apo-80-carotenal (IS)), according to each carotenoid absorption maximum. Quantification was achieved using the internal standard method.

6.3.6 Micelle size and zeta potential analysis

Aliquots of the aqueous micellar fraction were taken for analysis of the micelle size and zeta potential (indicator of the stability of the particles in solution). Aliquots were filtered prior to measurements, through a 0.2 µm syringe filter (mixed cellulose esters; Millipore, Molsheim, France). 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 25 °C with a Zetasizer Nano Zs instrument (Malvern Instruments, Malvern, UK).

6.3.7 Fatty acid titration

An acid-base titration was carried out to estimate the amount of FFA in the digesta at the end of simulated GI digestions. Free fatty acids were titrated with sodium hydroxide (NaOH 0.01 M), using

phenolphthalein as an indicator. In short, 5 drops of phenolphthalein (0.1% in ethanol) were added to 5 mL of previously centrifuged sample. Sodium hydroxide (NaOH 0.01 M) was then added quantitatively and slowly via a burette, ensuring adequate stirring of the sample, until a colour change to pink was observed that remained stable for at least 30 s.

6.3.8 Statistical analysis

Unless described otherwise, all values are expressed as mean ± SD. Normality of distribution and equality of variance of the data were tested by normality plots and box plots, respectively. When required, log-transformation was conducted. All carotenoid bioaccessibility data was normalized to a respective control which was run for every set of analyses, in order to minimize day-to-day variations. A general linear model was developed with the effect of mineral concentration, lipid source, bile extract concentration and pancreatin concentration as fixed factors and bioaccessibility of individual carotenoids (lutein, β-carotene, α-carotene, neoxanthin) as the observed dependent factor. A P-value below 0.05 (2-sided) was considered statistically significant. Following significant Fisher F-values, Bonferroni's post hoc-tests and Fisher-protected LSD tests (when comparing <4 groups) were conducted. The relation between the different tested outcomes (carotenoid bioaccessibility, particle size, particle zeta potential, moles of added NaOH) was evaluated by Pearson correlation analyses.

6.4 Results

6.4.1 General factors influencing carotenoid bioaccessibility from spinach

In the present experimental design (Part 6, Figure 1), we tested for the effects of: 1) different Mg2+ concentrations; 2) different combinations of bile (1 mM or 8 mM) and pancreatin (100 mg/L or 990 mg/L) concentrations; and 3) the effect of two different dietary lipid sources (canola oil and coffee creamer 10% fat), on the bioaccessibility of carotenoids from a spinach-based test meal.

As the model was fully factorial, all interactions were also studied. All aforementioned factors significantly ($P < 0.001$) affected the bioaccessibility of spinach-borne total carotenoids) in this fully factorial model (Part 6, Table 1).

In the original spinach matrix, we identified and quantified violaxanthin, neoxanthin, lutein, β-carotene and α-carotene (Part 6, Table 1). However, violaxanthin could not be detected after digestion. Data for neoxanthin and α-carotene bioaccessibility, due to their very low concentration, is not shown, even though they were considered for total carotenoid calculations.

Bioaccessibility of carotenoids, following GI digestion, was generally low. When no Mg^{2+} was added, bioaccessibility of total carotenoids ranged between 0.05 and 4.83% (Part 6, Figure 2), depending on the combination of bile and pancreatin concentration.

Part 6, Table 1 - Statistical evaluation of experiment: P-values for the investigated fixed factors, and fully factorial interactions associated with the outcome bioaccessibility, according to the general linear mixed model employed

Factor	Lutein	β-Carotene	Total Carotenoids
Mg^{2+} (mg/L)	<0.0001	<0.0001	<0.0001
Lipid	<0.0001	<0.0001	<0.0001
Bile (mM)	<0.0001	<0.0001	<0.0001
Pancreatin (mg/L)	<0.0001	<0.0001	<0.0001
Mg^{2+} x Lipid	<0.0001	<0.0001	<0.0001
Mg^{2+} x Bile	<0.0001	<0.0001	<0.0001
Mg^{2+} x Pancreatin	0.2196	<0.0001	0.4645
Lipid x Bile	<0.0001	<0.0001	<0.0001
Lipid x Pancreatin	0.5496	<0.0001	0.5360
Bile x Pancreatin	<0.0001	<0.0001	<0.0001
Mg^{2+} x Lipid x Bile	0.2477	<0.0001	<0.0001
Mg^{2+} x Lipid x Pancreatin	0.1682	<0.0001	0.0952
Mg^{2+} x Pancreatin x Bile	0.1682	<0.0001	0.4769
Lipid x Bile x Pancreatin	0.4974	<0.0001	0.5099
Mg^{2+} x Lipid x Bile x Pancreatin	0.2414	<0.0001	0.0749

In addition to bioaccessibility, the aqueous micellar fraction was also investigated for particle size and zeta potential, as well as the release of FFA. Pearson correlation analyses showed that bioaccessibility was positively correlated with particle size (Pearson's $r = 0.54$, $P < 0.0001$) and FFA released (Pearson's $r = 0.65$, $P < 0.0001$), and negatively correlated with absolute zeta potential (Pearson's $r = 0.60$, $P < 0.0001$).

6.4.2 Influence of the concentration of bile and pancreatin on the effect of magnesium on carotenoid bioaccessibility

The extent of the effect of Mg^{2+} on the bioaccessibility of total carotenoids was influenced by the concentration of bile ($P < 0.0001$ for magnesium-bile interaction), but not by pancreatin ($P = 0.46$ for magnesium-pancreatin interaction, Part 6, Table 1). Samples digested with 1 mM of bile were more susceptible to increasing concentrations of Mg^{2+} than samples digested with 8 mM, and Mg^{2+} concentrations of 200 mg/L and higher significantly ($P < 0.001$) reduced the bioaccessibility of carotenoids (Part 6, Figure 2), by up to 100%.

