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Adaption of an *in vitro* digestion method to screen carotenoid liberation and *in vitro* accessibility from differently processed spinach preparations

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ABSTRACT

Dark green leafy vegetables are primary food sources for lutein and β-carotene, however these bioactives have low bioavailability. The effects of mechanical and thermal processing as well as fat addition and fat type on lutein and β-carotene liberation and *in vitro* accessibility from spinach were investigated. Lutein liberation and *in vitro* accessibility were three-fold higher from spinach puree compared to whole leaves. Results for β-carotene liberation were similar, whereas that of β-carotene accessibility was only about two-fold. Steaming had no or a negative effect on carotenoid liberation. Fat addition increased β-carotene liberation from raw and steamed puree, but reduced lutein liberation from steamed leaves and raw puree. Fat types affected β-carotene differently. Butter addition led to a 2.5 fold increased liberation from raw spinach puree, while the effect of olive and peanut oil was significantly lower, but only minor effects were observed for lutein.

1. Introduction

Carotenoids are naturally occurring fat-soluble food pigments present in high concentrations in fruit and vegetables (Liu, 2013). Results obtained in epidemiological studies have indicated that dietary carotenoids could play a role in prevention of several diseases including certain types of cancer, cardiovascular disease and ophthalmic conditions (Krinsky & Johnson, 2005). Dietary intake of the xanthophylls lutein and zeaxanthin is hypothesised to be inversely related to the risk of Age-related Macular Degeneration (AMD), whereas β-carotene is associated to visual health through its role as a pro-vitamin A (Zampatti et al., 2014).

One of the primary dietary sources of lutein and β-carotene are dark green leafy vegetables (Liu, 2013). Large variations in carotenoid content of these vegetables have been observed following variations in genetic, cultivar and harvesting factors as well as post-harvest processing (Walsh, Bartlett, & Eperjesi, 2015).

Bioavailability of carotenoids from green leafy vegetables is relatively low and depends on a complex set of factors, which have been summarized in the acronym SLAMENGHI (Castenmiller & West, 1998). Bioavailability is defined as the fraction of ingested nutrients that is available for utilization in normal physiological functions or storage and is thus a key concept for nutritional effectiveness. Moreover, it includes bioaccessibility, which is the fraction of ingested nutrients released from the food matrix into the gastro-intestinal tract and thereby made available for intestinal absorption (Fernandez-Garcia, Carvajal-Lerida, & Perez-Galvez, 2009).

The first step in digestion of carotenoid containing vegetables comprises liberation of the components from the food matrix. The main prerequisites for this step include disintegration of the food matrix, i.e. reduction of particle size and cellular breakage, in order to release carotenoids (Bohn et al., 2015). In addition, food preparation and processing such as mechanical and thermal treatments as well as integration of the food item into whole meals, have been indicated to influence carotenoid liberation and accessibility (Bohn et al., 2015). Also, physiological factors during digestion such as pH values, enzyme activity and exposure time in the different intestinal compartments are known to be important (Alminger et al., 2014).

Accessibility of fat-soluble compounds like carotenoids depends on the type and amount of co-ingested fat available in the diet (van Het Hof, West, Weststrate, & Hautvast, 2000). Moreover, it is influenced by lipolysis facilitated by lipases and pancreatic juice, emulsification mediated by bile salts and incorporation into mixed micelles during their formation, before the compound can be taken up by intestinal mucosa (Castenmiller & West, 1998; Van Buggenhout et al., 2010). The specific carotenoid in question

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(Alminger et al., 2014) and the size of the mixed micelles formed have been indicated to be important factors (Corte-Real, Richling, Hoffmann, & Bohn, 2014) when evaluating the bioaccessibility potential.

Bioavailability and bioaccessibility are both complicated to assess *in vivo*. A simpler and faster approach, however, is offered by *in vitro* methods, which may be useful for screening the availability potential as a function of numerous factors such as physiological variations (age, pH, transit time etc.), matrices, food processing and meal effects. Various *in vitro* digestion methods have been described in the literature (Garrett, Failla, & Sarama, 1999; Hedren, Diaz, & Svanberg, 2002; Oomen et al., 2003). Thus, conflicting information and results have been reported (Van Buggenhout et al., 2010) making comparison of results difficult. A new European consensus *in vitro* digestion protocol has been elaborated within the COST action FA 1005 Infogest (Minekus et al., 2014). The goals were to standardize procedures and allow comparisons between different laboratories.

