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In vitro Bioaccessibility of Carotenes

Influence of microstructure in tomato and carrot as modified by processing

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ABSTRACT

Carotenes are a group of fat-soluble pigments in many fruits and vegetables associated with several important biological effects, like protection against the development of some forms of cancer and cardiovascular disease. The carotene bioavailability in plant foods, i.e. the fraction of ingested carotenes taken up and utilised in the body, is often increased by processing. The increased bioavailability is often ascribed to changes in the microstructure, but few studies have investigated this in detail.

The aim of the present work was to evaluate how different types of thermal and mechanical processing, and addition of dietary fat, affected the bioaccessibility of the carotenes lycopene, α-carotene and β-carotene in tomato and carrot, and whether this could be linked to structural changes. An *in vitro* approach was applied in order to screen a large number of processing parameters. The *in vitro* models mimicked different parts of the human gastrointestinal tract, and were used to assess the carotene release from the food matrix, micellar incorporation and cellular uptake. The predictive value of the *in vitro* models was evaluated by comparison with the *in vivo* bioavailability of β-carotene and lycopene from the same fruit and vegetable soups as measured in a human intervention study.

The *in vitro* bioaccessibility of carotenes from carrot and tomato was generally improved by thermal and mechanical processing, and this could be linked to a reduction in cell wall integrity. High-pressure homogenisation (HPH) of carrot, causing extensive cell wall disintegration, and addition of 5% olive oil gave the highest *in vitro* bioaccessibility of α- and β-carotene. For these samples, the micellar incorporation of β-carotene was up to 69% of the total content. In addition, *in vitro* results for β-carotene were consistent with *in vivo* bioavailability. A combination of thermal and mechanical processing of tomato significantly increased the *in vitro* release of lycopene, but attempts to further increase the bioaccessibility by a second thermal treatment or by HPH were not successful. Consequently, the micellar incorporation remained below 22%, even with an addition of 5% olive oil. Furthermore, the *in vitro* models used to assess lycopene bioaccessibility could not predict *in vivo* measurements.

In conclusion, processing conditions that reduced the cell wall integrity of tomato and carrot had a large positive effect on the *in vitro* bioaccessibility of carotenes and, with respect to β-carotene, also suggested an improved bioavailability as measured in humans.

Keywords: *Lycopene, β-carotene, α-carotene, processing, in vitro bioaccessibility, microstructure, tomato, carrot, blanching, high-pressure homogenisation, in vivo bioavailability, Caco-2 cell model*

LIST OF PUBLICATIONS

This doctoral thesis is based on the studies reported in the following papers, referred to in the summary by their Arabic numerals:

1. Tibäck E, Svelander C, Colle I, Altskär A, Alminger M, Hendrickx M, Ahrné L and Langton M (2009). Mechanical and thermal pretreatments of crushed tomatoes: Effects on consistency and *in vitro* accessibility of lycopene. Journal of Food Science, **74**(7): E386-E395.

2. Svelander C, Tibäck E, Ahrné L, Langton M, Svanberg U, Alminger M (2010). Processing of tomato: impact on *in vitro* accessibility of lycopene and textural properties. Journal of the Science of Food and Agriculture, **90**(10):1665-1672.

3. Svelander C, Lopez-Sanchez P, Pudney P, Schumm S, Alminger M. High pressure homogenisation (HPH) increase the *in vitro* bioaccessibility of α- and β-carotene in carrot emulsions, but not of lycopene in tomato emulsions. (Response to reviewers)

4. Alminger M, Svelander C, Wellner A, Martinez-Tomas R, Bialek L, Larque E, Perez-Llamas F. Applicability of *in vitro* models in assessing the *in vivo* bioavailability of carotenoids from soups containing carrot, tomato and broccoli. (Manuscript)

Related publications not included in the thesis

Lemmens L, Tibäck E, Svelander C, Smout C, Ahrné L, Langton M, Alminger M, Van Loey A, and Hendrickx M (2009). Thermal pretreatments of carrot pieces using different heating techniques: Effect on quality related aspects. Innovative Food Science and Emerging Technologies **10**(4):522-529.

Lopez-Sanchez P, Svelander C, Bialek L, Schumm S and Langton M (2010). Rheology and microstructure of carrot and tomato emulsions as a result of high-pressure homogenisation conditions. Journal of Food Science, **76**(1):E130-E140.

CONTRIBUTION REPORT

Paper 1: The author, Cecilia Svelander (CS), participated in designing the experiments and in sample preparation, performed the *in vitro* accessibility and total lycopene content analysis, interpreted the lycopene data and participated in writing the manuscript.

Paper 2: CS participated in designing experiments, sample preparation and laboratory work, and was responsible for interpreting the data and writing the manuscript.

Paper 3: CS participated in designing experiments and sample preparation, performed the carotene and ascorbic acid analyses and was responsible for interpreting the data and writing the manuscript.

Paper 4: CS participated in designing experiments, laboratory work, data analysis and writing the manuscript.

ABBREVIATIONS

AIR	Alcohol insoluble residue
BHT	Butylated hydroxytoluene
CVD	Cardiovascular disease
DHA	Dehydroascorbate
DLS	Dynamic light scattering
DM	Degree of methylation
DMEM	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
HPH	High pressure homogenisation
HPLC	High performance liquid chromatography
HTLT	High temperature, long time
HTST	High temperature, short time
L–AA	L-ascorbic acid
LM	Light microscopy
LTLT	Low temperature, long time
LDL	Low-density lipoproteins
PBS	Phosphate buffer solution
PG	Polygalacturonase
PME	Pectinmethylesterase
SD	Standard deviation
SEM	Scanning electron microscopy
TCEP	Tris[2-carboxyethyl]phosphine hydrochloride
TEM	Transmission electron microscopy
TIM	TNO gastro-intestinal model
TG	Triacylglycerol

TABLE OF CONTENTS

INTRODUCTION

A high intake of fruits and vegetables has been associated with a decreased risk of developing chronic diseases such cardiovascular disease (CVD) and different types of cancer (Steinmetz *et al.* 1996; Ness *et al.* 1997; Van Duyn *et al.* 2000; Wright *et al.* 2003; Singh *et al.* 2008). These protective effects have been partly attributed to carotenoids, a group of colourful, fat-soluble phytonutrients present in many fruits and vegetables (Khachik *et al.* 2002; Rao *et al.* 2007). Over 700 carotenoids have been identified in nature; the most prevalent carotenoids in human blood and tissues are α-carotene, β-carotene, lutein, lycopene, β-cryptoxanthin and zeaxanthin (Khachik *et al.* 1997; Maiani *et al.* 2009). Of these, β-carotene and lycopene account for about 50% to 70% of total carotenoid levels in the blood (O'Neill *et al.* 2001; Jenab *et al.* 2006). According to O'Neill *et al*. (2001), carrots are the main source of both α- and β-carotene in the European diet. Tomato and tomato products are the dominant dietary sources of lycopene and have been reported to supply over 85% of total dietary lycopene (Rao *et al.* 2000).

As a consequence of the epidemiological reports associating plant food intake with health benefits, much attention has focused on identifying specific bioactive components in fruits and vegetables. However, intervention trials with isolated compounds, such as β-carotene, have often failed to show positive results and have in some cases even resulted in negative effects (Omenn *et al.* 1996; Lin *et al.* 2009; Rytter *et al.* 2010a). This may be a matter of dosage, carotenes may for example have pro-oxidative properties at high concentrations, but it is also possible that the observed health benefits are due to a combination of different compounds in fruits and vegetables. Wright *et al*. (2003) investigated factors reducing the risk of lung cancer and found a stronger correlation for overall vegetable intake, compared with the intake of separate carotenoids. Similarly, tomato powder was more efficient than lycopene alone in reducing the risk of prostate cancer and tumour growth in model systems (Boileau *et al.* 2003; Canene-Adams *et al.* 2007). A whole food approach, based on intake of several fruits and vegetables, is therefore suggested as a better health promoting strategy compared with nutritional supplementation of specific compounds (Omoni *et al.* 2005; Åsgård *et al.* 2007; Tan *et al.* 2010).

Carrot and tomato are often subjected to domestic or industrial processing, which may reduce the nutrient content, but can at the same time improve nutrient bioavailability. Bioavailability is defined as the fraction of an ingested nutrient that is absorbed in the intestine and reaches systemic circulation. Human intervention studies have established that processing fruit and vegetables can increase carotene bioavailability, but the impact of different processing steps is still largely unknown (Gärtner *et al.* 1997; Rock *et al.* 1998; Livny *et al.* 2003; Maiani *et al.* 2009). Constraints with human studies, such as cost and number of subjects, limit the number of different samples that can be compared. As an alternative, several *in vitro* models mimicking the human gastrointestinal tract have been developed. Such models allow for the evaluation of a much larger number of

samples. Once processing solutions that favour an improved *in vitro* bioaccessibility of carotenes have been identified, the results can be evaluated in human studies.

The bioavailability of carotenes is related to their bioaccessibility, which is how much of the ingested carotene that is released into the gastrointestinal tract and made available for uptake. It has been suggested that the microstructure of fruits and vegetables has a large impact on bioaccessibility, and that processing increases carotene release by disrupting the food matrix (van het Hof *et al.* 2000b; Parada *et al.* 2007). The microstructure also has a large impact on the palatability and sensory perception of fruits and vegetable products, such as mouth feel and viscosity (Valencia *et al.* 2003). Understanding how processing affects sample microstructure, and how this in turn correlates with carotene bioaccessibility, could help in designing palatable fruit and vegetable products with a high nutritional value. Providing convenient, tasty and nutritious semi-manufactured or ready-to-eat products could in turn promote a healthier diet, especially among individuals with a low intake of fruit and vegetables.

Therefore the work presented in this thesis has focused on the correlation between processing, carotene bioaccessibility and structural properties. For this purpose, tomato and carrot were subjected to various types of mechanical and thermal processing. The carotene bioaccessibility of the processed samples was evaluated in different *in vitro* models and the results compared with information on sample micro- and macrostructure. This work was part of the European Union project "Nutritional and Structural Design of Natural Foods for Health and Vitality" with the overall aim to improve the nutritional and structural quality of ready-to-eat fruit and vegetable products.

OBJECTIVES

The overall aim of the work presented in this thesis was to identify processing conditions governing the *in vitro* bioaccessibility of carotenes from tomato and carrot and to investigate whether the bioaccessibility can be linked to structural properties.

The specific aims were:

- To evaluate how various thermal and mechanical pre-treatments, individually or in combination, affect nutrient retention and *in vitro* bioaccessibility of carotenes (paper 1-3)

- To study the relationship between *in vitro* bioaccessibility of carotenes and microstructure of tomato and carrot (papers 1-3)

- To assess whether macrostructural properties of the samples, such as consistency, are related to the *in vitro* bioaccessibility of carotenes (papers 1-3)

- To evaluate the Caco-2 cell line as a model to investigate cellular uptake of micellarised carotenes from *in vitro* digested samples (papers 3-4)

- To study how the chemical structure of different carotenes affects their relative *in vitro* release, micellar incorporation and uptake by Caco-2 cells (papers 3-4)

- To compare carotene bioaccessibility data obtained from different *in vitro* models to *in vivo* bioavailability (paper 4)

BACKGROUND

Carotenes

The work presented in this thesis focuses on carotenes, which belong to the carotenoids, a group of structurally related pigments produced by photosynthetic organisms (Olson *et al.* 1995). Based on their chemical composition, carotenoids are divided into carotenes, which only contain carbon and hydrogen atoms, and xanthophylls, which contain at least one oxygen atom. The discussion below deals with carotenes, but many of the properties described are applicable to the whole carotenoid group.

Chemical structure and properties

Carotenes are made from eight isoprenoid units forming a long, carbon chain of with conjugated double bonds (Figure 1). The completely planar C_{40} carbon skeleton of lycopene forms a parent structure from which all other carotenes can be derived, either by ring closure at the ends, or by changes in the hydrogenation level (Britton 1995). The ring closure will form one of six different cyclic end groups and, one of which being the β-ring marked in Figure 1. If the carotene contains at least one β-ring it can be cleaved to form retinol. The lack of polar groups make carotenes virtually insoluble in water, and the carotenes often form stable crystals owing to their elongated, symmetric chain structure (Craft *et al.* 1992; Britton 1995). The crystalline form significantly increases the stability of carotenes, which are otherwise sensitive to degradation and isomerisation, especially in the presence of oxygen or heat (Delgado-Vargas *et al.* 2000).
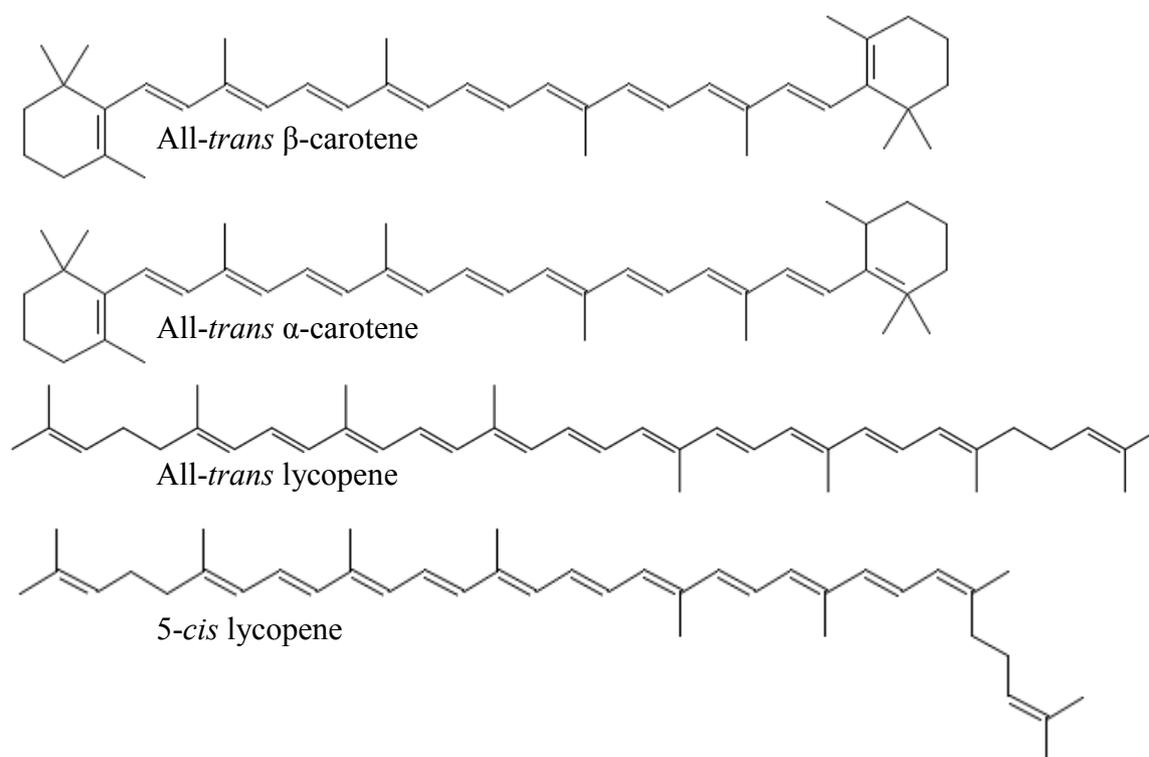

Figure 1. *Chemical structure of the all-trans forms of the three dominant carotenes in human plasma (β-carotene, α-carotene and lycopene) and of 5-cis lycopene. The square marks one of the two β-rings in β-carotene.*

The large number of conjugated double bonds in carotenes effectively delocalise π-electrons over the carbon chain and gives carotenes many of their trademark properties, as reviewed by Britton (1995). Due to the electron delocalisation, a relatively low energy corresponding to wavelengths of 400-500 nm is sufficient to bring about an excited state. In other words, carotenes absorb light in the visible region, which gives them an intense yellow to red coloration. The stability of the excited state also confers antioxidant properties of carotenes, making them efficient singlet oxygen quenchers.

The absence of cyclic end groups in lycopene further reinforces several of the general carotene properties, such as radical scavenging and hydrophobicity. Lycopene has two additional conjugated double bonds in the carbon chain, 11 compared with the maximum of 9 conjugated double bonds for carotenes that have undergone ring closure (Figure 1). This further strengthens the stability of the excited state of lycopene and makes it the most efficient singlet oxygen quencher of all common carotenes (Di Mascio *et al.* 1989). Cyclisation shortens the overall length of the molecule but increases its effective bulk. This means that lycopene molecules can be packed more tightly, giving extremely stable crystal structures, but also that their length may decrease their solubility in other media such as mixed micelles or cellular membranes (Britton 1995; Boileau *et al.* 1999).

The large number of double bonds in carotenes means that a great number of geometrical isomers are theoretically possible. For example, β-carotene has 272 possible isomers but, due to steric hindrance, only a few of these will ever be encountered in nature (Olson *et al.* 1995). Carotene *cis*-isomers have a bent structure and differ from their linear all-*trans* counterparts in several ways (Figure 1). The steric strain of *cis*-isomers makes them less thermodynamically stable, and the differences in shape may have a large impact on their incorporation into cellular structures. The bulkier structure of the *cis*-isomers also makes them less prone to aggregate or form crystals, which has a further negative impact on their stability during thermal processing but may also make them more available for solubilisation and uptake in the gastrointestinal tract (Britton 1995).

Carotenes in plants
Carotenes can be found in high concentrations in photosynthetic tissues, where they play dual roles as light harvesting pigments and protective agents (Britton 1995; Nagao 2009). The carotenes absorb and transfer energy to the chlorophyll, which generates the excited singlet state of chlorophyll that is active in photosynthesis (Britton 1995). At conditions of intense light, carotenoids can prevent radical formation both by accepting and dissipating excessive energy and by functioning as singlet oxygen quenchers (Britton 1995; Demmig-Adams *et al.* 1996). To perform these roles, the carotenes have to be in direct contact with chlorophyll, which is accomplished by specific chlorophyll/carotenoid-binding protein complexes of the two photosystems within the thylakiod membranes of the chloroplast (Demmig-Adams *et al.* 1996).

When fruits and vegetables ripen, chloroplasts differentiate into chromoplasts by degradation of photosynthetic structures in combination with extensive synthesis and accumulation of carotenes (Bathgate *et al.* 1985; Ljubesic *et al.* 1991). Carotenes in fruits

and vegetables are usually found in their all-*trans* form and may form crystalline structures when present in large numbers (Ljubesic *et al.* 1991). In tomato, lycopene is located in crystalline thylakoid structures in the chromoplasts, whereas β-carotene is believed to be dissolved in intracellular lipid material (Ben-Shaul *et al.* 1969; Ljubesic *et al.* 1991; Nguyen *et al.* 2001). In carrot, α- and β-carotene is in a crystalline form and bound to proteins (Ljubesic *et al.* 1991; Bryant *et al.* 1992; Kim *et al.* 2010).

The role of carotenes in human health
Over 700 carotenoids have been identified in nature and around 60 are found in the human diet, but only six of these constitute over 95% of total blood carotenoids (Khachik *et al.* 1997; Yonekura *et al.* 2007; Maiani *et al.* 2009). The dominant carotenoids in human blood are three xanthophylls, β-cryptoaxanthin, lutein and zeaxanthin, and three carotenes, α-carotene, β-carotene and lycopene (Maiani *et al.* 2009). Of the carotenes, both α- and β-carotene, but not lycopene, have pro-vitamin A activity, i.e. they can be metabolised into vitamin A in the body. However, all common carotenoids have been connected to health effects apart from their function as precursors of vitamin A. High intakes or plasma levels of α-carotene, β-carotene and lycopene have for example been associated with a reduced risk of developing several types of chronic diseases such as cancers and CVD (Kohlmeier *et al.* 1997; Giovannucci 1999; Rissanen *et al.* 2001; Wright *et al.* 2003; Omoni *et al.* 2005; Li *et al.* 2010).

