Enhancement of lycopene bioaccessibility from tomato juice using excipient emulsions: Influence of lipid droplet size

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Journal: Food Chemistry

Submitted: January 2016

Revised: April 2016

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Abstract

The use of excipient emulsions to increase the bioaccessibility of lycopene in tomato juice was studied by simulating gastrointestinal conditions. The influence of droplet diameter ($d = 0.17$ or $19\ \mu\text{m}$) and thermal treatment ($90\ ^\circ\text{C}$, $10\ \text{min}$) on lycopene bioaccessibility was evaluated. Lycopene bioaccessibility was relatively low ($< 8\%$) in the absence of excipient emulsions due to the crystalline nature of the carotenoids and their entrapment within chromoplasts. Emulsions containing small droplets were fully digested within the small intestine phase, and led to a higher bioaccessibility (12.5%) than emulsions containing large droplets (10.0%) or emulsion-free samples (7.5%). The relatively modest increase in bioaccessibility was attributed to the high level of entrapment in crystalline form. Thermal processing did not appreciably disrupt tomato cells, and therefore only led to a slight increase in lycopene bioaccessibility. Overall, this study shows that excipient emulsions may increase the bioaccessibility of carotenoids in tomato juices.

Keywords: tomato; lycopene; nanoemulsion; excipient foods; bioaccessibility; lipid digestibility

1 Introduction

Lycopene is a carotenoid that is responsible for the deep red color of fresh tomato fruit and tomato-based products (Shi & Maguer, 2000). Like other carotenoids, lycopene is a highly hydrophobic molecule with a long hydrocarbon structure containing many conjugated double bonds. Despite not having pro vitamin A activity, lycopene has been proposed to be responsible for several other health benefits associated with the intake of tomato products (Stahl & Sies, 1996). Lycopene functions as an antioxidant and its ability to quench singlet oxygen has been reported to be twice as high as β -carotene (Shi & Maguer, 2000). The consumption of lycopene-rich products has been suggested to provide protection against cardiovascular diseases and a broad range of cancers (Etminan, Takkouche, & Caamano-Isorna, 2004). Lycopene represents 90 % of the total carotenoid compounds in tomato fruit (Borguini & Torres, 2009). Thus, the consumption of tomato-based food products represents a major source of lycopene in the human diet. In plant tissues, carotenoids are typically located in chromoplasts inside the plant cells. Chromoplasts are specific organelles composed of lipids, proteins, and carotenoids that can have several different morphologies in plant tissues, such as crystalline, globular, fibrillar, membranous or tubular (Bartley & Scolnik, 1995). The nature of the chromoplasts in different plant tissues may influence their release within the gastrointestinal tract after ingestion, which would be expected to influence carotenoid bioaccessibility.

Lycopene bioaccessibility, which is usually taken to be the fraction incorporated into mixed micelles formed in the small intestine, is typically quite low in raw tomato products, ranging between about 0.1 and 3 % (Reboul, Richelle, Perrot, Desmoulins-Malezet, Pirisi, & Borel, 2006). This is a consequence of the lipophilic nature of carotenoids, their high melting point, and their entrapment in complex structures within tomato tissue. In fact, carotenoids have to be released from the food matrix after ingestion and be incorporated in the micelle fraction before becoming absorbable in the gastrointestinal tract. Therefore, the tomato tissue microstructure plays an important role in determining the bioaccessibility of lycopene in tomato products. However, there are also other factors that influence lycopene bioavailability in tomato products. Food processing often has a positive impact on carotenoid bioavailability due to its ability to disrupt plant tissue structure. The application of thermal treatment to tomato products might weaken the cell wall structure thus facilitating the diffusion and release of carotenoids from chromoplasts and therefore increasing their bioaccessibility

(Tibäck, Svelander, Colle, Altskär, Alminger, Hendrickx, et al., 2009). The presence of digestible lipids has also been shown to increase the *in vitro* bioaccessibility of carotenoids in plant-based products (I. J. P. Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, 2012). This effect can be attributed to the fact that the digestion of lipids within the gastrointestinal tract releases free fatty acids and monoacylglycerols that increase the solubilization capacity of mixed micelles thereby enhancing carotenoid solubilization. Therefore, the application of thermal processing in the presence of digestible lipids might be an effective strategy to enhance the bioactive properties of tomato products.

