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Particle Size Reduction Leading to Cell Wall Rupture Is More Important for the β-Carotene Bioaccessibility of Raw Compared to Thermally Processed Carrots

LIEN LEMMENS, SANDY VAN BUGGENHOUT, ANN M. VAN LOEY, AND MARC E. HENDRICKX*

Laboratory of Food Technology and Leuven Food Science and Nutrition Research Centre (LFoRCe), Department of Microbial and Molecular Systems (M^2S), Katholieke Universiteit Leuven, Kasteelpark Arenberg 22, 3001 Leuven, Belgium

The amount of nutrients that can be released from food products (i.e., nutrient *in vitro* bioaccessibility) is often studied as it is a starting point for investigating nutrient bioavailability, an indicator for the nutritional value of food products. However, the importance of mastication as a particle size reduction technique is poorly understood and is often neglected during *in vitro* procedures determining bioaccessibility. Therefore, the aim of the present work was to study the effect of mechanical breakdown on the β-carotene bioaccessibility of carrot samples, having different textural/structural characteristics (as a result of thermal processing). In the first part of this study, the *all-E-β*-carotene bioaccessibility of carrot particles of different sizes (ranging from cell fragments up to large cell clusters), generated from raw as well as from gently and intensely cooked carrot samples, was determined. In the second part of the study, the effect of human mastication on the particle size reduction of raw as well as of gently and intensely cooked carrot samples was investigated in order to allow identification and validation of a technique that could mimic mastication during *in vitro* procedures. Results showed a strong dependency of the *all-E-β*-carotene bioaccessibility on the particle size for raw and gently cooked carrots. After intense cooking, on the other hand, a considerable amount of *all-E-β*-carotene could be released from cell fragments (smaller than a cell) as well as from small and large cell clusters. Hence, the importance of mechanical breakdown, and thus also of (*in vitro*) mastication, is dependent on the carrot sample that is considered (i.e., the extent to which the carrot sample has been thermally processed prior to the particle size reduction). Structural changes occurring during mechanical and thermal processing are hereby key factors determining the *all-E-β*-carotene bioaccessibility. The average particle size distribution curves of raw and cooked carrots, which were chewed by 15 persons, could be mimicked by mixing 50 g of carrots using a Grindomix (Retsch) at 2500 rpm during 5 s. Based on this scientific knowledge, the identified *in vitro* mastication technique was successfully integrated in the *in vitro* digestion procedure determining the *all-E-β*-carotene bioaccessibility of carrot samples.

KEYWORDS: ***all-E-β*-Carotene bioaccessibility; *in vitro* digestion models; mastication; particle size; thermal processing**

INTRODUCTION

Fruit and vegetable based food products are often characterized in terms of nutritional content. However, it was emphasized by Fernández-García et al. (*1*) that it is (even more) relevant to investigate to what extent the bioactive nutrients present in the food products can be digested and effectively assimilated by the human body in order to eventually reach the target tissues where they can carry out their function. In this context, concepts as nutrient bioavailability and bioaccessibility are important. Nutrient bioavailability is defined as the fraction of an ingested nutrient that is available for utilization and for storage in the human body (*2*). Nutrient bioavailability incorporates availability for absorption, absorption, metabolism, tissue distribution and bioactivity of the nutrient (*1*, *3*, *4*). In order to be available for absorption, the ingested nutrients need to be released from the food matrix (*5*). This is referred to as the nutrient bioaccessibility (*2*). It is typically determined by *in vitro* methods simulating the human digestion process. Studying nutrient bioaccessibility is a good starting point for estimating or predicting nutrient bioavailability (*6*). However, it should be stressed that additional *in vivo* studies are necessary to validate the results obtained by *in vitro* analyses (*1*). To assess nutrient bioaccessibility, physiological conditions occurring during oral, gastric and intestinal

*Corresponding author. Tel: +32 16 32 15 85. Fax: +32 16 32 19 60. E-mail: marc.hendrickx@biw.kuleuven.be.