The aim of the present study was to apply the standardised COST Infogest protocol (Minekus et al., 2014) specifically to carotenoids. In order to test its usefulness as a screening tool to evaluate lutein and β-carotene liberation and *in vitro* accessibility from green leafy vegetables, differently prepared spinach was subjected to the standardised *in vitro* digestion model. To improve carotenoid liberation, the effects of pureeing, steaming, fat addition and type were studied in screening experiments. Moreover, two spinach preparations representing whole leaf salad and spinach puree were selected to compare carotenoid liberation and *in vitro* accessibility. The overall aim of the present study was to screen several influential parameters for carotenoid liberation and *in vitro* accessibility using a standardised protocol.

2. Materials and methods

2.1. Enzymes, chemicals and standards

Human α-amylase (300–1500 units/mg protein, A1031), porcine pepsin (⩾2500 units/mg protein, P7012), bovine/ovine bile acid mixture (B8381) and porcine pancreatin (8xUSP specifications, P7545) were purchased from Sigma Aldrich (Buchs SG, Switzerland) as were chemical compounds, buffer salts and 3,5-Di-*tert*-4-butylhydroxytoluene (BHT). Methanol, acetone, acetonitrile (all analytical grade, Carlo Erba, Val de Reuil, France) and dichloromethane (analytical grade, Honeywell) were procured from Thommen & Furler (Rüti BE, Switzerland). For filtration, Nylon syringe filters (pore size 0.22 μm, BGB SF 2503–1, BGB, Boeckten, Switzerland) were used. The carotenoid standards lutein (purity ⩾ 96%, No. 0133) and β-carotene (purity ⩾ 96%, No. 0003) were acquired from Carotenature (Lupsingen, Switzerland).

2.2. Samples, sample preparation and processing

Baby leaf spinach (*Spinacia oleracea*) was purchased fresh in one batch and further prepared in the laboratory: either used as whole leaf spinach (model for salad and steamed leaves) or processed into puree (model for smoothie and soup) by finely grinding whole leaves in a cutter at room temperature (La Moulinette DPA 1, Moulinex, Solingen, Germany).

Both spinach leaves and puree were divided into aliquots and further treated to mimic conventional domestic steaming. Briefly, spinach preparations were separated into eight aliquots of 75 g. Aliquot 1 was directly transferred to a plastic bag and immediately shock-frozen in liquid nitrogen (Messer Schweiz AG, Lenzburg, Switzerland) (referred to as raw without oil). For aliquots 2–4, three types of fat were added (referred to as raw with refined olive oil, peanut oil and butter, respectively; all oils purchased from the local retailer). 2.5 g of fat was added to 75 g of spinach (which has been recognised to improve β-carotene absorption from meals (van Het Hof et al., 2000). The mixture was transferred to plastic bags and shock-frozen as described above. In parallel, aliquots 5–8 (without and with the three types of fat, respectively) were steamed at 100 °C for 3 min in a household combi steamer (BOSCH HLHB34-2, Fust AG, Zug, Switzerland), transferred to plastic bags and shock-frozen (referred to as steamed). Samples were weighed before and after heating to calculate potential water loss. Each aliquot was processed in triplicates and all samples were stored at -20 °C in the dark for up to 1 month until *in vitro* experiments.

Samples to quantify the carotenoid content in starting materials were prepared for fresh and processed materials. An aliquot of each preparation was frozen immediately after processing in liquid nitrogen, ground to a fine powder in the cutter and stored at −20 °C in amber plastic bottles until analysis.

2.3. In vitro digestion

The *in vitro* digestion procedure was adopted according to the standardised COST Infogest protocol (Minekus et al., 2014) with electrolyte concentrations from Kopf-Bolanz et al. (2012). Digestive juices were prepared for mouth (Simulated Saliva Fluid, SSF), stomach (Simulated Gastric Fluid, SGF) and small intestinal (Simulated Duodenal Fluid, SDF) compartments according to Supplementary Table 1a. Addition of enzymes and final preparation of simulated fluids was done according to Supplementary Table 1b.