Recently, the concept of *excipient foods* has been introduced: these are foods that contain ingredients or structures that may not have bioactivity themselves, but that can promote the bioactivity of co-ingested bioactive agents by increasing their bioavailability (David Julian McClements & Xiao, 2014). Oil-in-water emulsions or nanoemulsions are particularly suitable candidates as excipient foods because their structure and composition can easily be manipulated. In particular, they can be designed to contain hydrophobic, hydrophilic, and amphiphilic ingredients that may promote bioavailability of different bioactive agents through different mechanisms. In this study, we focused on the utilization of nanoemulsions to increase the bioaccessibility of carotenoids from plant based-products. Nanoemulsions, consisting of lipid droplets ($d < 100$ nm) dispersed within an aqueous phase have several potential advantages over conventional emulsions for this purpose. Firstly, they tend to have a higher stability to gravitational separation and droplet aggregation, which may lead to a longer shelf-life (D. J. McClements & Rao, 2011). Second, the small size of the lipid droplets means that they may be able to more easily penetrate into plant tissues and solubilize carotenoids. Third, small droplets tend to be digested more rapidly within the gastrointestinal tract due to their large surface areas, which tends to promote the bioaccessibility of any encapsulated bioactive compounds (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Therefore, nanoemulsions might be a potential excipient food to be co-ingested with food products in order to enhance the bioavailability of naturally occurring bioactive compounds. However, the effectiveness of excipient nanoemulsions at increasing the bioavailability of specific bioactive compounds from specific food products still needs to be elucidated. In this context, the purpose of the present work was to study the influence of the addition of excipient emulsions and nanoemulsions on lycopene bioaccessibility from tomato

juice. These emulsion-based systems were designed to represent commercial products that could be mixed with tomato juices, such as sauces or creams.

The results of this study may be useful for developing a new range of excipient food products specifically designed to boost the bioavailability of health-promoting bioactive components in fresh and processed fruits and vegetables.

2 Material and methods

2.1 Materials

Corn oil purchased from a local supermarket was used as a source of digestible long chain triacylglycerols (LCT). Tomato juice was obtained from a local supermarket (Foodhold USA, LLC, Landover, MD, USA). The manufacturer claimed on the label that the tomato juice did not contain fat (0 % oil content). The initial concentration of lycopene in the tomato juice was determined to be 8.6 ± 0.3 mg/100 g juice. Tween 80, monobasic and dibasic phosphates, pepsin, bile salts and lipase were purchased from Sigma-Aldrich (St. Louis, MO). **The lipase used was purified lipase from porcine pancreas (type II) and the bile salts used were porcine bile extract.** Acetone, hexane and ethanol for the lycopene extraction were purchased from Fischer (Pittsburgh, PA, USA). All aqueous solutions were prepared using purified water from a Milli-Q filtration system (EMD Millipore, Merck KGaA, Darmstadt, Germany).

2.2 Methods

2.2.1 Emulsion and nanoemulsion formation

The oil phase consisted of pure corn oil and the aqueous phase consisted of a 5 mM phosphate buffer at pH 7. Conventional emulsions, also referred to as large emulsions (LE), were obtained by blending 10 % (w/w) of the oil phase (corn oil), 1 % (w/w) of Tween 80, and 89 % (w/w) of aqueous phase using a high-shear mixer at 20,000 rpm for 2 min. Nanoemulsions, also referred to as small emulsions (SE), were formed by passing the conventional emulsions three times through a high pressure homogenizer (Microfluidizer, Model M110-P, Microfluidics, Newton, MA) working at 20,000 psi.

2.2.2 Mixing and heat experiments

To simulate co-ingestion of tomato juice with an emulsion-based excipient food we mixed the two systems together prior to passing through the simulated gastrointestinal tract. Tomato juice (24 mL) and emulsion (6 mL LE or SE) were mixed together to achieve an oil concentration of 2 % (w/w) in the final samples. A control sample containing no added lipids was also prepared by mixing 24 mL tomato juice and 6 mL buffer solution (5 mM phosphate buffer, pH 7). The resulting mixtures were then stirred for 5 minutes. Moreover, to study the effect of thermal processing on the functionality of tomato juice, we submerged the mixed tomato-emulsion samples in a hot water bath at 90 °C for 10 minutes followed by an iced-water bath for 10 minutes. After mixing and thermal treatment, the tomato juice samples were subjected to *in vitro* gastrointestinal conditions.

2.2.3 *In vitro* digestion

An *in vitro* gastrointestinal tract (GIT) model consisting of mouth, stomach and small intestinal phases was used to simulate the gastrointestinal fate of ingested tomato juice samples. The parameters selected for the simulation of gastrointestinal conditions were based on previously reported recommendations (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, et al., 2014). Initially, the tomato juice-emulsion mixtures had 2 % (w/w) oil content.

Mouth phase: Simulated saliva fluid (SSF), containing mucin and various salts, was prepared according to a previous study (Sarkar, Goh, & Singh, 2009). A 10 mL-aliquot of the test samples was mixed with 10 mL of SSF, so that the final mixture contained 1% (w/w) oil. The pH of the mixture was adjusted to 6.8 and the sample was incubated at 37 °C for 10 min with continuous agitation at 100 rpm.

