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EFFECT OF ENZYMATIC TREATMENTS ON LYCOPENE *In vitro* BIOACCESSIBILITY IN HIGH PRESSURE HOMOGENIZED TOMATO PUREE AND CHROMOPLAST FRACTION

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ABSTRACT

Lycopene has been ascribed as a micro-nutrient which has many beneficial effects on human health due to its antioxidant activity. After high pressure homogenization, a decrease in lycopene in vitro bioaccessibility in tomato puree was reported. It was suggested that a new fiber network was formed and entrapped lycopene. In addition, the chromoplast sub-structure also constitutes important physical structural barriers for lycopene release from the matrix and subsequent incorporation into micelles. In order to increase the lycopene in vitro bioaccessibility in tomato puree, enzymatic treatments using different cell wall degrading enzymes (Pectinex Ultra SP-L and Viscozyme) were applied to the homogenized tomato puree. Enzymatic treatments using phospholipase D and protease were also applied to a fraction enriched with chromoplast in order to enhance lycopene in vitro bioaccessibility. An in vitro digestion assay was used to investigate the effect of enzymatic treatments on lycopene bioaccessibility. Results showed that the enzymes effectively catalyzed for solubilizing the new fiber network that was formed upon homogenization. However, no significant increase in lycopene in vitro bioaccessibility was found after any of the enzymatic treatments. By applying phospholipase D and protease enzymes on the fraction enriched with chromoplast, no further increase in lycopene in vitro bioaccessibility was found. Therefore, it is suggested that the chromoplast sub-structure, meaning how lycopene crystals are embedded within the chromoplast organelle, constitutes the most important barrier for lycopene bioaccessibility in tomato fruit.

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1 INTRODUCTION

Carotenoids are natural pigments that give distinctive red, orange, and yellow colours in many parts of plant such as roots, stems, leaves, flowers, and fruits. Synthesis reactions and accumulation of carotenoids take place in chromoplasts and chloroplasts of plants (Howitt and Pogson, 2006). In red tomato, lycopene is the most abundant pigment that gives the corresponding red colour characteristic of the fruit and tomato products (Martínez-Valverde *et al.*, 2002). Lycopene from tomatoes is well known as a strong antioxidant, that gives positive effects on human health such as preventing against cardiovascular diseases and carcinogenic diseases (Colle *et al.*, 2010b). Human body is not able to synthesize lycopene, therefore it is important to have a diet rich in lycopene from food sources like tomatoes and its diversified products.

An increase in the uptake of nutrients from plant-based foods can be accomplished by the applica-

tion of processing. However, high pressure homogenization in where most of the cell are broken, lycopene *in vitro* bioaccessibility has been found to be decreased (Colle *et al.*, 2010a). It was hypothesized that a new fiber network formed after high pressure homogenization and entrapped the lycopene in the plain tomato pulp, making it less accessible for digestive enzymes and bile salt (Clements *et al.,* 2009; Colle *et al*., 2010a).

In addition, lycopene is presented within the chromoplasts of tomatoes in a crystalline form (Holloway *et al.*, 2000). Schweiggert *et al.* (2012) illustrated that the chromoplast morphology had a big influence on the bioaccessibility of carotenoids and the physical form of carotenoids was one of the most important factors controlling the uptake of carotenoids.

In order to improve the bioaccessibility of lycopene in tomato puree after being high pressure homogenized, enzymatic treatments are attractive choices that could solubilize the new fiber network. Moreover, by applying enzymes to chromoplast enriched fraction from tomato, it would also be possible to investigate whether lycopene could be more readily solubilized and thus be more bioaccessibility.

2 MATERIALS AND METHODS

2.1 Materials

Red tomatoes (*Solanum lycopersicum c.v.* prunella*)* were bought in a shop in Belgium. Tomatoes were cut into thin slices (1cm) and vacuum packed in plastic bags. Tomatoes were further blanched at 95^{o}C for 8 minutes, with subsequent cooling in ice. Samples were frozen in liquid nitrogen and stored at -40^{o}C until analysis.

