Increased release of carotenoids and delayed *in vitro* lipid digestion of high pressure homogenized tomato and pepper emulsion.

Bente Kirkhus[a*], Nils Kristian Afseth[a], Grethe Iren A. Borge[a], Sveinung Grimsby[a], Christina Steppeler[a], Annika Krona[b], Maud Langton[c]

[a] Nofima - Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway

[b] RISE Research Institutes of Sweden, Gothenburg, Sweden

[c] Swedish University of Agricultural Sciences, Department of Food Science, Uppsala, Sweden

*Corresponding author: Bente Kirkhus, Nofima, Osloveien 1, NO-1430 Ås, Norway.

Phone +47 90036851, e-mail: bente.kirkhus@nofima.no

Short title: HPH and carotenoid release

Abstract

Carotenoids are lipophilic compounds that are digested and absorbed along with lipids. Emulsions based on a mixture of plum tomato and red sweet pepper, with 5% or 10% rapeseed oil, were obtained by high pressure homogenization, and the concentration of carotenoids in the emulsion oil droplets was quantified. The fraction of lycopene and beta-carotene released from the plant matrix into the oil droplets was highest in the 10% emulsion, which had larger oil droplets than the 5% emulsion. Xanthophylls were easily released into oil droplets in both 5% and 10% emulsions. The results suggest that the release of carotenoids made available for intestinal absorption depends on carotenoid type and can be significantly improved by increasing homogenization pressure and oil content. However, *in vitro* gastrointestinal digestion indicated the presence of constituents or structures in the emulsions, originating from tomato, that reduced pancreatic activity, which may delay micellarization and uptake of carotenoids.

Keywords: carotenoids, tomato, red sweet pepper, high pressure homogenization, *in vitro* lipid digestion, Raman spectroscopy

1. Introduction

Carotenoids are fat-soluble natural antioxidants with potential health benefits. Tomatoes and red sweet pepper are good sources of these phytochemicals, with lycopene being the most abundant carotenoid in ripe tomato, whereas capsanthin, beta-carotene and zeaxanthin are found in red pepper (U. Schweiggert, Kammerer, Carle, & Schieber, 2005; Shi & Le Maguer, 2000). Consumption of carotenoids has been associated with reduced risk of cardiac and heart disease, prostate cancer and age-related macular degeneration (Eggersdorfer & Wyss, 2018). However, the health effects of carotenoids depend on their bioaccessibility (the fraction that is released from the food matrix during digestion) which is considered relatively low for carotenoids in vegetables (R. M. Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012; van Het Hof, West, Weststrate, & Hautvast, 2000).

Food processing may improve the bioaccessibility of carotenoids by breaking down the bonding forces between carotenoids and the food matrix. Both mechanical and thermal processing have been shown to increase the release of lycopene from the chromoplasts of tomatoes (Gartner, Stahl, & Sies, 1997; Shi & Le Maguer, 2000; Tiback, et al., 2009), and thermal processing may as well cause isomerization of *trans*-lycopene to the more bioaccessible *cis* isomeric forms (Failla, Chitchumroonchokchai, & Ishida, 2008). However, thermal processing may also reduce carotenoid bioaccessibility, as indicated in some studies of red pepper (Pugliese, et al., 2014; Victoria-Campos, et al., 2013). During digestion released carotenoids are incorporated into micelles, allowing them to permeate the intestinal mucosa cells. The micellarization of carotenoids is strongly dependent on the presence of fat in the intestine (Colle, et al., 2013; van Het Hof, West, Weststrate, & Hautvast, 2000). Also the type of fat seems to affect the solubilization of carotenoids in micelles (Failla, Chitchumronchokchai, Ferruzzi, Goltz, & Campbell, 2014), as well as physicochemical

properties and the presence of other carotenoids in the food, since carotenoids compete for absorption (Wang, Roger Illingworth, Connor, Barton Duell, & Connor, 2010).

The bioaccessibility of carotenoids can be increased by incorporating them into the oil droplets of oil-in-water emulsions (Salvia-Trujillo & McClements, 2016). Such emulsions are suitable for introduction into a number of food products, adding nutritional value, as well as colour and taste to the final product. High pressure homogenization (HPH) is a well-known technique to produce stable oil-in-water emulsions, and combining heating and HPH of tomato may enhance carotenoid bioavailability in humans (van het Hof, et al., 2000). However, HPH may also release other phytochemicals, like polyphenols (Chanforan, Loonis, Mora, Caris-Veyrat, & Dufour, 2012) with the capability to inhibit pancreatic lipase and thereby lower carotenoid absorption (de la Garza, Milagro, Boque, Campion, & Martinez, 2011). Also different kinds of dietary fiber, e.g. pectin, may reduce the bioavailability of carotenoids by inhibiting lipid digestion (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014; Riedl, Linseisen, Hoffmann, & Wolfram, 1999). A positive linear relation has been found between lipid digestion products, e.g. free fatty acids (FFA), and the micellar incorporation of carotenoids (Mutsokoti, et al., 2017), suggesting that efficient lipid digestion (increase in FFA) is essential for the transport of carotenoids into micelles. The increase in FFA is rarely measured in carotenoid bioaccessibility studies. Therefore, one purpose of the present study was to determine how carotenoid rich HPH emulsions affect the lipase activity, by using a static *in vitro* digestion model. Another aim was to study the effect of increasing HPH pressure and oil content on the release of carotenoids from the plant matrix into emulsion oil droplets, by using both Raman spectroscopy directly on emulsion lipid droplets and quantification using Ultra High Performance Liquid Chromatography (UHPLC). An emulsion based on a mixture of 75% tomato and 25% red sweet pepper was chosen because it

adds both taste, colour and nutritional value with a broad range of carotenoids when included in food products, making it highly relevant for the food industry.

2. Materials and methods

2.1 Materials

Red ripe plum tomato (*Solanum lycopersicum*, 'Prunus') was grown and provided by Wiig Gartneri, Orre, Norway. Red sweet pepper (*Capsicum annuum*, "snack paprika" from Murgiverde, Spain) and rapeseed oil (Odelia, Norsk Matraps BA, Norway) were obtained from the local grocery. Trans-beta-apo-8'-carotenal (CAS Number 1107-26-2) and lycopene (CAS Number 502-65-8) standards were purchased from Cfm Oskar Tropitzsch GmbH (Marktredwitz, Germany), and beta-carotene (CAS Number 7235-40-7) from Sigma-Aldrich Co (St. Louis, MO, USA). Pepsin (porcine, P7000), pancreatin (porcine, P1750) and bile extract (bovine/ovine, B8381) were obtained from Sigma-Aldrich Co (St. Louis, MO, USA), and Pectinex Ultra SP-L from Novozymes Switzerland AG. Orlistat (Xenical® 120 mg) was obtained from Roche Pharma AG, Grenzach-Wyhlen, Germany. All chemicals were of analytical grade, and UHPLC solvents were of gradient grade.