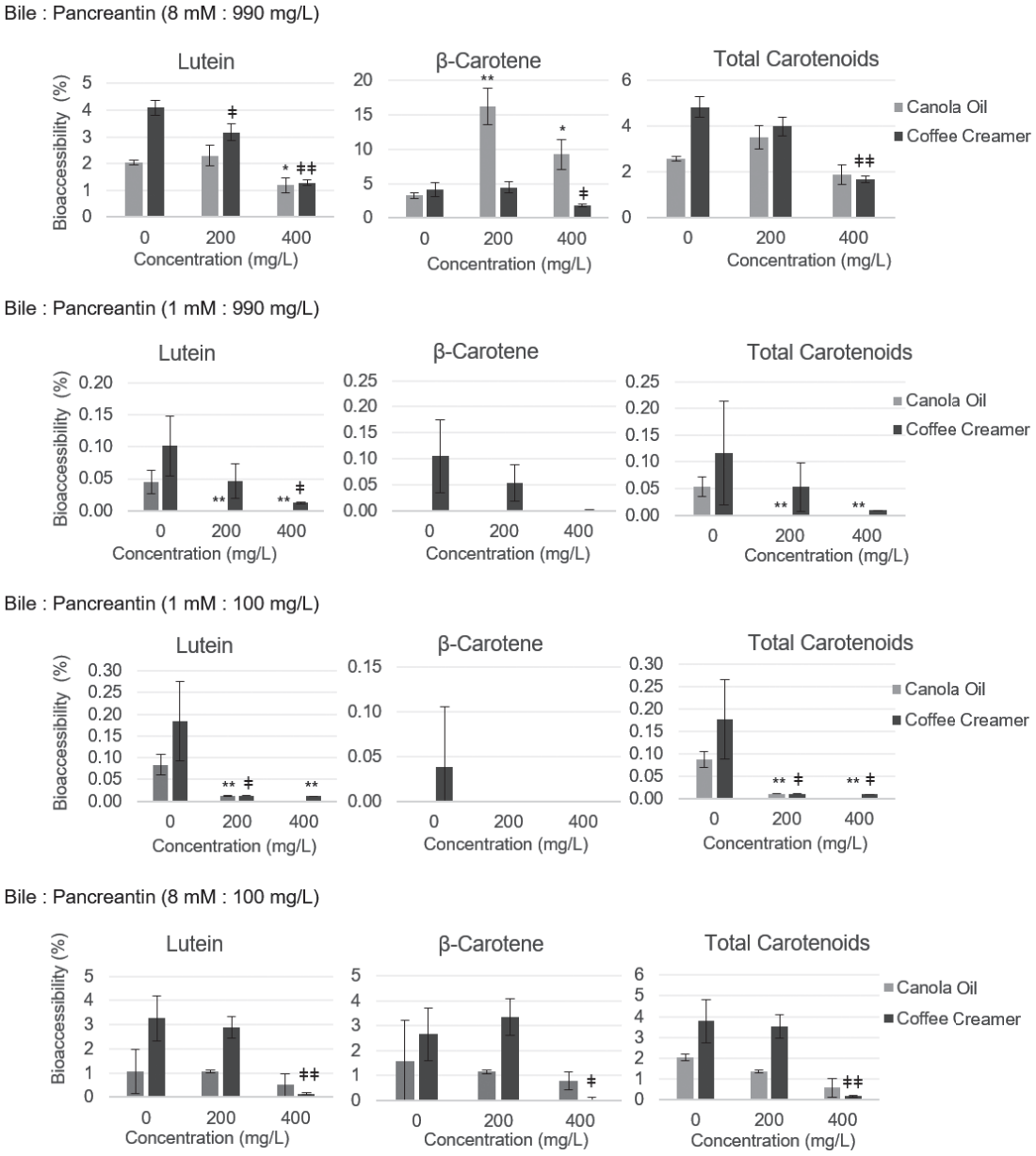
In the absence of Mg2+, the presence of bile extract at 1 mM resulted in a significantly lower percentage of total carotenoids recovered from the micellar phase when compared to 8 mM (0.11 ± 0.07% vs. 3.42 ± 1.22%, respectively). A similar effect was observed for lutein and β-carotene (Part 6, Figure 2). The effect of the concentration of pancreatin was less pronounced, and significant differences between samples digested with either 100 mg/L or 990 mg/L of pancreatin were only observed when bile acid concentration was 8 mM ($P < 0.001$). Nevertheless, the negative effect of Mg2+ on carotenoid bioaccessibility was observed at all bile:pancreatin ratios.

Part 6, Table 2 - Carotenoid content (mg/g wet weight) in the (raw and frozen) spinach (*Spinacea oleracea*) matrix

Vio	Neo	Lut	α-Car	β-Car	Total*
30.5 ± 10.8	4.8 ± 1.4	74.3 ± 13.2	0.9 ± 0.5	7.1 ± 1.0	117 ± 24

Values represent mean ± SD from n = 4 replicates.
Vio: violaxanthin; Neo: neoxanthin; Lut: lutein; α-Car: α-carotene; β-Car: β-carotene.
* Represents the sum of all measured carotenoids

6.4.3 Influence of lipid source on the bioaccessibility of carotenoids



Part 6, Figure 2 - Effect of varying magnesium concentrations on the bioaccessibility of carotenoids from spinach digested with different combinations of bile extract and pancreatin concentrations. Bile concentration is presented in mM, while pancreatin concentration is presented in mg/L. Values represent mean ± SD (n = 3). Total carotenoids comprise β- carotene, α-carotene, lutein and neoxanthin bioaccessibility. Statistical comparisons were carried out as described in the statistical section (6.3.8). Symbols * and ǂ represent statistically significant differences (P < 0.05) from the respective control (canola oil and coffee creamer with no added magnesium); Symbols ** and ǂǂ represent statistically significant differences (P < 0.01) from the respective control (canola oil and coffee creamer with no added magnesium).

Bioaccessibility differed significantly between samples digested with canola oil and those digested with coffee creamer, with a general tendency for a higher bioaccessibility of carotenoids from samples digested with coffee creamer (Part 6, Table 3).

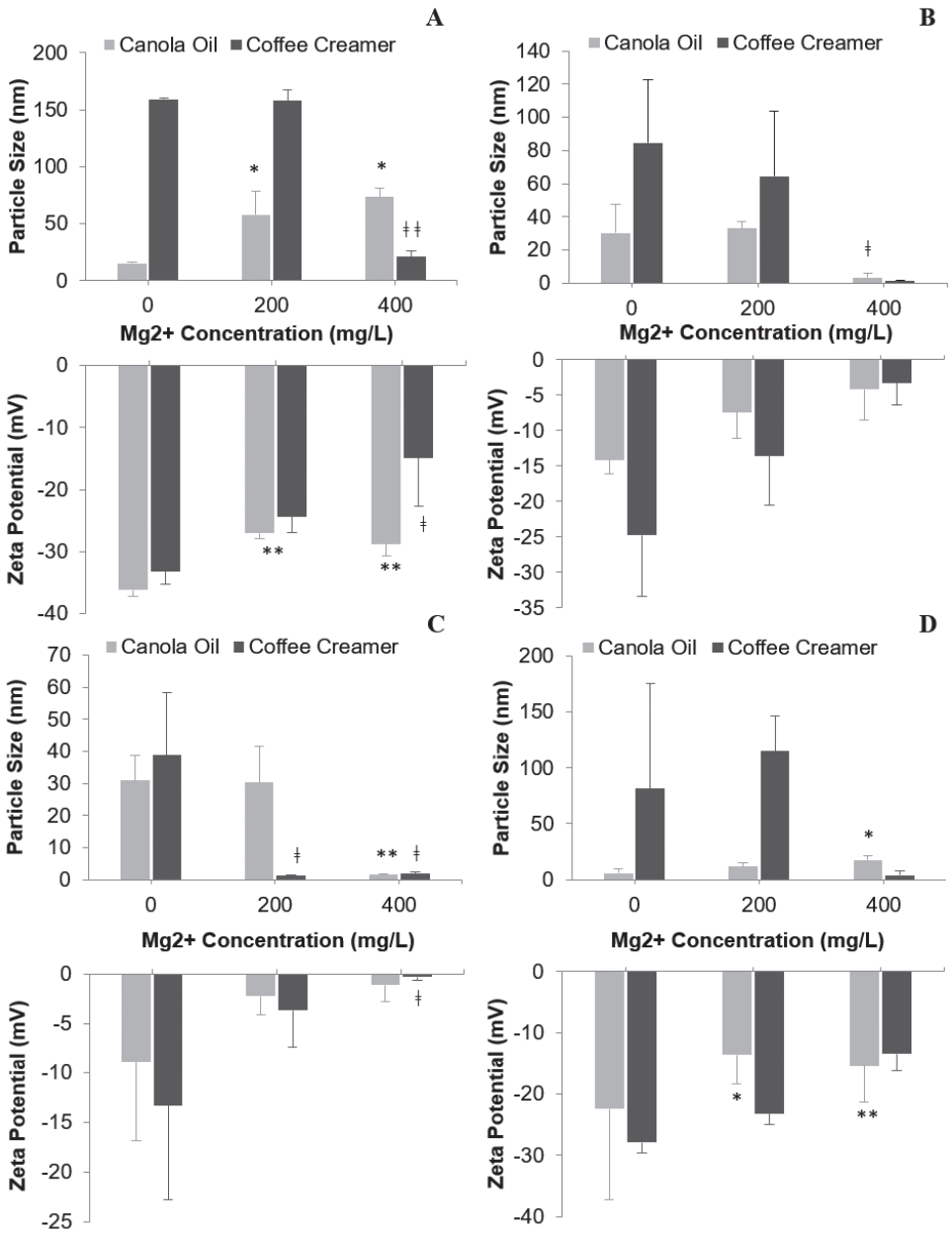
Part 6, Table 3 - Effect of the lipid source on the bioaccessibility (%) of spinach individual and total carotenoids, digested with either 1 mM or 8 mM of bile, without the influence of added magnesium