Pectinex Ultra SP-L (9500 Polygalacturonase U/ml) from *Aspergiluss aculeatus* was acquired from Novozymes. Viscozyme (Novozymes) is a multi-enzyme complex containing mostly Endo-1,3(4)-β glucanase (100 U/g) and has side activity including arabanase, hemicellulase and xylanase. Phospholipase D type I (175 U/mg at 30^{o}C and pH 5.6), from cabbage was purchased from Sigma-Aldrich, dissolved in 50 µM $CaCl_2$ solution before using. Protease type I from bovine pancreas (10 U/mg at 37^{o}C and pH 7.5) was acquired from Sigma-Aldrich.

2.2 Methods

2.2.1 Tomato puree preparation

Frozen tomatoes were thawed and the skin and seeds were removed. Tomatoes were mixed 3 times for 5 seconds, using a Büchi Mixer B- 400 (Flawil, Switzerland). Then, tomato puree was homogenized (Panda 2K, Gea Niro Soavi, Mechelen, Belgium) at 1000 bars for 1 cycle at 4^{o}C.

2.2.2 Cell wall degrading enzymatic treatment on high pressured homogenized tomato puree

The pH of tomato puree was adjusted to 5 using 1M $NaHCO_3$. Tomato puree was incubated with enzymes at different concentrations and time in a shaking water bath at 40^{o}C. After incubation, samples were treated at 95°C for 5 minutes to inactivate the enzymes. Tomato puree after being homogenized was used as control sample.

Pectinex Ultra SP-L treatments

The effect of pectinase on lycopene *in vitro* bioaccessibility was investigated by applying different concentrations of Pectinex Ultra SP-L (95, 190 and 285 U/g puree) for 30 minute, and different incubation time (30, 60, 120 minute) at a concentration of 95U/g puree.

2.2.3 Viscozyme treatments

The experiments were conducted to investigate the effect of Viscozyme on lycopene bioaccessibility. Different concentrations of enzyme (1.5, 2 and 2.5 U/g of puree) were added to the tomato puree. The samples were incubated in water bath at 40^{o}C for 30 minutes before enzyme inactivation step as mentioned above.

2.2.4 Combination of Viscozyme and Pectinex Ultra SP-L treatment

Viscozyme (2.5 U/g) and Pectinex Ultra SP-L (285 U/g) were also simultaneously added to tomato puree using the same conditions as mentioned above.

2.2.5 Chromoplast fraction preparation

Frozen tomatoes were thawed and the skin was removed. Tomatoes were then blended (Waring Commercial, Torrington, CT, USA) for 5 seconds (low speed) with 0.05M EDTA (1:1). The mixture was filtered using cheese clothes, and centrifuged (Beckman, J2 – HS centrifuge) at 27200 g for 30 minute at 4^{o}C. The chromoplast fraction was obtained by collecting the pellet and dissolving it again in distilled water (Hansen and Chiu, 2005; Palmero *et al.,* 2013).

2.2.6 Enzymatic treatment on chromoplast fraction

The chormoplast fraction pH was adjusted to 5.6 and 7.5 for enzymatic treatments with phospho

lipase D and protease, respectively. Sample was incubated with phospholipase D at 30°C and/or protease at 37°C for 30 minutes. The setting for chromoplast fraction experiment is shown in Table 1.

Table 1: Experimental set-up for enzymatic treatment on chromoplast fraction

Experiment	Incubation time (minutes)	Concentration (U/g)
Phospholipase D	30	10
Protease	30	20
Combination of enzymes		
- Phospholipase D	30	10
- Protease	30	20