2.2 Preparation of emulsions using high pressure homogenization (HPH)

Emulsions based on a mix of tomato and red sweet pepper (75% tomato + 25% red sweet pepper), containing 5% or 10% rapeseed oil, were obtained by high pressure homogenization (HPH) using a Panda PLUS 2000 (GEA Mechanical Equipment, GEA Niro

Soavi S.p.A., Parma, Italy). Fresh tomato (3 kg) and red sweet pepper (1 kg) were washed and cut into smaller pieces, and 15% water (v/w) was added before boiling in a saucepan for 20 minutes with the lid on. The content was then cooled on ice to approximately 15 °C, and the amount of evaporated water was replenished before addition of rapeseed oil (5% or 10%, w/w) and sodium benzoate (0.1%). The mixture was then homogenized in a blender (Wilfa BL 1200, Wilfa Norway) for 2 minutes (pre-emulsion). In order to remove seeds and larger particles the pre-emulsion was sieved using a separator with pore size 0.5 mm (Robot Coupe C80, Robot Coupe USA Inc.). Emulsions were produced by HPH at pressures 100, 200, 500, 1000 and 1500 bar. One part of each emulsion was filled in beakers (100 mL) with screw cap and stored at 4 °C for *in vitro* digestion studies, while the other part was frozen in tubes with screw cap (45 mL) at -20°C. Additional experiments with emulsions containing either tomato (100%) or red pepper (100%) were performed after the main experiment to find out whether the observed lipase inhibiting effect of the mixture originated from either tomato or pepper, or both. These emulsions were prepared with 10% rapeseed oil at 1500 bar, with samples also taken of the pre-emulsions. All emulsions were protected from light and analysed within 4 weeks.

2.3 Microstructure analysis of emulsions (CLSM)

Microstructure analysis of emulsions stored at 4 °C was performed with confocal laser scanning microscopy (CLSM) (Leica TCS SP5 II, Heidelberg, Germany). Oil droplets were stained with nile red (shown in green, Fig 1). The objective used was a HCX APO L-U-V with magnification 63 times and numerical aperture 0.90. Image size of the micrographs was 1024 x 1024 pixels. The light source was an Argon laser using λ_{ex} = 488 and signals from the samples were captured at wavelengths 600-675 nm. The size distribution of the oil droplets was

obtained by analysing micrographs using the image analysis processing program ImageJ. About 700-800 droplets were measured in each sample and the proportion (%) of each size category (0-50 μm^2) was calculated.

2.4. Quantification of carotenes by Ultra High Performance Liquid Chromatography (UHPLC)

2.4.1 Extraction of lycopene and beta-carotene from raw materials

Raw materials (tomato and red sweet pepper) were homogenized in a kitchen blender. Samples (5 g ± 0.05g) kept in 50 mL tubes were added 5 mL of an enzyme solution (Pectinex Ultra SP-L, 3% v/v in Milli Q water) plus 0.1 mL internal standard trans-beta-apo-8'-carotenal (1 mg/mL), and incubated at 50 °C for 2 h under agitation (350 rpm) in darkness. The samples were cooled to 20 °C before 5 mL 0.05% (w/v) butylated hydroxytoluene (BHT) in acetone and 30 mL n-hexane:acetone:ethanol (2:1:1, v/v) were added, and incubated for 2 hours with agitation (350 rpm) at 4 °C. Deionized water was added (5 mL) before shaking the samples vigorously by hand, and a phase separation was obtained by centrifugation at 2500 g for 10 min at 4 °C (Heraeus Multifuge 4KR Centrifuge, Thermo Fisher Scientific Inc., USA). The upper n-hexane layer was transferred to a new tube and the volume increased to 15 mL with the addition of n-hexane. The tubes were then capped and stored in darkness at 4 °C until UHPLC analysis. The extraction procedure was performed in triplicates in a dark room with a light source with red light to prevent degradation of the carotenoids.

2.4.2 Extraction of lycopene and beta-carotene from emulsions

Samples of emulsions (1 g ± 0.05g) stored at 4 °C were added 20 mL of n-hexane:acetone:ethanol (2:1:1) with 0.05% BHT plus 0.1 mL internal standard trans-beta-apo-8'-carotenal (1 mg/mL), and homogenized with a High-Speed UltraTurrax (IKA®-Werke

GmbH & Co. KG, Germany) at 15000 rpm for 1 minute on ice. The homogenate was filtered through a Whatman™ Grade 597 1/2 folded filter, and the filtrate was added 50 mL of a saturated NaCl solution and mixed in order to obtain a phase separation. The upper lipid phase was isolated and 10 mL of 10% methanolic KOH was added. The sample was then mixed and flushed with nitrogen before incubation at room temperature for 2 hours with agitation every 15-20 min. Using a separation funnel, the organic phase was washed twice with 20 mL ammonium acetate (50 mM) before being evaporated to dryness under nitrogen gas at max. 40 °C and resuspended in 3 mL THF:MeOH (1:4, v:v) with 0.05% BHT before UHPLC analysis. The extraction procedure was performed in triplicates in a dark room with a light source with red light to prevent degradation of the carotenoids.

2.4.3 Extraction of lycopene and beta-carotene from the emulsion oil phase

The content of carotenoids in the emulsion oil phase, i.e. lipid droplets, reflects the amount of carotenoids available for micellarization in the intestine. In order to estimate the contents of lycopene and beta-carotene in the emulsion oil phase, a representative lipid layer was obtained from emulsions frozen at -20 °C that were thawed and centrifuged at high speed (17000 g for 30 min at 4 °C). In other words, freezing was used to break the emulsion and complete a phase separation where the oil phase (obtained after centrifugation) could be subjected to UHPLC analysis of carotenoids. After centrifugation, the upper lipid layer was carefully transferred to a new tube and weighed before 10 mL n-hexane:ethanol (3:4, v:v) and 0.1 mL of internal standard trans-beta-apo-8'-carotenal (0.1 mg/mL) were added. Then 10 mL of 20% methanolic KOH was added and the sample was saponified in the dark at room temperature overnight. The sample was subsequently washed five times with 20 mL distilled water using a separation funnel based on the partition principle. Four mL of hexane was added to the funnel before 1/3 of the sample was added. Then 20 mL of distilled water was carefully

added along the inner edge of the funnel to avoid emulsification, and finally the rest of the sample was added. The lower phase was discarded and the washing procedure was repeated. The pH was 8-9 after the last washing step. The sample was evaporated to dryness under nitrogen gas and re-suspended in 3 mL THF:MeOH (1:4, v:v) with 0.05% BHT before UHPLC analysis. The extraction procedure was performed in triplicates in a dark room with a light source with red light to prevent degradation of the carotenoids.