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digestion are simulated by adjusting operational characteristics like temperature, pH, addition of enzymes, and so on (*7*). Nevertheless, only limited attention is given to the simulation of the mechanical breakdown of the food products during mastication (oral digestion). This is mainly important for solid food systems. If the imitation of the mechanical breakdown is specified (for most of the studies on bioaccessibility, this is not the case), it is very case-specific and, to the best of our knowledge, no scientific foundation for the method of choice can be found in currently existing literature. For example, techniques as "finely chopping" (*8*), "homogenizing with a kitchen blender for 1 minute" (*9*, *10*), "homogenizing with a kitchen blender for 15-second intervals" (*11*) and "mixing 2 times 5 seconds at 7500 rpm" (*12*) were reported to simulate mastication. Concerning carotenoid bioaccessibility in (processed) vegetable tissue, only few studies paid attention specifically to the mechanical breakdown during the oral phase in *in vitro* digestion protocols (e.g., Epriliati et al. (*13*)). It is clear that if mastication would have an effect on nutrient bioaccessibility, no unambiguous comparison between different studies can be made. These examples suggest that there are still some shortcomings for *in vitro* digestion protocols to determine nutrient bioaccessibility and that there is space for improvement, optimization and standardization facilitating the comparison of results of different studies and the improvement of the predictions for *in vivo* studies.

Generally, nutrient bioavailability is influenced by exogenous and endogenous factors (*3*), and the term "SLAMENGHI" combines the first letters of all these factors (*14*). The endogenous factors consist mainly of host related factors, while the exogenous factors are predominantly related to the food product, linking these factors also to nutrient bioaccessibility. In this case study on carrots, the major focus point was the effect of "the matrix in which the carotenoids are incorporated" ("M" in "SLAMENGHI") on the β-carotene bioaccessibility. Previous research (*15*) has shown that homogenization of raw and cooked carrot pieces can increase the β-carotene bioaccessibility. In line with the latter study, the present work aims for a more detailed insight into the relation between the carrot tissue particle size and the *all-E-β*-carotene bioaccessibility. Carrot pieces were mechanically processed and sieved in order to obtain 12 carrot particle fractions of different sizes. The *all-E-β*-carotene bioaccessibility of the different fractions was determined in order to have a clear view on the relation between the particle size and the β-carotene bioaccessibility. The influence of the textural/structural quality of the carrot material prior to mechanical processing was included. Hereto, carrot pieces were boiled in water during different time intervals. The resulting carrot samples, clearly differing in textural and thus structural quality, were treated and analyzed in the way as mentioned above (for raw samples). In the second part of the present work, it was the purpose to get a detailed insight into the particle size reduction effect of mastication. A small-scale human study was set up to determine the average particle size distribution of chewed carrot samples. Again this was performed for carrot samples differing in textural/structural quality in order to find out the effect of textural/structural quality on the chewing behavior. In a final part of the present work, a technique to *in vitro* mimic the particle size reduction during human mastication of carrot samples (with different textural/structural characteristics) was identified and validated. Based on this scientific knowledge, the identified *in vitro* mastication technique was successfully integrated in the *in vitro* digestion procedure determining the *all-E-β*-carotene bioaccessibility of carrot samples.

MATERIALS AND METHODS

Carrot Samples. Throughout the study, a single batch of carrots (*Daucus carota* cv. Nerac) was used (stored at 4 °C). Carrots were peeled, cut into pieces (around 1 cm^3) and used as such (raw carrots), cooked in boiling water for 3 min (gently cooked carrots) or cooked in boiling water for 25 min (intensely cooked carrots).

These three carrot samples were characterized in terms of hardness as an indication for the textural/structural quality. As explained in detail by Sila et al. (*16*), a compression test, using a TA-XT2i Texture Analyzer (Stable Micro Systems, Surrey, U.K.), was performed to examine the hardness of the carrots (calibrated carrot pieces, 10 mm height and 12 mm diameter). The residual hardness of the raw, gently and intensely cooked carrots was respectively 100%, 60% and 3%.