	Lutein (%)	β-Carotene (%)	Total Carotenoids (%)
Canola Oil			
Bile: 1 mM	0.06 ± 0.30	0.00 ± 0.00	0.07 ± 0.02
Bile: 8 mM	1.86 ± 0.25	1.32 ± 1.62	1.10 ± 1.21
Coffee Creamer (10% fat)			
Bile: 1 mM	0.143 ± 0.08	0.07 ± 0.08	0.15 ± 0.08
Bile: 8 mM	3.68 ± 0.76	3.40 ± 1.23	4.31 ± 0.91

Values represent mean ± SD of triplicates.
All pairwise comparisons between 1 mM and 8 mM of bile were found to be statistically significant ($P < 0.05$).
* Total carotenoids represents the sum of the sum of lutein, β-carotene, α-carotene and neoxanthin. Each carotenoid concentration was determined individually first by means of external standard calibration curves, and individual carotenoid concentrations were then added to yield total carotenoid concentration.

6.4.4 Factors influencing the size and zeta potential of the particles present in the micelle containing aqueous fraction

All factors investigated had a significant ($P < 0.001$) effect on the particle size of the aqueous micellar fraction (Part 6, Figure 3). Test meals digested with 8 mM of bile tended to produce larger micelles although only samples digested with a bile and pancreatin concentration combination of 8 mM and 990 mg/L, respectively, produced particles which were significantly ($P < 0.001$) larger than for other conditions. For all the other bile:pancreatin combinations, the particle sizes did not differ significantly. Samples digested with coffee creamer produced significantly ($P < 0.001$) bigger particles (60.9 ± 5.0 nm, mean ± SEM) than those digested with canola oil (25.8 ± 4.9 nm, mean ± SEM). Also, the concentration of Mg^{2+} affected the size of the particles found in the micellar fraction. The presence of Mg^{2+} at 400 mg/L resulted in significantly smaller particles at the end of the digestion ($P < 0.001$). Varying concentrations of Mg^{2+}, bile and pancreatin, but not lipid source, also significantly ($P < 0.001$) affected the zeta potential of the particles in solution.



Part 6, Figure 3 - Effect of varying magnesium concentrations on the particle size and zeta potential of aqueous micellar fractions from spinach digested with different combinations of bile and pancreatin concentrations, with 2 sources of lipids. Bile concentration is presented in mM, while pancreatin concentration is presented in mg/L. A: 8 mM: 990 mg/L; B: 1 mM: 990 mg/L; C: 1 mM: 100 mg/L; D: 8 mM: 100 mg/L. Values represent mean ± SD (n = 3). Statistical comparisons were carried out as described in the statistical section (2.8). Symbols * and ǂ represent statistically significant differences (P < 0.05) from the respective control (canola oil and coffee creamer with no added magnesium); Symbols ** and ǂǂ represent statistically significant differences (P < 0.01) from the respective control (canola oil and coffee creamer with no added magnesium

At a bile concentration of 1 mM and pancreatin concentration of 100 mg/L (Part 6, Figure 3 B, lower panel), the average zeta potential of the digesta was -4.9 mV, while at 8 mM of bile extract and 990

mg/L of pancreatin the average zeta potential was -27.4 mV (Part 6, Figure 3 A, lower panel). As the concentration of Mg^{2+} increased, the absolute value of the zeta potential decreased, indicative of a more unstable system prone to particle aggregation due to reduced repulsive forces. Zeta potential also negatively correlated with particle size (Pearson's r = -0.75, P < 0.0001).

6.4.5 Factors affecting the amount of free fatty acid released during digestion

Based on the amount of NaOH dispensed for the titration of the GI digesta, statistical analysis revealed that the concentration of bile, pancreatin, and lipid source, significantly influenced the amount of FFA in solution (P < 0.001).

Part 6, Table 4 - Amount (nmol) of 0.01 M NaOH required for the neutralization of liberated free fatty acids in the digesta following gastro-intestinal digestion. Phenolphthalein was used as a pH indicator. Samples were titrated until a color change to pink, remaining for at least 30 s, was observed.

	Magnesium concentration		
	0 mg/L	**200 mg/L**	**400 mg/L**
Canola oil			
Concentration bile : pancreatin			
1 mM : 100 mg/L	19 ± 2 ^{C}	21 ± 1 ^{D}	22 ± 3 ^{C}
8 mM : 100 mg/L	37 ± 1 ^{A}	36 ± 1 ^{C}	39 ± 6 ^{A}
1 mM : 990 mg/L	28 ± 3 ^{B}	27 ± 1 ^{B}	25 ± 1 ^{A,C}
8 mM : 990 mg/L	37 ± 1 $^{A;a}$	55 ± 1 $^{A;b}$	49 ± 2 $^{A;c}$
Coffee creamer			
Concentration bile: pancreatin			
1 mM : 100 mg/L	24 ± 3 $^{B;a}$	30 ± 3 $^{B;a,b}$	32 ± 1 $^{B;b}$
8 mM : 100 mg/L	52 ±5 $^{A;a}$	50 ± 1 $^{C;a,b}$	38 ± 2 $^{A,B;c}$
1 mM : 990 mg/L	42 ± 6 ^{A}	40 ± 6 ^{A,B}	39 ± 3 ^{A}
8 mM : 990 mg/L	47 ± 3 $^{A;a}$	50 ± 1 $^{A;a}$	40 ± 2 $^{A;b}$

Values represent mean ± SD of triplicates.
Values not sharing an upper-case superscript within one column are significantly different (P<0.05). Values not sharing a lower-case superscript within the same row are significantly different (P<0.05), unless no superscripts are indicated.

While the concentration of Mg^{2+} did not have any significant effect regarding fatty acid release on its own, a significant (P < 0.01) interaction with the other tested factors (concentration of bile, pancreatin, and lipid source) was observed. Samples digested with coffee creamer required on average more

NaOH (40 ± 1 nmol, mean ± SEM) than those digested with canola oil (32 ± 1 nmol) (Part 6, Table 4). Regarding the effects of bile and pancreatin concentrations in samples digested with 8 mM of bile, the average amount of NaOH (44 ± 1 nmol) was significantly ($P < 0.001$) higher than for samples digested with 1 mM of bile extract (29 ± 1 nmol), suggesting incomplete digestion of the latter. The effect of pancreatin concentration, though being significant ($P < 0.001$), appeared less influential on lipolysis than the concentration of bile, with a slightly higher average amount of NaOH being required for samples digested with 990 mg/L of pancreatin (40 ± 1 nmol) than with 100 mg/L (33 ± 1 nmol).

6.5 Discussion

Previously, we reported that varying concentrations of DM, including Mg^{2+}, can significantly affect the *in vitro* bioaccessibility of carotenoids, either of pure standards or from food sources (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2016; Corte-Real, Bertucci, et al., 2017), with the limitation that static *in vitro* models were used, with only 1 fixed concentration for bile extract and pancreatin, and thus digestive enzymes. For this reason, we here investigated whether altering the concentration of digestive enzymes and bile, with presumably crucial influence on carotenoid micelle formation (Bohn, 2012; Borel, 2003), would still yield similar negative influences of Mg^{2+} as a prominent dietary DM on carotenoid bioaccessibility, also in sight of recent contradictory results from human trials investigating the effect of Ca^{2+} on carotenoid bioavailability (Borel et al., 2016; Corte-Real, Guignard, et al., 2017). As less information was also available on Mg^{2+} compared to Ca^{2+} regarding its interaction with bile salts and its role on lipid digestion, we focused on the second most abundant dietary cation. Finally, as the interaction of Mg^{2+} and carotenoids could depend on the type of lipid, two different lipid sources (canola oil and coffee creamer) were employed.