Gastric phase: Simulated gastric fluid (SGF) was prepared using a method reported previously (Sarkar, Goh, Singh, & Singh, 2009) by dissolving 2 g of NaCl and 7 mL of HCl (37%) in 1 L of water and adjusting the pH to 1.2 using 1.0 M HCl. The “bolus” sample from the mouth phase was mixed with SGF at a 50:50 volume ratio so that the final mixture contained 0.5% (w/w) oil. The pH of the sample was adjusted to 2.5 using NaOH (1M) and incubated at 37° C for 2 hours with continuous agitation at 100 rpm.

Small intestinal phase: A pH-stat (Metrohm, 147 USA Inc.) was used to simulate the conditions in the small intestinal phase of the GIT. An aliquot (30 mL) of sample from the gastric phase was placed in a temperature-controlled (37 °C) chamber and the pH was set at

7.0 using NaOH solution. Then, 4 mL of bile extract (46.87 mg/mL) and 1 mL of calcium chloride (110 mg/mL) solutions dissolved in phosphate buffer were added to the sample and the pH was adjusted to 7.0 if necessary. Afterwards, 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in phosphate buffer was incorporated into the mixture. The pH of the mixture was monitored and the volume of 0.1 M NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from the lipid digestion (*i.e.*, to keep pH at 7.0) was recorded during 2 h. The amount of FFA released was calculated from the titration curves as described previously (Li & McClements, 2010).

It should be noted that the relatively simple static *in vitro* GIT model used in this study cannot simulate the complex conditions that occur within the human gastrointestinal tract, such as dynamic changes in pH, bile salt levels, enzyme levels, *etc.* Nevertheless, this method is useful for rapidly screening samples with different compositions and structures, which can then be tested in more detail using *in vivo* methods.

2.2.4 Particle characteristics

The physicochemical properties of the tomato juice with and without being mixed with small or large emulsion were determined in terms of the particle size, particle distribution and ζ -potential immediately after being mixed and in each phase during the simulated gastrointestinal conditions.

The mean particle size and particle size distribution of tomato juice samples were measured with a laser diffraction particle size analyzer (LS 13 320, Beckman Coulter Inc., USA) equipped with a universal liquid module. The instrument consists of a 5 mW laser diode with a wavelength of 750 nm and a secondary tungsten-halogen light source projected through a set of filters, which transmit three wavelengths (450 nm, 600 nm, and 900 nm), to measure particles that fall in the submicron range. The tomato juice samples were suspended in the liquid module in double distilled water prior to the particle size measurement to avoid multiple scattering effects. The particle size was reported as volume-weighted mean diameter in μm .

The ζ -potential of the particles was measured by phase-analysis light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Samples were diluted 1:10 with 5 mM phosphate buffer solution (pH 7.0) and then placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles.

2.2.5 Lycopene bioaccessibility

To determine the lycopene bioaccessibility (BA) in tomato samples, the fraction of solubilized lycopene in the micelle phase after the simulated gastrointestinal tract *versus* the concentration after the small intestine phase was reported. An aliquot of the raw digesta after the small intestine phase was centrifuged (CL10 centrifuge, Thermo Scientific) at $2600 \times g$ for 40 min at 25 °C. The supernatant was collected and was taken to be the “micelle fraction” in which the lycopene was solubilized.

The lycopene concentration in the initial tomato juice, digested tomato samples, and the micelle fraction was determined by a method reported previously (Fish, Perkins-Veazie, & Collins, 2002) with some modifications. Briefly, an aliquot of 0.3 g of the tomato sample was mixed with 2.5 mL of acetone containing a 0.05 % (w/v) of butylated hydroxytoluene (BHT), with 2.5 mL of ethanol and 5 mL of hexane in a 15 mL test tube with a plastic screw cap. The test tubes were vortexed for 10 sec twice and then further centrifuged at 320 g for 15 min. Then, 3 mL of double distilled water was added and left for 5 min at room temperature to allow phase separation to occur. The upper organic layer was collected and analyzed spectrophotometrically (Ultrospec 3000 pro, GE Health Sciences, USA) at 503 nm. A blank cuvette containing pure hexane was used as a reference cell. The concentration of lycopene in each sample was determined with the following equation:

$$\text{lycopene (mg/100g)} = \frac{Abs_{503} \times MW \times Vol \text{ hexane} \times 100}{\epsilon \times L \times g \text{ tomato}}$$

where Abs_{503} is the absorbance of the organic layer at 503 nm; MW is the lycopene molecular weight (536.9 g/mol); $Vol \text{ hexane}$ is the volume of hexane used (5 mL); ϵ is the lycopene extinction coefficient ($17.2 \times 10^4 \text{ (M/cm)}^{-1}$); L is the cuvette path length (1 cm) and $g \text{ tomato}$ is the sample weight (0.3 g).