The conjugated double bond system makes carotenes, particularly lycopene, potent antioxidants. This has been confirmed *in vitro*; for example carotenes significantly reduced the oxidative response of lymphocytes and low density lipoproteins (LDL) isolated from humans, as well as that of human cell lines (Martin *et al.* 1996; Oshima *et al.* 1996; Porrini *et al.* 2005). However, while there is accumulating evidence of antioxidative activity *in vitro,* the role of carotenes as antioxidants *in vivo* have been questioned (Rice-Evans *et al.* 1997; Erdman Jr *et al.* 2009; Pérez-Rodríguez 2009). For example, intervention trials with antioxidative compounds such as carotenes have often failed to show positive results (Omenn *et al.* 1996; Lin *et al.* 2009; Rytter *et al.* 2010b). The situation is further complicated by the fact that carotenes can act as pro-oxidants at high concentrations (El-Agamey *et al.* 2004). Based on the current state of knowledge, the potential antioxidant effects of carotenes in the human body are yet to be confirmed. Carotene metabolites are known to bind and activate nuclear receptors, such as retinoic acid receptors, and are for example highly active in systems regulating lipid metabolism (Hessel *et al.* 2007; Lefebvre *et al.* 2010). However, reviewing this in detail is beyond the scope of this thesis.

Carotene bioavailability and bioaccessibility
In order for ingested carotenes to have an effect on human health, they have to be taken up into the body, i.e. they have to be bioavailable. The general uptake and transport of fat and fat soluble nutrients such as carotenes can be divided into three major phases; intraluminal release and micellar incorporation, uptake by the intestinal mucosa and delivery to target sites (Castenmiller *et al.* 1997). Human studies on bioavailability often

investigate the final carotene levels in chylomicrons or serum, whereas *in vitro* models usually simulate specific parts of the process.

Carotene digestion, absorption and metabolism
The first step in carotene absorption is release of carotenes from the food into the small intestine and solubilisation in lipid droplets (Parker 1996). The release is enhanced by mechanical and enzymatic disruption of the food matrix during mastication and digestion (van het Hof *et al.* 2000b; Parada *et al.* 2007).

The released carotenes are then incorporated into mixed micelles in the duodenum. The mixed micelles are formed in the duodenum during digestion and consist largely of bile salt, free fatty acids, monoglycerids and phospholipids (Hernell *et al.* 1990). The mixed micelles have been described to have a discodial structure and a diameter of around 8 nm (Parker 1996).

Once carotenes are incorporated into mixed micelles, they reach the intestinal mucosa by diffusion through the unstirred water layer and are then absorbed into enterocytes (Castenmiller *et al.* 1997). The absorbed carotenes are then incorporated into lipoproteins such as chylomicrons and reach the blood via lymphatic structures.

Factors affecting carotene bioavailability
Carotene bioavailability is a complex process that can be affected by a number of proposed factors, summarised under the acronym SLAMANGHI (De Pee *et al.* 1996). The acronym stands for species of carotene, molecular linkage, amount of carotene in a meal, matrix in which the carotenoid is incorporated, absorption modifiers, nutrient status of the host, genetic factors, host-related factors and interactions. Some of the factors that can be evaluated *in vitro* are discussed below.

It has been suggested that the intactness of the food matrix is a major determining factor for the *in vitro* and *in vivo* accessibility of carotenes (van het Hof *et al.* 2000b; Parada *et al.* 2007; Tydeman *et al.* 2010a; Tydeman *et al.* 2010b). Processing is believed to increase the carotene release from the food matrix by disrupting cells and cell structures, as well as dissociate carotene-food matrix complexes.

The type of carotene as well as which iso-form may have a large impact on carotene bioavailability. Gärtner *et al.* (1997) reported higher relative concentrations of lycopene *cis*-isomers compared with the all-*trans* form in chylomicrons after ingestion of both fresh tomato and tomato paste. The proportion of lycopene *cis*-isomers was also higher in serum compared to the diet, which is in line with other studies (Schierle *et al.* 1997; Holloway *et al.* 2000; Hadley *et al.* 2003). It is still unclear whether the higher relative concentration of *cis*-isomers in the body compared with food is caused by a higher bioavailability of *cis*-isomers or *cis-trans* transformation *in vivo*, but recent evidence supports the suggestion that a combination of both may contribute to this effect (Unlu *et al.* 2007; Richelle *et al.* 2010).

Absorption modifiers can have both a negative and a positive impact on carotene bioavailability. Dietary fibre, such as pectin, has been shown to reduce the bioavailability of several carotenes, including β-carotene and lycopene (Erdman Jr *et al.* 1986; Rock *et al.* 1992; Riedl *et al.* 1999). Suggested mechanisms of action include an increased viscosity in the gastrointestinal tract, which reduces carotene migration and hampers micelle formation by binding bile acids and phospholipids. The presence of dietary fat has instead been shown to increase carotene bioavailability (Brown *et al.* 2004; Unlu *et al.* 2005; Yonekura *et al.* 2007). Dietary fat may improve carotene absorption in several ways; it can act as a bulk phase in which carotenes are solubilised prior to micellar incorporation, and provide a source of fatty acids for the mixed micelles. High levels of dietary fat in the small intestine may also promote micelle formation by increasing secretion of bile salts, pancreatic lipase and phospholipids, and will promote chylomicron assemblage by intestinal cells (Borel 2003).

If several different carotenes are present in the gastrointestinal tract at the same time, interactions between the different carotenes may also affect their bioavailability. However, different human studies have reported varying results, where simultaneous intake of carotenoids increased, decreased or did not affect the bioavailability depending on speciation and ratios (Van Den Berg 1999; Tyssandier *et al.* 2002; Yeum *et al.* 2002).

Methodology for assessing bioavailability in vivo
Measuring carotene bioavailability *in vivo* is a complex task and the results may differ depending on the choice of methodology. Human intervention studies are the "golden standard" but, due to practical limitations and high cost, animal models are sometimes employed. Human studies have also shown a large variation in carotene response, both for the same individual on different occasions and between different individuals (Borel *et al.* 1998). Bioavailability estimates are further complicated by carotene metabolism in the human body, for example formation of vitamin A.

Two common methods for evaluating carotene bioavailability are measurements of blood plasma/serum carotene levels or postprandial carotene content in the triacylglycerol (TG) rich fraction of serum/plasma. Blood plasma/serum levels of carotenes depend on long-term carotene intake and are commonly used to measure carotene response in blood samples collected after a period of continuous carotene intake. Since recently absorbed carotenes are first secreted into chylomicrons, which belong to the TG fraction, the TG carotene levels are a better measure of the bioavailability after a single meal. These methods have several benefits but can only be used to compare the relative impact of different carotene sources and do not provide information about the proportion of total ingested carotenes that is taken up into the body.

A number of different methods have been employed to assess the actual uptake of carotenes. These include the use of oral-faecal mass balance studies, radioactive or stable isotope tracers, total gastrointestinal wash-out and ileostomy mass-balance models. Mass-balance estimates are based on the difference between ingested and secreted carotenes, assuming that this difference constitutes the absorbed fraction. While these

9

models have the benefits of being relatively straightforward, they do not account for losses in the gastrointestinal tract due to *e.g.* oxidation, isomerisation or microbial activity, and may therefore overestimate the true absorption. In a study by Livny *et al.* (2003) for example, where ileostomy volunteers ingested a carrot and compared with all-*trans* β-carotene, a significantly higher proportion of β-carotene *cis*-isomers were recovered in the ileal effluents. This could have been due to a lower uptake of *cis*-isomers, but may also be an indication of isomerisation of the ingested all-*trans* β-carotene.

In vitro methods for estimating bioaccessibility

Due to the limitations of *in vivo* models, *in vitro* models have been developed as a more simple, inexpensive and reproducible alternative. The models are applied to study digestive stability, release from the food matrix, micellarization, intestinal uptake or transport and metabolism of different food components, and to estimate their bioavailability. *In vitro* models mimicking the conditions of the gastrointestinal tract can be either static or dynamic.

The work presented in this thesis used the simpler, static *in vitro* digestion model. There is currently no standardised model for static *in vitro* digestion of carotenes, and methodological details will differ between different studies, but the general steps are similar in most models (Garrett *et al.* 1999a; Hedrén *et al.* 2002; Reboul *et al.* 2006). Normally, the stomach and upper part of the small intestine are simulated by addition of physiological amounts of salts and enzymes, adjustment of pH and incubation at 37°C. During an initial gastric phase, samples are incubated at gastric pH in the presence of gastric enzymes such as pepsin. The upper part of the small intestine, the duodenum, is thereafter simulated by increasing the pH and adding pancreatic enzymes and bile salts. Some models will also include an oral digestion phase, which simulates mastication in the presence of *e.g.* amylase, and there are examples of colon fermentation being included in the model (Serrano *et al.* 2005; Bengtsson *et al.* 2009a).

In a dynamic model, such as the TNO gastro-intestinal model (TIM) the basic principles are the same, but there is a higher degree of complexity more closely compatible to the conditions in the gastrointestinal tract (Minekus *et al.* 1999). The TIM model comprises four compartments, representing the stomach, duodenum, jejunum and ileum, and can also be combined with a colonic compartment. The main differences compared with the static models are the continuous removal of nutrients, simulation of peristalsis and more controlled, gradual changes in pH, enzyme levels etc.

The Caco-2 model is based on a human colon adenocarcinoma cell line, which has many characteristics of small intestinal epithelium. It was originally established to measure the bioavailability and toxicity of drugs but is increasingly used within food research to study the uptake and transport of various compounds such as iron, zinc and carotenoids (Hilgers *et al.* 1990; Garrett *et al.* 1999a; Haraldsson *et al.* 2005; Ryan *et al.* 2008). The Caco-2 cells will grow to form a monolayer which then spontaneously differentiates to create many structural and functional components of intestinal cells. These components include

microvilli, tight junctions between the cells and brush border enzymes, which means that the Caco-2 cell line can be used to study active and passive uptake and transport of nutrients. Two main types of cell models are applied for these kinds of studies, one being a simpler model where cells are grown on plastic and only cellular uptake of nutrients is measured and the other a more complex membrane insert model. The insert model is used to study both uptake and basolateral secretion, and here the cells are instead grown on a porous membrane in an insert. Growing the cells on inserts mimics the *in vivo* situation more closely, but the higher complexity of the model also makes it more demanding with regard to time and cost. An issue with the Caco-2 cell line is that cellular uptake of nutrients will vary between different experiments, depending on *e.g.* the general condition and passage number of the cells. Results can therefore only be compared within the same experiments.

Plant materials

The botanical classification of tomato is as a fruit and carrot as a root vegetable, and they differ in their nutritional composition and structural properties. The cells of tomato flesh are around ten times larger than those of carrot and the cell walls differ in molecular and enzymatic composition. This has a large impact on both tissue hardness and response to processing for carrot and tomato.

Tomato

Tomato and tomato products are the major source of lycopene in the Western diet (Shi *et al.* 2000; O'Neill *et al.* 2001). In North America, they have for example been reported to provide over 85% of the total dietary lycopene (Rao *et al.* 2000). All-*trans* lycopene is the predominant carotene in tomato, but it also contains detectable amounts of other carotenoids such as β-carotene and lutein (Abushita *et al.* 2000). In addition to carotenes, tomato has a high content of other bioactive compounds such as vitamins C and E (Abushita *et al.* 2000).

The worldwide production of tomato was estimated at over 141 million tonnes in 2009, making it one of the most important agricultural commodities in the world (FAO 2011). Tomatoes are grown and consumed in most of the world, and the largest producers are China, USA, India and Turkey (FAO 2011). However, tomato consumption has only been common in the last 200-300 years, making it a relatively new addition to the human diet (Costa *et al.* 2005).

A large proportion of the yearly tomato harvest is processed into canned tomatoes, tomato sauce and purée, or used as ingredients in soups and stews. For example, the annual per capita consumption in the USA was around 8.5 kg for fresh tomatoes and 33 kg for processed tomatoes (United States Department of Agriculture 2009). Tomatoes used in processing are grown specifically for that purpose and differ from the salad varieties that are available in the supermarkets (Costa *et al.* 2005). For example, since colour is a major quality parameter in tomato products, plant breeding has favoured higher lycopene levels in processing tomatoes compared with salad varieties (Hayes *et al.* 1998; Costa *et al.* 2005). To facilitate transport over long distances salad tomatoes are often harvested

before they are completely ripe, whereas the processing varieties are usually harvested when fully ripe and subjected to an initial processing in close proximity to the fields.

Carrot

Regular orange carrots do not contain lycopene and only low amounts of vitamin C but are rich in α- and β-carotene. O'Neill *et al*. (2001) compared the carotenoid intake in five European countries and found that carrots contributed to 24-60% of total β-carotene intake, and 60-95% of total α-carotene in the diet. Carrots are also a good source of *e.g.* phenolic compounds and dietary fibre (Chantaro *et al*. 2008). The orange carrot was developed in the Netherlands in the 18th century and predominates in the west, but differently coloured varieties are consumed in *e.g.* India, China and Japan. The difference in coloration between carrot varieties stems from their carotenoid and phenolic composition, for example is lycopene responsible for the colour of red carrots and anthocyanins, a group of polyphenolic compounds, for the colour of purple carrots (Arscott *et al.* 2010).

According to the food and agricultural database of the United Nations, about 28 million tonnes of carrots and turnips were produced worldwide in 2009 (FAO 2011). The annual carrot production in Europe is over 6 million tonnes (European Commission 2011). Compared to tomato, carrots have a longer history of human consumption. Documentation from Iran and surrounding regions describes the presence of purple and yellow carrot varieties in the 10th century, which then spread west to Europe and east to China during the 15th century (Arscott *et al.* 2010).

Structural and nutritional components of tomato and carrot

Vitamin C or ascorbic acid is found in most fruits and vegetables, and pectins are one of the main structural components of the cell wall. Since ascorbic acid is easily degraded it is sometimes regarded as a "marker" compound for nutrient loss during processing. Pectins can have a large impact on structural properties of tomato and carrot, and often undergo large changes during processing.

Ascorbic acid

Historically, ascorbic acid has mainly been connected with its role in preventing scurvy, but recent epidemiological evidence suggests that it may also affect human health in other ways, such as enhancing several aspects of the immune system, increasing iron absorption and potentially reducing the risk of some cancers and cardiovascular disease (Davey *et al.* 2000; Naidu 2003). In fresh fruits and vegetables, ascorbic acid is present mainly as L-ascorbic acid (L-AA) but is readily oxidised to dehydroascorbate (DHA) in the presence of oxygen.

L-AA is a labile molecule and can be both enzymatically and non-enzymatically degraded during industrial and domestic cooking, as well as lost by leakage into the cooking water (Davey *et al.* 2000). Crushing fruit and vegetable tissue brings L-AA degrading enzymes into contact with their substrate, normally causing extensive degradation of L-AA.

Factors determining the extent of non-enzymatic degradation of L-AA are oxygen levels, light, pressure, temperature, presence of metal ions and pH.

Pectins and pectin degrading enzymes
The surrounding cell wall is a characteristic feature of plant cells, providing structure to the plant. Pectins are a group of polysaccharides that act as structural components in the cell wall and help glue the cells together in the middle lamella. The structure of pectins makes them efficient in binding water and, under the right conditions, they can form gels. The exact architecture of pectins is still under debate, but one of the main structural features is a backbone of linked galacturonic acid residues, which are esterified with a varying degree of methylation (DM) (Willats *et al.* 2001). Pectins with a high DM forms gels through cross-linking via hydrophobic interactions and hydrogen bonds. Pectins with a low DM form gels in the presence of divalent ions through aggregation and helix formation.

Pectin is degraded through β-elimination, acid hydrolysis or enzymatic activity. The major enzymes responsible for pectin degradation are pectinmethylesterase (PME) and polygalacturonase (PG) (Thakur *et al.* 1996; Lopez *et al.* 1997; Crelier *et al.* 2001; Verlent *et al.* 2007). PME catalyses de-esterification of pectin, which lowers the DM. Low DM pectins can thereafter be depolymerised by either PG or, in the acidic environment of *e.g.* tomato purée, by acid hydrolysis (Krall *et al.* 1998; Fachin *et al.* 2003; Fraeye *et al.* 2007). Pectin demethylation strengthens the polymer network, whereas depolymerisation causes a decrease in viscosity. The temperature optimum for PME and PG is 50-60°C, and the enzymes are inactivated at temperatures around 80°C (Hayes *et al.* 1998; Verlent *et al.* 2007)

Processing of fruits and vegetables
Plant foods are often subjected to thermal and mechanical processing prior to ingestion and this can have a major impact on both the content and bioavailability of nutrients. The word processing is often associated with large-scale industrial production but also includes domestic cooking.

Plant foods contain a large number of quality degrading enzymes, and a blanching step is often included early during industrial processing to inactivate these enzymes (Hayes *et al.* 1998). The blanching temperature is chosen depending on the desired properties of the final product. A higher temperature of around 90°C will rapidly inactivate most quality degrading enzymes but may produce undesired changes in colour and flavour due to chemical reaction such as the Maillard reactions. To avoid this, a lower temperature of around 60°C can be employed, but this will not inactivate all enzymes. For example, the pectin degrading enzymes PME and PG will be highly active at these temperatures. Heat treatments at 100°C or above are commonly used both as a sterilisation step during industrial production and in domestic cooking.

Tomato contains both PME and PG, but carrot only contains PME, and the effect of heat treatments on structural properties will differ. In intact fruits and vegetables, PME and PG

are located in the cell walls but are released during mechanical processing such as crushing. Heat treatments may also cause leakage of pectin from the cell walls (Greve *et al.* 1994). In tomato, the combined action of PME and PG during low temperature blanching will cause depolymerisation of the released pectins, which is reported to reduce the final viscosity (Fachin *et al.* 2003). This type of blanching is therefore used in the production of for example tomato juice (Hayes *et al.* 1998). Carrot, which lacks PG, can instead obtain a harder texture when subjected to blanching at around 60°C. This is because PME reduces the degree of methylation of the pectins, promoting crosslinking between pectins via salt bridges formed in the presence of divalent metal ions such as calcium.

High-pressure homogenisation (HPH) is commonly used to generate emulsions in the pharmaceutical and food industries, but is less commonly used in the production of plant based foods (Lopez-Sanchez *et al.* 2010). During HPH, the material is forced through a small constriction by the use of pressure deforming and breaking of the particles. In the present thesis, HPH was used to obtain a more thorough disruption of the plant matrix compared with crushing in household or industrial blenders.

METHODS AND METHODOLOGICAL CONSIDERATIONS

Study overview

This thesis is based on the four major studies presented in papers 1-4 and on the results of several smaller pilot studies. The impact of processing on carotene release from the food matrix, which is the first requirement for carotene bioavailability, is investigated in papers 1-2. In paper 3, the next steps, micellar incorporation and cellular uptake, are added to the analysis and finally in paper 4 the predictive value of the applied *in vitro* models with regard to *in vivo* bioavailability is assessed. The thermal treatments investigated were boiling at 100°C, as well as several types of blanching at temperatures below 100°C. The blanching treatments were named HTST (high temperature – short time), LTLT (low temperature – long time) and HTLT (high temperature – long time). Short times were 4-10 min and long times were 40 min and upwards, temperatures around 60°C were considered low and high temperatures were 90-95°C. Mechanical treatments included crushing by various blenders and high pressure homogenisation (HPH).