Similar as for earlier *in vitro* studies with Ca^{2+} (Biehler, Hoffmann, et al., 2011; Corte-Real et al., 2016; Corte-Real, Bertucci, et al., 2017), it was found that increasing the concentration of Mg^{2+} significantly decreased the bioaccessibility of all individual and total carotenoids, especially at the highest concentration of 400 mg/L, chosen to reflect a supplement containing approximately the RDA (400 mg/d for adult men). The observed effects were in line with a decreased absolute zeta potential

of the micellar fraction, suggestive of rather less stable particles. The results indicate that Mg^{2+} reduced carotenoid bioaccessibility by interfering with the ability of the system to form stable mixed micelles. Nevertheless, the action of Mg^{2+} on carotenoid bioaccessibility was largely influenced by the concentration of bile. In the presence of 1 mM of bile, 200 mg/L of Mg^{2+} was sufficient to significantly reduce the micellarization of total carotenoids from spinach, while at 8 mM of bile the same effect was only observed at 400 mg/L of Mg^{2+}. Contrarily, concentrations of pancreatin affected bioaccessibility of carotenoids to a lesser extent, and no significant differences were observed for total carotenoids between lowest (100 mg/L) and highest (990 mg/L) concentration when sufficient bile was present. This indicates that the negative effects persisted for a range of physiologically plausible concentrations of bile salts and pancreatin, and thus digestive enzymes such as lipase.

Previous studies have emphasized that *in vitro* bioaccessibility of carotenoids is dependent on bile (Tyssandier et al., 2001) and pancreatic enzyme concentration (Biehler, Hoffmann, et al., 2011). The capacity of the system to form mixed micelles is related to the bile salt critical micelle concentration (CMC). Below the CMC, transfer of carotenoids from the matrix into the lipid oil phase is inhibited (Rich, Faulks, et al., 2003). Most bile salts have a CMC above 2 mM, so a bile concentration of 1 mM may have been insufficient to initiate micellar formation. Also, a lower concentration of bile would have been more susceptible to the effects of Mg^{2+}, removing the majority of available bile salts via precipitation. The inhibitory effect of a low concentration of bile and, to a lesser extent, of pancreatin on carotenoid bioaccessibility, was also observed by the extent of lipolysis, as indicated by the amount of NaOH needed for fatty acid neutralization (Part 6, Table 4). Neutralization of fatty acids required significantly less NaOH in samples digested with 1 mM than with 8 mM, and in samples digested with 100 mg/ L of pancreatin versus 990 mg/L. In fact, after digestion with 1mM of bile extract and coffee creamer, we observed a thick layer of undigested fatty material floating in the test tubes. This layer was not present in samples digested with 8 mM of bile.

The presence of dietary fat can significantly improve carotenoid bioaccessibility, but may depend on the amount and type of lipid added (Gleize et al., 2013; Goltz et al., 2012). Here, we tested for differences in carotenoid bioaccessibility digested with or without Mg^{2+} when using either a vegetable

or an animal source of fat, containing approximately the same amount of lipids. Significant differences between the two types of fat were encountered, with a somewhat higher bioaccessibility with coffee creamer. Also, there was a significant interaction of Mg^{2+} and lipid type, indicating that the effect of Mg^{2+} depended on the type of lipid added. In milk, the lipid phase exists in the form of an oil-in-water emulsion composed of triglycerides, fatty acids, phospholipids and proteins, also termed as a milk fat globule membrane (MFGM) (Contarini & Povolo, 2013). The properties of the MFGM as an emulsifier may give an advantage to dairy products for the transfer of lipid-soluble microconstituents including carotenoids into bile salts and mixed micelles (Bezelgues, Morgan, Palomo, Crosset-Perrotin, & Ducret, 2009). As a crucial stabilizing element of mixed micelles, bile salts decrease the surface tension of the water-lipid interface, stabilizing emulsions (Hofmann & Mysels, 1987), giving them a negative charge. In our experiments, the zeta potential of the micellar fractions depended on the bile concentration. Micellar fractions had smaller absolute zeta potentials when samples were digested with 1 mM compared to those digested with 8 mM of bile, similar to an earlier study (Wickham et al., 1998). Increasing concentrations of Mg^{2+} also decreased the absolute zeta potential, for both lipid sources. In a study by Wickham et al. (1998), the authors showed that at bile concentrations of 6 mM, increasing Ca^{2+} concentration reduced the absolute zeta potential of the emulsions, suggesting that this might be due to the role of Ca^{2+} to shield the surface charge of the emulsion droplets.

However, binding to bile salts might not entirely explain the effect of Mg^{2+} on carotenoid bioaccessibility, as Mg^{2+} also binds FFA to form soaps of low solubility. In early studies with rodent models, it was found that both Ca^{2+} and Mg^{2+} lowered the digestibility of poorly absorbable fats with a marked increase in the excretion of fat in form of soaps (Cheng et al., 1949; Tadayyon & Lutwak, 1969). Thus, the observed decrease in bioaccessibility at high concentrations of Mg^{2+} might have been the result of its binding to both fatty acids and bile acids, resulting in the formation of soaps and precipitated bile salts.

6.6 Conclusion

In summary, Mg^{2+}, at high physiological concentrations, negatively affected the bioaccessibility of carotenoids from a spinach matrix *in vitro*, at 2 different concentrations of bile (1 or 8 mM) and pancreatin (100 and 900 mg/L). This suggests an inhibitory effect of Mg^{2+} on carotenoid bioaccessibility over a range of different digestive conditions (at least at highest concentrations of Mg^{2+} studied), possibly more reflective of an *in vivo* adaptive situation. The results of this study are thus partly in support of results obtained earlier including other minerals, employing a *static in vitro* digestion model at fixed concentrations of bile extract and pancreatin, as suggested in a recent consensus model (Minekus et al., 2014).

However, the effect of Mg^{2+} was especially accentuated in the presence of low bile concentrations (1 mM) and, to a lesser extent, at low concentrations of pancreatin (100 mg/L), indicating that under such critical concentrations, the negative effect of Mg^{2+}, and perhaps, though not studied here, other DM, is more pronounced. Although bile concentrations as low as 1 mM are not normally found *in vivo* in healthy subjects, we could imagine this happening in e.g. cholestasis, where fat soluble micronutrient malabsorption is known to be an issue (Silva et al., 2015). Further *in vivo* investigations are necessary to fully assess the extent to which Mg^{2+} could potentially affect carotenoid bioavailability.

6.7 Conflict of interest

The authors declare no conflict of interest.

6.8 Acknowledgments

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7. GENERAL DISCUSSION

7.1 General considerations

In light of recent results reporting the negative effects of Ca^{2+}, Mg^{2+}, Zn^{2+} and iron on the bioaccessibility and cellular uptake of carotenoids from a spinach matrix (Biehler, Hoffmann, Krause, & Bohn, 2011; Biehler, Kaulmann, Hoffmann, Krause, & Bohn, 2011), it was hypothesized that these DM were likely to bind bile salts and FFA during the intestinal digestion, forming mineral bile salts and fatty acid soaps of poor solubility and prone to precipitate, preventing the formation of bile-lipid mixed micelles and, hence, reducing carotenoid bioaccessibility.