The lycopene bioaccessibility was calculated using the following equation:

$$\text{Bioaccessibility} = \frac{C_{micelle}}{C_{RawDigesta}} \times 100$$

where $C_{micelle}$ and $C_{RawDigesta}$ are the concentrations of lycopene in the micelle fraction and in the overall sample (raw digesta) after the *in vitro* digestion, respectively. **In this study, we did not take into account the transformation (chemical or biochemical) or absorption of the lycopene, which would also be expected to contribute to the overall bioavailability.**

2.2.6 Tomato juice microstructure

The tomato juice microstructure was determined by microscopy. The overall appearance of tomato cells was observed by optical microscopy, whereas to detect the location of the carotenoids in the tomato chromoplasts confocal fluorescence microscopy was used. Carotenoids naturally fluoresce and so it is possible to detect their location in these organelles (D'Andrea, Amenos, & Rodriguez-Concepcion, 2014; Kilcrease, Collins, Richins, Timlin, & O'Connell, 2013). Moreover, by the same principle, it is possible to track the possible migration of carotenoids from the tomato chromoplasts to the oil droplets after mixing with the emulsions. For the optical microscopy images, a Nikon Microscope (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) was used with a 20× objective lens. A small aliquot of each sample was placed on a microscope slide and covered with a cover slip prior to analysis. For confocal microscopy images, an air-cooled argon ion laser Model IMA1010 BOS (Melles Griot, Carlsbad, CA) was used to excite Nile red at 488 nm. The resulting fluorescent spectra of carotenoids were detected in the 515 nm channel equipped with a narrow pass filter (HQ 515/30 m) with a pinhole size of 150 μm . The images generated had a size of 512 $\times$ 512 pixels, with a pixel size of 414 nm, and a pixel dwell time of 61.44 μs . All images were taken and processed using the instrument software program (EZ- CS1 version 3.8, Nikon, Melville, NY).

2.2.7 Statistical analysis

Experiments were carried out in duplicate, and the results were expressed as the mean and the standard deviation. A statistical analysis software program (JMP 8, SAS Institute Inc.) was used to perform the analysis of variance. The Student's t test was run to determine significant differences at a 5% significance level ($p < 0.05$).

3 Results and discussion

3.1 Particle size and particle size distribution

In this section we studied the particle size of tomato juice samples during *in vitro* digestion and the influence of adding excipient large or small emulsions, with oil droplet sizes of $19.0 \pm 0.1 \mu\text{m}$ and $0.167 \pm 0.001 \mu\text{m}$, respectively. Moreover we assessed the impact of the application of a heat treatment (90 $^{\circ}\text{C}$ for 10 min) on the particle size of tomato juice to mimic a cooking process. The initial mean particle diameter of the raw tomato juice was around 390

μm and it remained practically unchanged during passage through the mouth, stomach, and small intestine phases (**Figure 1 A**). This observation was supported by the particle size distributions, which showed a population of large particles around 400 μm that remained present throughout *in vitro* digestion (**Figure 2**). Typically, tomato-derived products can be considered to be suspensions of plant-tissue-based particles in an aqueous phase that contains dissolved pectin, sugars, and organic acids (Anthon, Diaz, & Barrett, 2008). The plant-based particles consist of mechanically disrupted parenchyma tissue, which are cells in spherical or polyhedral shape, with a diameter of 50 to 500 μm and are held together by the pectin of the middle lamella (Aguilera & Stanley, 1990). The particle size of vegetable purees or juices largely depends on the mechanical treatment applied and it will vary according to the presence of cell clusters or cell fragments (Katlijn R. N. Moelants, Cardinaels, Jolie, Verrijssen, Van Buggenhout, Zumalacarregui, et al., 2013). Our results are in agreement with other studies reporting that tomato juice or tomato puree products have mean particle diameters between about 350 to 700 μm (K. R. N. Moelants, Cardinaels, Van Buggenhout, Van Loey, Moldenaers, & Hendrickx, 2014). Nevertheless, there is little information regarding the influence of the passage of foods through the different gastrointestinal phases on their particle size. In agreement with our results, it has been reported that the structure of carrot tissues remained unaffected after *in vitro* digestion with intact carotene bodies remaining within the interior of the plant cells (Tydeman, Parker, Wickham, Rich, Faulks, Gidley, et al., 2010).