The aim of study 1 was to separately investigate how thermal and mechanical processing of tomato affected the *in vitro* bioaccessibility of lycopene. To achieve this, a 3 x 2 study design with three crushing times (15, 30 and 120 s) and two thermal treatments (HTST and boiling) was used. Unheated tomatoes crushed for the same time periods, as well as subjected to crushing in an Ultra Thurrax, were also included in the analysis. In total 10 different treatment combinations were investigated as summarised in Figure 2. The total content and *in vitro* release of lycopene was measured, as was sample density, Bostwick consistency, the activity of pectin degrading enzymes (PME and PG) and the pectin degree of methylation (DM). Microstructural characterisation was done with light microscopy (LM) and transmission electron microscopy (TEM).
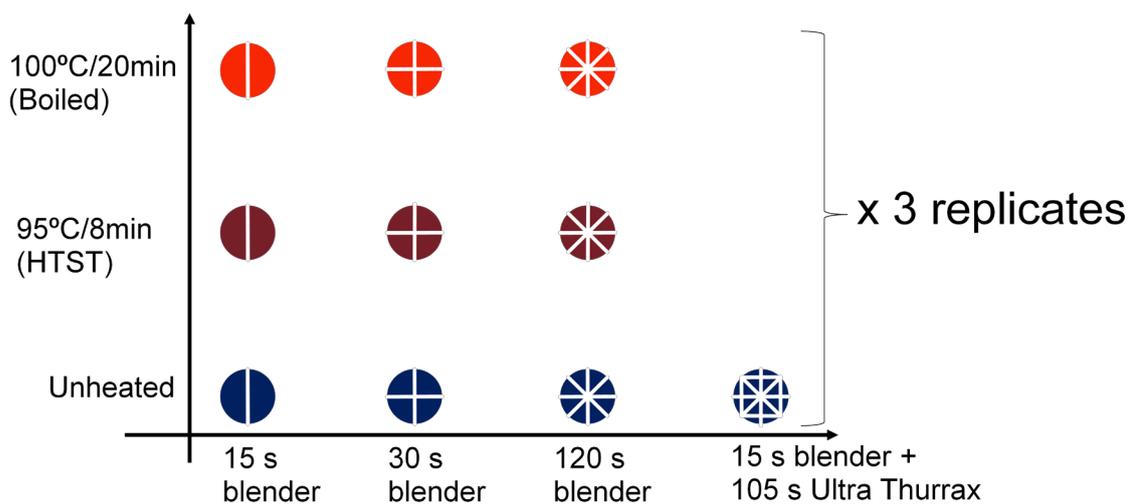

Figure 2. *Experimental design for study 1, in total 10 different treatments were compared. Sample preparation was repeated three times for each treatment, giving three independent replicates.*

The objective of study 2 was to compare how different heat treatments affected nutritional and structural properties of crushed tomato samples. Repeated heat treatments were used, where samples were first subjected to LTLT or HTST blanching, then cooled in an ice batch followed by boiling. The retention and *in vitro* bioaccessibility of lycopene and β-carotene was measured, sample microstructure was investigated with LM and the Bostwick consistency index was used as a measure of sample macrostructure. The experimental work was divided into two parts. The first part, 2a, was a screening experiment to evaluate the effect of different processing combinations on the final product. In total 8 different sample treatments were investigated, as summarised in Table 1. The results from 2a were then used to select 4 treatments for the second part (2b). The impact of each processing step was investigated in 2b, as illustrated in Figure 3.

Cut → Crush
Cut → Crush → Boil
Cut → Crush → LTLT → Boil
Cut → LTLT → Crush → Boil
Cut → Ca^{2+} → Crush → LTLT → Boil
Cut → LTLT → Ca^{2+} → Crush → Boil
Cut → Crush → HTST → Boil
Cut → HTST → Crush → Boil

Table 1. *Experimental design in study 2a, in total 8 different treatments were compared. Sample preparation was repeated three times for each treatment, yielding three independent replicates. Boiling was done at 100°C for 10 min, LTLT blanching at 60°C for 40 min and HTST blanching at 90°C for 4 min. The treatments selected for study 2b are in italic.*
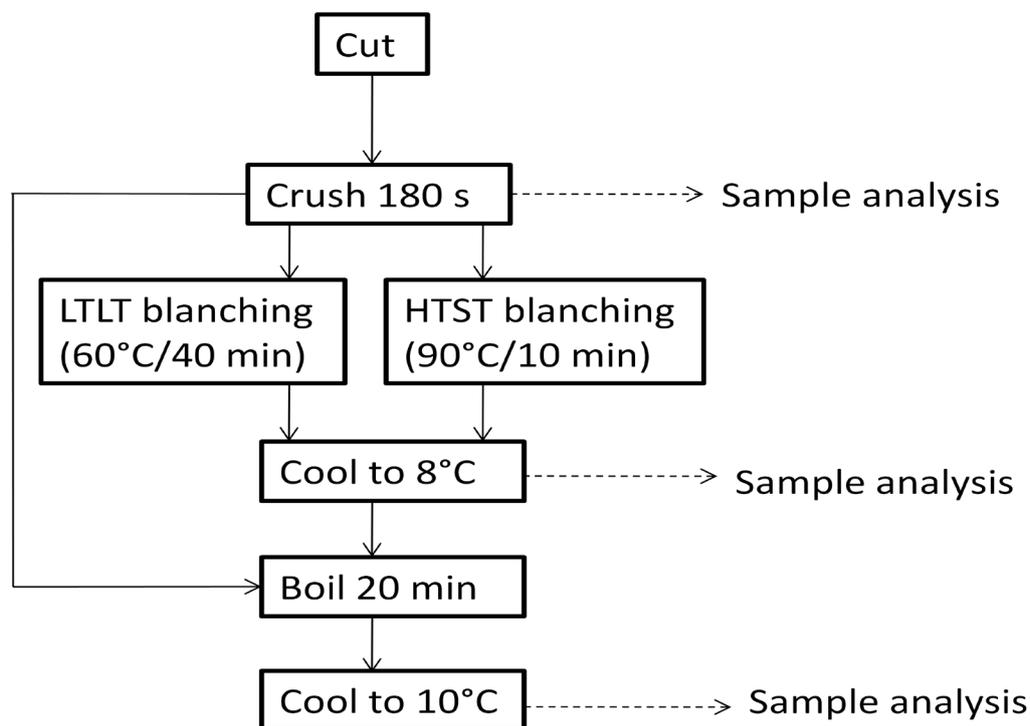

Figure 3. *Experimental design for study 2b, in total 4 different treatments were compared and samples collected throughout sample production to evaluate the impact of the separate processing steps. Sample preparation was repeated three times for each treatment, yielding three independent replicates.*

In study 3, high pressure homogenisation (HPH) was used to obtain a thorough disruption of the carrot and tomato matrices. The samples in studies 1 and 2 contained only tomato, but olive oil was added in study 3 to increase carotene bioaccessibility and to facilitate micelle formation during *in vitro* digestion. The *in vitro* bioaccessibility of carotenes was estimated by measuring the release from the food matrix, incorporation into mixed micelles and uptake by Caco-2 cells. The microstructure of the different samples was characterised by LM, scanning electron microscopy of frozen samples (cryo-SEM) and Raman spectroscopy. Sample treatments are summarised in Table 2. The micro- and macrostructural characterisations of the samples have been presented in detail by Lopez-Sanchez *et al.* (2010).

Sample notation	Processing conditions
Carrot emulsions	
C	Carrot H+ B
C10/1	Carrot H+ B + 10 MPa 1 cycle
C10/10	Carrot H+ B + 10 MPa 10 cycles
C100/1	Carrot H+ B + 100 MPa 1 cycle
Tomato emulsions	
T	Tomato H + B
T10/1	Tomato H + B + 10 MPa 1 cycle
T10/10	Tomato H + B + 10 MPa 10 cycles
T100/1	Tomato H + B + 100 MPa 1 cycle
Mixed emulsions	
M	C + T
M10/1	M + 10 MPa 1 cycle
M10/10	M + 10 MPa 10 cycles
M100/1	M + 100 MPa 1 cycle
MA10/1	C10/1 + T10/1
MA10/10	C10/10 + T10/10
MA100/1	C100/1 + T100/1

Table 2. *Experimental design for study 3, in total 15 different treatments were compared. All samples contained 5% olive oil. H=heated at 90±5°C for 40 min; B=blending at maximum speed for 3 min.*

The aim of study 4 was to evaluate the predictive value of the *in vitro* models used in studies 1-3 by comparing *in vitro* and *in vivo* data. The same soups that had been used in two human studies were also characterised *in vitro*, and the results were compared. In total three soups were compared, the soups were all made from 20% each of tomato, carrot and broccoli, but processed in different ways. The *in vitro* characterisations comprised carotene release from the food matrix, micellar incorporation and cellular uptake. The *in vivo* data was the based on the measured plasma response of β-carotene and lycopene after 4 weeks ingestion of 300 ml soup/day. The soups were evaluated in two separate human trials, the first was a smaller pilot study where 14 male subjects consumed the soup named "pilot" (Martínez-Tomás *et al.* 2011a). This was followed by a larger, parallel study where a total of 69 male and female subjects consumed either a "reference" or an "optimised" soup daily (Martínez-Tomás *et al.* 2011b).

Raw material

Most of the raw materials used in studies 1-3 were obtained during the summer season and purchased from local supermarkets. The exceptions were the tomatoes used in study 2a, which were bought in the winter, and the processing tomatoes in study 3, which were

imported directly from the producer. Due to the large amounts required in study 4, all raw materials were obtained directly from producers in Europe during the summer season.

For study 1, red, ripe, round salad tomatoes on the vine of the variety Bella Donna were bought from a local supermarket in Sweden on one occasion. The same variety was used for the TEM images, but the tomatoes were obtained a few weeks later.

For study 2, the experimental work in the two parts was done on two separate batches of red, round salad tomatoes. Tomatoes used in 2a were of an unknown variety from Gran Canarias, Spain. In 2b, the tomatoes were of the same variety as in study 1, Bella Donna.

For study 3, sample preparation was performed in The Netherlands. Carrots of the Nantes variety were purchased from a local supplier, and red processing tomatoes were obtained from Acorex, Spain.

In study 4, carrots from the UK (variety Nairobi), tomatoes from Spain (variety Heinz 9997) and broccoli from Germany (variety Ironman) were used for production of the reference and optimised soups. A similar raw material was used for production of the pilot soup, except that tomato paste was used instead of processing tomatoes due to seasonal unavailability.

Sample preparation

In study 1, 0.7 kg of fresh tomato were cut into wedges and crushed in a kitchen blender for 15, 30 or 120 s. The crushed samples were either analysed as they were, subjected to HTST blanching at 95°C for 8 min or boiled (100°C) for 20 min. An additional mechanical treatment was included for unheated tomatoes; they were first crushed for 15 s in the blender and then subjected to ultra-high speed homogenisation in an Ultra Thurrax for 105 s. The experimental design is shown in Figure 2. The whole sample preparation was repeated three times for each treatment, giving three independent replicates. To ensure the same time for enzymatic conversions, the time from the start of crushing to the heat treatment was standardized to 4 min.

For study 2a, tomatoes were cut into wedges and either blanched as wedges or crushed 120 s in a kitchen blender. The wedges or pulp were then subjected to either HTST (90°C/4 min) or LTLT (60°C/40 min) blanching. The samples were cooled to 8°C in an ice bath and remaining tomato wedges were crushed for 120 s. All samples were boiled (100°C) for 10 min. The LTLT samples were crushed with or without the addition of 0.5% $CaCl_2$. Two reference samples, one unheated and one boiled without previous blanching, were also made. Sample production is summarised in Table 1.

For study 2b, the HTST and LTLT blanching of crushed tomato followed by boiling were repeated, as were the reference treatments (Figure 3). As discussed below, some modifications were made in order to further standardise the samples and obtain a more homogenous material. The crushing time was increased to 180 s and, to ensure the same

time for enzymatic reactions, a holding time of 300 s from the start of crushing to the initiation of thermal treatment was introduced. To ensure a temperature of ≥85°C for ≥4 min, which would be more than sufficient to completely inactivate of PME and PG, the time for HTST blanching was increased to 10 min. The boiled reference samples in 2a did not deviate significantly from the unheated reference samples. Thus the boiling time was increased to that used in study 1, namely 20 min. To assess the effect of different processing steps, aliquots of the samples were collected prior to and after each thermal treatment.

In study 3, tomato and carrot samples were prepared using similar processing conditions. The raw material was cut into pieces of approximately 2 cm^3 and deionised water, containing sorbic acid as a preservative, was added. The carrot samples contained 50% (w/w) added water and the tomato samples 10% (w/w) added water. The final concentration of sorbic acid was around 0.1% (w/v). Samples were heated to 90±5°C and kept at this temperature for 40 min. After the thermal treatment, samples were immediately cooled in ice water, followed by crushing for 3 min in the presence of 5% (w/w) olive oil using a kitchen blender. Blended tomato samples were passed through a fine screen (1 mm) to remove seeds and large skin pieces that would otherwise block the homogeniser. To reduce the effect of biological variation in the raw material as encountered in studies 1 and 2, and to isolate the effect of HPH, one single large batch of carrot and tomato, respectively, was produced. Samples were homogenised at a pressure of 10 MPa 1 and 10 times (cycles through the homogenator), and at a pressure of 100 MPa 1 time. Samples were either pure tomato or carrot emulsions, or a 50/50 mix of the two emulsions. The mixing of the tomato and carrot emulsions was done either prior to or after HPH. All sample treatments are listed in Table 2.

Three different soups were used in the studies presented in paper 4. One was produced on a pilot plant scale and is called the "pilot" soup. The other two soups were produced later on a factory scale and are called the "reference" and "optimised" soups. All soups contained 20% each of carrot, tomato and broccoli. For the reference soup, a traditional all-in-one approach was used. The optimised and pilot soups were instead produced in a split stream process, where sample pre-treatments could be optimised for each of the raw materials. The optimised soup was subjected to HPH at 10 MPa, which had been shown in study 3 to increase the *in vitro* bioaccessibility of α- and β-carotene while not negatively affecting lycopene accessibility. In addition to carrot, broccoli and tomato, the soups contained lemon juice, some herbs and spices and olive oil, 2.5% in the reference soup and 5% in the other two soups. All soups were produced in three separate batches.

In vitro models for estimating carotene bioaccessibility

Two different *in vitro* models were used to investigate carotene bioaccessibility. A static *in vitro* digestion mimicking the conditions in the stomach and upper part of the duodenum was used to assess the carotene release from the food matrix and micellar incorporation. To study cellular uptake of carotenes, Caco-2 cells were incubated in the presence of micellarised carotenes. The mixed micelles used in the cell model were either

collected after a static *in vitro* digestion or synthesised directly from micellar components.

Model development for static in vitro digestion
There is currently no consensus or standardised method for estimating carotene bioaccessibility *in vitro* and numerous varieties of the model are implemented in different research groups. The static *in vitro* digestion procedure used in the present thesis is mainly based on those developed by Hedrén *et al.* (2002) and Garrett *et al.* (1999a) but with several modifications as discussed below.

Many *in vitro* models simulate a fasted state in the human gastrointestinal tract, but these conditions often differ from those during the fed state (Reboul *et al.* 2006). For example, a rapid rise in the gastric pH is often observed after ingestion of food, which is then followed by a slow decrease in pH over time (Tyssandier *et al.* 2003). To mimic these conditions in the *in vitro* model, the gastric incubation was initiated at pH 4 but then lowered to pH 2 for the second half of gastric incubation.

Static *in vitro* models are usually 2- or 3-phase models, where the 3-phase model includes an oral phase. The purpose of the oral phase is to simulate mastication and mixing of food with saliva, usually by addition of amylase (Garrett *et al.* 1999a; Bengtsson *et al.* 2009a). Amylase catalyses the reduction of starch into sugars. Tomato and carrot do not contain large amounts of starch, and the inclusion of an oral phase during *in vitro* digestion did not have a discernable impact on the final carotene bioaccessibility. It was therefore decided not to include an oral phase. However, for other samples with higher starch content, such as the sweet potato investigated by Bengtsson *et al.* (2009a), inclusion of an oral digestion step may be vital.

Several precautions were taken in order to reduce oxidation of carotenes during the *in vitro* digestion. Ascorbic acid was used as an antioxidant during the gastric phase and pyrogallol and α-tocopherol were added before the intestinal phase. Samples were also blanketed with nitrogen to reduce the amount of oxygen present. The retention of the final model with these safety measures was around 90% for α- and β-carotene and about 85% for lycopene. During initial model development, the presence of antioxidants was shown to improve carotene retention. However, for the *in vitro* digestions made prior to addition of the micellar fraction to Caco-2, the pyrogallol was excluded due to its potential cell toxic effects, and this was found to not affect the carotene retention. The addition of antioxidants may not correspond to the situation in the gastrointestinal tract but was considered an appropriate measure in the current application. The aim was to standardise the method and to isolate the effect of processing on carotene release, micellar incorporation and uptake as much as possible without losses during digestion that would obscure the results.

Two different methods are commonly used to isolate the micellar fraction, microfiltration and ultracentrifugation (Garrett *et al.* 1999a; Veda *et al.* 2006; Thakkar *et al.* 2007;

Bengtsson *et al.* 2010). For practical reasons, microfiltration was used to collect the micellar phase in the present work. The methods have been demonstrated to be interchangeable with regard to carotene content in the micellar fraction (Failla *et al.* 2008b).

The static *in vitro* digestion method allowed for analysis of 4 duplicate samples/day, which meant that sample storage prior to *in vitro* digestion was unavoidable in larger experiments. For studies 1 and 2, samples were frozen in liquid nitrogen directly after sample preparation and stored at -80°C until the day of analysis. This assured that all samples had received similar treatment prior to digestion, but comparisons between fresh and frozen samples indicated that freezing had a negative impact on the *in vitro* bioaccessibility of carotenes for both the tomato and carrot samples. The samples were therefore not frozen prior to *in vitro* digestion in studies 3 and 4. However, this might instead have induced structural changes during storage.

Final static in vitro digestion method
A static 2-phase *in vitro* model simulating the stomach and upper part of the small intestine was used. The samples to be digested were added to foil covered tubes together with solutions containing ascorbic acid and physiological amounts of sodium, potassium, calcium, phosphate and magnesium. To simulate the gastric phase, the pH was adjusted to 4 and a pepsin solution was added. The samples were blanketed with nitrogen and incubated for 30 min at 37°C on a rotary shaking plate. The pH was then lowered to 2 and samples were incubated for an additional 30 min. To simulate the gastric phase, the pH of the samples was increased to 6.9 and a gastric solution containing pancreatin, bile extract, pyrogallol and α-tocopherol was added. Samples were blanketed with nitrogen and incubated for 2 h. After intestinal incubation, the tubes were centrifuged and the resulting supernatant vacuum filtered into amber Erlenmeyer flasks. The carotene content of the vacuum filtered digesta was considered a measure of the release from the food matrix. To isolate the micellar fraction of the samples, the digesta was microfiltered (0.22 μm) to remove crystalline carotenes and carotenes bound to small structures.

Lycopene solubility
The solubilisation of carotenes in dietary fat prior to micellar incorporation has been suggested to be an important step for carotene absorption, and lycopene solubility in olive oil was therefore investigated (Rich *et al.* 2003). Lycopene is a planar molecule that easily forms thermodynamically stable crystals. To make sure that true solubility in the oil was measured, and to avoid the energy barrier of breaking the crystals, lycopene was solubilised in organic solution before determination of solubility in oil. Varying amounts of a stock solution of lycopene in hexane (containing 1 g L^{-1} BHT) were added to olive oil, giving final concentrations of 0.001-2.0 mg lycopene g^{-1} olive oil. The hexane was then evaporated under N_2, the oil centrifuged and microfiltrated (0.22 μm) to remove precipitated carotenes and the carotene content of diluted oil samples analysed by HPLC.

Model development for carotene uptake into Caco-2 cells

The Caco-2 cell model was mainly based on the publications of Garrett *et al.* and the standardized protocol developed by the initiative of HarvestPlus (Garrett *et al.* 1999a; Garrett *et al.* 1999b; Garrett *et al.* 2000).

The major concern during initial cell experiments was low levels of carotenes in the lysed cells, which made detection difficult. Several changes were therefore implemented in order to increase the carotene content in the cell lysate. Two of the most important changes were increasing the carotene concentration of the digesta by using a larger amount of sample for the static *in vitro* digestion and switching from 12-well plates to 6-well plates. For 12-well plates the number of cells/well was not always sufficient to achieve detectable carotene content. The switch to 6-well plates increased the total carotene content in the cell lysate simply by increasing the cell growth area, and thereby the total cell content. There are examples of even larger growth areas being used, but the trade-off for increasing cell growth areas is a decrease in replication (Thakkar *et al.* 2007).

Caco-2 cells are sensitive to the bile salts and digestive enzymes present in the digesta as a result of the static *in vitro* digestion, and the digesta was therefore diluted. However, this leads to a further reduction of the already low carotene concentration. An experiment in which the cells were exposed to digesta with a varying degree of dilution was therefore carried out to investigate the concentration at which cell vitality was severely reduced. Extensive cell death occurred when cells were incubated with undiluted digesta or digesta diluted 1:2, clearly demonstrating that dilutions of at least 1:3 are necessary. The 1:3 dilutions did not cause visible cell death, but a lower relative carotene uptake was obtained compared with the 1:4 dilutions. The lower uptake may have been an indication of a reduced fitness of the cells, and a 1:4 dilution was therefore used.