However, this hypothesis was still to be tested *in vitro* in a systematic manner for other food matrices, and additional physico-chemical parameters needed to be determined to support the proposed mechanism. Also some prominent questions, reflecting a gap on existing scientific knowledge, remained to be investigated, including:

- Which dietary DM and/or trace elements hamper carotenoid bioaccessibility and at what concentrations?
- What are the effects of increasing DM concentrations on the mixed micelle formation?
- How do these effects translate into carotenoid micellarization?
- How would this affect carotenoid cellular uptake?
- Are the effects equal for all species of carotenoids?
- Can this hypothesis be validated *in vivo*?

The goal of this project was therefore to investigate the mechanistic aspects occurring during GI digestion that might explain the observed interactions between DM and carotenoids.

To do so, this project was organized in different work-packages/experimental setups with the following objectives:

i. To optimize/define conditions for an *in vitro* digestion protocol to investigate the bioaccessibility of pure carotenoids.

ii. To test the effect of DM on the bioaccessibility of pure individual carotenoids, during GI digestion, and on the physico-chemical properties of the simulated digestive fluids, namely viscosity and surface tension.

iii. To test the effect of DM on the bioaccessibility, during GI digestion, and cellular uptake of dietary carotenoids from different food matrices, and to assess changes in physico-chemical properties of the simulated GI fluids.

iv. To perform a human trial that would allow to test the hypothesis *in vivo* and validating, or not, the results obtained in the *in vitro* trials.

v. Finally, to assess the importance of bile and pancreatin on the possible mechanism of action of DM, by testing the former at low concentrations, somewhat mimicking an insufficiency scenario.

To investigate the *in vitro* bioaccessibility of carotenoids we have employed a static model of GI digestion mimicking conditions in humans. The amount carotenoids collected and extracted from the middle aqueous micellar phase of the centrifuged digesta was defined as the bioaccessible fraction. Pure commercial carotenoids were quantified via spectrophotometry. Dietary carotenoids from the digested food matrices, extracted from blood samples, and those extracted from cellular uptake trials, were separated via liquid chromatography. Changes in macroviscosity were assessed by steady state shear flow measurements of micellar fractions, and surface tension was determined via the weight-drop method as previously described by Permprasert and Devahastin (2005). Particle size and zeta-potential were determined by dynamic light scattering, and laser Doppler micro-electrophoresis, respectively. The effect of DM on carotenoid bioavailability was evaluated *in vitro* via cellular uptake trials, using a Caco-2 TC7 subclone cellular model, while evaluation *in vivo* was done by means of a human trial. For this purpose, 25 healthy male participants were recruited for a postprandial, randomised, crossover, double-blinded trial, to test the effect of three Ca^{2+} supplementation doses

(500 mg or 1000 mg of Ca^{2+} as calcium carbonate, or 0 mg in the form of a mannitol placebo) on the postprandial response of the TRL-associated carotenoid concentration over time.

7.2 Insights from *in vitro* studies

7.2.1 Methodological issues

In order to define the best conditions for the *in vitro* digestion of pure commercial carotenoids, we tested the following factors, as described in Part 2: (i) type of lipid matrix employed (milk 4% fat, cream 18% fat, or pure canola oil), (ii) presence/absence of emulsifiers (e.g. lecithin and taurocholate); (iii) addition of a gastric lipase; and (iv) final filtration (20 nm or 200 nm) of the micellar fractions.

We observed how changing certain conditions in the protocol could dramatically affect the bioaccessibility of carotenoids and, specifically in this case, of pure β-carotene. Including a lipid source in the protocol was of the uttermost importance to insure a proper solubilisation of the pure carotenoids. Adding either cream (18% fat) or canola oil resulted in a bioaccessibility that was significantly higher compared to the same protocol, but to which the same volume (150 µL) of milk (4% fat) was added instead (Part 2, Figure 1). Another factor that improved significantly the bioaccessibility of pure β-carotene was the presence of an emulsifier mix (10 mg lecithin + 50 mg monoolein + 5 mg oleic acid). Lecithin is a source of phospholipids, which are also part of the composition of mixed-micelles (Parker et al., 1999), and have been shown to stabilize biological emulsions, resulting in the increased bioaccessibility of pure carotenoids (Borel et al., 1996). The transfer of carotenoids from lipid droplets into mixed micelles is dependent, among other factors, on the extent of lipid digestion (Salvia-Trujillo et al., 2017). While the breakdown of triglycerides occurs mainly in the small intestine, a smaller percentage (around 30%) of lipid hydrolysis occurs trough the action of the human lingual and gastric lipases (Wilde & Chu, 2011). Still, *in vitro* digestion protocols do not normally include the addition of a gastric lipase, mainly because of the difficulty to find a suitable commercially available alternative to the human lipase (Alminger et al., 2014). Based on the observation of a supernatant layer of lipids at the end of the digestion, we knew that lipolysis was

incomplete, which was possibly limiting carotenoid bioaccessibility. Thus, we wondered whether the addition of a lipase to the gastric phase would improve lipid digestion and carotenoid bioaccessibility. However, the addition of the fungal lipase from *Rhizopus oryzae* as a replacement for the human gastric lipase did not appear to have any impact on the bioaccessibility of pure β-carotene. These results either reflected a limited capacity of the system to incorporate β-carotene, which was independent of fungal lipase activity, or that enzymatic activity of the fungal lipase, and hence gastric lipolysis, was limited by the pH conditions of the gastric phase, i.e pH = 3. Free *R oryzae* lipase is active between pH 3 and 11, nevertheless its activity decreases substantially at a pH less than 5 (Ghamgui et al., 2007; Ray et al., 2013). In either case, these results highlight a limitation in current GI digestion protocols, and the need for further investigation to find better commercial alternatives for the human gastric lipase.

Finally, we also tested the impact of different filtration cut-offs on the amount of carotenoids recovered from the micellar fraction. It is a standard practice in simulated digestion protocols to filter the aqueous micellar fraction at 200 nm prior to carotenoid extraction, as a way to isolate true mixed-micelles from other larger crystalline structures (Alminger et al., 2014; Failla & Chitchumronchokchai, 2005). However, the size of bile-lipid mixed-micelles is said to be approximately 8 nm in diameter (Hernell, Staggers, & Carey, 1990; Sy et al., 2012), and there was a possibility that larger droplets, with diameters > 8 nm and < 200 nm and enclosing carotenoids, though not necessarily bioaccessible, would be present in the filtered micellar fraction, leading to an overestimation of the bioaccessible fraction of carotenoids. Applying an additional filtration step, through a 20 nm membrane filter, to the previously filtered micellar fraction, resulted in the loss of approximately 28% of β-carotene, as it can be seen in Figure 2 of Part 2. This suggested that the standard single 200 nm filtration might indeed result in some degree of carotenoid bioaccessibility overestimation, even though losses to the filter membranes, due to the sequential filtration steps, should also be considered. Although the filtration of samples at a 20 nm cutoff, instead of 200 nm, would be advisable to ensure the isolation of lipid-bile micelles and avoid overestimation of carotenoid bioaccessibiliy, the additional filtration

represented additional costs and time to the project, and for this reason it was not used in the following experiments.

Having all the previous results in mind, we selected the conditions that we deemed to, on one hand be closer to physiological conditions, and on the other hand be important for the solubilisation of pure carotenoids: i) canola oil was chosen as a lipid source; ii) the addition of an emulsifier-mix was kept; iii) no gastric lipase was included; iv) and samples were filtered only once, through a standard 200 nm membrane filter.