The digestion principle is presented in Fig. 1 and described here in short. Prior to digestion, samples were defrosted and 5 g of sample was weighted into an amber screw-capped bottle. 5 mL of SSF fluid containing human α-amylase was added and the sample incubated at pH 7.0 ±0.2 for 10 min. Following this, 10 mL of SGF containing porcine gastric pepsin was added, pH adjusted to 3.0 ± 0.2, and the sample re-incubated for 2 h. The final step in the digestion procedure included the addition of 20 mL SDF

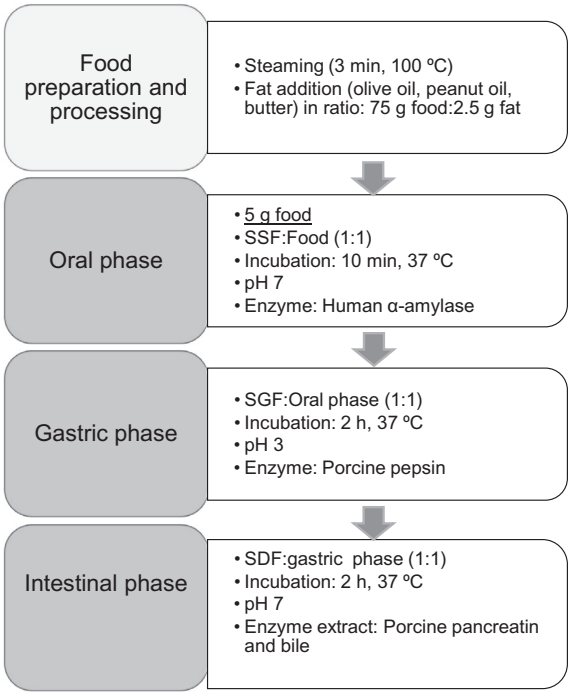

Fig. 1. Preparation and digestion principle. SFF: simulated saliva fluid; SGF: simulated gastric fluid; SDF: simulated duodenal fluid.

containing porcine pancreatin and bile from bovine and ovine sources to the content from the gastric compartment. The pH was adjusted to 7.0 ± 0.2 and samples incubated for another 2 h.

All samples were digested in triplicate. The pH was monitored throughout the digestion procedure and adapted if values differentiated from target values beyond ±0.2 pH units. Samples were covered with nitrogen gas before incubation for both the gastric and duodenal step to mimic human physiological reduction of oxygen levels during digestion. The digestion procedure was performed under red light and all incubations were carried out at 37 °C in a shaking water bath at 90 stroke/min.

After incubation, the samples were immediately transferred to 50 mL falcon tubes on ice to minimise enzyme activity. Samples were immediately centrifuged for 10 min at ⩽5 °C (4495*g*, Haereus Multifuge 3RS+, Thermo Fischer Scientific, Reinach, Switzerland) and the middle phase containing liberated carotenoids was separated from undigested material in the residue and oil droplets on the surface. Centrifugation and separation were repeated. For quantification of carotenoid liberation, 5 mL of the aqueous supernatant was freeze-dried immediately under exclusion of light and oxygen, whereas for *in vitro* accessibility analysis aliquots were filtered through 0.22 μm syringe filters before freeze-drying. Exactly 1 mL of the filtered *in vitro* accessibility fraction was freeze-dried. Freeze-drying was done overnight in the dark.

2.4. Extraction of carotenoids

Lutein and β-carotene were extracted from starting materials, fat sources and digested samples with methanol/acetone (1:1, v/v) containing 0.01% BHT. Starting materials (1.5 g) were weighed in triplicates into brown bottles. Approx. 80 mL of extraction solvent was added and extracts were homogenised for 30 s in a Polytron blender at full speed under constant nitrogen supply followed by ultrasonic treatment for 30 min. Extracts were transferred to a 100 mL volumetric flask and filled up to the mark. Fat sources (100 mg) were extracted with 10 mL of extraction solvent. To quantify carotenoid liberation and *in vitro* accessibility in digested samples, freeze-dried supernatants were re-dissolved in extraction solvent in a ratio of 1:1. After adding small glass beads, samples were vortexed and subsequently subjected to 30 min ultrasonic treatment. Aliquots of the extracts from starting material and digested samples were filtered (pore size 0.22 μm) into brown UPLC vials and immediately analysed. All extraction procedures were performed under red light.