Cellular carotene uptake appears to be largely dependent on incubation time. For example, Garret *et al.* (1999b) noted an increase in cellular β-carotene concentration with time for incubation times up to 30 h. The choice of incubation time in the model is therefore an important consideration and influenced by many different parameters, including cell survival, cell vitality, carotene detectability, practical feasibility and the actual holding time of carotenes in the gastrointestinal tract. Considering these parameters, an incubation time of 4 or 6 h was suggested and the two times were compared. The intracellular carotene content was higher after 6 h of incubation, and there were no differences in cell survival between the two incubation times, which was in line with previously reported results (Garrett *et al.* 1999a). In the study by Garrett *et al.* no adverse effects were detected after up to 6 h of incubation with diluted digesta (1:4) when cytotoxicity was evaluated by several different methods.

Methodology for the final Caco-2 cell model

Stock cultures of Caco-2 cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS), amino acids and antibiotics. For the cellular

uptake experiments, cells were seeded at passage 32 in 6-well plates at a density of 65 000 cells cm^{-2}.

The uptake experiments were initiated by washing the cells with phosphate buffer solution (PBS), adding micellar carotene solutions to the cells and incubating the cells for 4 or 6 h. The carotene solutions were either diluted micellar isolates from static *in vitro* digestions or solutions of synthetic mixed micelles. Cell viability during incubation was visually assessed by LM. After incubation, the micelle solutions were removed and the cells washed once with PBS containing 1% (v/v) FBS and twice with PBS alone. Cells were thereafter removed from the plate by the addition of either 0.5 M NaOH or trypsin solution, collected and stored at -80°C until analysis. Protein content of the lysed cell samples, considered a measure of cell count, was analysed with the Pierce BCA Protein assay kit.

Synthetic micelle production
The Caco-2 cell model was initially used as an extension of the static *in vitro* digestion, but the many confounding factors, such as the concentration-dependent uptake of carotenes, obstructed interpretation of the results. In order to get a more controlled system with known composition and pre-determined carotene levels, a method was developed for producing carotene containing synthetic mixed micelles. The micelle production method was mainly based on Staggers *et al.* (1990) Canfield *et al.* (1990), Sugawara *et al.* (2001) and Chitchumroonchokchai *et al.* (2004). The toxicity of synthetic mixed micelles and their components was determined using an MTT assay, and no effect on cell metabolic activity was detected for the concentrations used in the present study.

In brief, synthetic micelle production was initiated by sonicating Dulbecco's modified Eagle medium (DMEM) containing oleic acid, monoolein, lyso-phosphatidylcholine, sodium taurocholate and carotenes for 60 min. Samples were then microfiltrated to remove crystalline carotene, and micellar carotene incorporation was confirmed spectrophotometrically. Canfield *et al.* (1990) reported a shift in the absorption maximum from 452 to 460 nm for β-carotene in micelles compared with organic solvents. A similar shift in the absorption maximum from 472 to 481 nm was observed for lycopene.

Carotene analysis
Determination of the total carotene content in the samples was a process in four steps. First, the sample matrix had to be disrupted enough to obtain a complete carotenes release during the next step, which was extraction of carotenes into an organic phase. A more polar solvent was then added to obtain phase separation, removing the water-soluble compounds in the sample. Finally, the carotenes were separated by HPLC and quantified by diode array detection. While carotenes appear to be relatively stable within the protective food matrix, they are highly susceptible to isomerisation and oxidation in solution (Britton 1995). To prevent loss as a result of these reactions, all carotene analyses were made in subdued light and butylated hydroxytoluene (BHT) was used as an antioxidant. It was also discussed whether sample preparation at ambient temperature could cause carotene degradation, but using chilled solutions and working in a cold room

(4°C) did not increase the carotene recovery, and these precautions were deemed redundant.

Determination of total carotene content

Carotene extraction from the raw material and processed samples was based on the method presented by Sadler *et al.* (1990) with some modifications. All volumes were halved to decrease the amount of organic waste, and the extraction times were doubled as a full carotene extraction was not obtained during the shorter times initially used. In brief, samples were mixed with an extraction solution of hexane:acetone:ethanol (50:25:25) containing 1 g BHT L^{-1}. The mix was stirred for 20 min and to facilitate phase separation water or NaCl solution was added. The solution was stirred for an additional 10 min, and the samples were then left to stand to allow phase separation before an aliquot of the upper organic phase was collected and analysed by HPLC as described below. In studies 1 and 2, distilled water was used to separate the phases. Substituting the water for a NaCl solution increased the polarity of the polar phase and gave a better phase separation. It was therefore used in the later studies, except when samples already contained NaCl.

Carotene extraction from in vitro digesta

Carotenes were extracted from *in vitro* samples according to one of two methods. When release from the food matrix was the only parameter of interest, the method used for the determination of total carotene content described above was used. For the samples where the digesta was needed for further studies, *e.g.* analysis of carotenes in the micellar fraction or uptake studies in Caco-2 cells, a scaled-down version of the method described above was developed. In this version, an aliquot the digesta was vortexed together with a small amount of the extraction solution in a test tube. NaCl solution was then added and the tubes vortexed a second time, after which the phases separated by centrifugation. The upper organic phase was collected for analysis by HPLC as described below.

Carotene extraction from cell samples

Cells that had been removed by trypsin were sonicated prior to carotene extraction in order to break cell structures, whereas cells removed by NaOH were already lysed and thus not sonicated. Carotenes were extracted by vortexing the cell lysates with hexane containing 1 g L^{-1} BHT for 15 s. Methanol was added to the test tubes and the tubes were vortexed a second time. For cells removed by trypsin, NaCl solution was then added to the extract and the tube vortexed a third time before centrifugation. The phases were separated by centrifugation and the upper hexane phase collected. The extraction procedure was repeated 1-2 times. The collected organic phase was evaporated under a stream of nitrogen, and carotenes were redissolved in a small volume of the mobile phase and analysed by HPLC.

Carotene extraction from plasma and chylomicrons

The samples were denatured by addition of ethanol, followed by vortexing and addition of hexane. Both the ethanol and hexane contained 1 g BHT L^{-1}. After repeated vortexing and sonication, the samples were centrifuged and the upper hexane phase collected. The extraction procedure was repeated once. The collected hexane phase was evaporated

under a stream of nitrogen, and residues were redissolved in mobile phase and analysed by HPLC according to the method described below.

HPLC analysis of carotenes

Carotenes were separated using reversed-phase elution on a C_{30} column (YMC, 250×4.6 mm, particle size 5 μm) and identified and detected using a photodiode array detector. A gradient elution method with methanol and increasing concentrations of methyl *tert*-butyl ether was used. The gradient allowed for the separation of all-*trans* β-carotene and its isomers, as well as all-*trans* lycopene and most of its geometrical isomers (Figure 4). All-*trans* lycopene and 5-*cis* lycopene displayed some overlap, but the interference of the *cis*-isomer with quantification of all-*trans* lycopene was considered insignificant.
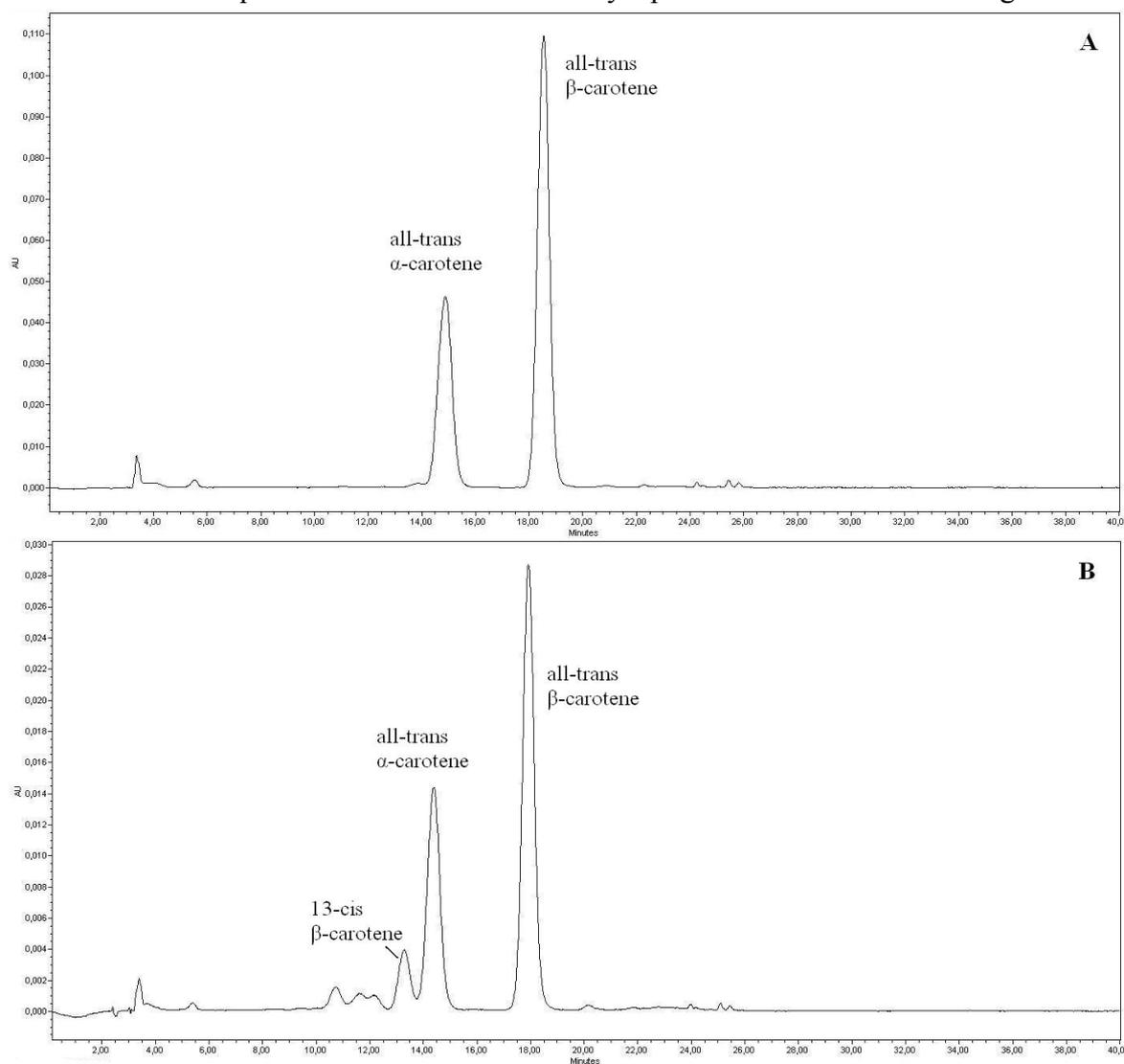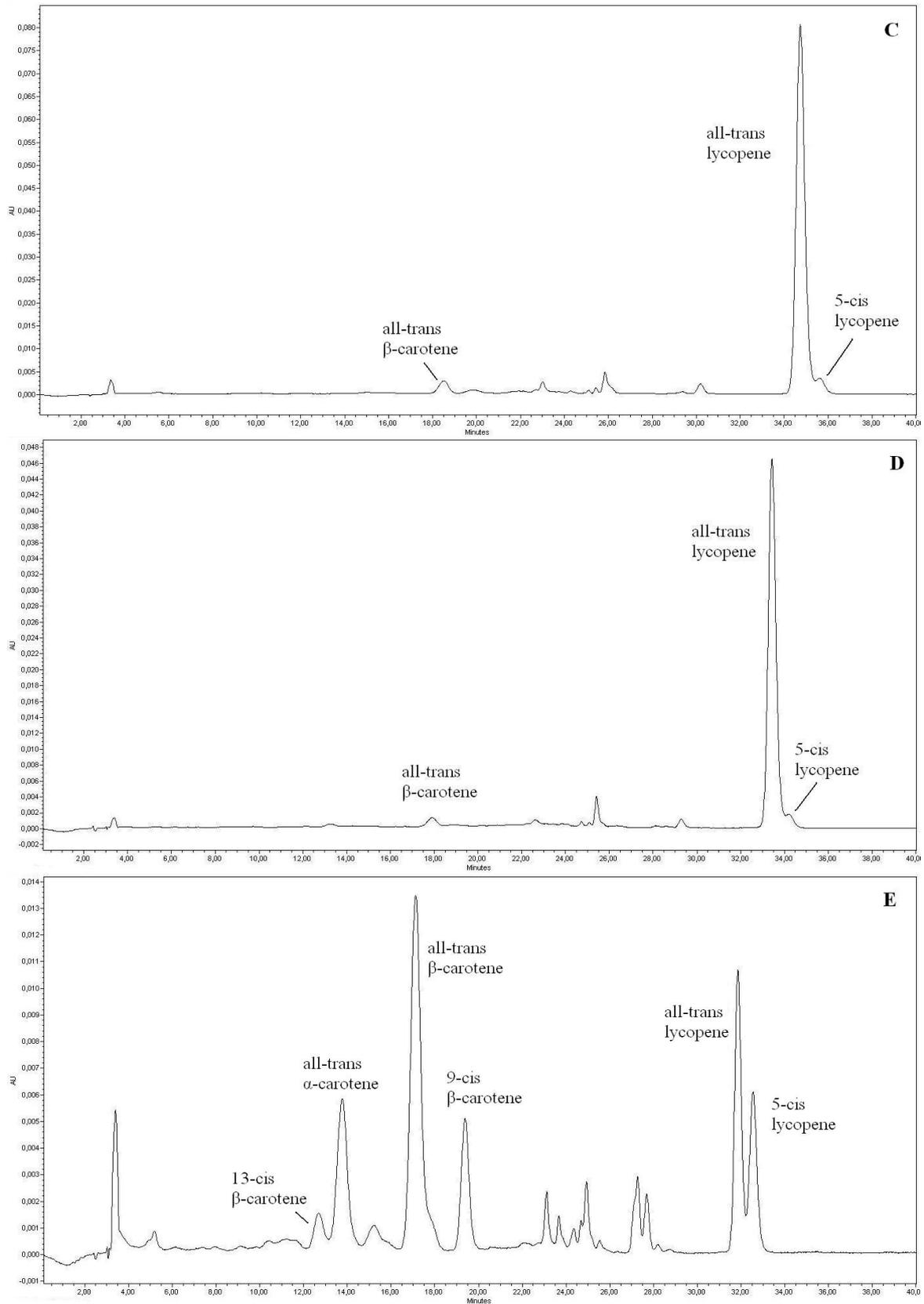
Figure 4. *HPLC profile of raw carrot (A), carrot pieces heated 90°C/40 min (B), raw tomato (C), tomato pieces heated 90°C/40 min (D) and the reference soup for the human study (E) as separated by the developed gradient.*

Ascorbic acid analysis

The extraction procedure and methodological considerations were largely based on the review by Davey *et al.* (2000). Ascorbic acid is highly susceptible to oxidation, which must be taken into account during sample handling, extraction and analysis if an underestimation of the true content as a result of ascorbic acid degradation is to be avoided. All analyses and sample handling were therefore done under subdued light, and containers were covered with aluminium foil. To minimise oxidative losses, the water activity (a_w) was also reduced by performing the analyses on lyophilised material. Lyophilisation was even more vital when initial ascorbic acid levels were analysed in the raw material, since ascorbic acid oxidase was not inactivated by thermal treatment. These samples were frozen as intact as possible and were not ground until after lyophilisation. To facilitate the extraction, samples were milled into very small particles in a coffee grinder. On the basis of information given in the publication of Lykkesfeldt (2000), TCEP (tris[2-carboxyethyl]phosphine hydrochloride) was used to reduce oxidised ascorbic acid, allowing for the quantification of both DHA and L-AA.

Ascorbic acid was extracted from lyophilised material by dissolving the samples in a meta-phosphoric acid solution and sonicating them for 2 x 5 min. The tubes were then centrifuged and the resulting supernatant diluted in buffer with and without TCEP and analysed by HPLC. Samples were separated using reversed phase HPLC with isocratic elution with phosphate buffer on a Thermo Aquasil C_{18} column (150 × 4.6 mm, particle size 3 μm). Ascorbic acid was quantified by electrochemical detection in a glassy carbon flow cell against freshly made reference solutions containing known concentrations of L-ascorbic acid.

Micro- and macrostructural characterisation

Structural characterisation was done with several different methods, as described below. Owing to its relative ease of use, light microscopy was the most commonly employed method, but other types of microscopy were necessary for more detailed studies and higher magnification. Different rheological methods were used for macrostructural characterisation but, due to a weaker link between macrostructure and *in vitro* bioaccessibility, only two are presented in the summary.

Light microscopy was performed on samples in studies 1-3. The samples were diluted to enable a clearer visualisation of the cells and cell clusters. To better visualise the cells, tomato samples in studies 1 and 2 were stained with toludine blue. Samples for studies 1 and 2 were examined using a Microphot FXA light microscope and, in study 3, with a Zeiss Axioplan microscope.

For cryo-scanning electron microscopy (cryo-SEM), samples were frozen in liquid ethane immediately after processing and kept frozen during subsequent sample handling and analysis. Thin sections of the sample were obtained by cryo-planing with a diamond knife in a cryoultramicrotome. For SEM analysis, the sample was briefly sublimated (freeze-etched) at −90°C in the cryopreparation chamber of the microscope and covered by a layer of Pt that was a few nanometres thin. The samples were then analysed at

−125°C, at 3 kV and a working distance of about 10 mm, using the in-lens secondary electron detector.

For transmission electron microscopy (TEM), the samples were fixed in 2% glutaraldehyde, dehydrated in increasing concentrations (30-99.5%) of ethanol, embedded in plastic and cut in ultra-thin slices (60-70 nm). The slices were stained with silver proteinate to visualize the polysaccharides. Staining was done by inversion on droplets of the following solutions: 1% periodic acid for 20 min, 1% thiocarbohydrazide in 10% acetic acid for 1 h and 1% silver proteinate for 30 min in darkness. Stained samples were examined using a LEO 906e transmission electron microscope at 100 kV.

The carotene/oil ratio of oil droplets in the emulsions from study 3 was determined with a Raman spectrometer (785 nm laser excitation) optically coupled to an Olympus BX60 microscope. Spectra showing solid carotenes present in the oil droplets were omitted from data analysis.

The Bostwick consistometer was developed by E.P. Bostwick especially for tomato products and is frequently used in industry (Barrett *et al.* 1998). The Bostwick consistency index is experimentally related to flow behaviour at low shear rates, and the results depend on the viscosity, density and yield stress of the sample (Barringer *et al.* 1998). The Bostwick consistometer is a sloped stainless steel tray with a 0.5 cm grading and a gate-operated sample reservoir at the upper end of the slope (Figure 5). To make a measurement, the sample is poured into the reservoir and the gate is opened. The Bostwick index of consistency is determined as the distance the sample has flowed down the slope under its own weight after a set time. The Bostwick consistency results were compared with viscosity measurements in a vane but, apart from showing the well-known fact that tomato is shear thinning; the rheological data did not provide additional information. The more straightforward Bostwick method was therefore used for structural analysis in studies 1 and 2.

Figure 5. *The Bostwick consistometer used in studies 1 and 2. The gate to the left has been opened and tomato pulp is running down the graded slope. The Bostwick consistency index is determined as the distance the pulp has run down the slope after a set time.*

The texture of carrot pieces was measured in several pilot studies by using a compression test with an aluminium probe. The hardness of the carrot pieces was defined as the maximum stress needed to compress the samples to 70% of their original height.

The methods for pectin analysis and activity of pectin degrading enzymes are described in brief below; details of methods and references are given in paper 1. PME activity was determined titrimetrically by measuring the acid release when extracted PME from the samples was added to a pectin solution. The activity of PG extracted from the samples

was measured by spectrophotometrical monitoring of the formation of reducing sugars. The alcohol-insoluble residue (AIR) that contained the pectin fraction was isolated by filtration of samples dissolved in 95% ethanol. Pectin DM was estimated by comparing the molar content of methoxyl and galacturonic acid in the AIR.