2.4.4 Analysis of lycopene and beta-carotene by UHPLC

Lycopene and beta-carotene were analyzed by UHPLC using an Agilent 1290 UHPLC system equipped with diode array detector (DAD) and a thermostated autosampler (4 °C). Carotenoids were separated with an Acquity BEH Shield RP18 column (1.7 µm, 2.1x100 mm) (Waters Corporation, Massachusetts, USA). Eluent A was a mixture of acetonitrile:methanol:aqueous Tris-HCl buffer (0.1 M pH 8.0) (72:8:3, v:v:v) while eluent B was methanol:ethyl acetate (68:32, v:v). Flow rate was 0.3 mL/min and injection volume was 5 µL. The gradient elution profile used was as follows: 0% B for 3 min, 0-100% B in 5 min, 100% B for 3 min, 100-0% B in 0.5 min and 0% B for 3.5 min; in total 15 min run time. Identification of all-*trans* and *cis* lycopene and beta-carotene peaks was performed by comparing retention times and spectral characteristics with those of the representative standards. Lycopene and beta-carotene were quantified on a basis of external standard curves for all-*trans* lycopene and beta-carotene, with UV detection at 503 and 452 nm, respectively. The results were calculated based on three replicates and expressed as milligram per 100 g of fresh sample or oil.

2.4.5 Calculation of the fraction of carotenoids released into emulsion oil droplets

The fraction (%) of carotenoids released from the plant matrix into emulsion lipid droplets in emulsions containing 5% or 10% rapeseed oil was calculated by the formula: *%released = (concentration in the oil phase/whole emulsion) x %oil*, where the concentrations of carotenoids in the oil phase and whole emulsion are given in mg/100g oil and emulsion, respectively.

2.5 Analysis of total carotenoids by spectrophotometry

Raw materials (tomato and red sweet pepper) were homogenized in a kitchen blender. Samples (5 g) of homogenates and HPH emulsions were added 5 mL of an enzyme solution containing Pectinex Ultra SP-L (40 μL/mL water), and incubated at 40 °C for 4 hours in order to release carotenoids from the plant matrix. Extraction of carotenoids was performed on ice for 2 hours under constant shaking in the dark after addition of 30 mL n-hexane:acetone:ethanol (2:1:1) and 5 mL 0.05% BHT in acetone. Deionized water (5 mL) was added and the samples left at room temperature for 5 min to allow for phase separation. The absorbance (A) of the upper n-hexane layer was measured at 503 nm in a spectrophotometer (Ultrospec® 3000, Pharmacia Biotech, NJ, USA). The total concentration of carotenoids was calculated based on three replicates, from the formula: carotenoids (mg/kg) = (A_{503} * 31.2)/g sample (Fish, Perkins-Veazie, & Collins, 2002).

2.6 Analysis of total carotenoids by Raman spectroscopy

Raman spectroscopy was used for rapid estimation of carotenoid/lipid ratios (relative units) directly in lipid droplets of emulsion samples stored at 4 °C. Before Raman analysis, emulsion aliquots were placed on an aluminum plate. Raman spectra were recorded on a LabRam HR 800 confocal Raman microscope (Horiba Scientific, France). The excitation

wavelength of 785 nm was generated by a single frequency diode laser (Toptica photonics, Germany). A 50 X fluotar objective (0.55 NA, Leica, Germany) was used for focusing, and collection of Raman scattered light. The confocal hole was set at 1000 mm and an exposure time of 3 times 10 s was used. The Raman scattering was dispersed with a 600 lines per mm grating, which resulted in spectra in the range 200 cm^{-1} to 2000 cm^{-1}. Five Raman spectra were obtained from lipid droplets of each emulsion. Background correction was performed using an approach adopted from Lieber et al. 2003 (Lieber & Mahadevan-Jansen, 2003) based on polynomial fitting and subsequent polynomial subtraction. Carotenoid/lipid ratios (relative units) were calculated by identifying the highest Raman intensity values in the following two spectral regions: 1) Carotenoid region: 1510 cm^{-1} – 1520 cm^{-1} (i.e. carotenoid C=C stretching); and 2) lipid region: 1650 cm^{-1} – 1660 cm^{-1} (i.e. lipid C=C stretching). Carotenoid/lipid ratios were calculated as averages of the five replicates.

2.7 In vitro digestion

2.7.1 In vitro digestion model

Fresh emulsions were subjected to a static *in vitro* digestion model simulating the oral-, gastric- and duodenal phases. The model is based on the EU Cost Action 1005 INFOGEST harmonized digestion method with standardized electrolyte solutions for the preparation of simulated salivary, gastric- and intestinal fluids, respectively (Minekus, et al., 2014). Emulsions (1.0 g) were placed in tubes and 1 mL of an electrolyte solution containing salivary amylase (50 U/ml) was added and kept at 37 °C for 2 min. The gastric phase was simulated by adding 2.0 mL of an electrolyte solution containing pepsin (4000 U/mL). The pH was adjusted to 3.0 with 10 M HCl before incubation in a rotary incubator (Innova® 40/40R, New Brunswick Scientific, Edison, NJ, USA) at 37 °C and 215 rpm for 120 min. In order to simulate the intestinal phase,

tubes were added 4 mL of simulated duodenal fluid containing 0.07 mM $NaHCO_3$, porcine pancreatin and bile resulting in a bile salt concentration of 10 mM and pancreatin concentration of 1.25 mg/mL in the final volume. After adjustment of the pH to 7 with 10 M NaOH, the samples were incubated at 37 °C and 215 rpm for 20, 40, 80 and 140 minutes in intestinal phase. Tubes were placed on ice after withdrawal, and 15 mL of $CHCl_3$: MeOH (2:1, v:v) was immediately added in order to stop the lipid hydrolysis. The lipid digestion of 1 g emulsion containing 10% rapeseed oil was compared with the digestion of 1 g of a mixture of 10% rapeseed oil and 90% water (control), i.e. water replacing tomato and pepper, and 1 g of the same mixture (10% rapeseed oil in water) with added Orlistat (1/400 capsule, 0.3 mg), a lipase inhibitor used to treat obesity. All experiments were repeated three times (n=3), and analysed in duplicate at each time point.