2.5. Quantification of carotenoids

Quantification of lutein and β-carotene was achieved on an Acquity UHPLC-PDA based system (UPLC™, Waters Corporation, Milford, USA) following a methodology described by Chauveau-Duriot, Doreau, Nozière, and Graulet (2010) and modified by Eriksen et al. (under revision). Separation was accomplished on an Acquity UPLC column (HSS C18, 1.8 μm, 2.1 × 150 mm) with a Vanguard pre-column (HSS C18, 1.8 μm, 2.1 × 50 mm column, Waters). A gradient of 25% 0.05 mol/L ammonium acetate in water with 75% acetonitrile:dichloromethane:methanol (75:10:15, v/v/v) (B) and acetonitrile:dichloromethane:methanol (60:10:30, v/v/v) (A) was applied. Separation was achieved using the following gradient: 0–4.5 min 100% B, 4.5–7.5 min 100% B → 100% A, 7.5–12.5 min 100% A, 12.5–16 min 100% A → 100% B, where after the system was stabilised on 100% B from 16–19 min. The flow rate was 0.5 mL/min, column temperature 35 °C and injection volume was 5 μL. Carotenoids were detected at 450 nm and identified by comparing retention time and absorption spectra to that of known standards. Quantification was based on a seven-point external calibration curve for lutein and β-carotene. Standards were dissolved in chloroform to produce stock solutions of 50 mg/L, which were further diluted in methanol:acetone (1:1, v/v) to produce standard working solutions in the range of 0.125 to 5.0 mg/L (Eriksen et al., under revision).

2.6. Data analysis and definitions

Carotenoid content of lutein and β-carotene in starting materials is presented as mg carotenoid/100 g fresh material. Liberation and *in vitro* accessibility are calculated as the fraction (%) of carotenoid released from the matrix to the total carotenoid content in the respective starting material. Results are presented as mean ± SD. Data variables were assessed for normality using Kolmogorov-Smirnow Test and by visual inspection of boxplots. Statistical differences between pair-wise comparisons were calculated by student's *t* test, while ANOVA followed by Tukey-Kramer test was applied for multiple comparisons. P values < 0.05 were considered significant.

3. Results and discussion

Bioavailability of carotenoids from dark green leafy plant matrices has been associated with several influential factors including the carotenoid species in question, intrinsic matrix effects, co-ingested fat as well as physiological variations connected to digestion (Castenmiller & West, 1998). Therefore, knowledge about the influence of these factors on *in vitro* accessibility of carotenoids is fundamental, when evaluating the bioavailability potential of carotenoids from green leafy vegetable matrices based on *in vitro* results.

3.1. Carotenoid content in raw and processed starting materials

Carotenoid contents in baby leaf spinach before and after processing are presented in Table 1. These concentrations are in good agreements with earlier published data on a range of spinach cultivars grown over two seasons in New Hampshire, US (Kopsell & Lefsrud, 2006).

As expected, the addition of fat to non-heated whole leaf and pureed spinach and the specific fat type added did not affect carotenoid content in starting material (results not shown). Butter contained traces of lutein and β-carotene, while only traces of β-carotene were detected in olive oil. No traces of carotenoids were found in peanut oil (results not shown). These results rule out a significant "addition effect" mediated by the three fat sources.

Steaming resulted in varying effects on the starting content of carotenoids for each spinach preparation (Table 1). Both carotenoids in whole leaf spinach were significantly reduced after steaming. In contrast, the contents of lutein and β-carotene in spinach puree were not influenced by steaming. In accordance to these results, steaming has been reported in the literature to result in varying effects on different green leafy vegetables and specific carotenoids in question. Kidmose et al. investigated the effects of 3, 6 and 9 min of steam-blanching at 90 °C on three cultivars of fresh organic spinach leaves (Kidmose, Edelenbos, Christensen, & Hegelund, 2005). A significant increase in lutein was found after 3 min steaming, while β-carotene remained unchanged. The authors hypothesised that the increase in lutein could be due to reduction in intrinsic enzymatic degradation during storage when comparing steamed samples to unprocessed raw material and enhanced extractability due to structural changes at a cellular level (Kidmose et al., 2005). In contrast to these findings, Mazzeo et al. showed a significant increase in β-carotene from industrial pre-processed spinach (particle size unknown) after steaming for 20 min at 100 °C (Mazzeo et al., 2011).

Table 1
Carotenoid content (mg/100 g edible matter) in raw and differently processed spinach.