2.2.7 Lycopene bioaccessibility

In vitro digestion

The *in vitro* procedure was based on the method described by Hedren *et al.,* 2002 and adjusted by Colle *et al.*, (2010b) and Lemmens *et al.*, (2010). The stomach digestion was simulated by adding 5ml of NaCl/ascorbic acid solution (0.9% NaCl, 1% ascorbic acid), 5ml stomach electrolyte solution (0.30% NaCl, 0.11% KCl, 0.15% $CaCl_2.2H_2O$, 0.05% $KHPO_4$, 0.07% $MgCl_2.6H_2O$ in water) and 10 ml of oil emulsion (5% olive oil, 1% phosphattidylcholine). The pH was adjusted to 4±0.05 (using HCl or $NaHCO_3$ 1 M) before adding 5ml of pepsin solution (0.52% porcine pepsin (Sigma-Adrich) in electrolyte solution). The headspace of the samples was flushed with nitrogen and incubated for 30 minutes at 37°C during shaking end-over-end. The second stage of stomach digestion was performed by adjusting the pH to 2, and then again flushing the headspace with nitrogen and incubating for 30 minutes at 37°C while shaking end-over-end. To simulate the small intestine digestion, the pH of the samples was adjusted 6.9 and added duodenal juice (0.4% porcine pancreatin (Sigma-Aldrich), 0.2% porcine lipase (Sigma-Aldrich), 2.5% porcine bile extract (Sigma-Aldrich), 0.5% pyrogallol (Sigma-Aldrich) and 1% tocopherol (Sigma-Aldrich). The headspace of samples was flushed again with nitrogen and the incubation continued for 2 hours at 37°C while shaking end-over-end. Incorporation into micelles in the small intestine was imitated by using ultracentrifuge (L7 Ultracentrifuge, Beckman, Namen, Belgium) at 165000 g for 1 hour and 7 minutes. Digested juice was collected, and filtered (Chromafil PET filters, 0.20 µm pore size) into a brown Erlenmeyer for subsequent carotenoid extraction.

2.2.8 Carotenoid extraction and quantification

The extraction procedure was followed as described by Colle *et al.* (2010a) and Lemmens *et al.* (2009). Samples (about 5 gram) were mixed with 0.5g NaCl. Then, 50 ml of extraction solution (hexane:acetone:ethanol [50:25:25] and 0.1% butylated hydroxytoluene) was added. Samples were stirred at 4°C for 20 minutes. Afterwards, 15 ml of MiliQ water was added and the stirring was continued for 10 minutes. The apolar phase (hexane) which contains carotenoids was separated from the polar phase by using separation funnels. Discarding aqueous layer, apolar phase (carotenoid extract) was collected, and filtered (Chromafil PET filters, 0.20 µm pore size) into brown tube.

Lycopene quantification was performed using a spectrophotometer (Ultrospec 2100 pro UV/Visible) at 472 nm. Lycopene content was calculated by the following formula (Yeum and Russell, 2002; Rodriguez-Amaya and Kimura, 2004):

$$C = \frac{A \times volume \times 10^4}{A_{1cm}^{1\%} \times sample\,weight}$$

where: C is lycopene content (µg/g), A is absorbance, volume is total volume of extract (25 mL), $A_{1cm}^{1\%}$ *is absorption coefficient of lycopene (3450), and sample weight (g)*

Calculation of lycopene *in vitro* bioaccessibility

Lycopene *in vitro* bioaccessibility was calculated as a ratio of the bioaccessible lycopene content to the corresponding lycopene content of the original sample. Samples were analyzed in triplicate.

2.2.9 Bostwick consistency index

The tomato puree (before/after enzymatic treatments) was placed in the reservoir of the Bostwick consistometer. The gate was opened and the flow length (cm) of the sample was measured after 30 seconds at ambient temperature. A high Bostwick consistency index corresponds to a puree with a low consistency having therefore a low resistance to flow. In contrast, a low Bostwick consistency index pulp fraction stands for high consistency

purees having a high resistance to flow (Christiaens *et al.*, 2012).

2.3 Data analysis

In order to evaluate significant differences among lycopene *in vitro* bioaccessibility (B/C) of the sample treated with different enzymes, a Tukey's Standarized Range Test (SAS version 9.3, SAS Inst. Inc., Cary, NC, USA) was used. The level of significance was considered at $P<0.05$. Microsoft excel 2007 was used to calculate standard deviation of the obtained results.

3 RESULTS

3.1 Application of cell wall degrading enzyme on high pressure homogenized tomato puree

3.1.1 Effect of cell wall degrading enzyme on consistency index of high pressure homogenized tomato puree

In order to evaluate effectiveness of the cell wall degrading enzymes acted on the substrate, the viscosity of the sample was measured. The Bostwick consistency index of enzymatic treated samples and the control sample are presented in Table 2. After being homogenized, the fiber network formed causes an increase of viscosity, low consistency index (20cm) was found on the homogenized tomato puree. The results showed that tomato puree samples which were treated with Pectinex Ultra SP-L and Viscozyme separately or simultaneously had higher consistency index compared to homogenized puree.