2.7.2 Lipid extraction and analysis of free fatty acids (FFA) in digested samples

The rate of lipid digestion was determined by measuring the formation of free fatty acids (FFA) in digested samples by solid phase extraction (SPE) and gas chromatography with flame ionization detection (GC-FID). An internal standard, C23:0 (methyl tricosanoate, Larodan Fine Chemicals AB, Sweden) was used for the quantification of fatty acids in the FFA fraction. Lipids were extracted from the digesta (Bligh & Dyer, 1959) and separated into lipid classes, i.e. free fatty acids (FFA), neutral lipids (mono-, di- and triacylglycerols) and polar lipids using an automated solid phase extraction (SPE) system (Gerstel MPS Autosampler, Gerstel GmbH, Switzerland) with a modified and in-house validated method based on Ruiz et al. (Ruiz, Antequera, Andres, Petron, & Muriel, 2004). FFA were eluted with diethyl ether:acetic acid (99:1, v:v), and the solvent was removed by evaporation under N_2 before the fatty acids were derivatized using 3M methanolic HCl. The methyl esters were analyzed using an Agilent 6890 capillary GC equipped with a BPX-70 column, 60 m x 0.25 mm i.d., 0.25 μm

film (SGE Analytical Science Pty Ltd, Ringwood, Australia) and flame ionization detector. The temperature program started at 70 °C for 1 min, increased by 30 °C/min to 170 °C, 1.5 °C/min to 200 °C and 3 °C/min to 220 °C with a final hold time of 5 minutes. Peaks were integrated using Agilent GC ChemStation software (rev. A.05.02) (Agilent Technologies, Little Falls, DE), and fatty acids identified by use of external standards. Coefficients of variation were < 5%. Total lipid hydrolysis was measured as mg FFA per g oil in the emulsions.

2.8 Design of experiments (DOE) and statistical analyses

Statistical analyses of designed experiments were performed with Unscrambler® v 9.8 (Camo Inc., Norway) in order to establish the effects of HPH pressure (200 and 1500 bar) and emulsion oil content (5% and 10%) on the release of beta-carotene and lycopene into emulsion oil droplets. Significant ($p<0.05$) main effects and interaction effects were analyzed by classical DOE analysis using multiple linear regression (MLR) and Scheffé formulas. In other experiments significant differences between means were estimated by either Student's t test or by one-way analysis of variance (ANOVA) followed by the Tukey method using Minitab16 statistical software (Minitab Ltd., Coventry, UK). P values < 0.05 denoted significance.

The effect of HPH pressure (100, 200, 500, 100 and 1500 bar) on the concentration of total carotenoids in emulsion oil droplets (Raman spectroscopy) was measured in 5% and 10% emulsions. Furthermore, the effect of HPH pressure (200 and 1500 bar) on lipolytic activity during *in vitro* digestion was measured in 10% HPH emulsions, and compared with 10% rapeseed oil in water. Whether the effect on lipolytic activity originated from tomato or red pepper was investigated in 10% emulsions (pre-emulsion and HPH 1500 bar) based on either tomato or red sweet pepper.

3. Results and discussion

3.1 Microstructure of emulsions (CLSM)

The micrographs in Fig. 1a show the oil droplets (green) in emulsions (75% tomato and 25% pepper) with 5% or 10% rapeseed oil, produced at various HPH pressures, and Fig. 2 shows the droplet size distributions. At 200 bar the average droplet size was 5.3 m^2 in 10% emulsion, compared to 2.5 m^2 in 5% emulsion, and the droplet size decreased to 2.3 m^2 and 0.7 m^2, respectively, at 1500 bar. The reduction in droplet size and clustering of droplets into larger aggregates was observed in both emulsions when the homogenization pressure increased from 100 to 1500 bar (Fig. 1a). This was probably due to a more severe disruption and restructuring of the food matrix at higher pressures, causing the liberation of cell wall materials that may form a network that immobilize and stabilize the oil droplets (Lopez-Sanchez, Svelander, Bialek, Schumm, & Langton, 2011; Wallecan, McCrae, Debon, Dong, & Mazoyer, 2015). The larger oil droplets in 10% emulsions compared to 5% emulsions may be due to a smaller emulsifier/oil ratio, making it easier for the oil droplets to coalesce. Larger fat droplets have also been found in samples with the highest fat content when making cream cheese (Wendin, Langton, Caous, & Hall, 2000). One reason could be due to the higher fat content itself, or that the higher fat content was more difficult to disperse in the matrix.

In order to study the oil phase of the emulsion, emulsions were frozen at -20 °C and thawed, thereby letting oil droplets to disintegrate. After centrifugation, the emulsions separated into four phases (Fig. 1b). Phase 1 (on top) consisted of a red oil layer, revealing a high content of carotenoids. Phase 2 (below phase 1) and phase 4 (on bottom) were intact emulsions, with

phase 4 containing more cell wall material than phase 2. Phase 3 was mostly water. Micrographs of phases 2 and 4 showed that phase 2 comprised more and larger oil droplets and was less homogenous than phase 4. As expected, a significant fraction of the emulsion oil droplets was broken during the freezing process. The following centrifugation seemed to force the oil as well as the larger oil droplets to the top, whereas smaller droplets tightly bound to the food matrix, remained at the bottom. Raman spectrophotometry of the emulsions (see section 2.6) indicated that the carotenoid/lipid ratio was similar in droplets of various sizes (results not shown). Hence, the oil layer at the top was representative for the oil droplet content, and UHPLC could easily be used to determine the concentration of dissolved carotenoids made available for intestinal micellarization and absorption.

3.2 Release of carotenoids into emulsion oil droplets

The contents of lycopene, beta-carotene and total carotenoids in raw materials and emulsions are shown in Table 1. Spectrophotometry was used to measure the total carotenoid content, whereas UHPLC was used to measure the contents of lycopene and beta-carotene specifically. As expected, the spectrophotometric measurements of lycopene and beta-carotene in tomato corresponded well with the UHPLC measurements, whereas in red sweet pepper, which is high in other carotenoids than lycopene and beta-carotene (e.g. capsanthin, zeaxanthin), there was a clear discrepancy (Table 1). The fraction (%) of lycopene and beta-carotene released into the emulsion oil phase (oil droplets) was estimated based on the contents in the emulsion oil phase (Table 2) and the whole emulsion (Table 1). The fraction released varied from 25.4 to 50.9 % for lycopene and from 39.8 to 72.4% for beta-carotene (Table 2), and DOE analysis showed significant main effects of HPH pressure, emulsion oil content and carotenoid type, as well as significant interaction effects between HPH pressure × oil content