7.2.2 Influence of divalent minerals on aspects of carotenoid of bioavilability

Following the optimisation of the digestion protocol, the next step was to investigate the project's principal hypothesis. In a first instance, we investigated the effect of DM on the bioaccessibility of pure carotenoids, avoiding potential confounding factors such as complexity of the food matrix and possible competition between carotenoids species. We wondered whether the effect of DM would be dependent on the type of carotenoids, i.e. xanthophylls or carotenes. As seen in the results of Part 3, the addition of DM to the digesta significantly reduced carotenoid bioaccessibility. However, the strength of the effect depended on the both type and concentration of the cation. While it was clear that high concentrations of DM prevented carotenoid micellarization, the effect of the cations appeared less straightforward at lower concentrations, especially in the case of Zn^{2+}, for which a concentration of 50 mg/L led to a slight yet significant improvement of xanthophyll (lutein and neoxanthin) bioaccessibility.

A similar response was observed in Part 4 when testing the effect of DM on the bioaccessibility of carotenoids from food matrices; i) at DM concentrations equivalent or higher than the RDA, bioaccessibility of dietary carotenoids was significantly reduced; ii) at lower concentrations, Zn^{2+} significantly improved the bioaccessibility of carotenoids from carrot juice, field salad and spinach. Curiously, the addition of Ca^{2+} at lower concentrations, i.e. 250 mg/L, also significantly improved the bioaccessibility of dietary carotenoids in all the tested matrices, with the exception of spinach. Although we cannot explain as to why this happened, it is most likely due to a matrix related factor,

such as the biding of Ca^{2+} to dietary fibre or oxalic acid (Blaney, Zee, Mongeau, & Marin, 1996; C. W. Weber, Kohlhepp, Idouraine, & Ochoa, 1993), since the same was not seen when testing pure carotenoids. It would be important to understand how the concentration of DM determines the occurrence of these opposing effects on carotenoid bioaccessibility, and whether this phenomenon is relevant *in vivo*.

We were also interested in knowing how the varying concentrations of DM would affect uptake by Caco-2 cells. Due to time related reasons, we decided to limit the cellular uptake trials to two different matrices, one liquid (carrot juice) and a green leafy matrix (spinach), and one divalent mineral, in this case Ca^{2+}. Due perhaps to already low bioaccessibility of β-carotene, in addition to the dilution factor of the digesta used in the cellular trial, this carotenoid was not detected. This finding was also corroborated by the following *in vivo* study, resulting in low β-carotene bioavailability.

Instead, lutein and neoxanthin (in the case of spinach) were the main carotenoids taken up by the Caco-2 cells. The negative effect of Ca^{2+} on the bioaccessibility of lutein and neoxanthin from spinach, also resulted in a significantly lower uptake at the cellular level, especially at Ca^{2+} concentrations ≥ 500 mg/L. Yet, the same was not observed for the lutein from carrot juice. Despite the negative effect on Ca^{2+} (1000 mg/L) on the bioaccessibility of lutein, only a small not significant decrease was measured for cellular uptake. It should be noted that, even without the addition of Ca^{2+}, cellular uptake of lutein from carrot juice was only approximately 4%, while less than 1% of lutein from spinach was taken up. Also it is hard to predict whether similar results were to be found *in vivo*, given the limitations of current cellular uptake models: i) being a simplistic model simulating only enterocyte-like cells, while other naturally existing cells, such as mucus producing cells, are not represented; ii) the absence of an unstirred water layer and mucus layer, which might affect the distribution of mixed-micelles to enterocytes; iii) an incubation period of 4 h which might be insufficient considering the existence of studies showing that cellular uptake can take place over longer periods of time; among others (Biehler & Bohn, 2010).

Additionally, to the effect on carotenoid bioaccessibility, the hypothesis was stated that DM bind bile salts and fatty acids to form insoluble complexes that will precipitate, hence reducing the formation of

mixed-micelles. Thus, we have looked into physico-chemical properties of the digesta including macroviscosity, surface tension, and zeta-potencial, factors which could potentially be affected by the precipitation of bile salts and FFA.

The measurements of macroviscosity and surface tension of the digesta showed that by increasing the concentration of DM, the surface tension tended to increase while macroviscosity decreased. This effect was significantly more pronounced for the digestion of pure carotenoids (Figure 3 and Table 2, Part 3). In the case of the digestion of food matrices, though the same trend was present, this was not significant, most likely due to a buffering effect of food matrix constituents, such as fibres. One important property of bile is its ability to act as a surfactant agent, i.e. to reduce the surface tension between the aqueous milieu and dietary fats, hence promoting the solubilisation and digestion of the latter. Another property of bile is its negative electrostatic charge that confers a negative zeta potential value to the digestive fluid. In general, we can say that the higher the absolute value of the zeta potential, the more stable a colloid dispersion becomes as repulsive forces between particles are also higher. Inversely, the weaker the zeta potential, the weaker the repulsive forces and thus the stability of the dispersion, making it more likely that molecules will tend to aggregate. By observing the variation of the zeta potential (Figure 5 and 6, Part 4), and how its absolute value decreased as the concentration of DM increased, we could assume that the negative effects on the bioaccessibility of carotenoids are the result of the removal of bile salts and other surfactant molecules (e.g. phospholipids) from the solution, which would explain the increase in surface tension and the reduction of the electrostatic charge of colloid dispersion. However, these results do not tell us what happens to the free fatty acids, nor the impact the DM had on lipid digestion. Additionally, after filtration of the digesta, we were able to observe the presence of larger precipitates and a visibly clearer aqueous fraction in samples digested with higher concentrations of DM, especially Ca^{2+}. This observation, together with results for surface tension and macroviscosity, strongly indicated that the presence of DM induced the formation of insoluble complexes and its precipitation.

However, the physico-chemical variation measurements could not explain the increases in bioaccessibility observed for lower concentrations of Zn^{2+} and Ca^{2+}. One possible explanation is

based on the results from Hu and colleagues (2010), reporting an increase of %FFA release from the *in vitro* digestion of corn oil-in-water emulsions stabilized with β-lactoglobulin, with increasing concentrations of calcium chloride up to 20 mM (equivalent to 0.72 mg/L). The authors proposed that Ca^{2+} would bind to the newly formed FFA originating from lipolysis, removing them from the surface of undigested lipid droplets, which creates accesses to lipases and allows lipolysis to proceed due to product removal, shifting the equilibrium toward the product side. However, this hypothesis remained to be verified in our system, and was for this reason investigated in Part 6, by determining the effect of Mg^{2+} on the extent of lipolysis.

Other aspects that prevented us from fully understanding the mechanism by which DM hampered carotenoid micellarization includeding the complexity of the food matrix, and potential interactions between DM and food constituents, such as fibers, phytates and oxalic acid.

One interesting, and unexpected effect, was that of Zn^{2+}. This trace element had in general a positive effect on the bioaccessibility of carotenoids, and was the only cation that significantly improved the bioaccessibility of β-carotene from spinach and field salad. To our knowledge, there is no existing evidence reporting the binding of Zn^{2+} to free fatty acids, that could explain a similar mechanism to calcium-biding to FFA and allowing lipolysis to proceed. However, Zn^{2+} presumably binds to FFA in a similar fashion to Ca^{2+}. Nevertheless, one study by Hedemann and colleagues (2006) reported an increased activity of certain pancreatic enzymes in weaned pigs fed supplementary Zn^{2+} (100 or 2500 ppm), suggesting a role for Zn^{2+} in pancreatic enzyme activity modulation. Similarly, Ca^{2+} ions may also regulate lipolysis by acting as cofactors of pancreatic lipase (Alvarez & Stella, 1989; Kimura, Futami, Tarui, & Shinomiya, 1982). Based on this, we wanted to assess whether DM, specifically Mg^{2+}, was able to modulate pancreatic enzyme activity and lipid digestion, as described in Part 5. Magnesium is the second most abundant dietary mineral after Ca^{2+}, and although previous studies have pointed towards the binding of Mg^{2+} to FFA resulting in precipitation, similarly to Ca^{2+} (Cheng et al., 1949; Tadayyon & Lutwak, 1969), we were not able to find any information regarding a potential effect of Mg^{2+} on lipid digestion. Additionally, we wanted to investigate to what extent different Mg^{2+} concentrations affected carotenoid bioaccessibility when varying bile and pancreatic enzyme

concentration to mimic bile and/or pancreatic insufficiency (Part 5, Figure 2). Variations in the concentration of FFA in the digesta were assessed by acid-based titration, using NaOH at 0.01 M. Bile concentration was key to the micellarization of spinach carotenoids, as well as lipolysis. At 1 mM of bile, not only was bioaccessibility drastically reduced (Part 5, Figure 2), but also the amount of titrated FFA was significantly lower than for samples digested with 8 mM of bile extract (Part 5, Table 2). These results are in accordance to those found by others (Biehler, Kaulmann, et al., 2011; Tyssandier et al., 2001), in which transfer of carotenoids into mixed micelles was significantly compromised when bile was used at low concentrations (< 2 mM) or even being absent.