We did not detect a significant change in the mean particle diameter or particle size distribution of tomato juice after adding large or small excipient emulsions (Figure 1), which can be attributed to the fact that large particles scatter light much more intensity than smaller ones, and therefore dominate the overall light scattering signal. Thus, the oil droplets remain undetected when they are mixed with tomato juice containing much larger particles. Applying a heat treatment (90 °C, 10 min) to the tomato juice (with or without excipient emulsions) did not have a significant impact on the measured particle size, which suggests that the plant tissue remained intact. Conversely, other authors have reported that applying heat to plant-based products might induce cell wall softening and structural changes causing a particle size reduction (Lemmens, Van Buggenhout, Oey, Van Loey, & Hendrickx, 2009). However, morphology changes of plant-based suspensions induced by thermal treatment largely depend on the nature of the plant and the heat treatment conditions (K. R. N. Moelants, Cardinaels,

Jolie, Verrijssen, Van Buggenhout, Van Loey, et al., 2014). Other authors have also reported that thermal degradation of pectin in the cell wall and middle lamella weakens the intercellular adhesion leading to cell separation rather than cell rupture (Ormerod, Ralfs, Jackson, Milne, & Gidley, 2004). On the other hand, we observed a slight increase in particle size of tomato juice in the small intestine phase after adding a small emulsion with or without the application of heat, presenting values near 500 μm . This increase in particle size may be an indication of some loosening of the plant tissue structure in the presence of lipid digestion products. For example, free fatty acids or monoacylglycerols may penetrate into the plant tissue and increase the gaps between the cells. Alternatively, the observed increase in particle size may be due to the formation of complexes of plant tissues and mixed micelles.

3.2 ζ -potential

The ζ -potential of initial raw tomato juice (T) was around -35 mV (**Figure 1 B**). The negative charge of the tomato juice samples observed in the present work might be attributed to the presence soluble anionic polysaccharides (such as pectin) at the surfaces of the plant-tissue particles or dissolved within the aqueous phase. Mechanical homogenization processing to obtain the juice induces the solubilization of structural compounds of the plant cell wall such as pectin. Pectin consists of a group of polymers with linear regions of $\alpha(1-4)$ linked galacturonic acid units separated by branched regions of neutral sugars (Sila, Van Buggenhout, Duvetter, Fraeye, De Roeck, Van Loey, et al., 2009). A fraction of the galacturonic acid groups are esterified with methyl groups, thus the overall molecular charge depends on the ratio of esterified to nonesterified groups, as well as the pH relative to the pK_a of the acid groups ($\text{pK}_a \approx 3.5$). Thus, pectin molecules tend to be negatively charged at high pH, but lose their charge at pH values appreciably below pH 3.5 (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014). Other authors have also reported a negative charge (-26 mV) for carrot juice (Chen, Zhao, Yang, & Zhang, 2012).

After adding excipient small or large emulsions the ζ -potential of the tomato juice samples became less negative (-24 and -25 mV), respectively. This change in particle charge might be due to a contribution of the lipid droplets to the overall signal, or due to an interaction of the anionic particles or polymers in the tomato juice with the lipid droplets.

The ζ -potential of tomato juice with or without excipient emulsions remained negative in the mouth and stomach phases (**Figure 1 B**), with values between -24 and -26 mV. The

aqueous phase used to dilute these samples was phosphate buffer (pH 7). Nevertheless, when we measured the ζ -potential of the same samples using simulated saliva fluid for the mouth (pH 6.8) or simulated stomach fluid for the stomach (pH 2.5) as dispersants, the ζ -potential was -21.5 and +0.64 mV respectively. These differences in ζ -potential depending on dispersant type can be attributed to the different pH, which significantly impacts the electrical charge of anionic polysaccharides (such as pectin). A pronounced increase in the magnitude of the negative charge was observed after exposure to simulated small intestine conditions, which was more pronounced in the tomato juice samples containing large or small excipient emulsions compared to tomato juice. The digestion of triglycerides by pancreatic lipase results in the release of anionic free fatty acids (Singh, Ye, & Horne, 2009), which might alter the overall charge of the suspended food particles. On the other hand, heat-treated (90 °C 10 min) tomato juice containing small or large excipient emulsions presented a significantly more negative ζ -potential after the small intestine phase compared to the non-treated juice (**Figure 1 B**). The lower ζ -potential observed in heat-treated samples might be due to several reasons: (i) thermal treatments disrupt the cell membrane allowing pectin and ions in the cell to migrate to the external aqueous phase (K. R. N. Moelants, Cardinaels, Van Buggenhout, Van Loey, Moldenaers, & Hendrickx, 2014); and (ii) pectin present in the cell wall might be degraded due to β -elimination processes or acid hydrolysis induced by thermal processing thereby altering its charge characteristics (Greve, McArdle, Gohlke, & Labavitch, 1994).