and HPH pressure × carotenoid type. In summary, the results indicated that higher amounts of both lycopene and beta-carotene were released with increasing homogenization pressures in 10% emulsions, but not in 5% emulsions. This could be due to larger oil droplets in 10% emulsions (Fig. 2) being more optimal for incorporating these carotenes. The results are in accordance with the study of Svelander et al. (Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011) showing no effect of HPH intensity on the release of lycopene in tomato emulsions containing 5% olive oil. The present results further showed that beta-carotene was somewhat better released and solubilized in the oil droplets than lycopene, not only was a higher percentage release observed in both 5% and 10% emulsions, but the increase caused by increasing HPH pressure in 10% emulsions was also larger (Table 2). One explanation may be a less tight binding of beta-carotene to the red pepper matrix than lycopene in tomato, as differences in the morphology of chromoplasts and the physical deposition form of carotenoids may play a major role in their bioaccessibility (R. M. Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012). Also differences in hydrophobicity, with lycopene being more hydrophobic, may explain a lower solubility of lycopene in rapeseed oil droplets (Colle, et al., 2013; Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011; Tyssandier, Lyan, & Borel, 2001). The lycopene concentration in the emulsion droplets ranged from 37 to 53 mg/100g (Table 1), which is higher than the reported solubility of lycopene in olive oil (22 mg/100g) (Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011). A maximum concentration of lycopene in rapeseed oil (53 mg/100g) may have been reached in the 5% emulsion (Table 2). However, by increasing the oil content to 10%, and increasing the HPH pressure, the fraction of total lycopene released from the plant matrix into the emulsion oil droplets could be significantly improved (almost two-fold). Increasing the HPH pressure from 200 to 1500 bar in 10% emulsions resulted in a 34% increase in the fraction released. This is in the same range as the increased *in vivo* bioavailability reported by van het Hof et al. (van het

Hof, et al., 2000) when homogenized tomato was included in meals containing about 20% fat, showing a 23% increase in the postprandial content of lycopene in plasma lipids when changing from mild to severe homogenization, and a 62% increase when changing from no homogenization.

Raman is becoming a frequently used technique for characterization of carotenoids (Baranska, Roman, Dobrowolski, Schulz, & Baranski, 2013; Pudney, Gambelli, & Gidley, 2011), and several studies have shown how Raman spectroscopy can be used for relative quantification of pigments and lipids (Pilat, et al., 2012, Li, et al., 2017). In this study, Raman spectroscopy was used as a rapid method for estimating carotenoid/lipid ratios in emulsion lipid droplets. Fig. 3 shows that an increase in homogenization pressure from 100 to 1500 bar resulted in a two-fold increase in amount of total carotenoids released from the plant matrices into the oil droplets. The largest increase was observed between 100 bar and 500 bar. Results further indicate that the release was not affected by the fat content of the emulsion, since the oil droplet concentration of carotenoids in 5 % emulsions were about twice as high as in 10% emulsions (Fig. 3). This is in contrast to what was observed for lycopene and beta-carotene measured individually by UHPLC. Hence, the results indicate that the xanthophylls in red sweet pepper, which dominates the mix of carotenoids in the emulsions are more easily released and solubilized in the emulsion oil droplets than the carotenes (lycopene and beta-carotene). Polar xanthophylls act differently than the nonpolar carotenes in lipid emulsions, as well as in micelles, which probably make them more bioaccessible (Furr & Clark, 1997).

3.3 In vitro lipid digestion

The lipid digestion took place only in the intestinal phase, since the *in vitro* digestion model (section 2.7.1) did not include gastric lipase. As demonstrated in Fig. 4a, a delay in

intestinal lipid digestion was observed for emulsions containing 10% rapeseed oil compared to the control (10% rapeseed oil in water). The delay was characterized by an initially slower rate of digestion resulting in lower levels of free fatty acids (FFA) at 20, 40 and 80 minutes. However, at 140 minutes the amount of FAA reached almost the same level as the control. Whether this pattern will also be present *in vivo* is not known. A major drawback of the static digestion model is that digestive products are not removed during the digestion process, which may cause product inhibition of digestive enzymes. However, the initial decrease in lipid digestion compared to rapeseed oil alone (control) clearly indicates that the tomato and pepper emulsion has the potential to reduce pancreatic lipase activity. No emulsifier was added to the control, as this was not needed in order to obtain a satisfactory lipid digestion rate, probably due to adequate amounts of emulsifying components, e.g. bile acids, in the intestinal phase. If emulsifiers had been added it would most probably have caused an even faster lipid digestion rate, providing a more pronounced difference between the vegetable emulsions and the control.

There was no significant difference between emulsions produced at 200 bar versus 1500 bar despite different microstructures, e.g. smaller droplet size and larger droplet aggregates at the highest pressure of 1500 bar (Fig. 1a). A possible explanation for this is that droplet aggregates or oil droplets embedded in plant matrix material may make it more difficult for the lipase to access the oil droplet surfaces, whereas on the other side, smaller droplet sizes provide a larger oil droplet surface available for the lipase. The net effect of these two phenomena may explain why the rate of lipid digestion was just the same for emulsions produced at 200 and 1500 bar.

In vitro digestion of HPH emulsions (1500 bar) based on either tomato or pepper alone (Fig. 4b) indicated that the lipid digestion was delayed only in tomato emulsions (and not pepper emulsions), and that the delay was present already in the pre-emulsion. The reduced lipolytic activity could be due to the presence of fiber (pectin), polyphenols or cell wall

fragments. Pectin, the most common fiber in tomato and red sweet pepper, has been shown to inhibit digestion of lipids under simulated gastrointestinal conditions (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014), as well as inhibit absorption of carotenoids in humans (Cervantes-Paz, et al., 2017; Riedl, Linseisen, Hoffmann, & Wolfram, 1999). Other components that may have the potential to inhibit pancreatic lipase activity are polyphenols (de la Garza, Milagro, Boque, Campion, & Martinez, 2011) and thylakoids (Albertsson, et al., 2007). Lycopene aggregates on thylakoid membranes and fragments may be released together with lycopene and hence reduce the lipid digestibility rate, but this needs further investigation. Although the observed reduction in lipolytic activity was lower compared to that caused by the lipase inhibitor Orlistat, the results indicate that there are components in tomato that may affect pancreatic lipase activity, either directly by inhibiting the active site of the lipase, or through the formation of structures forming a physical barrier between the lipase and lipid droplet surface. Whether this only applies to the variety (plum tomato) used in the present study or to tomatoes in general is not known.