Part 1. *Generation of Carrot Particle Fractions of Different Sizes.* Raw, gently cooked and intensely cooked carrot pieces were mechanically processed, i.e. mixed or blended (Grindomix GM 200, Retsch, Haan, Germany), and by using the technique of wet sieving (Retsch, Aartselaar, Belgium), fractions of 12 different particle size ranges were obtained (<40 μm, 41−80 μm, 81−125 μm, 126−160 μm, 161−250 μm, 251−500 μm, 501−800 μm, 801−1000 μm, 1001−1400 μm, 1401−2000 μm, 2001−4000 μm, and 4001−6300 μm). The amount of sample needed to generate sufficient material of each particle size fraction, in order to be able to perform nutritional analyses, depended on the textural/structural characteristics of the sample (raw, gently cooked and intensely cooked).

all-E-β-Carotene Bioaccessibility and Concentration. The *all-E-β*-carotene bioaccessibility of the different carrot particle fractions was determined using a static *in vitro* digestion model. The procedure was based on the method of Hedrén et al. (*15*) with some modifications as described by Lemmens et al. (*12*). Briefly, NaCl (0.9% in water)/ascorbic acid (1% in water) solution and stomach electrolyte solution were added to the carrot samples. The stomach electrolyte consists of NaCl (0.30%), KCl (0.11%), $CaCl_2 \cdot 2H_2O$ (0.15%), KH_2PO_4 (0.05%) and $MgCl_2 \cdot 6H_2O$ (0.07%) dissolved in water. Before addition of gastric juice (0.52% porcine pepsin (Sigma-Aldrich) in electrolyte solution), the pH of the samples was adjusted to pH 4. The samples were incubated for 30 min at 37 °C while shaking end-over-end. The further stay in the stomach was imitated by adjusting the pH to pH 2, followed by an incubation step for 30 min at 37 °C while shaking end-over-end. To mimic the passage through the gut, the pH was increased to pH 6.9, duodenal juice (containing 0.4% porcine pancreatin (Sigma-Aldrich), 2.5% porcine bile extract (Sigma-Aldrich), 0.5% pyrogallol (Sigma-Aldrich) and 1% tocopherol (Sigma-Adrich) in water) was added and the samples were incubated for 2 h at 37 °C while shaking end-over-end. Prior to all incubation steps, the headspace of the samples was flushed with nitrogen in order to minimize the contact with oxygen. To separate digested and undigested material, the samples were filtered (Macherey-Nagel 615 1/4, folded filters, 185 mm diameter) and β-carotene was extracted from the filtrate. For each sample, the β-carotene bioaccessibility was determined in triplicate.

The *all-E-β*-carotene concentration was determined according to the procedure of Sadler et al. (*17*) with minor modifications as described by Lemmens et al. (*12*). Concisely, extraction solvent (containing hexane, acetone, ethanol and buthylated hydroxytoluene), $CaCl_2$ and water were added, and after stirring at 4 °C, an organic phase and an aqueous phase could be separated. β-Carotene, being dissolved in the organic phase, was separated and quantified by a RP-HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium), equipped with a C_{30}-column (25 °C) (5 μm × 250 mm × 4.6 mm, YMC Europe, Dinslaken, Germany) and a diode array detector (DAD) (450 nm). Gradient elution (increasing the fraction of methyl *tert*-butyl ether (15% to 55%) at the expense of the fraction of methanol, while keeping the water fraction constant at 4%) was applied. For the quantification, a calibration curve of *all-E-β*-carotene (CaroteNature, Lupsingen, Switzerland) was prepared. For each sample, the extraction of β-carotene and the subsequent determination of the concentration were performed in duplicate.

By measuring the dry matter content of the samples, the β-carotene concentration and the β-carotene bioaccessibility could be expressed on dry weight basis.

Statistical Analysis. Significant differences among the absolute and relative *all-E-β*-carotene bioaccessibility means for carrot particle fractions of different sizes were analyzed for each carrot sample type (raw, gently and intensely cooked) using Tukey's Studentized range test (statistical software package SAS, version 9.2, Cary, NC). The level of significance was set at $P < 0.05$.