Processing	Lutein					β-Carotene				
	Raw	Steamed				Raw	Steamed			
Fat	No fat Mean ± SD	No fat Mean ± SD	Olive oil Mean ± SD	Peanut oil Mean ± SD	Butter Mean ± SD	No fat Mean ± SD	No fat Mean ± SD	Olive oil Mean ± SD	Peanut oil Mean ± SD	Butter Mean ± SD
Whole leaves	11.08 ± 0.11^{c}	9.73 ± 0.15^{b}	9.86 ± 0.35^{b}	8.02 ± 0.50^{a}	10.10 ± 0.13^{b}	6.00 ± 0.11^{b}	5.16 ± 0.11^{a}	$5.78 \pm 0.36^{a,b}$	4.80 ± 0.27^{a}	5.92 ± 0.19^{b}
Puree	$10.42 \pm 0.26^{a,b}$	10.76 ± 0.10^{b}	$9.52 \pm 1.23^{a,b}$	8.98 ± 0.46^{a}	$9.74 \pm 0.19^{a,b}$	5.83 ± 0.15^{a}	5.63 ± 0.35^{a}	5.41 ± 0.92^{a}	5.26 ± 0.39^{a}	5.71 ± 0.21^{a}

Data represent mean values ± SD of triplicates. Significant differences ($P < 0.05$) of means between raw spinach preparation and steaming with or without fat are indicated with different letters.

Steaming when combined with different fat types correspondingly resulted in varying effects on the carotenoid content of the two spinach preparations (Table 1). The lutein content in whole leaf spinach was significantly diminished after steaming in combination with peanut oil, when compared to steamed samples without fat, or with olive oil or butter. In contrast, the combination of butter and steaming led to an increase in β-carotene, when compared to steamed whole leaves without fat and with peanut oil. For spinach puree, only a significant reduction in lutein was observed after steaming with peanut oil compared to steamed samples without fat addition. To our knowledge, results on combined effects of fat and steaming on starting contents of carotenoids in green leafy vegetables have not yet been published in the literature.

3.2. Influence of domestic processing on carotenoid liberation and in vitro accessibility after in vitro digestion

Liberation of carotenoids from the food matrix is the first step in the absorption process. Following liberation, carotenoids are emulsified in lipid droplets that develop into micelles in the intestinal lumen before uptake into enterocytes (Alminger et al., 2014). Liberation and incorporation into micelles is anticipated to be influenced by factors such as domestic processing, fat addition, the specific matrix, and the carotenoid in question (Alminger et al., 2014).

Liberation of lutein and β-carotene after domestic processing and *in vitro* digestion of whole leafs and spinach puree is presented in Fig. 2 while differences between liberation and *in vitro* accessibility are shown in Table 2.

Mechanical disruption of the food matrix was the dominating influencing factor for carotenoid liberation resulting in significantly higher values of both carotenoids from puree (see Fig. 2B) compared to whole leaf spinach (see Fig. 2A) independent of additional processing ($P < 0.05$). These results are in accordance with earlier results found in our laboratory for carotenoid liberation from spinach (Eriksen, Luu, Dragsted, & Arrigoni, 2016) and is further supported by results on *in vitro* accessibility of carotenoids from carrots (Moelants et al., 2012). To our knowledge, no studies investigating the effect of mechanical processing have been published previously on green leafy vegetable matrices. The effects of particle size reduction are expected to arise from disintegration of the food matrix and reduction in the potential plant cell wall barrier towards digestion (Bohn et al., 2015). Tissue particle size seems, based on the present results, to be a main influencing factor, clearly highlighting the importance of standardising food preparation conditions and/or mastication before comparing results from vegetable sources between static *in vitro* models. Furthermore, the combined effect of mastication and blending of food and saliva following pre-processing is difficult to imitate *in vitro*.

Liberation and *in vitro* accessibility of lutein was generally higher than β-carotene (Fig. 2 and Table 2). Several articles reported in the literature confirm higher accessibility of the less polar lutein compared to β-carotene from spinach (Chitchumroonchokchai, Schwartz, & Failla, 2004; Courraud, Berger, Cristol, & Avallone, 2013) and vegetables in mixed meals (Garrett, Failla, & Sarama, 2000; Garrett et al., 1999; Kaulmann, André, Schneider, Hoffmann, & Bohn, 2016; Reboul et al., 2006). Liberation and *in vitro* accessibility of the two carotenoids were influenced differently by the domestic procedures applied in this study as described below.