A positive linear relation between lipid digestion (increase in FFA) and the micellar incorporation of carotenoids has been suggested (Mutsokoti, et al., 2017). FFA may therefore be a relevant measure when studying carotenoid *in vitro* bioaccessibility. However, bioaccessibility is not only determined by the increase in FFA (formation of micelles), since micelles may be attached to plant matrix material and bile binding components present in the intestine, and therefore not be available for uptake in the body. *In vitro* digestion models have been frequently used for studying bioaccessibility of carotenoids, sometimes in combination with CaCo2 cell studies (O'Sullivan, Jiwan, Daly, O'Brien, & Aherne, 2010; Pugliese, et al., 2014; Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011). The *in vitro* bioaccessibility is usually estimated by measuring the fraction of carotenoids that is present in the micellar (water) phase after centrifugation and filtration of the digesta. In the present study,

we experienced that centrifuged samples of the digesta could be divided into similar phases as for undigested emulsions (Fig. 1b). Moreover, the amount of carotenoids and lipids in the micellar phase was very low (results not shown). We therefore suspect that micelles were associated with the plant debris during centrifugation (phases 2 and 4). Entrapment of micelles in the plant matrix network formed during HPH of tomato has been suggested as a plausible explanation for the reduced *in vitro* bioaccessibility of lycopene observed in some studies (Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011), but whether this gives a true picture of the *in vivo* situation is not known. A study by Alminger et al. (Alminger, et al., 2012) showed poor consistency between measured *in vitro* and *in vivo* bioaccessibility of lycopene in HPH soups. However, there are many studies showing that *in vitro* bioaccessibility (carotenoid concentration in the micellar phase) is consistent with *in vivo* data, in particular for beta-carotene (Alminger, et al., 2012; Van Loo-Bouwman, et al., 2014). As shown in the present study, components in tomato-based emulsions may delay lipid digestion, which may reduce the fraction of lycopene transferred to a micellar phase and hence decrease the *in vitro* bioaccessibility. The observed reduction in lipase activity cannot however explain the markedly reduced *in vitro* bioaccessibility in HPH tomato products reported by others (Colle, et al., 2013; Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011). Whether the present reduction in lipase activity has any impact on *in vivo* bioavailability needs further investigation.

4. Conclusions

An adequate amount of oil in tomato and pepper emulsions is crucial for some carotenoids to be released from the plant matrix into the oil droplets during HPH. An emulsion containing 10% rapeseed oil had larger oil droplets than a 5% emulsion, and seemed more optimal for solubilizing and incorporating lycopene and beta-carotene into the oil, thus making

them more bioaccessible. Other carotenoids typical for red sweet pepper, e.g. the more polar xanthophylls like capsanthin, seemed to be more easily released from the plant matrix into the oil droplets, also in 5% emulsions. The results suggest that carotenoid release into oil droplets in HPH emulsions can be optimized by increasing both the oil content and the homogenization pressure. However, pancreatic lipase activity was initially reduced and further research should identify which components were responsible for the delay in lipid digestion and whether it influences carotenoid uptake *in vivo.*

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Conflict of interest

There are no conflicts of interest

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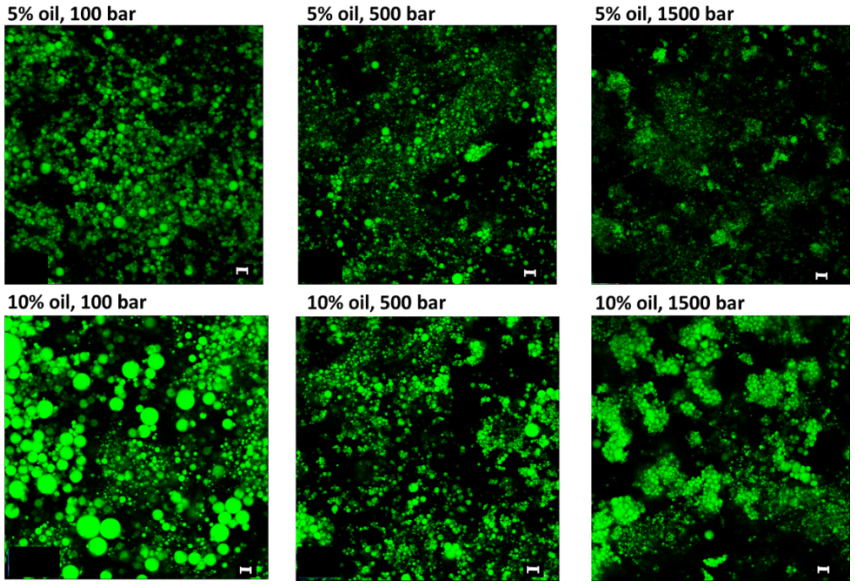
Fig. 1. Microstructure of emulsions **a)** Micrographs of HPH emulsions (75% tomato and 25% pepper) with 5% and 10% rapeseed oil, homogenized at 100, 500 and 1500 bar. Oil droplets in green. Scale bar 10 µm, **b)** Illustration of the lipid distribution in freeze stored emulsions. *Left:* frozen and thawed emulsions (10% oil) after centrifugation separated into four phases with an oil layer at the top (phase 1). *Right:* Micrographs of phase 2 and 4. Oil droplets in green. Scale bar 50 µm.

a)



b)

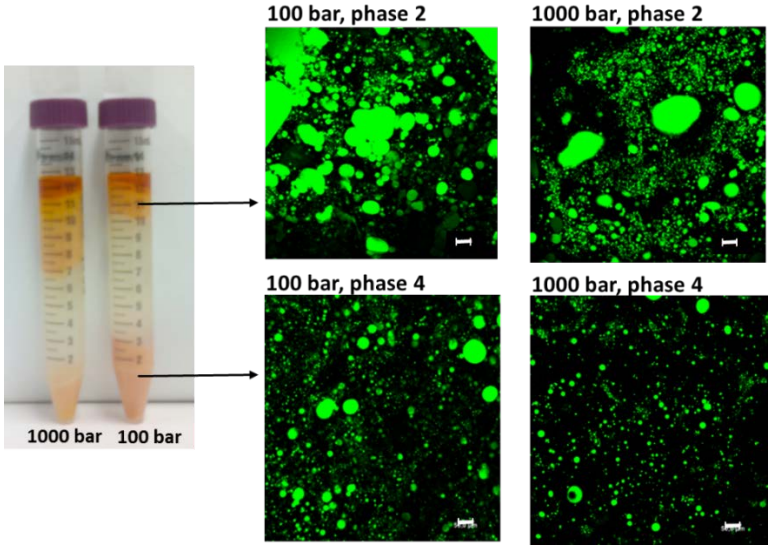


Fig. 2. Size distributions of oil droplets in HPH emulsions (75% tomato and 25% pepper) containing **a)** 5% oil, homogenized at 200 bar, **b)** 5% oil, homogenized at 1500 bar, **c)** 10% oil, homogenized at 200 bar and **d**) 10% oil, homogenized at 1500 bar. The diagrams show the proportion (%) of droplets in each size intervals from 0-50 μm^2, and the mean and median values of the oil droplet area (μm^2) is given for each droplet size distribution.