Table 2: Bostwick consistency index of tomato puree after enzymatic treatment

Enzymatic treatment	Bostwick consistency index (cm)
No enzymatic treatment	20
Pectinex Ultra SP-L (95 U/g)	> 24
Viscozyme (2 U/g)	> 24
Combination of Pectinex Ultra SP-L (95 U/g) and Viscozyme (2U/g)	> 24

3.1.2 Effect of cell wall degrading enzyme on lycopene in vitro bioaccessibility

Pectinex Ultra SP-L treatments

The results of lycopene *in vitro* bioaccessibility of tomato puree incubated with pectinases (Pectinex Ultra SP-L) at different incubation time (30, 60, and 120 minutes) and concentration (90, 190 and 285 U/g) were presented in the Figure 1. No significant differences ($p>0.05$) in lycopene *in vitro* bioaccessibility was found between samples incubated with pectinases at different incubation time (from 30 to 120 minutes). Figure 1b showed that by using a range of pectinases concentration (from 95 to 285 U/g of puree), the lycopene *in vitro* bioaccessibility did not change. These results suggested that the use of pectinase did not improve the lycopene *in vitro* bioaccessibility on tomato puree after high pressure homogenization.

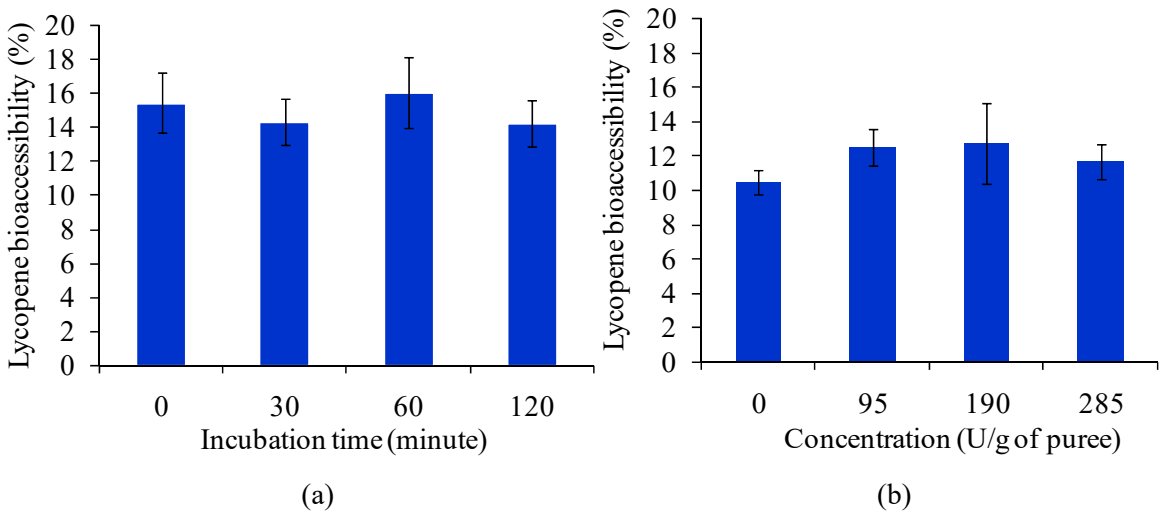

Fig. 1: Lycopene *in vitro* bioaccessibility of tomato puree treated with pectinases (Pectinex Ultra SP-L) at (a) different incubation time and (b) concentration

Data are expressed as mean ± standard deviation (n = 3)

Viscozyme treatment

The results of lycopene *in vitro* bioaccessibility of tomato puree previously treated with different concentrations were shown in Figure 2. The treated samples with different concentrations of Viscozyme, ranging from 1.5 to 2.5 U/g of puree, did not result on any significant difference ($p<0.05$) on lycopene *in vitro* bioaccessibility.