Statistical methods

All data were statistically evaluated with two versions of the SPSS software, SPSS 16 or PASW 18. Values are presented as mean ± standard deviation (SD) of 3 sample replicates unless otherwise stated. A normal distribution was assumed for the data, and treatments were compared using one-way analysis of variance (ANOVA) followed by Duncan's post hoc test when means were significantly different. Differences were considered significant at $p < 0.05$. Linear regression analysis was done to determine linear relationships between macrostructural properties and carotene *in vitro* bioaccessibility.

RESULTS AND DISCUSSION

Several human studies have demonstrated that processing of fruits and vegetables can have a large effect on carotene bioavailability, but information about the impact of specific processing steps is still lacking. The topics investigated in this thesis include how properties of the raw material, choice of thermal treatment and the processing order affect the *in vitro* bioaccessibility of carotenes. Other questions of interest were whether thermal and mechanical treatments were interchangeable and whether the carotene bioaccessibility of samples already subjected to extensive thermal and mechanical processing can be further improved by additional processing, either in the form of a second heating step or by mechanical disruption with HPH.

The present work evaluated the changes in nutrient content, carotene bioaccessibility and structural properties as a result of thermal and mechanical processing of tomato and carrot. The carotene content of the raw material showed a large biological variation, and there was minor degradation of carotenes during processing. The microstructure of all samples was characterised to try to explain the impact of processing on carotene *in vitro* bioaccessibility. Tomato and carrot were used as model materials, and their response to processing differed in several ways. The variety and degree of ripeness had a large impact on the physical properties of tomato, such as tissue rigidity, and this had a great influence on the *in vitro* bioaccessibility of carotenes. For both tomato and carrot, relatively extensive crushing in combination with thermal treatment significantly improved *in vitro* release of carotenes. After this type of processing, the tomato samples contained mostly single, intact cells whereas carrot samples mainly contained larger cell clusters. Further disruption of the cell structure of a pre-processed material by high pressure homogenisation enhanced the *in vitro* bioaccessibility of α- and β-carotene from carrot, but not of lycopene from tomato. Prolonged heating or higher temperatures often reduced the *in vitro* bioaccessibility of carotenes, showing that increased processing not always results in higher carotene bioaccessibility. Lycopene had a significantly lower micellar incorporation and solubility in dietary lipid compared with other carotenes, such as β-carotene. Cellular uptake of carotenes was linearly dependent on the amount of carotenes added to the cells, and was lower for lycopene as compared with β-carotene. The *in vitro* and *in vivo* results correlated well for β-carotene but not as well for lycopene. This may be because the amount of dietary fat was a major limiting factor for the *in vitro* bioaccessibility of lycopene, whereas other factors, such as carotene release from the food matrix, may have a larger impact on lycopene bioavailability *in vivo*.

Nutrient content and retention

The final nutrient content of a vegetable based product varies extensively depending on the raw material and processing conditions. The nutrient content of the raw material is influenced by a number of factors, including variety, growing conditions, maturity at time of harvest and postharvest storage (Thompson *et al.* 2000; Maiani *et al.* 2009; Tsukakoshi *et al.* 2009). Nutrient retention after processing are largely influenced by the type of processing, holding times and temperature conditions (Davey *et al.* 2000; Maiani *et al.* 2009).

Carotene content

The lycopene content of the tomatoes used as raw material varied greatly depending on variety and season (studies 1-4). Tomatoes of processing varieties had the highest lycopene content (*e.g.* 86 mg kg^{-1} in study 3) and the tomatoes harvested during the winter season had the lowest lycopene content (11 mg kg^{-1}, study 2a). The salad tomatoes used in study 1 and study 2b both had average lycopene contents slightly above 40 mg kg^{-1}. Literature values on lycopene levels in tomato range from 19 to 85 mg kg^{-1} for salad tomatoes and from 51 to 132 mg kg^{-1} for processing tomatoes (Hart *et al.* 1995; Abushita *et al.* 2000; Thompson *et al.* 2000; Anese *et al.* 2002; Akbudak 2010).

The β-carotene levels in carrots also varied extensively, both n the average content of the carrot samples in the different studies and between different carrot roots of the same variety and origin. The individual variation between carrot roots was investigated by measuring the β-carotene content of three carrot roots obtained from the same producer and batch on the same day. The carrot roots contained 73, 89 and 97 mg β-carotene kg^{-1} respectively. The carrot variety used in the soups for the human trial (paper 4) and in the emulsions subjected to HPH (paper 3) was selected for its high β-carotene content, and it contained about 90 mg β-carotene kg^{-1}. The carrots used in other studies were obtained from local producers or supermarkets and contained between 35 and 56 mg kg^{-1}. The β-carotene levels obtained in the present studies were similar to those reported in the literature (O'Neill *et al.* 2001; Lemmens *et al.* 2009; Maiani *et al.* 2009; Tsukakoshi *et al.* 2009; Imsic *et al.* 2010).

Carotene retention

Carotenes are sensitive to heat, light, oxygen and extremes in pH and are easily lost due to oxidation or isomerisation when dissolved in lipid or an organic solution, but appear to be more stable in a food matrix (Britton 1995; Nguyen *et al.* 1998).

Lycopene was relatively stable during thermal processing; the retention was ≥80% for all treatments investigated. When losses did occur, it was during thermal treatments in the higher temperature range of 90-100°C. According to Nugyen *et al.* (2001), lycopene is likely to remain in a crystalline state during thermal treatment, which offers a high protection against thermal degradation. However, several studies have reported higher lycopene content in pre-processed compared with raw tomato samples, which indicates an increased extractability of lycopene in the chemical analysis. It is therefore possible that losses of lycopene during thermal processing were more extensive, but were masked by an increased extractability (Thompson *et al.* 2000; Sánchez-Moreno *et al.* 2006).

The heat treatments of tomato that had no or little impact on lycopene caused significant losses of β-carotene. For example, after LTLT blanching followed by 20 min boiling, the retention was 95% for lycopene and 60% for β-carotene (study 2). Heating pieces of tomato at 90°C for 40 min caused no detectable loss of lycopene, but β-carotene retention was only 65% (study 3). The large losses of β-carotene from tomato were most likely a result of differences in physical structure. Unlike the more stable crystal form in carrot,

β-carotene in tomato is dissolved in intracellular lipids, making it more susceptible to isomerisation and degradation (Nguyen *et al.* 2001).

The α- and β-carotene in carrot were also relatively stable during thermal processing. Pieces of carrot heated at 90±5°C for 40 min retained over 90% of their all-*trans* β-carotene and 97% of the all-*trans* α-carotene (study 3). Heating the soups used in the human trial according to the instructions given to the participants, which was to bring the soup to a slow boil in a pot, did not cause detectable losses of lycopene or β-carotene. Similar to lycopene in tomato, β-carotene, and probably also α-carotene, is present in a crystalline form in carrot chromoplasts (Ljubesic *et al.* 1991). This crystalline form may have contributed to the high stability during thermal processing.

In general, carotenes appeared to be more stable during heat treatment of larger pieces as compared with crushed samples. For example, crushed tomatoes heated at 90°C for 10 min retained 83% of the original lycopene content (study 2), but no detectable losses of lycopene occurred when tomato pieces were heated at 90±5°C for 40 min (study 3). Heating of the more intact pieces could have protected the carotenes in several ways, such as retaining the structural integrity of the chromoplasts and preventing solubilisation of carotenes into cellular lipid structures.

Loss of all-*trans* carotenes during processing can be caused either by isomerisation or oxidative degradation (Delgado-Vargas *et al.* 2000). Several *cis*-isomers of β-carotene were detected in samples subjected to thermal processing; indicating that loss of all-*trans* β-carotene was at least partly due to isomerisation (Figure 4 A, B and E). Lycopene isomerisation was generally not detected, except for the soups produced for the human study, where a high content of 5-*cis* lycopene was detected (Figure 4 C-E). The soups in the human trial were the only samples subjected to thermal treatments in the presence of dietary fat, which could explain the comparatively large amounts of *cis*-isomers in these samples. Presence of dietary lipid during thermal treatment of carotene containing vegetables has been shown to increase the formation of carotene *cis*-isomers, probably by solubilising the carotenes (Marx *et al.* 2003; Bengtsson *et al.* 2010).

The mechanical treatments used in the different studies did not appear to cause carotene degradation. Neither the different crushing times used in study 1 nor the high pressure homogenisation (HPH) in study 3 had a detectable impact on the lycopene content in the samples. This is in agreement with previous studies where HPH did not have an impact on the lycopene content in tomato (Pérez-Conesa *et al.* 2009; Colle *et al.* 2010). Mechanical treatments provide some energy input in the form of shearing, but only minor heat develops and the shearing forces mainly act on larger structures such as cell walls. The HPH used in study 3 was a relatively fast process, just a few minutes in total. It is possible that the longer homogenisation times used because of the much larger sample size in study 4 could have led to greater heat production and caused *cis-trans* isomerisation or oxidative degradation.

Both β-carotene and lycopene were fairly stable during storage. No losses were detected after up to 16 weeks storage at 8°C or -80°C (paper 3). When the soups in study 4 were stored at ambient temperature, lycopene initially appeared to be more stable than β-carotene but was reduced to a higher degree after prolonged storage (paper 4). After 3 months of storage, the lycopene retention was 100%, whereas the β-carotene retention was 67-84% in the 3 batches of optimised soup, and 89-98% for the reference soup. After 6 months additional storage, β-carotene retention was 86±1% for the reference soup, and 74±2% in the optimised soup. The lycopene retention was similar for both soups, between 69 and 77% in the different batches.

Ascorbic acid retention
Since ascorbic acid is highly water soluble and easily lost by leakage, as well as by chemical and enzymatic oxidation, it has been used as an indicator of processing intensity (Davey *et al.* 2000). The ascorbic acid levels of the samples in studies 3 and 4 were measured to get a rough estimate of the effect of processing on the general nutritional value. Similar losses of ascorbic acid in mg kg^{-1} were obtained for carrot and tomato during the initial thermal and mechanical processing in study 3. However, since the initial ascorbic acid content of carrot and tomato differed considerably, large differences were found for the relative retention of ascorbic acid. About 95% of the L-AA was lost during processing of carrot samples, compared with ~30% in the tomato samples. The decrease in ascorbic acid content after sample preparation was most likely caused by a combination of enzymatic activity and thermal degradation. Since the samples were crushed in the water used for heating, no losses caused by leakage were expected. Once the emulsions had been produced, HPH did not appear to cause extensive additional loss of ascorbic acid. This is in agreement with the results of Pérez-Conesa *et al.* (2009), who showed a high retention of ascorbic acid in tomato purée after HPH. The ascorbic acid content of the soups in study 4 varied greatly between the different batches. The three batches of reference soup contained between <1 and 9.4 mg ascorbic acid kg^{-1}, and the ascorbic acid content of the optimised soups was even lower, between <1 and 1.3 mg kg^{-1}. The ascorbic acid content in the pilot soup was generally higher, between 25 and 39 mg kg^{-1}, except in one batch where the ascorbic acid level was below the detection limit of 1 mg kg^{-1}. The low ascorbic acid levels and large variation between batches were caused by several technical difficulties during the up-scaled industrial-like production, and illustrate the importance of standardising and controlling processing parameters.

Processing, structure and *in vitro* release of carotenes
The increased carotene bioavailability observed after processing of plant material is often suggested to be caused by changes in structural properties after processing, for example disruption of the food matrix. However, only a limited number of studies offer information about both bioavailability or bioaccessibility and structural properties of the same samples (Parada *et al.* 2007; Tydeman *et al.* 2010a; Tydeman *et al.* 2010b). In the present thesis, all samples that were subjected to an *in vitro* digestion and estimation of carotene bioaccessibility were also characterised structurally. The emphasis has been on the relation between carotene bioaccessibility and sample microstructure, but some

attempts have also been made to correlate the *in vitro* bioaccessibility with the macrostructure of the samples. The macrostructural properties of a sample are affected by its microstructure, but also by other factors that could have an impact on carotene bioaccessibility, such as the presence of dietary fibre (Hayes *et al.* 1998; Rao 2006). If rheological properties could be correlated with carotene accessibility, such information would be a valuable tool for estimating the carotene accessibility of different food products.

Influence of raw material

The *in vitro* release of lycopene varied greatly depending on the type of tomatoes that were used as raw material. The tomatoes in studies 1 and 2b were of the same variety, origin and season, although obtained at two different occasions, and samples produced from these tomatoes had a similar *in vitro* release of lycopene. For the unheated samples, the relative *in vitro* bioaccessibility of lycopene was between 10 and 17% of total lycopene content in study 1 and between 12 and 17% of the lycopene content in study 2b. The tomatoes used in study 2a, which were of a different variety and harvested during the winter season, had a significantly lower *in vitro* release of lycopene, only 5±1%. The apparent impact of the raw material on lycopene bioaccessibility remained after thermal treatments. For example, the relative *in vitro* bioaccessibility of lycopene after 20 min of boiling was in the range of 27 to 38% in study 1 and 12 to 33% in study 2b. Boiling the less ripe tomatoes used in study 2a for 10 min had no effect on the *in vitro* bioaccessibility; it remained around 5%. Similarly, the samples in study 2 subjected to HTST blanching followed by boiling had a relative *in vitro* bioaccessibility of 3.7±1.6% and 29.3±7.3% in parts 2a and 2b respectively. While the thermal and mechanical treatments were not identical in the different studies, the results are still an indication that the raw material has a large impact on the bioaccessibility of the final product.

The tomatoes used in study 2a had both significantly lower relative *in vitro* bioaccessibility and total content of lycopene, where the latter indicates a lower degree of ripeness (Thompson *et al.* 2000). As the ripening effects of tomato include a softening of the cell walls, it is proposed that the lycopene *in vitro* release in study 2a was impaired by more rigid cell walls (Barrett *et al.* 1998). This would explain why the only thermal treatment found to increase the *in vitro* bioaccessibility of lycopene in this study was LTLT blanching of crushed tomatoes followed by boiling. This thermal treatment causes extensive enzymatic degradation of pectins, potentially reducing the structural integrity of the cell walls (Verlent *et al.* 2006).

Mechanical pre-treatments of tomato

Four different crushing intensities were compared, both alone and in combination with thermal treatments, to assess the individual contribution of thermal and mechanical processing to changes in bioaccessibility (study 1). The different types of crushing gave tomato samples ranging from roughly crushed tomatoes with a wide range of particle distributions to a smooth and uniform tomato paste. In spite of the visible differences in macrostructure, the unheated tomato samples all had a similar lycopene bioaccessibility (Figure 6). Investigating sample microstructure by LM revealed that there was a difference in the size distribution of the cell clusters but as shown in Figure 7, all samples mainly contained relatively undamaged cells.
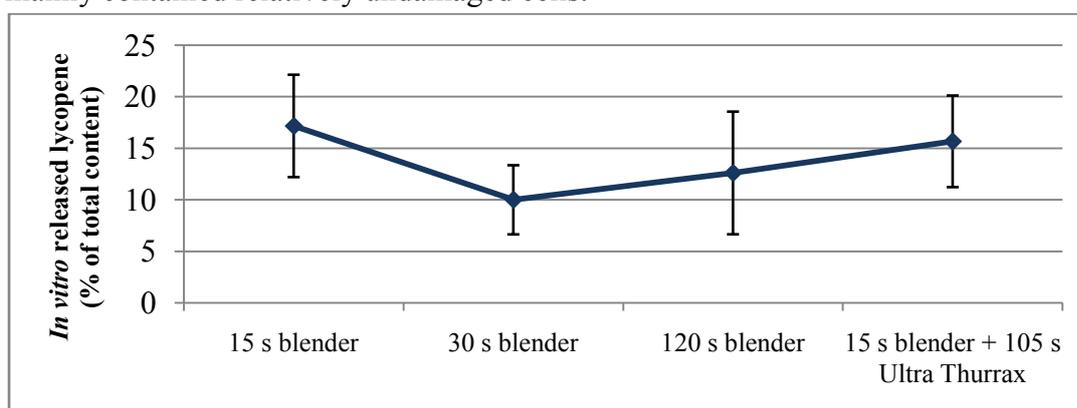

Figure 6. *The relative in vitro release of lycopene from the unheated tomato samples in study 1 given as mean±SD of three independent treatment replicates. The lycopene in vitro release was not significantly different for any of the crushing conditions investigated. LM images of these samples are shown in Figure 7 below.*
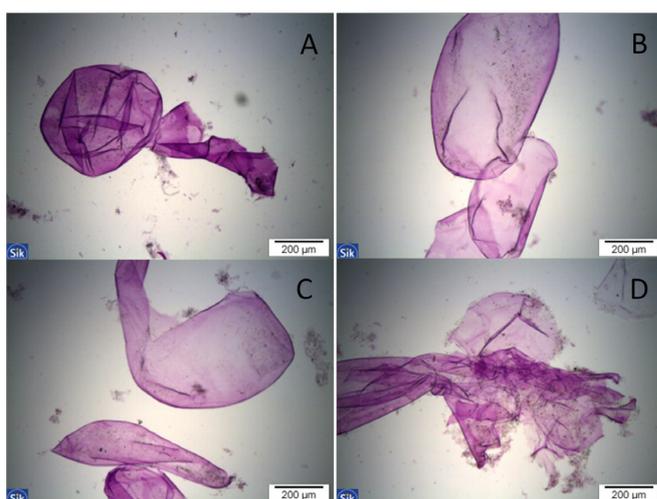

Figure 7. *Light micrographs of unheated crushed tomato in study 1, bars = 200 μm. Crushing times were 15 s (A), 30 s (B), 120 s (C) and 15 s followed by 105 s ultra high speed homogenisation (D). A majority of the tomato cells remained intact after all crushing treatments. The in vitro release of lycopene from these samples is shown in Figure 6.*
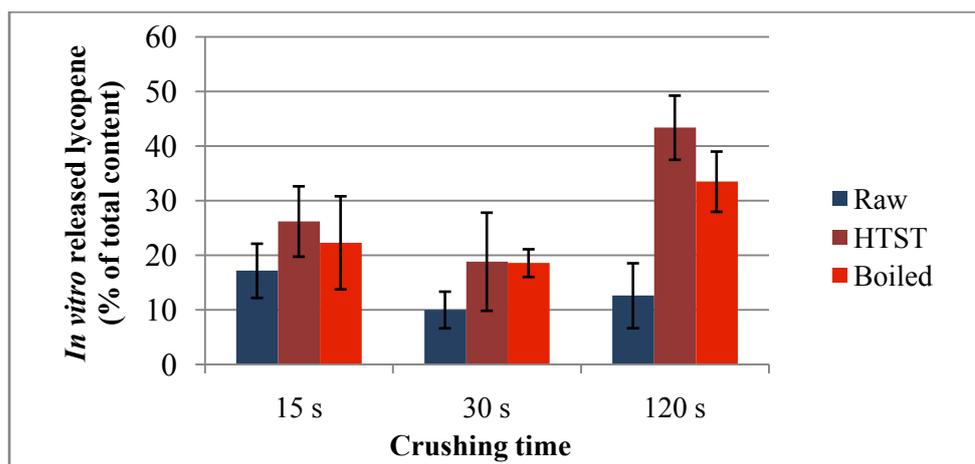
36

Figure 8. *The relative in vitro release of lycopene from thermally and mechanically pre-treated tomato in study 1. Values are given as mean±SD of three independent treatment replicates. The in vitro release of lycopene was significantly (p < 0.05) improved by HTST blanching (95°C/8 min) and by boiling (100°C/20 min) of samples crushed for 120 s in a household blender, but not by thermal treatment of samples crushed for the shorter times.*