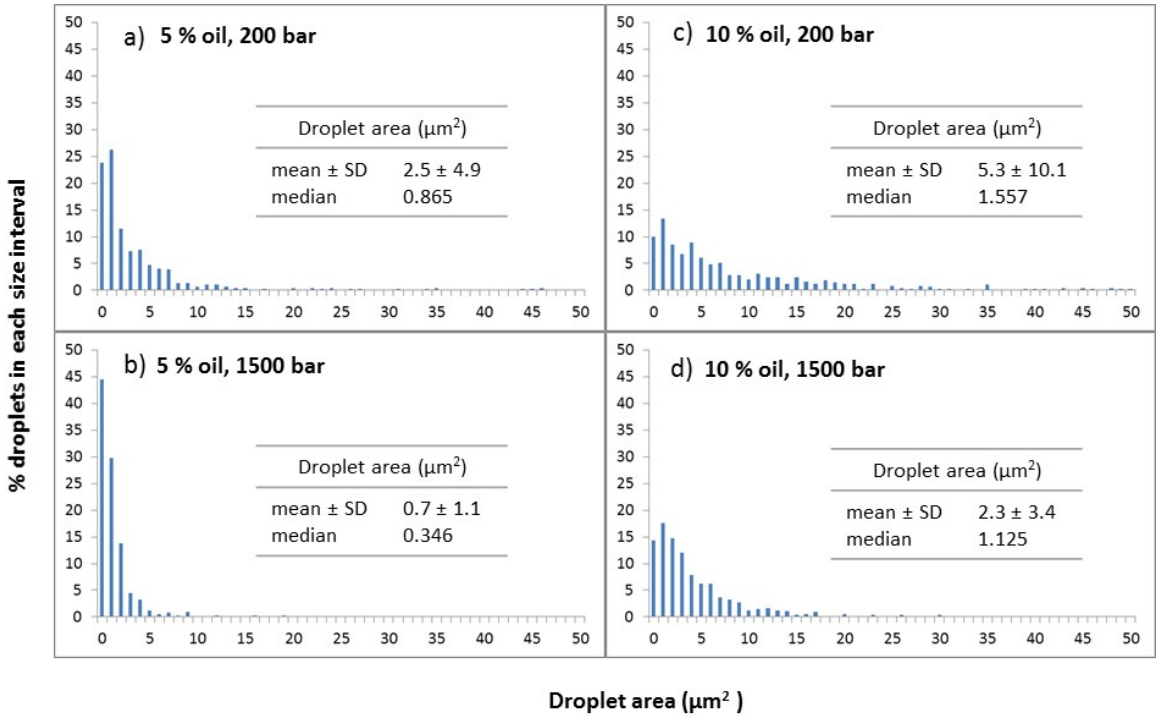


Fig. 3. Raman spectroscopic measurements of total carotenoids in oil droplets of HPH emulsions (75% tomato and 25% red pepper). Carotenoid/oil ratios (relative units, estimation error = 0.42) are plotted for emulsions with 5% rapeseed oil (—) and 10% rapeseed oil (- - -) at increasing homogenization pressures (100, 200, 500, 1000 and 1500 bar).

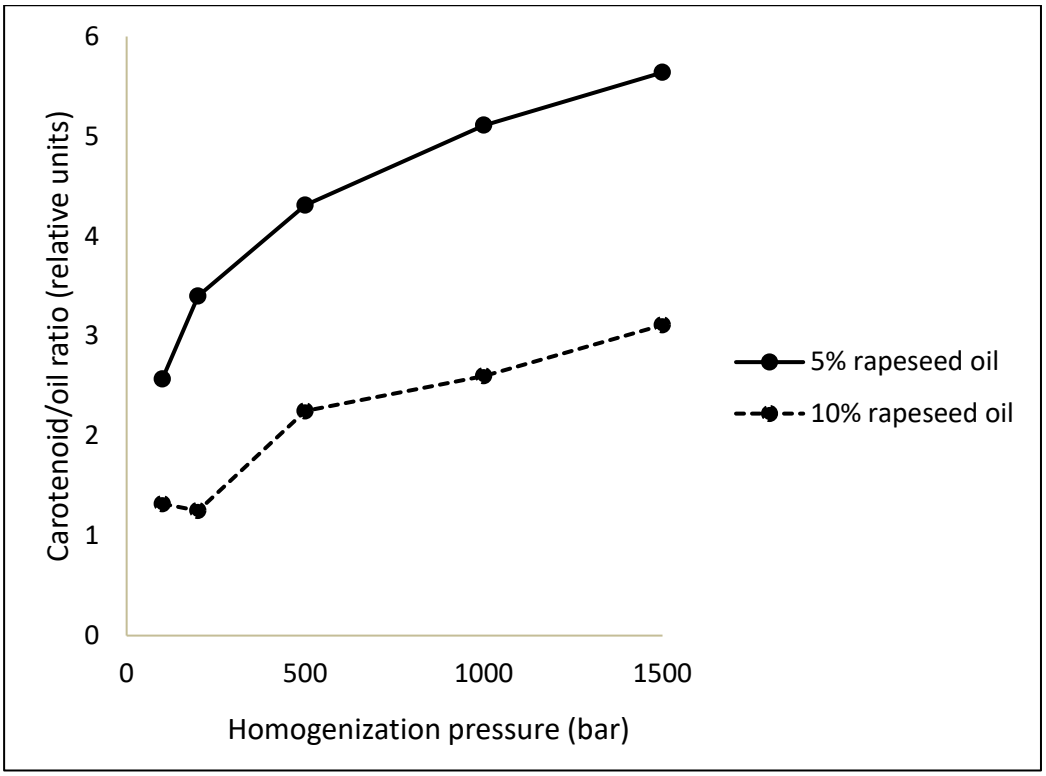
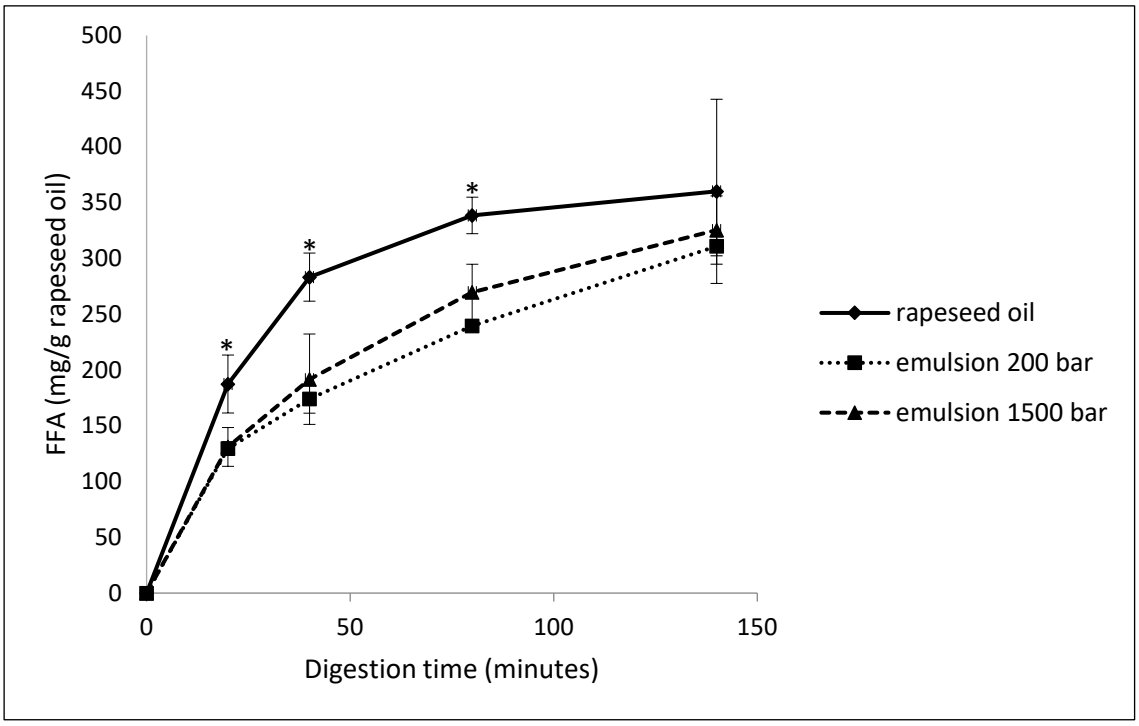