3.3 Oil digestibility

The FFA release in the small intestine phase of the samples largely depended on the type of excipient emulsion added and also on the application of a thermal treatment (90 °C, 10 min) after mixing with the excipient emulsions (**Figure 3**). As expected, tomato juice and heat-treated tomato juice without excipient emulsions did not show a significant release of FFA since they did not contain oil, as indicated by the manufacturer. On the other hand, tomato juice containing the small excipient emulsion exhibited rapid lipid digestion: 85% of the FFAs were released after 10 min, and complete lipid digestion had occurred after 2 hours. However, the tomato juice containing the large excipient emulsion exhibited much slower lipid digestibility: only 40% FFAs were released after 10 min and only 85% FFAs has been released after 2 hours. The differences between the rate of lipid digestion in small and large excipient emulsions can be attributed to differences in their initial droplet size. The small emulsion had an initial mean droplet diameter around 0.17 μm , whereas the large emulsion

had one around 19 μm . Emulsions with smaller droplet sizes are digested faster than those with larger droplet sizes due to the higher active surface area of lipid exposed to digestive enzymes (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Moreover, the fact that the small or large emulsions were mixed with a tomato juice did appear to interfere with their digestibility. On the other hand, the tomato juice containing excipient emulsions that were thermally treated (90 °C 10 min) had a lower FFA release compared to the corresponding non-thermally treated samples. For example, the heat-treated tomato juice containing the small excipient emulsion only released 50 % FFAs after 10 min of *in vitro* digestion and only reached 80 % FFAs release after 2 hours. This could be possibly due to the destabilization of the oil droplets during the heat treatment, which led to an increase in the oil droplet size and therefore a decrease in the surface area available for the lipase to adsorb. In fact, we detected a significant increase in the oil droplet size after heat treatment in the optical microscope images as described in the following section (**Figure 5 B**). Other authors have reported droplet growth in nanoemulsions stabilized with non-ionic surfactants (Tween 80) after being exposed to temperatures above 70 °C and then cooled to ambient temperature (Saberri, Fang, & McClements, 2013). Droplet instability was mainly attributed to an increase in droplet coalescence as the temperature was increased, associated with dehydration of the non-ionic surfactant head-group leading to a reduction in phase inversion temperature. The thermal stability of emulsion-based delivery systems mixed with food products is of particular importance as they are often exposed to elevated temperatures during preservation operations.

3.4 Tomato juice microstructure

Changes in the microstructure of the tomato juice samples during passage through the simulated gastrointestinal tract were studied by optical and confocal microscopy. Raw tomato juice was observed to contain both whole and disrupted chromoplasts suspended in an aqueous serum phase (**Figure 4 A**). These chromoplasts appeared to contain crystalline carotenoid granules entrapped inside the cell membrane. It is known that carotenoids found in vegetable tissues have complex structures that depend on tissue type. Moreover, throughout the gastrointestinal phases, the structure of the chromoplasts appeared to stay unchanged and the carotenoid granules remained in the interior of the tomato tissue cells. After adding large or small excipient emulsions the oil droplets remained outside the chromoplasts and were not able to penetrate into the interior of the tomato cells (**Figure 4 A**). In the case of tomato juice mixed with the large excipient emulsion, the oil droplets stayed in the vicinity of the tomato

chromoplasts in the mouth and stomach phases and some oil droplets were detected in the small intestine phase, which can be attributed to the fact that not all of the oil phase was digested for this sample (see previous section). For the tomato juice mixed with the small excipient emulsion, the oil droplets were undetectable throughout the *in vitro* digestion phases since their droplet size was below the limit of resolution of the microscope used.

The microstructure of the thermally-treated tomato juice (with or without excipient emulsions) was monitored by confocal microscopy (**Figure 4 B**). Confocal images showed the presence of carotenoid (lycopene) granules in the interior of the chromoplasts, which appeared as red spots since carotenoids naturally fluoresce. We did not detect any noticeable tomato cell breakage by the action of thermal processing. Moreover, there appeared to be no increase in tomato cell permeability, since the lycopene globules remained in the interior of the tomato cells in the mouth and stomach phases. Nevertheless, the carotenoid bodies appeared partially disrupted in the small intestine phase, and the undigested oil droplets (LE) were seen to be loaded with lycopene (**Figure 4 B**). The microscopy images therefore support the results of the pH-stat *in vitro* digestion experiments, where some undigested oil was detected in a number of the tomato samples: T+LE, T+SE+Heat, and T+LE+Heat (**Figure 3**). Additionally, we compared optical and confocal images of the tomato juice samples after mixing with excipient emulsions and being heat-treated (**Figure 5**). Initially, the oil droplets did not have any fluorescence after the thermal treatment, whereas they did fluoresce after the small intestine phase. This indicates that the lycopene was not transferred into the lipid droplets after simple mixing of the tomato juice with the excipient emulsions, nor after thermal processing. Nevertheless, the undigested oil after the small intestine phase was loaded with lycopene, which suggested that passage of the samples through the simulated gastrointestinal tract did facilitate the transfer of lycopene into the lipid droplets. **This phenomenon may have occurred because the mixed micelles formed by lipid digestion facilitated the disruption of the lycopene-containing structures within the plant cells, and were also capable of solubilizing them.**