Applying thermal treatment to the crushed samples increased the *in vitro* release of lycopene from samples crushed for 120 s, but not for the shorter crushing times of 15 and 30 s (Figure 8). These results are similar to those reported in the human study by van het Hof *et al*. (2000a), where tomato samples subjected to both mechanical and thermal pre-treatments gave a significantly higher plasma response as compared with only thermally pre-treated samples. An apparent decrease in cell wall density after thermal treatment could be observed by TEM; this was probably due to leakage of pectins and cell wall fibre into the surrounding liquid phase (Figure 9). Similarly, a reduction in cell wall integrity was observed in cryo-SEM images of carrot pieces after heating at 90°C for 40 min (Figure 10). Gärtner *et al*. (1997) suggested that heat treatment might yield the same structural changes as mechanical disruption, but the results in the present study did not support this suggestion. Even after extensive crushing, such as ultra-high-speed homogenisation, most tomato cells remained intact and retained lycopene within the cells. After thermal treatment, that reduced cell wall integrity, however, the *in vitro* release of lycopene from the extensively crushed tomato was significantly enhanced. In short, a combination of both crushing and thermal treatment was necessary to significantly improve the *in vitro* release of lycopene from the tomato matrix.
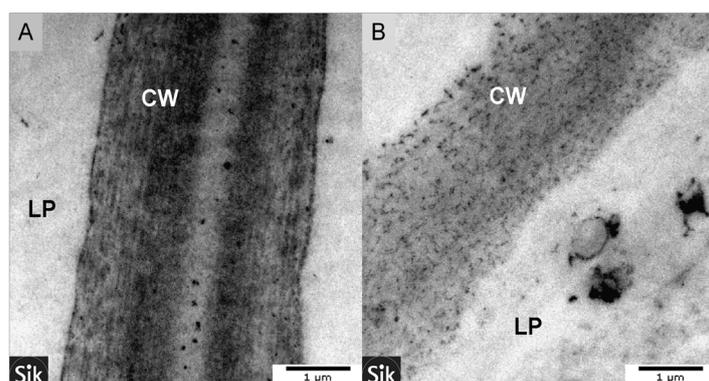

Figure 9. *TEM micrographs of the cell wall of unheated (A) and boiled (B) tomato pulp, stained for carbohydrates. Bars = 1 μm. A lower density of the cell wall (CW) was observed after boiling, probably due to leakage of structural components of the cell wall, such as pectin, into the surrounding liquid phase (LP).*
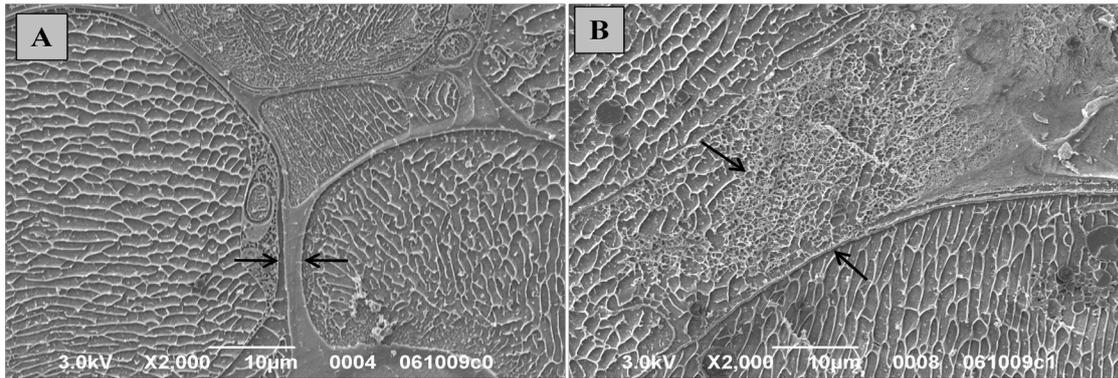
37

Figure 10. *Cryo-SEM images of carrot pieces before (A) and after (B) thermal treatment at 90°C for 40 min. Bars = 10 μm. A thicker and more porous cell wall was observed in the thermally treated carrot (arrows).*

Thermal treatments of tomato

Common thermal treatments include low or high temperature blanching and boiling. HTST blanching (90°C/10 min), LTLT blanching (60°C/40 min) and boiling (100°C/20 min) were compared in study 2b. The *in vitro* release of lycopene from the tomato matrix was significantly improved from 5.1±0.2 to 9.2±1.8 mg kg^{-1} after LTLT and to 9.7±0.6 after HTST blanching (Figure 11). Boiling only slightly increased the lycopene release to 6.3±1.9 mg kg^{-1} (n.s.). It is possible that cell wall degradation contributed to the increased lycopene release from the food matrix for both HTST and LTLT blanching, but as a result of two different mechanisms of action. In LTLT blanched samples, pectins were probably enzymatically degraded. A thermally induced decrease in the structural integrity of the cell wall may instead have occurred in samples subjected to HTST blanching, similar to what was observed in study 1.
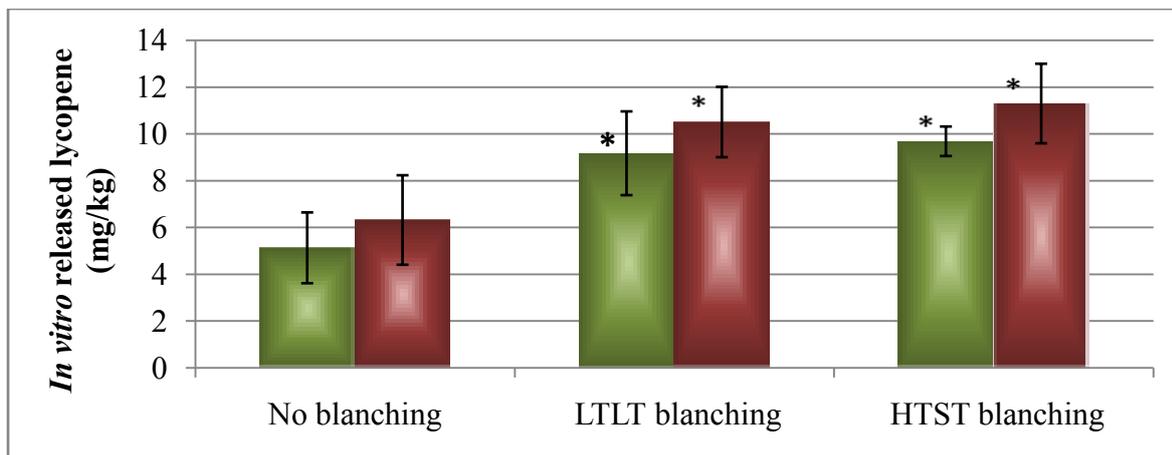

Figure 11. *The relative in vitro release of lycopene from crushed tomato subjected to HTST blanching (90°C/10 min) or LTLT blanching (60°C/40 min) alone (green bars) and followed by boiling at 100°C for 20 min (red bars) as measured in study 2b. Values are given as mean±SD of three independent treatment replicates. As denoted by asterisks, all blanched samples, both before and after boiling, had a significantly (p<0.05) higher in vitro release of lycopene compared to the unheated sample, but the boiling treatment alone did not significantly improve the lycopene release.*

Disrupting the tomato matrix by crushing will immediately release PME and PG, which through their combined action will cause pectin degradation and a loss of consistency (Hayes *et al.* 1998). No detectable PME or PG activity remained after HTST or boiling (study 1), but the enzymes are usually highly active during LTLT blanching as reviewed by Hayes *et al.* (1998). If the common processing order of first crushing the tomato followed by blanching is reversed, so that tomato pieces instead of tomato pulp are subjected to LTLT, most of the pectin degrading enzymes will still be retained within the cell walls. This would lead to reduced pectin degradation in the liquid phase and a higher consistency of the final sample, as shown in study 2a where tomatoes were crushed either before or after LTLT blanching. The samples that were crushed after blanching, and then immediately boiled to inactivate PME and PG, retained a much higher consistency compared to the tomatoes that were crushed before the LTLT blanching.
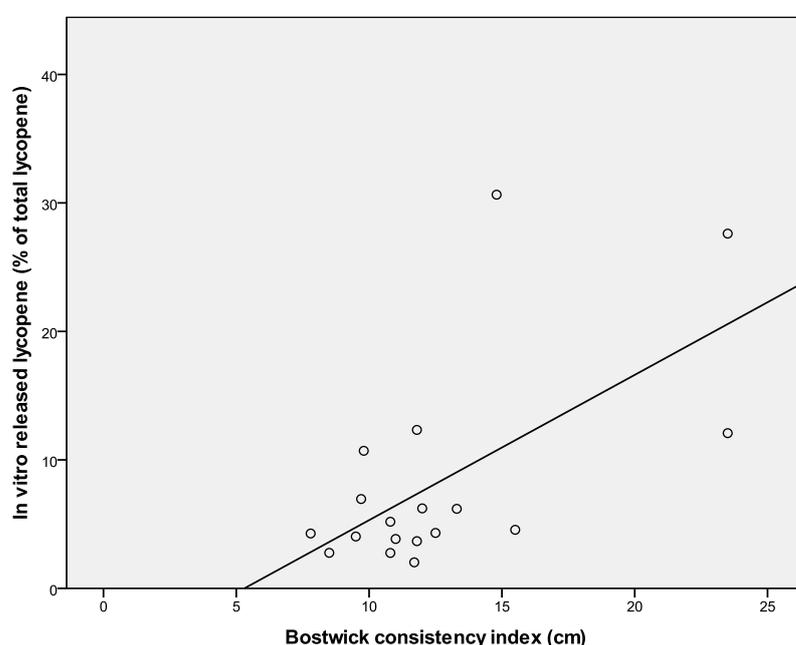

Figure 12. *Correlation between the in vitro release of lycopene and the Bostwick consistency index (study 2a). A weak trend of increasing in vitro release with decreasing consistency, as indicated by the sample travelling a longer distance in cm down the slope of the Bostwick consistometer, was observed (R^2=0.37). However, the variation in both consistency index and in vitro bioaccessibility was relatively low, and comparison of samples with a larger spread is necessary to properly investigate the relation between consistency and bioaccessibility.*

The higher viscosity of the samples that were crushed after LTLT blanching was correlated with a lower *in vitro* release of carotenes, which may in turn be related to a higher content and molecular weight of the pectin in this sample (Erdman Jr *et al.* 1986). However, when plotting the Bostwick consistency against the *in vitro* bioaccessibility of lycopene as measured in studies 1 and 2 there was only a weak linear correlation between the two (Figure 12). For example, boiling the LTLT blanched samples in study 2b caused a further significant reduction in consistency, but as shown in Figure 11 it only had a minor impact on the *in vitro* bioaccessibility of lycopene. It is therefore suggested that other factors than viscosity, such as absence of dietary lipid or formation of cellular

aggregates, also affected the lycopene bioaccessibility. It should be noted though, that both the Bostwick and *in vitro* bioaccessibility values were rather similar for most of the samples (Figure 12). An experiment designed specifically to produce a wide range of consistencies and *in vitro* bioaccessibility values would be necessary to make definite conclusions on the potential correlation between consistency and bioaccessibility.
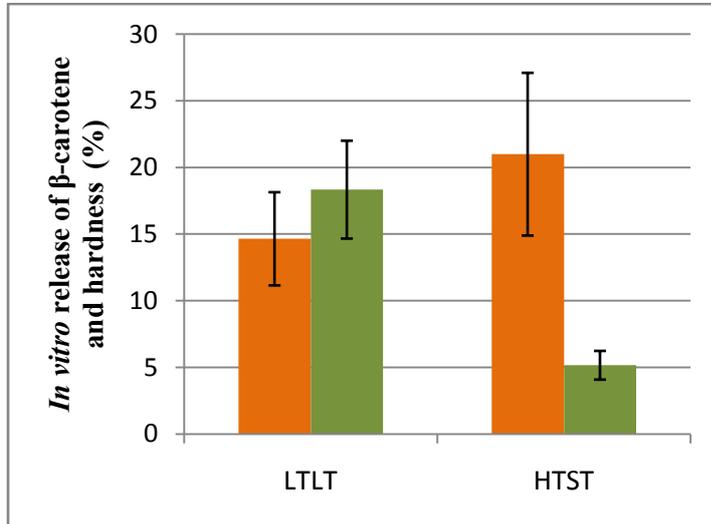

Figure 13. *The relative in vitro release of β-carotene (orange bars,% of total content) and relative hardness (green bars,% compression stress compared with raw pieces) of carrot pieces subjected to LTLT (60°C/40 min) or HTST (90°C/4 min) blanching followed by boiling (100°C/20 min). Results from a pilot study (unpublished).*

For carrot, the crushing/blanching order and blanching temperature had a large impact on *in vitro* bioaccessibility and structure, but often in opposite directions compared with tomato. The absence of PG in carrot means that LTLT blanching can be used to strengthen the pectin network. When carrot pieces subjected to HTST and LTLT blanching followed by boiling were compared, the LTLT blanched pieces were harder and had a lower *in vitro* release of β-carotene compared with HTST blanched pieces (Figure 13). A higher *in vitro* bioaccessibility was also obtained when carrot pieces were subjected to HTLT blanching and then crushed, compared with crushed carrot that was subjected to HTLT blanching (Figure 14). However, considering the high temperature used, enzymatic explanations are unlikely; instead, aggregation of cellular components may have influenced the bioaccessibility as discussed under "Excessive processing".
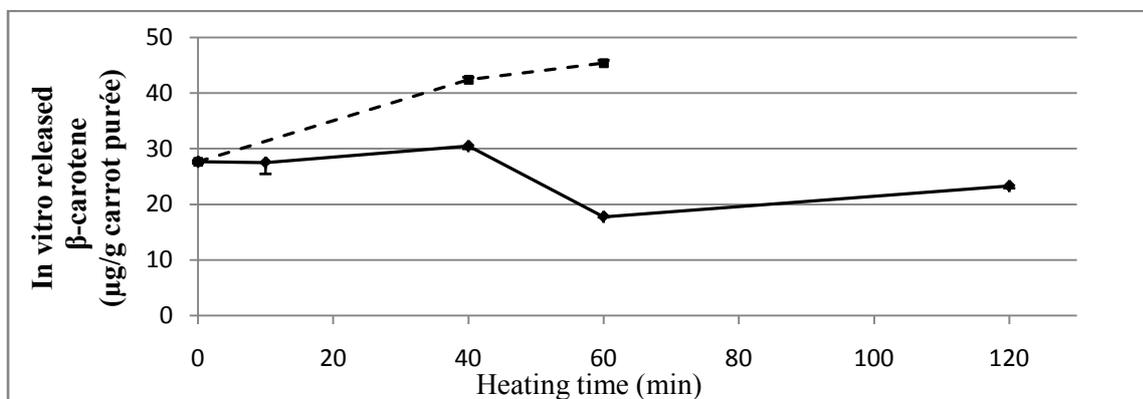

Figure 14. *The in vitro release of β-carotene from crushed carrot. Carrot pieces were crushed before (solid line) or after (dashed line) heating at 90°C up to 120 min. Results from a pilot study (unpublished).*

High-pressure homogenisation

The different thermal and mechanical treatments investigated in papers 1 and 2 and the various pilot studies were all found to produce a material that contained a large proportion of intact cells and cell clusters. High pressure homogenisation (HPH) was used in the work described in paper 3 to get a more thorough disruption of the cells. The starting material was an emulsion made from crushing thermally pre-treated carrot (C) or tomato (T) together with 5% (w/w) olive oil. To create mixed samples, 50% of the carrot and 50% of the tomato emulsions were combined before (M) or after (MA) HPH. Three homogenisation treatments with an increasing degree of intensity were used: homogenisation at 10 MPa 1 and 10 times (10/1 and 10/10) and at 100 MPa 1 time (100/1).
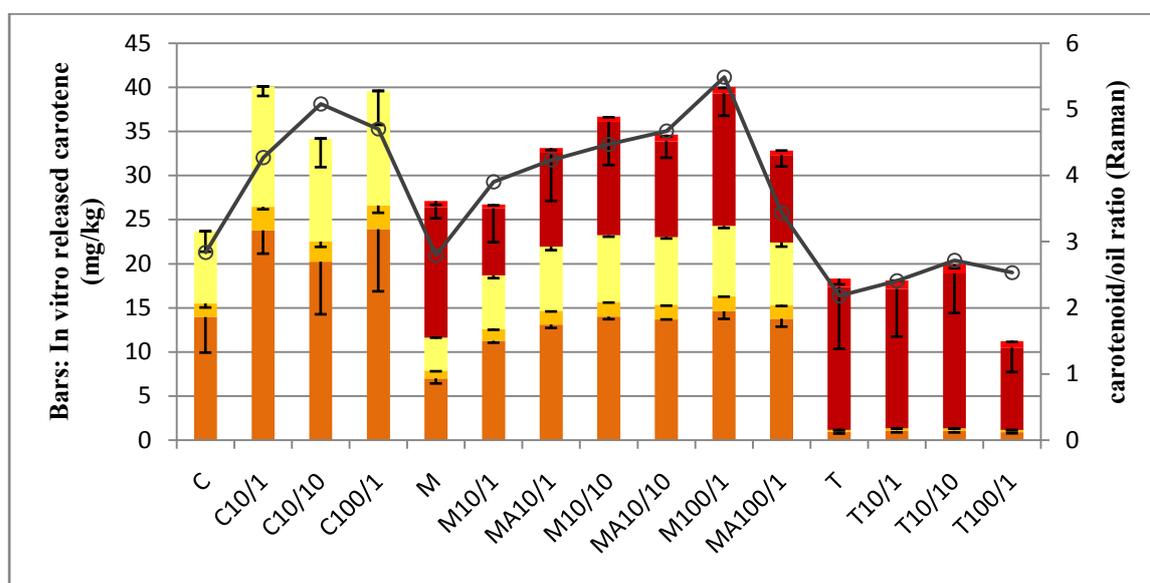

Figure 15. *Total amount of different carotenes released during in vitro digestion (bars, left axis) and the carotenoid/oil ratio as measured by Raman spectroscopy (line, right axis) in the emulsions subjected to HPH at increasing concentrations (study 3). HPH significantly (p< 0.05) increased the in vitro release of carotenes from carrot and mixed emulsions, but not from the tomato emulsions. Sample notation is given in Table 2. Bar colour (bottom up) represents all-trans β-carotene (dark orange), 13-cis β-carotene (light orange), all-trans α-carotene (yellow), all-trans lycopene (dark red), 5-cis lycopene (bright red). In vitro values are mean±SD of 2-3 independent treatment replicates.*

HPH significantly ($p<0.05$) enhanced the *in vitro* bioaccessibility of α- and β-carotene in carrot and mixed emulsions but did not appear to increase lycopene bioaccessibility (Figure 15). The lycopene results were in line with those in the literature, where neither the *in vitro* release nor the human plasma levels of lycopene were significantly increased by HPH of tomato (Van Het Hof *et al.* 2000a; Colle *et al.* 2010). To my knowledge, this is the first study on the effect of HPH on carotene bioaccessibility in carrots. The *in vitro* results closely corresponded with the carotene/oil ratio in the dispersed oil, indicating that the amount of carotenes dissolved in the oil phase is an important factor in carotene *in vitro* bioaccessibility (Figure 15). The correlation between degree of tissue disruption and

41

in vitro bioaccessibility of carotenes was not as clear. Although the gradually increasing intensity of homogenisation led to progressively more ruptured carrot cells, the *in vitro* bioaccessibility of carotenes was similar in all HPH treated samples (Figure 16 and 17). There was also a difference in degree of cell disruption in the different tomato samples but, in spite of this, the *in vitro* bioaccessibility of lycopene appeared to be similar in all samples (Figure 15 and 17).