Fig. 4. *In vitro* lipid digestion of vegetable emulsions and controls, all containing 10% rapeseed oil **a)** The formation of free fatty acids (FFA) during intestinal digestion (0-140 min) of rapeseed oil in water (control) and HPH emulsions (75% tomato and 25% pepper) homogenized at 200 bar and 1500 bar, **b)** The formation of FFA after 40 min intestinal

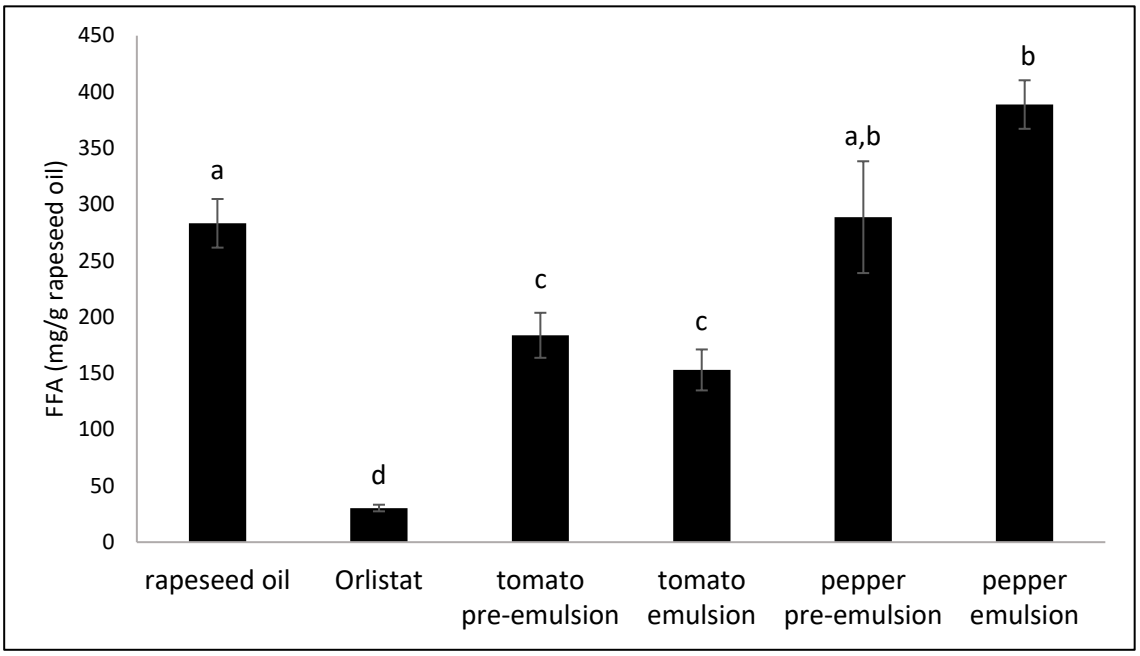
digestion of rapeseed oil in water (control), rapeseed oil in water with added Orlistat, and pre- and HPH emulsions (1500 bar) of either tomato or red sweet pepper.

a)



*Significantly different at a given time point ($p < 0.05$, one-way ANOVA)

b)



a,b,c,d - different letters indicate significant ($p<0.05$) differences between means (one-way ANOVA)

Table 1. Carotenoid contents (mg/100g) in raw materials (homogenates of red ripe plum tomato and red sweet pepper) and HPH emulsions (75% tomato and 25% pepper) containing 5% or 10% rapeseed oil, respectively. Total carotenoids were measured by spectrophotometry, and lycopene and beta-carotene by UHPLC.

	total carotenoids (mg/100g)	lycopene (mg/100g)	beta-carotene (mg/100g)
Tomato	14.3 ± 2.9	13.8 ± 0.6	0.6 ± 0.1
Red sweet pepper	43.6 ± 6.9	0.0	4.2 ± 0.4
5 % emulsion	17.8 ± 0.6	10.1 ± 0.3	2.0 ± 0.4
10 % emulsion	16.6 ± 0.5	9.8 ± 0.3	1.7 ± 0.1

Table 2. Concentrations of lycopene and beta-carotene in HPH emulsion oil droplets (rapeseed oil), analysed by UHPLC. The fraction (%) of carotenoids released from the plant matrix into the lipid droplets is calculated for emulsions (75% tomato and 25% pepper) with 5% and 10% rapeseed oil homogenized at 200 and 1500 bar.

% oil in emulsion	HPH pressure (bar)	Carotenoid type	Concentration (mg/100g oil)	% released to oil droplets
5	200	lycopene	53.1 ± 1.8	26.3 ± 1.3[a]
5	1500	lycopene	51.3 ± 0.9	25.4 ± 0.4[a]
10	200	lycopene	36.8 ± 2.4	37.6 ± 2.5[b]
10	1500	lycopene	49.9 ± 1.1	50.9 ± 1.2[c]
5	200	beta-carotene	17.0 ± 2.9	42.5 ± 6.8[a]
5	1500	beta-carotene	15.9 ± 2.3	39.8 ± 5.8[a]
10	200	beta-carotene	8.6 ± 0.7	50.6 ± 4.2[a]

10	1500	beta-carotene	12.3 ± 0.4	72.4 ± 2.5[b]

740 a,b,c - different letters indicate significant ($p<0.05$) differences between means obtained for each carotenoid type

741 (one-way ANOVA)

742