Although the concentration of pancreatin also significantly affected both carotenoid micellarization and FFA concentration, it did so to a lesser extent. Samples digested with 100 mg/L of pancreatin elicited lower carotenoid bioaccessibility and a lower volume of titration with NaOH. The effect of the low concentration of bile and pancreatin on carotenoid bioaccessibility was especially accentuated by the addition of Mg^{2+}, and in conditions with 1 mM bile the addition of this divalent mineral hampered carotenoid micellarization, in most cases up to 100%. We could speculate that if a similar effect of low bile and pancreatic enzyme concentrations would take place *in vivo*, that this would consequently translate into the hindered absorption of carotenoids, and possibly other food constituents and nutrients, as it happens in chronic conditions such as pancreatic insufficiency, and cholestasis in which nutrient deficiencies are present (Dutta et al., 1982; Shneider et al., 2012).

Conversely, addition of Mg^{2+} had overall no significant effect on the concentration of FFA. Still, when we split the data by the type of lipid source used, canola oil or coffee creamer, we observed that, for some conditions digested with coffee creamer, the titrated amount of FFA was significantly affected by the concentration of Mg^{2+}. At 300 mg/L of Mg^{2+}, the amount of titrated FFA was significantly lower compared to the control (i.e. no addition of Mg^{2+}). Yet, for reasons that we cannot explain, no significant effect was observed for samples digested with canola oil. However, based on the fact that results were different depending on the source of dietary lipids, we could speculate that the effect of Mg^{2+} was not directly related to the regulation of enzymatic activity, but rather to possible interactions

between the cation and other food constituents present in the coffee creamer, such as whey proteins and caseins (Oh & Deeth, 2017).

In summary, results from the *in vitro* experiments were consistent and in line with previous results, and appear to clearly indicate that DM do have the potential to modulate carotenoid bioaccessibility by: i) binding to bile salts and other surfactant agents, changing their solubility and rendering them unavailable to form mixed-micelles; and ii) affecting the extent of lipolysis.

7.2.3 Insights from an in vivo study

In spite the *in vitro* results, the hypothesis of DM – carotenoid interaction remained to be validated *in vivo*. For this purpose, we designed and carried out a human trial to study the effect of different doses of Ca^{2+} supplementation (500 mg or 1000 mg, and a placebo) on the bioavailability of carotenoids from a spinach based meal. Twenty-five healthy and free living males were recruited to participate in a postprandial, randomized, placebo controlled, crossover, double-blinded human trial. Participants were given 270 g of whole spinach leaves seasoned with 18 mL of canola oil for breakfast. The Ca^{2+} supplement was given at the end of meal, as calcium carbonate in gelatine capsules, together with 300 mL of water. Each participant was scheduled for 3 clinical visits and assigned randomly to a treatment group. The bioavailability of carotenoids was assessed in function of the variation over time of the postprandial concentration of carotenoids in the TRL plasma fraction (representing newly absorbed carotenoids), and the baseline corrected AUC was used as an outcome measure. Based on the statistical analysis of the average AUCs of lutein, β-carotene, β-cryptoxanthin and also triglycerides following each Ca^{2+} supplementation dose, no statistical significance was found between them (Part 5, Figure 3), which suggests that Ca^{2+} supplementation had no effect on carotenoid bioavailability, hence not validating our hypothesis *in vivo*. However, it could not be completely rejected that the comparatively limited amounts of carotenoids, the rather high fiber content of spinach, and the possibly rate-limited dissolution kinetics of the calcium carbonate during digestion contributed to the non-significant findings.

Yet, a similar recently published research study has found opposite results. The authors investigated the effect of 500 mg of Ca^{2+} supplementation on the bioavailability of lycopene from a tomato paste based meal, and results showed an 83% reduction of lycopene bioavailability upon supplementation of the test meal with Ca^{2+}. It should be highlighted, nonetheless, that there were some differences between the two studies that could have, at the end, resulted in the contradictory results, as for example: the choice of the more homogeneous tomato paste versus whole leaf spinach based test meal; Ca^{2+} supplement was dissolved in water as opposed to the gelatine capsule form used in our trial; assessment of plasma lycopene levels over a limited time frame instead of measuring newly absorbed carotenoids present in the TRL fraction.

Still, in light of the differing results between these two studies, we believe that the hypothesis presented in this project is not to be discarded just yet; that DM may indeed be considered as one of the factors influencing carotenoid bioavailability, and that this factor deserves further investigation.

7.2.4 Future Perspectives

The work developed and the results that were derived from this project have provided some more insights about the interactions of DM and carotenoids during digestion, and it has opened the door for future research questions. On one hand we have seen *in vitro* how the results varied according to the type of food matrix that was being tested. This same food matrix dependent effect might explain to some extent the results obtained in the human trial, where we detected no significant effect of Ca^{2+}, while another research group has found opposing results when using a different type of food matrix, results that were coincidentally published almost in parallel. This highlights the need to better understand the interactions that occur between DM and other food constituents such as soluble fibers, polysaccharides, proteins, and other phytochemicals.

It should be mentioned that the DM and trace element content in a meal it is probably not high enough to reach the concentrations needed to see a negative effect on carotenoid micellarization and intestinal uptake. Still, this could be possible with the regular intake of mineral supplements. Hence, a longer human trial, with a minimum duration of several weeks up to several months, looking at the

effect of daily intake of mineral supplements on plasma carotenoid concentration, could be envisioned to assess whether higher concentrations of DM have long term effects on the body's carotenoid status and other liposoluble nutrients and microconstituents.

Another important aspect brought to light by the *in vitro* results here presented is how crucial the concentration of bile and pancreatin are to the micellarization of carotenoids, and how bioaccessibility is more prone to being compromised in a low bile or pancreatin : high mineral ratio scenario. In fact, patients suffering from GI disorders such as pancreatic insufficiency were found to be deficient in liposoluble vitamins (Dutta et al., 1982). The question arises as to whether low carotenoid status of these patients was also to be found, however, this had not been investigated.

Finally, the results from this study could also be of relevance for the industry, especially concerning the formulation of dietary supplements. With the existing number of multivitamin and multimineral products on the market, it would be important to evaluate the full extent of the influence of DM of the intestinal uptake of other nutrients present in the formulations, so that concentrations could be adjusted accordingly to insure minimum negative effects on bioavailability.