Figure 16. *Light micrographs of the carrot emulsions in study 3. Inserts show cryo-SEM images of the same samples and arrows indicate cell walls or cell wall fragments in these images. The unhomogenised carrot emulsions (A) consisted mainly of larger clusters of intact cells, homogenisation 1 time at 10 MPa (B) reduced the cell clusters to smaller units but most cells walls remained intact. After homogenisation 10 times at 10 MPa (C) the emulsions consisted mainly of single, intact cells, whereas no intact cells remained after homogenisation 1 time at 100 MPa (D) and only cell wall fragments were observed by cryo-SEM.*
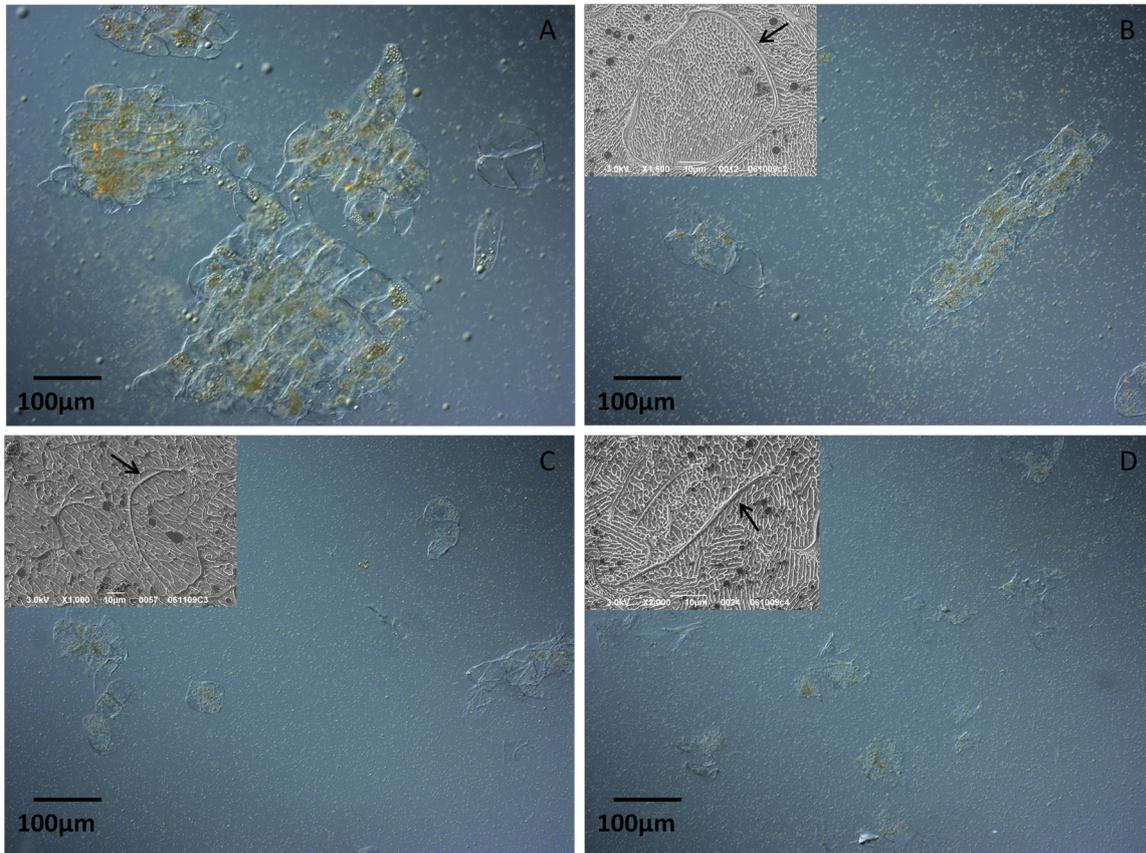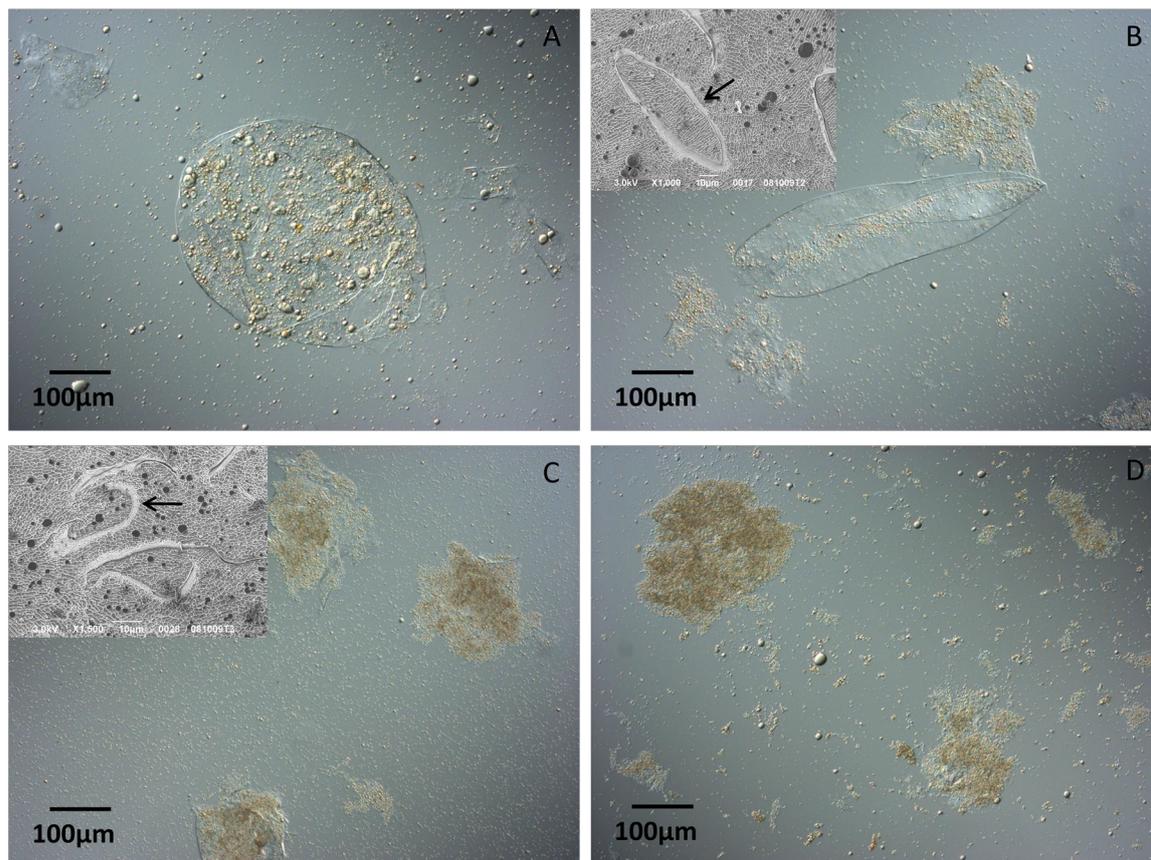
Figure 17. *Light micrographs of the tomato emulsions in study 3. Inserts show cryo-SEM images of the same samples and arrows indicate cell walls or cell wall fragments in these images. The unhomogenised (A) emulsions and the emulsions homgenised 1 time at 10 MPa (B) consisted mainly of single, intact cells. Emulsions subjected to homogeinsation 10 times at 10 MPa (C) or 1 time at 100 MPa (D) contained no intact cells.*

The results presented in this thesis indicate that cell wall integrity has a large impact on the release of carotenes from the food matrix, which is also described in previous studies (Bengtsson *et al.* 2010; Tydeman *et al.* 2010b). However, it did not appear to be the only factor affecting the *in vitro* bioaccessibility of carotenes in the emulsions. It is suggested that the 5% olive oil present was not sufficient to dissolve all carotenes. This is supported by the bioaccessibility data obtained from measurements of the mixed emulsions, which contained 50% each of carrot and tomato emulsion. In the mixed emulsions, the concentrations of α-carotene, β-carotene and lycopene were lower compared to the emulsions containing only carrot or tomato. In the mixed samples, the *in vitro* bioaccessibility of α- and β-carotene gradually increased with an increasing degree of tissue disruption (Figure 15). Furthermore, the relative *in vitro* release of lycopene was higher in the mixed emulsions, 19-38% compared with 12-22% in the tomato emulsions. Assessment of lycopene solubility in olive oil confirmed that the 5% (w/w) used in the emulsions was only sufficient to dissolve around 15% of the lycopene in the tomato emulsion. Similarly, when comparing literature values of β-carotene solubility in dietary fat with the β-carotene/oil ratios in the emulsions, it was concluded that the amount of

olive oil was most likely sufficient to dissolve all β-carotene present in the mixed emulsion. However, for the carrot emulsion, in which the β-carotene concentration was higher, the amount of olive oil may not have been sufficient to dissolve all β-carotene (Borel *et al.* 1996). It is therefore suggested that degree of cellular disruption has a large influence on carotene *in vitro* bioaccessibility from carrot, provided that the samples contain sufficient amounts of dietary lipid.

Excessive processing
Fruits and vegetables are often subjected to excessive processing in industrial food production. This may significantly reduce the content of many easily degraded nutrients, and the results of the present studies also indicated that overly extensive processing did not further increase the *in vitro* release of carotenes.

Tomato products are often subjected to more than one thermal treatment. For example, an initial blanching to inactivate quality degrading enzymes may be followed by a later pasteurisation or sterilisation step during packaging (Hayes *et al.* 1998). It is also common that pre-processed tomato products are heated again during domestic cooking. The impact of repeated thermal processing of tomato pulp was therefore investigated in study 2 by applying either LTLT or HTST blanching followed by boiling for 20 min (Figure 11). The additional boiling step resulted only in a small, non-significant increase of the *in vitro* release. The results of the present study are in agreement with data from a human study by van het Hof *et al.* (2000a), where an extensive second boiling treatment of thermally pre-processed material was compared with minor additional heating. The extensive boiling gave a higher plasma and postprandial lycopene response, but the differences were not significant. The studies are not completely comparable, however, as the thermal pre-processing in the study by van het Hof *et al.* was more extensive; sterilisation at 100°C for 55 min.

Heat treatment of both crushed tomato and carrot resulted in the formation of intra- and extracellular aggregates, where the more extensive heat treatments led to larger aggregates (Figure 18). A similar aggregation of cellular material after HPH was indicated in the LM images, but in this case it may have been an artefact of the dilution done during preparation of the samples for LM (Figure 17 C and D). Blanching carrots has previously been reported to induce a tendency for aggregation of cellular material (Rich *et al.* 2003). Rich *et al.* suggested that the precipitated structures were formed by aggregation of coagulated protein and lipid. A close-up of the aggregates revealed clear orange and red coloration, indicating the presence of lycopene and β-carotene (Figure 18 C). The presence of aggregates was usually coupled with a reduced *in vitro* bioaccessibility, possibly because carotenes are retained within the aggregates during digestion. It would therefore be of value to further investigate whether this type of aggregation is indeed detrimental for carotene bioaccessibility and whether it can be prevented by the use of milder thermal treatments.
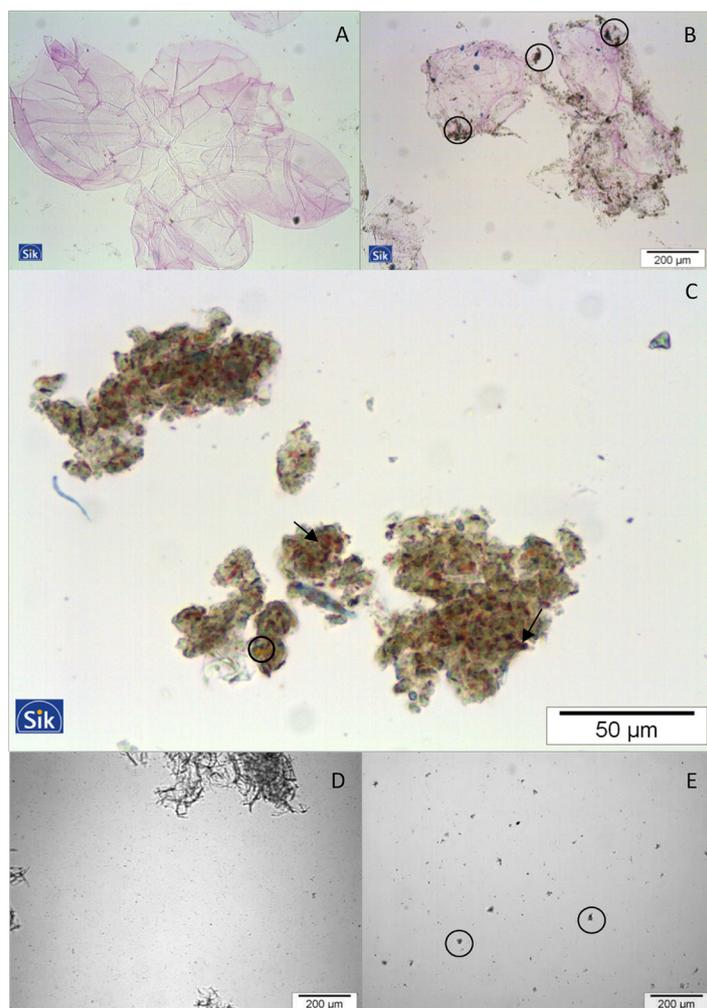
Figure 18. *Aggregation of cellular material after thermal treatments of crushed tomato from study 2b (A-C) and crushed carrot from the pilot study presented in Figure 14 (D and E). No aggregates were found in the unheated samples (A and D), but extensive aggregation (circles) could be observed after boiling the tomato at 100°C for 20 min (B) and blanching the carrot at 90-95°C for 40 min (E). Image C is a close-up of some of the aggregates in image B showing orange (circles) and red (arrows) coloration.*

HPH treatment of tomato emulsions at the highest pressure, 100 MPa, resulted in the lowest lycopene bioaccessibility (Figure 15). A similar observation was made by Colle *et al*. (2010), who reported an inverse relationship between homogenisation pressure and *in vitro* bioaccessibility of lycopene. Colle *et al.* suggested that HPH caused release of pectins and other structural components of the cell walls into the liquid phase, which improved the strength of the fibre network. It has previously been hypothesized that the presence of a strong polymer network might entrap lycopene, which reduces its bioaccessibility (McClements *et al.* 2009). Since most cells were already broken by less intense pressure treatments, it is suggested that higher pressures may therefore not only be unnecessary but even counterproductive (Figure 17).

Micellar incorporation and cellular uptake of carotenes

Once carotenes have been released from the food matrix, they have to be incorporated into mixed micelles in the small intestine in order to be taken up by the mucosa (Castenmiller *et al.* 1997). The micellar fractions obtained during simulated *in vitro* digestion experiments were isolated in studies 3 and 4 to assess the relationship between *in vitro* release and micellar incorporation of carotenes and to investigate carotene uptake by Caco-2 cells. The release from the food matrix was estimated by measuring the amount of carotenes available in a digesta isolated by centrifugation and vacuum filtration

45

(pore size >10 µm) of *in vitro* digested samples. The micellarisation efficiency was assessed by measuring the amounts of carotenes recovered in the micellar fraction, which was isolated by microfiltration (0.22 µm) of the digesta.

Micelle formation and carotene incorporation

To facilitate micelle formation *in vitro* and improve the bioavailability of carotenes, 2.5-5% olive oil was added to the samples in studies 3 and 4. Fatty acids are a vital component of mixed micelles and can be provided by enzymatic lipolysis during *in vitro* digestion. A pilot study showed that the addition of 5% olive oil to thermally treated carrot had a positive impact of the *in vitro* release of β-carotene, and that it was essential for the micellar incorporation (Figure 19). The *in vitro* release was around 1.8 times higher and the micellar incorporation 11 times higher in samples containing oil. *In vitro* studies investigating the effect of dietary fat have shown varying results, even though the addition of dietary fat has in most cases been reported to improve the extent of carotene micellarisation (Huo *et al.* 2007; O'Connell *et al.* 2008; Ornelas-Paz *et al.* 2008; Bengtsson *et al.* 2009a). For example, Bengtsson *et al.* (2009a) reported around 20 times higher β-carotene concentrations in the micellar fraction when 2.5% sunflower oil was added to thermally processed orange fleshed sweet potato. On the other hand, O'Connell *et al.* (2008) reported a significantly reduced micellar incorporation of β-carotene in several cases when 5 or 20% olive oil, peanut oil or rapeseed oil was added to mixes of raw fruits and vegetables. The varying results may be due to a number of factors, including properties of the raw material, such as the endogenous lipid content, choice of processing, the carotene composition of the samples and the *in vitro* model itself, i.e. how well the model is adapted to the presence of lipid.
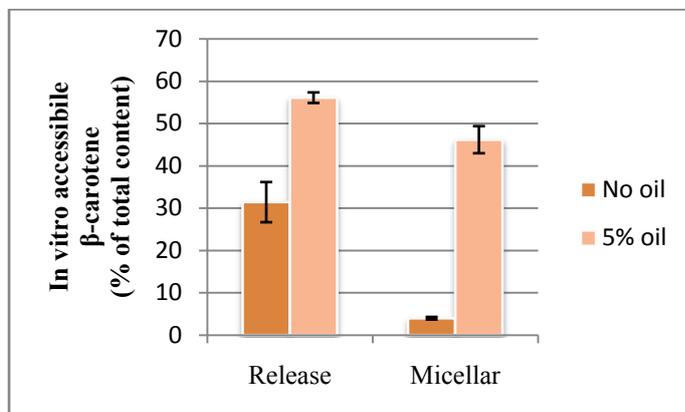
Figure 19. *The relative in vitro release and micellar incorporation of β-carotene in heat treated (90°C/40 min) carrot pieces homogenised with and without the addition of 5% (w/w) olive oil. Results from a pilot study (unpublished).*

Many *in vitro* digestion experiments are performed using conditions that simulate the fasted state. Both the pH conditions and secretions in the stomach and small intestine are influenced by the presence of food, and *in vivo* responses to high amounts of lipid in the duodenum include increased secretion of bile salts and lipolytic enzymes and chylomicron synthesis (Borel 2003; Tyssandier *et al.* 2003). Differences in lipid, bile and pancreatin concentrations are expected to influence the transfer of lipophilic molecules to the micellar phase. The importance of model evaluation and possible adaptation was illustrated in a pilot experiment where an additional 5% olive oil was added to a mix of tomato and carrot already containing 5% oil. In the samples with 10% olive oil, a

coloured lipid phase, most likely containing a large amount of dissolved carotenes, could be observed after *in vitro* digestion. The lipid phase was then removed during isolation of the digesta, and the *in vitro* micellarisation of β-carotene was reduced from 49±10 to 7±1% in samples containing additional olive oil. It is suggested that the amounts of bile salts and pancreatic enzymes used in the *in vitro* model were sufficient to manage 5% dietary fat, but that the model has to be adapted for investigation of higher concentrations of dietary lipid.
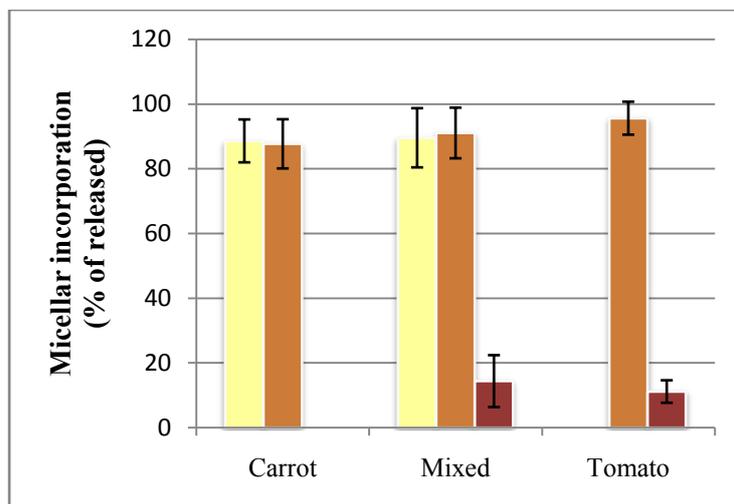

Figure 20. *The proportion of in vitro released carotenes that were recovered in the micellar fraction (study 3). Values are mean±SD for all carrot, mixed and tomato emulsions produced with the different types of HPH summarised in Table 2. The bars denote α-carotene (yellow), β-carotene (orange) and lycopene (red).*