Different carotenoid-containing structures have been found in vegetable products, including plastoglobules, crystalline and microfibrillar structures, and internal membranous structures (Egea, Bian, Barsan, Jauneau, Pech, Latche, et al., 2011). In tomatoes, carotenoids are found as solid crystalline substructures inside the chromoplasts (Jeffery, Holzenburg, & King, 2012). The chromoplasts structure, as well as the cell wall composition and

organization of the specific plant tissue, may therefore have a major impact on the release of carotenoids in the gastrointestinal tract. In our study, the crystalloid globular structure of carotenoids in tomato chromoplasts may account for the relatively low bioaccessibility of lycopene observed in raw tomato juice (7.5 %) after *in vitro* digestion. It has been reported that the bioaccessibility of lycopene is much lower from tomatoes than from carrots (Palmero, Lemmens, Ribas-Agusti, Sosa, Met, Umutoni, et al., 2013). Even though the carotenoids in both vegetables had similar solid crystalline morphologies, they attributed the lower lycopene bioaccessibility in tomato to differences in the chromoplast sub-organization as well as other plant matrix effects that might decrease the release of the carotenoids into the mixed micelle phase.

3.5 Lycopene BA

We assessed the lycopene bioaccessibility from tomato juice after exposure of the samples to the simulated gastrointestinal conditions before and after being mixed with small or large excipient emulsions and with or without heat-treatment (90 °C 10 min) (**Figure 6**). The lowest lycopene bioaccessibility was observed in the raw tomato juice without containing any excipient emulsion, which was 7.5 %. Mixing an excipient emulsion with the tomato juice significantly increased the lycopene bioaccessibility with the effect being greater for the emulsion with the smallest droplets: BA = 10.1 % for large droplets and 12.5 % for small droplets. This effect can be attributed to the higher lipid digestibility for the small excipient emulsions mixed with the tomato juice (**Figure 3**), as this would lead to more mixed micelles to solubilize the carotenoids. Other studies have also reported that the addition of a lipid source to plant-based products will increase the bioaccessibility of lipophilic compounds due to the presence of a higher amount of mixed micelles (Periago, Bravo, García-Alonso, & Rincón, 2013). However, the increase in lycopene bioaccessibility from tomato products has been reported to vary depending on the type and concentration of oil added (I. J. P. Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, 2012). Conversely, it has been reported that the addition of high concentrations of digestible lipids (*e.g.* olive oil, sunflower oil and fish oil) might decrease the lycopene bioaccessibility in tomato products due to an incomplete hydrolysis of the triglycerides (Anese, Bot, Panozzo, Mirolo, & Lippe, 2015). It has also been reported that adding an oil-in-water emulsion might lead to a lower increase in lycopene bioaccessibility from tomato products compared to adding a bulk oil if the emulsifier

interferes with the transfer of lycopene into the lipid droplets or mixed micelles (Degrou, Georgé, Renard, & Page, 2013).

Overall, our results suggest that the addition of a digestible lipid source in the form of an excipient emulsion only causes a limited increase in lycopene bioaccessibility after digestion of tomato products, which is probably due to the high level of entrapment of the carotenoids in the tissue cells. Lycopene is a highly lipophilic molecule which, in order to be isolated from the matrix, has to be transferred first from the innermost physical barrier (chromoplast), across the cell wall and through the aqueous environment surrounding the cells, which might be considered an additional physical barrier for carotenoid incorporation into the oil phase (Palmero, et al., 2013). The embedment of chromoplasts in complex structures within the tomato tissue cells makes it difficult to be released within the gastrointestinal tract, therefore remaining entrapped after digestion, thus being difficult to enhance its bioaccessibility.

It is generally assumed that thermally treated plant-based products have a higher carotenoid bioaccessibility due to an increase in the cell wall permeability and therefore an easier release of lipophilic compounds during digestion (Netzel, Netzel, Zabaras, Lundin, Day, Addepalli, et al., 2011). Our results indicate that a thermal treatment (90 °C 10 min) of tomato juice only led to a small but significant increase in lycopene bioaccessibility after *in vitro* digestion compared to raw tomato juice (**Figure 6**). However, there were no significant differences between the heat-treated tomato juices mixed with small or large excipient emulsions compared to the respective raw tomato juices mixed with excipient emulsions. Nonetheless, the thermally treated tomato juice mixed with a small excipient emulsion showed the highest values of lycopene BA after *in vitro* digestion (13.8 %). This result suggests that heat treatment may enhance the release of lycopene in tomato products, but it has a minimal effect when combined with the presence of excipient emulsions. Other authors have reported that the addition of lipid prior to processing might enhance carotenoid bioaccessibility in vegetables (I. Colle, Van Buggenhout, Van Loey, & Hendrickx, 2010). Nevertheless, the type of carotenoid seems to play a crucial role in this aspect. There is evidence indicating that the application of heat after mixing with lipids might even decrease lycopene bioaccessibility of tomato products (Ines J. P. Colle, Lemmens, Van Buggenhout, Met, Van Loey, & Hendrickx, 2013). Thermal processing might lead to the solubilization of pectin, therefore creating a polysaccharide network within the gastrointestinal fluids that entraps the carotenoids and limits their incorporation into the mixed micelles. Our initial