Addition of fat is, based on the fat-soluble nature of carotenoids and *in vivo* results (Brown et al., 2004), suggested to influence micellarization and absorption positively (Bohn, 2008). In the present study, the addition of different fat types to raw whole leaf spinach affected neither carotenoid liberation nor *in vitro* accessibility significantly ($P > 0.1$, results not shown). Whether this may be related to an incomplete oil emulsification and thus entrapment of carotenoids remains speculative. In contrast, lutein liberation was significantly decreased, while that of β-carotene was significantly increased with addition of fat to raw pureed material (Fig. 2B). In accordance with these observations, several studies reported in the literature have shown increased *in vitro* accessibility of β-carotene from vegetable sources, but limited effect on lutein in the presence of various fat types (Failla, Chitchumronchokchai, Ferruzzi, Goltz, & Campbell, 2014; Nagao, Kotake-Nara, & Hase, 2013). To our knowledge, no studies reported a reduced accessibility of lutein in the presence of fat. However, it has been hypothesised, that low amounts of fat in a meal could favour micellarization and/or absorption of the less polar carotenes in the presence of both carotenoids (Biehler, Kaulmann, Hoffmann, Krause, & Bohn, 2011; Bohn, 2008).

The specific origin and composition of fat sources, e.g. triglyceride content (Gleize et al., 2012), fatty acid chain length (Huo, Ferruzzi, Schwartz, & Failla, 2007) and degree of saturation (Gleize et al., 2012), have been suggested to influence specific carotenoids differently and further studies to verify this assumption are currently needed (Bohn, 2008). In the present study, olive oil had the most pronounced effect on lutein liberation from raw spinach puree leading to a reduction of around one third, when compared to the sample without fat. As described above, no studies reported a reduction in accessibility of lutein after addition of fat. Similarly to the liberation observed in our study for raw spinach puree (Fig. 2B), however, Gleize et al. found a significantly higher *in vitro* accessibility of lutein from a chopped spinach containing mixed meal in presence of butter compared to olive oil (Gleize et al., 2012). Effects of fat addition on β-carotene in puree were found to be much stronger. Addition of butter to raw spinach puree resulted in increased liberation of around 25 and 150%, when compared to other fat types and sample without fat, respectively. These results indicate a superior effect on β-carotene liberation with addition of an emulsion of animal fat, which is high in saturated fatty acids and contains a complex variety of short and medium chain monounsaturated fatty acids (Saxholt et al., 2008). These results contradict to some degree those of Failla et al. who found an increased micellarization of β-carotene from mixed salad