For lycopene, the micellar incorporation was very low in spite of the presence of oil, only 1-3 mg kg^{-1} for the different samples (papers 3 and 4). For the tomato emulsions in study 3, which had the highest lycopene content of all samples investigated, this corresponded to a relative micellar incorporation below 4% of the total lycopene content, or 6-14% of the released carotenes (Figure 20). This can be compared with other carotenes in the same samples; for example 88-96% of the released α- and β-carotene was recovered in the micellar fraction. Similarly, during production of synthetic mixed micelles for use in cellular uptake experiments, the maximum micellar incorporation of all-*trans* β-carotene was 17 times higher compared to that of all-*trans* lycopene. An impaired micellar incorporation of all-*trans* lycopene has also been reported in the literature (Boileau *et al.* 1999; Sugawara *et al.* 2001). This might be due to the longer effective chain length of the planar lycopene molecule compared to other carotenes, which would physically hinder micellar incorporation (Boileau *et al.* 1999). That explanation is partly supported by a higher relative micellar incorporation and bioavailability of the bulkier, but shorter, lycopene *cis*-isomers, which has been observed both in the present and previous studies (Boileau *et al.* 1999; Unlu *et al.* 2007; Failla *et al.* 2008a). To further investigate the relation between micellar size and carotene incorporation, the size range of synthetic mixed micelles containing different combinations of β-carotene and lycopene was measured with dynamic light scattering (DLS). The micelles in which lycopene was present were significantly larger than those only containing β-carotene (Figure 21). Mixed micelles have a diameter of around 8 nm, but the average size was around 160 nm when only lycopene was present, suggesting that micelles were not the dominating structure in these samples (Parker 1996).
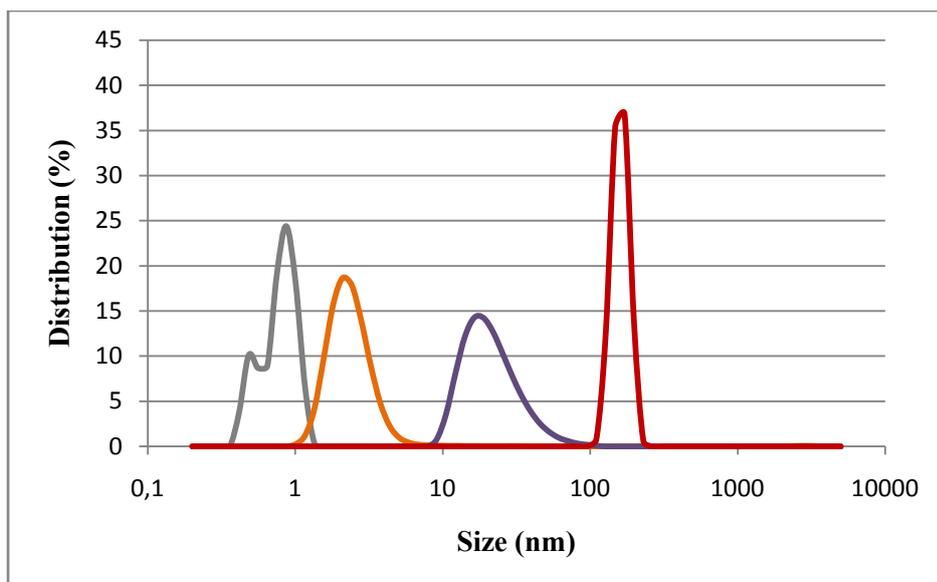
Figure 21. *The size distribution of synthetic mixed micelles containing no carotenes (grey), β-carotene (orange), both β-carotene and lycopene (purple) or lycopene (red) as measured by DLS. Results from a pilot study (unpublished).*

Carotene uptake in the Caco-2 cell model

The cellular uptake experiments with the Caco-2 cell models were performed both with micellar fractions isolated after *in vitro* digestion and with synthetic mixed micelles produced from a mix of micellar components. All uptake experiments, regardless of micellar source, showed a clear linear correlation between the carotene concentration in the micellar fraction and the uptake by the cells (Figure 22). A linear correlation between concentration and uptake has also been reported in the literature (Garrett *et al.* 1999b; During *et al.* 2002; Thakkar *et al.* 2007; Bengtsson *et al.* 2009b). Another clear trend was that the relative uptake of lycopene was consistently lower compared to that of β-carotene, both when the carotenes were present together and separately. The results are similar to those reported in the literature for Caco-2 cells (Sugawara *et al.* 2001; During *et al.* 2002).

Figure 22. *Cellular uptake of β-carotene provided by the micellar fraction isolated after static in vitro digestion (A) and by synthetic mixed micelles (B). Results in A are from paper 3 and results in B are from a pilot study (unpublished).*
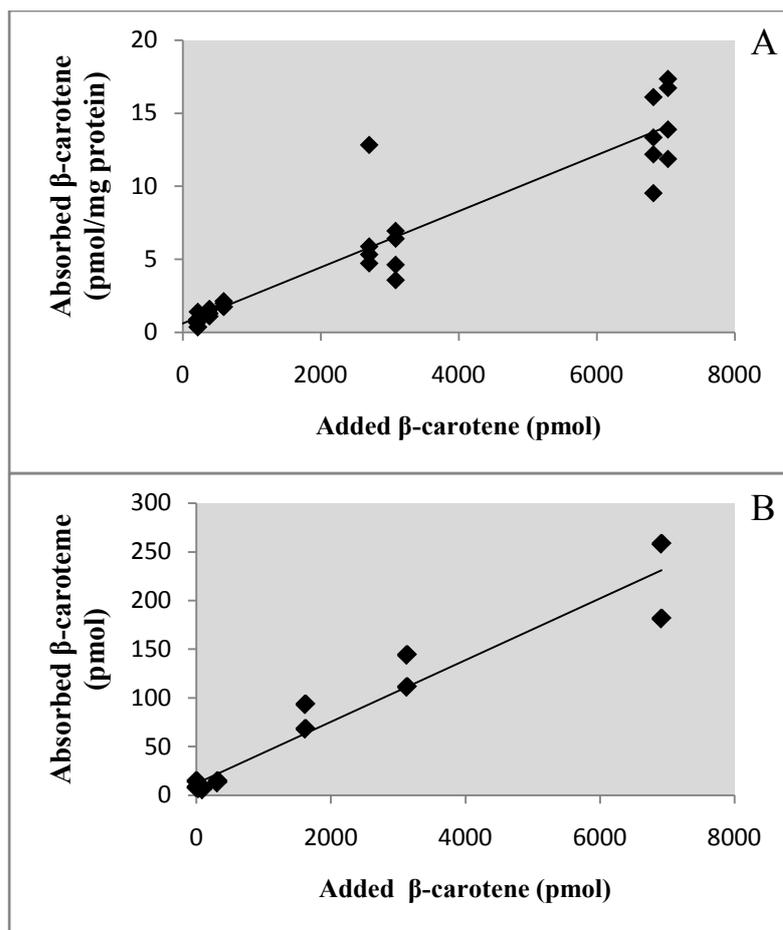
The linear uptake makes it difficult to compare different types of processing and food samples, but the cell model can be a useful tool for investigating for example possible interactions between different carotenes. In the present study, some initial uptake experiments were done to investigate possible synergistic effects, but no such trends were detected. For example, the uptake of β-carotene in synthetic mixed micelles appeared to be unaffected by the presence of lycopene. In study 3, the linear correlation between concentration and uptake was not affected by whether the β-carotene came from digesta of carrot emulsion, tomato emulsion or a 50/50 mix of both carrot and tomato emulsion, even though the digests varied in carotene composition. However, data in the literature on possible interactions or synergistic effects have given inconsistent results (Van Den Berg 1999). The potential impact on micelle size also indicates that different combinations of carotenes might affect the uptake and transport of carotenes (Figure 21). A more systematic approach, where both the uptake and transport models are utilised, could help to clarify whether these kinds of interactions have an impact on carotene bioavailability.

Comparison of *in vitro* and *in vivo* results

There is presently no clear consensus on the best approach for estimating carotene bioavailability. Human trials represent the "golden standard" but are limited by several factors such as high cost and complicated methodologies; only a low number of samples can thus be evaluated by human studies. In addition, both inter and intra-individual responses have been reported to vary greatly, making reproducibility an issue and a large

number of subjects a requirement (Stahl *et al.* 1995; Borel *et al.* 1998). Compared with *in vivo* studies, *in vitro* models are rapid, reproducible and cost efficient, allowing for the screening of a large number of samples. However, in order for the *in vitro* models to be of any practical value, it is vital to evaluate how well they correspond with *in vivo* results. A high correlation between *in vitro* and *in vivo* results has been reported for β-carotene by Tydeman *et al.* (2010a) and for several carotenes, including β-carotene and lycopene, by Reboul *et al.* (2006). However, as concluded by Granado *et al.* (2006), food related factors affecting the bioavailability of carotenoids may be estimated using *in vitro* methods, but host-related factors, different physiologic processes and homeostatic control limit the comparability.

To assess the predictive value of the different *in vitro* models used in this thesis, data from these models were therefore compared with results of human studies in paper 4. The human studies are presented in detail in the papers by Martínes-Tomás *et al.* (2011a; 2011b) A major advantage of the study presented in paper 4 is that it includes several steps of the digestion and absorption process, from release to cellular uptake. Another benefit is that the same soups were characterised both *in vitro* and *in vivo*, as opposed to comparing similar samples produced at different times from different raw materials. The bioavailability ratios were calculated by dividing the average plasma increase in human subjects after consumption of the optimised soup with that of subjects consuming one of the other soups. Similarly, the bioaccessibility ratio was calculated by dividing the *in vitro* release, micellar incorporation or cellular uptake of the optimised soup with that of the other two soups. Cellular uptake of carotenes from the optimised and pilot soups was not compared as these experiments were made on different occasions. The carotene content was similar in the optimised and pilot soups but lower in the reference soup. Since this most likely affected both the carotene content of the digesta after *in vitro* digestion and the plasma carotene levels, the different carotene content of the different soups was not compensated for during calculation of the ratios. The comparison between β-carotene and lycopene was made by dividing the relative plasma increase or *in vitro* bioaccessibility of all-*trans* β-carotene with that of all-*trans* lycopene in the same soups.

Table 3. *A comparison between the "optimised" and "reference" soups given as the ratios between the increase in plasma carotene levels in human subjects after 4 weeks consumption of the two soups (In vivo plasma), the amount of carotenes recovered in the digesta (in vitro release) or micellar fraction (in vitro micellar) after a static in vitro digestion or as the uptake in the Caco-2 cell model (Cell uptake).*

Carotene	*In vivo* plasma	*In vitro* release	*In vitro* micellar	Cell uptake
All-trans β-carotene	1.83	1.31	1.34	1.84
Total lycopene	0.37	1.12	1.09	1.66
5-cis lycopene	0.31	0.77	0.73	1.19

Table 4. *A comparison between the "optimised" and "pilot" soups given as the ratios between the increase in plasma carotene levels in human subjects after 4 weeks consumption of the two soups (in vivo plasma), the amount of carotenes recovered in the digesta (in vitro release) or micellar fraction (in vitro micellar) after a static in vitro digestion.*

Carotene	*In vivo*	*In vitro* release	*In vitro* micellar
All-trans β-carotene	1.00	1.03	1.03
Total lycopene	0.24	0.55	0.56
5-cis lycopene	0.19	0.62	0.67

In the present study, the *in vitro* and *in vivo* data correlated well for β-carotene but not as well for lycopene (Table 3 and 4).The largest discrepancy was observed in the comparison of the *in vitro* and *in vivo* data for lycopene in the optimised and reference soups (Table 3). For these samples, the *in vitro* data indicated a higher lycopene bioaccessibility from the optimised soup but, in the human study, the reference soup induced the highest increase in lycopene plasma levels. However, these ratios were based on total lycopene bioaccessibility in the soups. When compensating for the higher lycopene content in the optimised soup by calculating the *in vitro* release relative to the total lycopene content, it appeared to be slightly higher in the reference soup compared to the optimised soup (Figure 23). As previously discussed, lycopene has a very low solubility in dietary fat, and lycopene *in vitro* bioaccessibility may be largely dependent on the lycopene/fat ratio in the samples. The reference soups contained 2.5% olive oil and the other two soups contained 5% olive oil. The lower amount of fat in the reference soup may have been the main factor limiting lycopene bioaccessibility during static *in vitro* digestion. For the more dynamic conditions in human subjects, which include a continuous decrease of lycopene levels due to cellular uptake, the low solubility of lycopene may not be as decisive. Instead, other factors, such as the higher relative release from the food matrix, might have been more important for lycopene bioavailability.
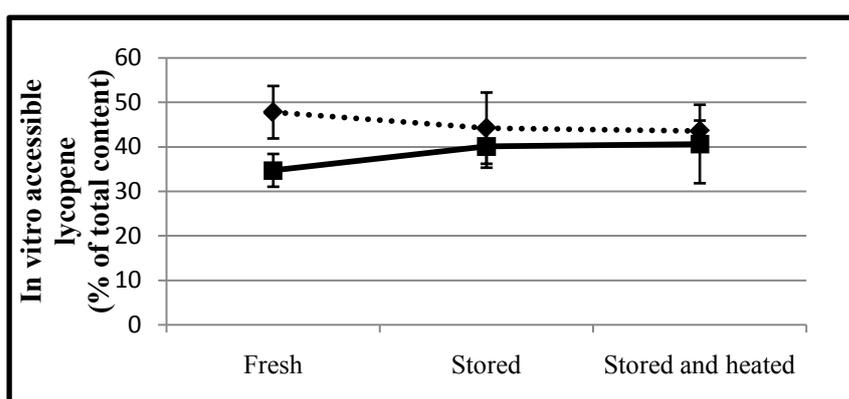
Figure 23. *The relative in vitro release of lycopene from the reference (dashed line) and optimised (solid line) soups that used in a human trial (paper 4). In vitro digestions were performed on freshly made soups ("Fresh") and on soups that had been stored at ambient temperature for the duration of the human trial, which was 3 months ("Stored"). To investigate whether this had an impact on the in vitro bioaccessibility, the stored soups were also heated according to the instructions given to the participants in the human study ("Stored and heated"). Values are average ±SD of three independent batches.*

When comparing *in vitro* and *in vivo* results, both the bioaccessibility and bioavailability was higher for β-carotene compared with lycopene (Table 5). In fact, all work presented in this thesis has shown a lower relative bioaccessibility of all-*trans* lycopene compared with all-*trans* β-carotene. The difference is apparent in all steps of the digestion process, namely release from the food matrix, solubilisation in dietary lipid, micellar incorporation and cellular uptake, and gives an accumulating effect. The limited bioaccessibility of lycopene *in vitro* is in agreement with data from both the present and previous human studies (Oshima *et al.* 1996; Edwards *et al.* 2003; Porrini *et al.* 2005).

Table 5. *The ratio of all-trans β-carotene and lycopene bioavailability in vivo and bioaccessibility in vitro for the three soups compared in study 4. The ratios were obtained by dividing relative[a] plasma response or in vitro bioaccessibility of β-carotene with that of lycopene.*

Soup	*In vivo* plasma	*In vitro* release	*In vitro* micellar	Cell uptake
Pilot	1.71	0.78	1.08	1.58
Optimised	6.85	1.74	2.44	2.95
Reference	1.35	1.23	1.58	2.05

[a] Relative to the total content of β-carotene and lycopene respectively in the soups

In summary, the results in the present study support the use of *in vitro* models as a screening tool with the aim to study pre-absorptive factors affecting β-carotene bioaccessibility, but the *in vitro* models were less suitable for predicting lycopene bioavailability. The explanation may be that the static *in vitro* models are more affected by differences in total lycopene content and the amount of dietary fat in the samples as compared to the more dynamic *in vivo* situation.

CONCLUSIONS

The major conclusion from the studies presented in this thesis is that choice of processing conditions had a large impact on the *in vitro* bioaccessibility of carotenes, and that this was related to microstructural properties of the samples.

Specific conclusions are:

- Thermal and mechanical processing of tomato affected the microstructure in different ways, and a combination of both was required to increase the *in vitro* release of lycopene. The thermal processing reduced cell wall density, whereas mechanical crushing disrupted the tomato matrix into single cells and small cell clusters.

- Blanching treatments of tomato at temperatures between 60 and 95°C resulted in a higher *in vitro* release of lycopene compared with boiling at 100°C. The more extensive formation of carotene-containing aggregates after the boiling treatment might have contributed to this effect.

- A second thermal (boiling) or mechanical (HPH) treatment of pre-processed tomato did not further enhance the *in vitro* bioaccessibility of lycopene, indicating that the integrity of the cell matrix was not the only factor that affected lycopene bioaccessibility. Other factors may have included the low lycopene solubility in dietary fat and in mixed micelles, aggregation of cellular components and formation of a fibre network.

- In carrots, high pressure homogenisation (HPH) was shown to thoroughly disrupt the cell matrix, which in turn significantly increased the *in vitro* release of α- and β-carotene.

- Addition of olive oil to thermally processed carrot almost doubled the *in vitro* release of β-carotene and increased the amount of β-carotene recovered in the micellar fraction more than 10 times.

- Carotene retention after processing was generally high, whereas processing substantially reduced ascorbic acid levels in several cases. Since the results presented in this thesis indicated that excessive processing, which normally reduces the total nutrient content, was not beneficial for carotene *in vitro* bioaccessibility, a higher nutrient retention may be obtainable without compromising *in vitro* bioaccessibility.

- Macrostructural analysis showed a weak trend of higher carotene bioaccessibility in samples with a lower viscosity or hardness, but no general conclusions could be made on the basis of sample macrostructure.

- The carotene uptake by Caco-2 cells was linearly dependent on the amount of carotenes added to the cells, and combining an *in vitro* digestion with the cell model did not provide much additional information compared with the *in vitro* digestion alone. However, the cell model may be a useful tool for investigating differences in relative uptake and possible synergistic effects between different carotenes.

- The relative bioaccessibility of all-*trans* lycopene was consistently lower than that of the other carotenes investigated in all steps of the *in vitro* digestion procedure, namely release from the food matrix, solubility in dietary fat, micellar incorporation and cellular uptake. Similarly, a higher relative plasma response was obtained for all-*trans* β-carotene compared with all-*trans* lycopene in the human studies.

- The positive correlation between the bioaccessibility ratios of β-carotene measured with the *in vitro* models and the bioavailability ratios measured in humans suggest that the models can give an estimate of β-carotene *in vivo* bioavailability, whereas the predictive value was lower for lycopene bioavailability.

FUTURE OUTLOOK

The ultimate goal of the work presented in this thesis is to identify and control the factors governing carotene bioavailability, and thus to be able to design plant food based products with a high nutritional value. Some suggestions for continued work towards that goal are given below. Most of the suggestions require input from experts in different fields, which I believe is vital for fully understanding the factors controlling carotene bioavailability. All papers presented in this thesis come from such collaborations, and this has helped me reach understandings and conclusions I could not have obtained otherwise.

The predictive value and correlation between *in vitro* models and the *in vivo* situation needs to be further evaluated. Ideally, the material used in future human studies would also be assessed by different *in vitro* models. There is also a need to further develop and refine the *in vitro* models, for example adapting them to varying amounts of dietary lipid or adding a colon fermentation step. A few studies have investigated microstructural changes after *in vitro* and/or *in vivo* digestion. Continuing this line of research would give valuable information about the correspondence between the *in vitro* and *in vivo* situations.

Other treatments than mechanical and thermal processing may also have a large impact on carotene bioaccessibility. Post harvest freezing of fruits and vegetables are commonly used to improve storage stability, but pilot experiments indicated this may decrease the *in vitro* release of carotenes from both tomato and carrot. Calcium salts are often added to maintain the structure of *e.g.* whole canned tomatoes, and the results in paper 2 indicated that this may reduce lycopene bioaccessibility.

Two different types of Caco-2 models are currently used by different groups, the simpler model presented in this thesis where cells are grown on the bottom of a plate and a more complex insert model where cells are grown on porous membranes. The higher complexity of the insert models makes them a closer estimate of the *in vivo* situation, but the simpler models are more cost and time effective. It would therefore be of great value to evaluate whether the more complex insert model provides additional information.

Factors affecting lycopene bioaccessibility and bioavailability are still poorly understood. Suggested topics to be investigated are the solubility of lycopene in different types of dietary lipid and mixed micelles, and the impact of lycopene on micellar size. It would be interesting to investigate whether higher concentrations of dietary lipid would increase lycopene micellarisation *in vitro*, but these types of studies would have to be done in combination with adaptation of the *in vitro* model. More knowledge is also needed about the difference between *cis*- and *trans*-isomers of lycopene, both with regard to uptake and effects in the human body.

It appears that β-carotene *in vitro* release from carrot is mainly determined by the intactness of the cell wall, whereas lycopene release from tomato is a more complex matter. It would therefore be interesting to compare these results with the impact of cell wall integrity on lycopene and β-carotene release from other matrices, *e.g.* red carrots, which contain lycopene, or a tomato variety where β-carotene is the dominating carotene.

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