theory was that nanoemulsions with a small droplet size would be more effective in enhancing the tomato lycopene BA compared with larger droplet size excipient emulsions. Nevertheless, our results indicate that the high level of entrapment of the lycopene-rich particles inside chromoplasts inhibits their transfer to the lipid phase during gastrointestinal conditions. Nonetheless, the lycopene BA was enhanced to a higher extent by mixing it with a small emulsion prior to a heat treatment.

4 Conclusions

The current work showed that excipient emulsions containing small digestible lipid droplets ($d < 200$ nm) can increase the bioaccessibility of lycopene from tomato products. Small excipient emulsions mixed with tomato juice were completely digested under simulated gastrointestinal conditions, which facilitates the transfer of lycopene into the mixed micelles in the small intestine phase. Nevertheless, the lycopene bioaccessibility of tomato juice samples was limited by the strong level of natural entrapment within the chromoplasts, which remained intact during the *in vitro* digestion, thus preventing the lycopene transfer into the lipid droplets. Moreover the lycopene in tomato is present in a crystalloid form, which impedes its liberation and solubilization within the aqueous phase. **The application of a thermal treatment after mixing of the excipient emulsions with the tomato juice, simulating a cooking process, did not lead to a significant increase in the lycopene bioaccessibility. These results suggest that fact that lycopene is trapped within the chromoplasts of tomato products is a major obstacle limiting the release and subsequent incorporation of lycopene within the micelle fraction after digestion.** Overall, this study has provided valuable information for the rational design of excipient foods to be co-ingested with fruit- or vegetable-based food products to enhance their potential health benefits.

Acknowledgements

This material is based upon work supported by the Cooperative State Research, Extension, Education Service, United State Department of Agriculture, Massachusetts Agricultural Experiment Station (Project No. 831) and by the United States Department of Agriculture, NRI Grants (2011-03539, 2013-03795, and 2014-67021).

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Figure captions

Figure 1 Volume-weighted mean particle size (μm) (A) and ζ -potential (mV) (B) of tomato juice (T) mixed with a large emulsion (T+LE) or a small emulsion (T+SE) and after the application of heat ($90\text{ }^{\circ}\text{C}$ 10 min) (T+H, T+LE+H, T+SE+H) during the *in vitro* digestion. Different capital letters represent significant differences ($p < 0.05$) between *in vitro* digestion phases for the same sample. Different lower case letters represent significant differences ($p < 0.05$) between different samples in a given *in vitro* digestion phase (initial, mouth, stomach or small intestine)

Figure 2 Particle size distribution of raw tomato juice (T) during the *in vitro* digestion phases (initial, mouth, stomach and small intestine)

Figure 3. Calculated free fatty acids released (%) from the tomato juice (T) containing small (SE) or large (LE) excipient emulsions with or without applying heat ($90\text{ }^{\circ}\text{C}$ for 10 min) over time during the course of the *in vitro* small intestine stage.

Figure 4 Optical images (20X) of tomato juice (T), tomato juice with large (T+LE) or small (T+SE) excipient emulsion (A) and confocal images (60X) of heated samples ($90\text{ }^{\circ}\text{C}$ 10 min) of tomato juice (T+Heat), tomato juice with large emulsion (T+LE+Heat) and tomato juice with small emulsion (T+SE+Heat) (B) under simulated gastrointestinal conditions. Scale bar is $100\text{ }\mu\text{m}$ (A) and $50\text{ }\mu\text{m}$ (B)

Figure 5 Comparison of optical and confocal images (60X) of tomato juice with large (A) (T+LE+Heat) or small (B) (T+SE+Heat) emulsion immediately after heat treatment ($90\text{ }^{\circ}\text{C}$ 10 min) and after *in vitro* small intestine phase. Scale bar is $50\text{ }\mu\text{m}$.

Figure 6 Lycopene bioaccessibility (BA; %) of tomato juice (T) mixed with large (LE) or small (SE) excipient emulsions with or without a posterior heat treatment ($90\text{ }^{\circ}\text{C}$ 10 min).

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Figure 6 Lycopene bioaccessibility (BA; %) of tomato juice (T) mixed with large (LE) or small (SE) excipient emulsions with or without a posterior heat treatment (90 °C 10 min). Different capital letters represent significant differences ($p < 0.05$) between no addition of oil or adding large or small excipient emulsions. Different lower case letters represent significant differences ($p < 0.05$) between non-treated and heat-treated tomato within the same sample.