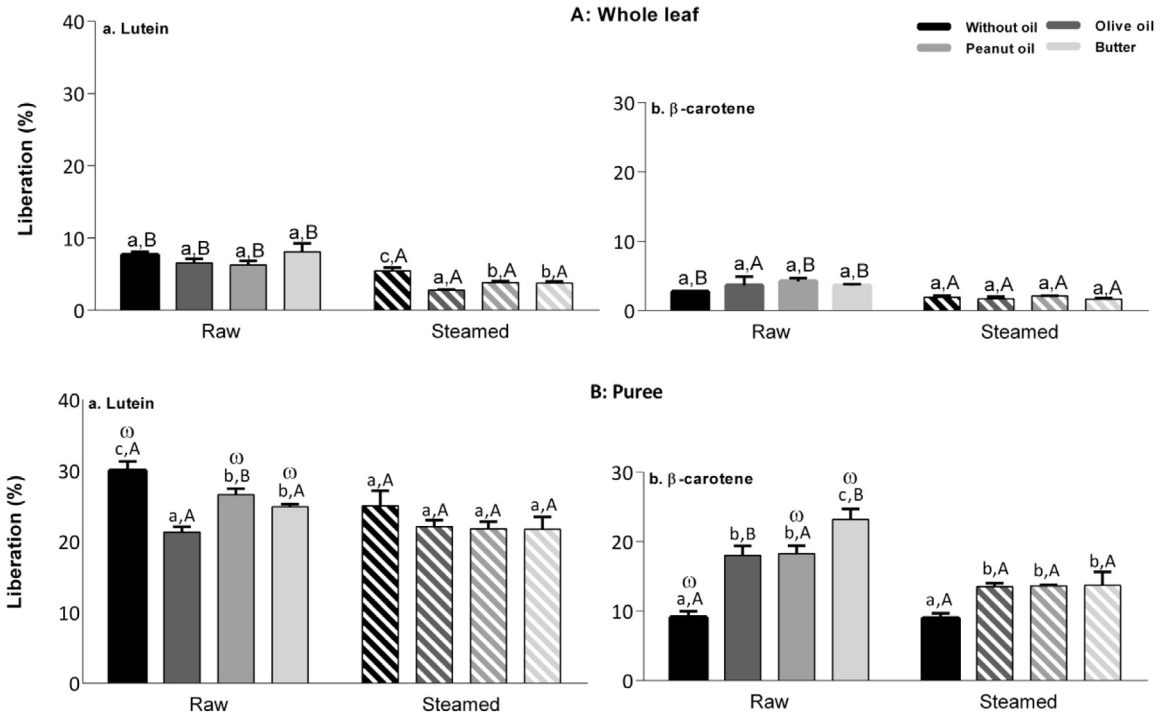

Fig. 2. Effect of domestic heat treatment and particle size on lutein (a) and β-carotene (b) liberation from spinach after *in vitro* digestion in the present of different fat types. Data represent mean values ± SD of triplicates, except for ω: n = 2. Significant differences ($P < 0.05$) of means showing the effect of fat are indicated with different lowercase letters, different capital letters show the effect of steaming.

Table 2
Liberation versus *in vitro* accessibility (micellarization) of lutein and β-carotene from raw whole leaves and steamed spinach puree.

	Lutein		β-Carotene	
	Liberation Mean ± SD	*In vitro* accessibility Mean ± SD	Liberation Mean ± SD	*In vitro* accessibility Mean ± SD
Whole leaf, raw				
No fat	7.7 ± 0.5^{a}	6.1 ± 1.3^{a}	2.7 ± 0.1^{b}	2.0 ± 0.1^{*,a}
Olive oil	6.5 ± 0.6^{b}	5.3 ± 0.3^{a}	3.6 ± 1.3^{a}	2.8 ± 0.9^{a}
Peanut oil	6.3 ± 0.6^{a}	5.3 ± 0.4^{a}	4.3 ± 0.5^{a}	3.5 ± 0.4^{a}
Butter	8.1 ± 1.2^{a}	6.4 ± 1.1^{a}	3.6 ± 0.2^{b}	2.7 ± 0.4^{a}
Puree, steamed				
No fat	25.0 ± 2.1^{a}	20.9 ± 3.6^{*,a}	9.0 ± 0.7^{b}	5.6 ± 1.4^{*,a}
Olive oil	22.1 ± 0.9^{b}	17.0 ± 2.7^{a}	13.5 ± 0.5^{b}	5.6 ± 0.7^{a}
Peanut oil	21.8 ± 1.0^{b}	16.8 ± 1.0^{a}	13.6 ± 0.2^{b}	6.1 ± 0.8^{a}
Butter	21.7 ± 1.7^{a}	21.7 ± 2.8^{a}	13.7 ± 1.9^{b}	6.2 ± 0.6^{a}

Data represent mean values ± SD of triplicates, except for *: n = 2.
Significant differences (P < 0.05) of means between liberation and *in vitro* accessibility from starting material are indicated with different letters.

vegetables in the presence of fat sources dominated by unsaturated fatty acids, i.e. soybean oil > olive > canola > butter (Failla et al., 2014). Huo et al. on the other hand did not find an effect of fatty acid saturation degree on *in vitro* accessibility of carotenes from a mixed salad, but instead reported a reduction with reduced acyl chain length of the fatty acids (Huo et al., 2007). Our present results are further contradicted by results from an *in vivo* study reporting the addition of canola oil to a salad being superior to butter, when evaluating carotenoids in the postprandial TLR fraction (Goltz, Campbell, Chitchumroonchokchai, Failla, & Ferruzzi, 2012). Obviously, the effect of fats may be governed by other experimental factors, e.g. *in vitro* digestion parameters and enzyme activity, leading to this inconsistent picture.