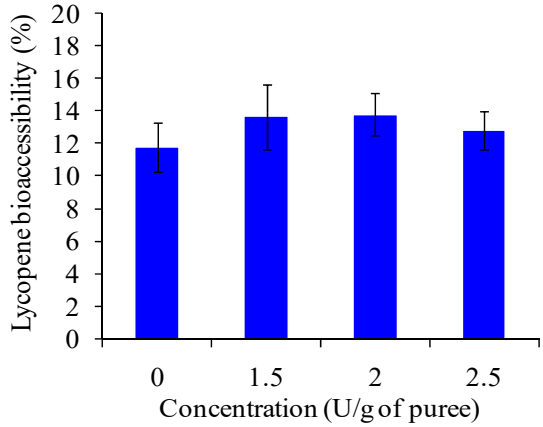

Fig. 2: Lycopene *in vitro* bioaccessibility of tomato puree treated with different concentrations of Viscozyme

Data are expressed as mean ± standard deviation (n = 3)

In short, separately using cell walls degrading enzymes (Pectinex Ultra SP-L and Viscozyme) could not improve lycopene *in vitro* bioaccessibility on tomato puree after high pressure homogenized. Therefore, a mixture of cell wall degrading enzymes might be needed to completely solubilize the new network formed and thus enhancing lycopene release.

Combination of Pectinex Ultra SP-L and Viscozyme treatment

There was no significant difference in lycopene *in vitro* bioaccessibility in homogenized tomato purees which were treated separately and simultaneously two commercial enzymes (Pectinex Ultra SP-L and Viscozyme). Tomato puree were treated with Pectinex Ultra SP-L, Viscozyme and a combination of these two enzymes, the lycopene *in vitro* bioaccessibility corresponded to 13.2 ± 1.14%, 15.2 ± 2.23% and 11.8 ± 0.87% while for the homogenized tomato puree, it was 12.8 ± 0.18% (Figure 3).

The experiments with Pectinex Ultra SP-L and Viscozyme confirmed that cell wall degrading enzyme did not have a positive effect on lycopene *in vitro* bioaccessibility. The maximum value of lycopene *in vitro* bioaccessibility obtained after applying cell wall degrading enzymes was between 12 and 15%.

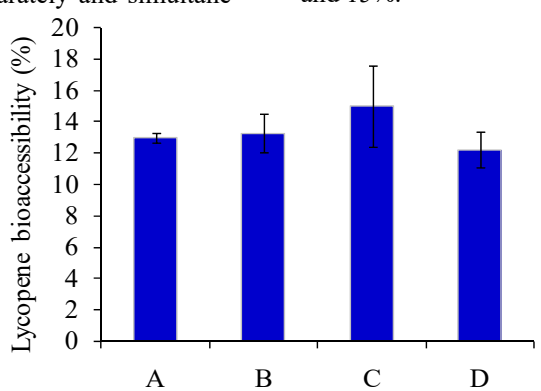

Fig. 3: Lycopene *in vitro* bioaccessibility of tomato puree treated cell wall degrading enzyme. A: No enzymatic treatment; B: Pectinex Ultra SP-L 95 U/g; C: Viscozyme 2 U/g; D: Pectinex Ultra SP-L 95 U/g and Viscozyme 2 U/g

Data are expressed as mean ± standard deviation (n = 3)

3.2 Effect of enzymatic treatments on lycopene *in vitro* bioaccessibility in the chromoplast fraction

The results of lycopene *in vitro* bioaccessibility in the fraction enriched with chromoplast after enzymatic treatments were shown in Figure 4. No significant difference in lycopene *in vitro* bioaccessibility in chomoplast fractions that were treated with difference type of enzymes was found.

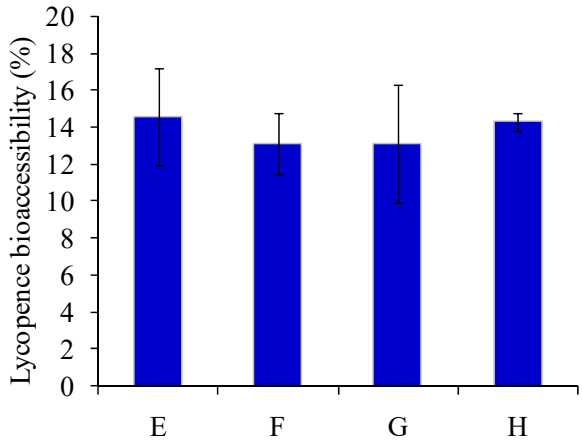

Fig. 4: Lycopene *in vitro* bioaccessibility of chromoplast fraction treated with enzymes. E: Control, F: Phospholipase D (10 U/g), G: Protease (20U/ 1g), and H: Mixture of Phospholipase D and proteas