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9. CURRICULUM VITAE

JOANA CORTE-REAL

EDUCATION

PhD in Nutritional Chemistry | (2012 –)

Luxembourg Institute of Health (LIH), Luxembourg (L)
Luxembourg Institute of Science and Technology (LIST), Belvaux (L)
Technischen Universität Kaiserslautern (D)

Master in Integrated Systems Biology | (2010)

Faculty of Sciences, Technology and Communication, University of Luxembourg (L)

License (Bachelor+1) in Marine Biology and Biotechnology | (2006)

School of Tourism and Maritime Technology (ESTM), Polytechnic Institute of Leiria (IPL), Peniche (P)

RESEARCH

PhD Thesis | Bioavailability of Carotenoids

Luxembourg Institute of Health (LIH), Luxembourg (L)
Luxembourg Institute of Science and Technology (LIST), Belvaux (L)

Preparation and conduction of a clinical trial; carotenoid extraction and analysis; gastro-intestinal digestion models; Caco-2 cellular models

Master Thesis | Microarray Analysis of reactive astrocytes

Neuro Inflammation Laboratory, University of Luxembourg (L)
Former Microarray Center, Luxembourg Institute of Health (L)

Bioinformatic analysis of microarray data; Inference of gene networks; Data mining

Research Assistant | Neuro Inflammation Laboratory

Neuro Inflammation Laboratory, University of Luxembourg (L)

RNA extraction; Real-time PCR (qPCR); Data analysis

Undergraduate Thesis | Effects of Stress on Aquaculture Fish Rearing

Aquaculture Group, Centre of Marine Sciences (CCMAR), Faro (P)

Zootechnical (sampling and handling) assays; Laboratory assays (glucose and cortisol)

EXPERIENCE

Participating member of the EUROCAROTEN COST Action (CA15136)

Representative of the Cost Action's Think Tank for the year 2016-2017

Collaborative development and production of the EUROCAROTEN COST Action Newsletter

LANGUAGES

Portuguese	●●●●●
English	●●●●○
French	●●●○○
Spanish	●●○○○
Luxembourgish	●○○○○

COMPETENCES

Human Trial
Chromatography
Carotenoid analysis
qPCR
Cellular Culture
SPSS Statistical Software

PERSONAL SKILLS

Communication ●●●●○
Team Work ●●●●○
Management ●●●○○
Creativity ●●●●○
Adaptability ●●●●●

INTERESTS

Nutrition & Health
Psychology
Reading
Swimming and Yoga

EXPERIENCE (continued)

Organising Committee's Member | Sep-Dez 2015
Luxembourg Institute of Science and Technology (LIST), Luxembourg (L)

Organisation of the Institute's ERIN Department 1st PhD Day Event

Supervision of Master Students | 2013-2015
Luxembourg Institute of Science and Technology (LIST), Luxembourg (L)

Training in skills and methods; Scientific discussion; Guidance throughout the assigned tasks; Review of final report

Veterinary Assistant | (2007 – 2008)
Veterinary Clinic *Vale do Coto*, Caldas da Rainha (P)

Front desk; Patient scheduling and billing; Assistance to animal health and care services; Cleaning and maintenance of animal facilities.

ACHIEVEMENTS

1st Place for Best Poster Award
5th Clinical Research Days (October 2013) | Organised by the Clinical and Epidemiological Investigation Centre (CIEC), Luxembourg Institute of Health (LIH), Luxembourg (L)

2nd Place Award for Outstanding Student Speaker Presentation and Student Scholarship Award
17th International Carotenoid Symposium (June 2014) | Park City (Utah), USA

2nd Place for Best Poster Award
Macular Carotenoid Conference (June 2015) | Cambridge (UK)

1st Place for Best Oral Presentation
1st ERIN PhD Day (December 2015) | Luxembourg Institute of Science and Technology (LIST), Belvaux (L)

PUBLICATIONS

Corte-Real J, Iddir M, Soukoulis C, Richling E, Hoffmann L, Bohn T (2016); Effect of divalent minerals on the bioaccessibility of pure carotenoids and on physical properties of gastro-intestinal fluids; Food Chem. 17 (Pt A): 546-53

Benlloch-Tinoco M, Kaulmann A, **Corte-Real J**, Rodrigo D, Martínez-Navarrete N, Bohn T. (2015); Chlorophylls and carotenoids of kiwifruit puree are affected similarly or less by microwave than by conventional heat processing and storage; Food Chem. 187: 254-62

Corte-Real J, Richling E, Hoffmann L, Bohn T (2014); Selective factors governing *in vitro* β-carotene bioaccessibility: negative effects of low filtration cutoffs and alterations by emulsifiers and food matrices; Nutr Res. 34(12): 1101-10

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10. LIST OF PUBLICATIONS

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11. POSTERS AND ORAL PRESENTATIONS

ORAL PRESENTATION: **Corte-Real J.**, Guignard C., Gatenbeim M., Bernard W., Burgard K., Hoffmann L., Richling E., Bohn T. No influence of supplemental dietary calcium intake on the bioavailability of spinach carotenoids in humans. 18th International Symposium on Carotenoids, 9th – 14th July 2017, Lucerne (Switzerland).

POSTER: **Corte-Real J.**, Iddir M., Soukoulis C., Richling E., Hoffmann L., Bohn T. Effect of divalent minerals on the bioaccessibility of pure carotenoids. 12th European Nutrition Conference, 20th – 23rd October 2015, Berlin (Germany).

POSTER: **Corte-Real J.**, Iddir M., Soukoulis C., Richling E, Hoffmann L., Bohn, T. Effect of divalent minerals on the bioaccessibility of lutein and other carotenoids. Macular Carotenoid Conference, 9th – 14th July 2015, Cambridge (UK). **2nd Place for Best Poster Award**

POSTER: **Corte-Real J.**, Gantenbein M., Chioti A., Hoffmann L., Bohn T. BIOCAR - A clinical trial on the impact of calcium supplementation on the BIOavailability of CARotenoids. Journée Portes Ouvertes CIEC, 19th May 2015, Luxembourg (Luxembourg).

ORAL PRESENTATION: **Corte-Real J**, Richling E., Hoffmann L., Bohn T. Setting up models for studying the effect of dietary minerals on bioavailability aspects of carotenoids. 17th International Symposium on Carotenoids, 29th June – 4th July 2014, Park City (Utah, USA). **2nd Place Award for Outstanding Student Speaker Presentation and Student Scholarship Award**

POSTER: **Corte-Real J.**; Gantenbein M., Chioti A.; Hoffmann L., Bohn T. BIOCAR – A clinical trial on the impact of calcium supplementation on the bioavailability of carotenoids. Luxembourgish Nutrition Conference (NuLux), 25th October 2013, Luxembourg (Luxembourg).

POSTER: **Corte-Real J.**; Richling E.; Hoffmann L.; Bohn T. Setting up models for studying the effect of dietary minerals on the bioavailability aspects of carotenoids. Luxembourgish Nutrition Conference (NuLux), 25th October 2013, Luxembourg (Luxembourg).

POSTER: **Corte-Real J.**; Bohn T.; Velez T.; Gantenbein M.; Chioti A. BIOCAR – A Clinical trial on the impact of calcium supplementation on BIOavailability of CARotenoids. 5th Clinicar Research Day, 23rd October 2013, Lxembourg (Luxembourg). **1st Place for Best Poster Award**

POSTER: **Corte-Real J.**, Richling E.; Hoffmann L.; Bohn T. Setting up models for studying the effect of dietary minerals on the bioavailability aspects of carotenoids. 12th International Congress of Nutrition, 15th – 20th September, Granada (Spain).

POSTER: Corte-Real J.; Richling E.; Hoffmann L.; Bohn T. BIOCAR – Bioavailability of Carotenoids. PhD Days University of Luxembourg, 9th – 10th September 2013, Luxembourg (Luxembourg).

13. STATUATORY DECLARATION

I, Joana Corte-Real, hereby declare that the present work was written and compiled by myself, accounting the aid of mentioned sources and authors, and that it follows the doctoral regulations of the Department of Chemistry of the Technical University of Kaiserslautern.

Kaiserslautern, the 17th October 2018 Joana Corte-Real