Steaming of whole leaf spinach generally resulted in reduced liberation of both lutein and β-carotene (Fig. 2A). These results could indicate an increased vulnerability of carotenoids to oxidation and possible degradation after steaming, although no significant effect of steaming was observed for pureed materials (Fig. 2B). To our knowledge, no studies have been published previously on the effect of steaming on liberation and *in vitro* accessibility of carotenoids from green leafy vegetables.

3.3. Combined effects of domestic processing on carotenoid liberation and in vitro accessibility after in vitro digestion

Steaming combined with fat addition resulted in reduced liberation of lutein from whole leaf spinach, when compared to both raw samples with added fat and steamed samples without fat (Fig. 2A). In contrast, the combined effect of steaming and fat addition did not influence or counteract β-carotene liberation from whole leaf spinach, when compared to steamed samples without fat. The specific type of fat added to steamed whole leaf spinach had no significant effect, except for a reduction in lutein liberation from steamed samples containing olive oil.

As for spinach puree, steaming combined with fat addition had no effect on lutein liberation, when compared to steamed samples without fat (Fig. 2B). In contrast, β-carotene liberation was increased around 35% by the combined effect of fat and steaming, when compared to steamed samples without fat. When fat was added to raw puree, however, β-carotene liberation was found to be more than two-fold compared to fat-free raw spinach. The specific type of fat combined with steaming to puree spinach did influence neither carotenoid liberation (Fig. 2B) nor *in vitro* accessibility (Table 2, $P > 0.1$).

3.4. In vitro accessibility versus carotenoid liberation after in vitro digestion

Method parameters for both *in vitro* digestion as well as post-digestion processing and assessment of carotenoid liberation and *in vitro* accessibility vary between published methods (Alminger et al., 2014). Liberation is often not reported in the literature, although it is comparable to that applied by Hedren et al. for analysis of carotenoids from Tanzanian green leafy vegetables (Hedren, Mulokozi, & Svanberg, 2002). The method for obtaining values for *in vitro* accessibility of carotenoids from spinach matrices is better described. However, large variations in method parameters are reported for both centrifugation (2500–167'000*g* at 4–20 °C for 20 min–18 h) and filtration (filter pore sizes: 0.02–0.45 μm) (Chitchumroonchokchai et al., 2004; Corte-Real et al., 2014; Courraud et al., 2013; Ferruzzi, Failla, & Schwartz, 2001; O'Sullivan, Ryan, Aherne, & O'Brien, 2008; Reboul et al., 2006; Sy et al., 2012). Therefore, results are difficult to compare without knowledge of the influence of these factors.

Inclusion of a filtration step had only minor effects on *in vitro* accessibility of carotenoids from raw whole leaf spinach when compared to levels of liberation (Table 2). In contrast, *in vitro* accessibility of lutein from steamed spinach puree was reduced significantly when olive oil and peanut oil were used as fat sources. As for β-carotene, *in vitro* accessibility was reduced by up to 55% in the presence of any fat type when compared to liberation (Table 2). Differences in hydrophobicity have been shown *in vitro* to influence the transfer of carotenoids between triglycerides and the mixed micelles (Sy et al., 2012). We therefore hypothesise, that the higher hydrophobicity and the location of the β-carotene molecule in the core of the micelle, is differentiating it from lutein after filtration.

4. Conclusions and outlook

Our results confirm that bioavailability of carotenoids from plant material is generally low and depends on a complex set of factors. Mechanical and thermal processing are strategies towards increasing the bioaccessibility potential of carotenoid containing matrices, though these processes should be chosen carefully. Disintegration of the matrix was shown to be a good approach to increase carotenoid liberation. Steaming as a mild heating process reduced carotenoid liberation from whole leaf spinach, while both liberation and *in vitro* accessibility of β-carotene from spinach puree were positively modulated by addition of different fat types with or without steaming.

Overall, the present study highlights the effectiveness of *in vitro* digestion protocols as screening tools for *in vitro* accessibility. However, some shortcomings remain. Filtration to obtain carotenoid containing micelles led to significantly reduced β-carotene *in vitro* accessibility values from steamed puree but not from raw whole leaves. Whether mechanical or the thermal treatment has a higher impact on micelle formation remains to be elucidated. Generally, the authors consider reporting of micellarization values useful to mimic *in vivo* conditions. In order to evaluate the physiological usefulness of *in vitro* results as prediction for *in vivo* outcomes, a validation study is currently ongoing.

Conflict of interest and funding

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.11.146.

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