Data are expressed as mean ± standard deviation (n = 3)

4 DISCUSSION

Cell wall components (pectin, cellulose, hemicelluloses) are carbohydrate polymers that have a high water holding capacity, thus increasing viscosity of the media. Even if the network was not formed after being homogenized, the polymers retain water from its structure. The increase in consistency index (both in separation and combination of enzyme) due to the fact that the fiber network was degraded. Pectin chains were hydrolyzed by Pectinex Ultra SP-L and in the same way for Viscozyme treated sample, cellulose and hemicellulose contributing in the network were degraded. Therefore, these results implied that the puree treated with enzymes effectively degraded cellulose/hemicelluloses and pectin components. By hydrolyzing these polymer, the water holding capacity is lost leading to a release of free water to the system, and therefore increasing the consistency index (Singh *et al.*, 2012).

It has been previously mentioned that there are two main physical barriers for carotenoid release from the food matrix which are the cell wall and the chromoplast sub-structure (Lemmens *et al.*, 2010; Jeffery *et al.*, 2012). It has been also suggested that disruption of plant cell walls may have a positive impact on carotenoid bioaccessibility (Castenmiller *et al.*, 1999). According to Diaz *et al.* (2004), there was a significant increase in β-carotene *in vitro* bioaccessibility on carrot by using mixture of pectinases (Pectinex Ultra SP-L) and cellulases (Cellubrix^{TM} L). However, there was no significant increase in lycopene *in vitro* bioaccessibility on tomato puree, previously homogenized and treated with different cell wall degrading enzymes. This may be explained by the differences in cell wall structure. The cell walls in carrot are rich fiber and compact, while the tomato cell walls less in fiber and thinner (Jeffery *et al.*, 2012). Palmero *et al.* (2013) also showed that there is no significant difference between lycopene bioaccessibility between tomato cell clusters and chormoplast fraction. It implies that tomato cell wall components and the fiber network formed after high pressure homogenized do not contribute as the major boundary for lycopene release. After applying high pressure homogenization and cell wall degrading enzymes, the maximum lycopene *in vitro* bioaccessibility value obtained was 15% that corresponds to the chromoplast lycopene *in vitro* bioaccessibility. Therefore, the fiber network might have been solubilizied, thus the matrix was reached the chromoplast level.

Lycopene in tomato is stored within chromoplasts as crystals. It has been illustrated that lycopene is presented in membrane-shaped structures as caro-tenoid-protein complexes or membrane bound

in tomato fruit (Egea *et al.*, 2010). The envelope of the chromoplast organelle consists of two membranes constructed by lipid bilayers. Composition of tomato fruit chromoplast membranes were investigated and was suggested that they are mainly composed of galactolipids (monogalactosyl diglycerides, and digalactosyl diglycerides) and phospholipids (phosphatidyl choline and phosphatidyl etha-nolamine) (Whitaker, 1986). It was also suggested by Vishnevetsky *et al.* (1999) that carotenoids associated to proteins appear during plastoglobule formation. Phospholipase D (10 U/g chromoplast fraction) and protease (20 U/g chromoplast fraction) were separately and simultaneously used to try to degrade the chromoplast membrane and breakdown the linkage between carotenoids and protein. Palmero *et al.* (2013) indicated that isolated chromophast in oil fraction had a significant increase in the lycopene *in vitro* bioaccessibility compared to chromoplast fraction (without oil). Organelle membranes could already be damaged during the isolation of the fraction, which may explain for the enzymatic treatments not having any additional effects on lycopene *in vitro* bioaccessibility. The results confirm that chromoplast substructure is the most important barrier for lycopene *in vitro* bioaccessibility in tomato fruit.

5 CONCLUSIONS

It can be concluded that the fiber network was not the main barrier for lycopene bioaccessibility. The maximum value of lycopene in vitro bioaccessibility obtained after enzymatic treatments corresponds to the chromoplast fraction. Phospholipase D and protease treatments on chromoplast fraction could not enhance the lycopene *in vitro* bioaccessibility in tomato chromoplast fraction. It suggested that chromoplast membrances or lycopene-protein associations did not prevent lycopene accessible. In that case, lycopene crystals and their sub-localization within the chromoplast would be the main factors governing lycopene *in vitro* bioaccessibility.

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