Part 2. *Human Mastication*. The protocol for the human study was approved by the Commission for Medical Ethics (K.U.Leuven), and all participants signed a consent form prior to the start of the study. In total, 15 healthy subjects were asked to chew a certain amount of carrot pieces until they would normally swallow it. At that moment, the chewed carrots were expectorated in a plastic recipient, the subjects rinsed their mouth with water and the water, with remaining carrot particles, was collected in the same recipient. Sufficient carrot material was provided so that the subjects had the possibility to swallow some carrot material initially in order to accurately detect the moment of swallowing. In total, at least 50 g of chewed carrots was needed for further particle size distribution analysis. The same protocol was applied for the three carrot samples.

In Vitro Mastication. Carrot pieces (around 1 cm^3) were mixed using a Grindomix (5 s at 2500 rpm for 50 g of carrot material, Grindomix GM200, Retsch, Haan, Germany) in order to imitate human mastication *in vitro*. Based on the particle size distribution of the mixed carrot samples, this specific *in vitro* mechanical processing step was selected from diverse mixing conditions (using the Grindomix) with following variables: (i) amount of carrot material, (ii) mixing speed and (iii) duration and cycles of mixing.

Determination of Particle Size Distribution. A sieve shaker (Retsch, Aartselaar, Belgium) was used to determine the carrot tissue particle size distribution of the (*in vitro*) masticated samples. Hereto, the sieve shaker, equipped with 12 sieves (40, 80, 125, 160, 250, 500, 800, 1000, 1400, 2000, 4000, 6300 μm), was loaded with 50 g of the sample and by wet sieving during 2 min (shaking amplitude 0.5 mm), different carrot tissue particle size fractions were obtained. Each sieve was then manually dried with paper, and the fractions retained on the different sieves were weighed. The outlet, i.e. the fraction containing the washing water and the carrot particles smaller than 40 μm, was also collected. In order to convert the wet weight of the samples into dry weight, the dry matter content of the different fractions was determined.

all-E-β-Carotene Bioaccessibility and Concentration. The *all-E-β*-carotene bioaccessibility of the different carrot samples (raw, gently cooked and intensely cooked) was determined by combining the *in vitro* mastication (as described above) and the static *in vitro* digestion procedure (as described in Part 1 of Materials and Methods).

The *all-E-β*-carotene concentration of the carrots samples was determined as described in Part 1 of Materials and Methods.

RESULTS AND DISCUSSION

Part 1. Carrot Tissue Particle Size in Relation to *all-E-β*-Carotene Bioaccessibility. In this study, fractions with different particle sizes (cell fragments up to large cell clusters) were generated from the three carrot samples (raw, gently cooked and intensely cooked). It was investigated how the β-carotene bioaccessibility was influenced by the carrot tissue particle size and whether thermal processing before mechanical processing influenced the relationship between these two parameters. Focus was given on the bioaccessibility of *all-E-β*-carotene since isomerization in intact carrot pieces during the thermal processes applied in this study was shown to be limited (*18*, *19*) and changes for *all-E*-α-carotene were expected to be similar to changes for *all-E-β*-carotene. The absolute *all-E-β*-carotene bioaccessibility and the *all-E-β*-carotene bioaccessibility as a percentage of the *all-E-β*-carotene concentration in each specific fraction are plotted as a function of carrot tissue particle size for the three carrot samples in respectively **Figure 1A** and **Figure 1B**. Since microscopy analyses (*20*) of carrot core and cortex tissue revealed that 95% of the carrot cells had a mean diameter smaller than 125 μm, all particles smaller than 125 μm are grouped and represented as a single fraction in **Figure 1**. It clearly illustrates the difference between particles smaller than a carrot cell and particles containing carrot cell clusters as this criterion will be used as a starting point to explain the observed differences.

By analogy with Ellis et al. (*21*), it was anticipated that the release of β-carotene from the carrot tissue is dependent on the

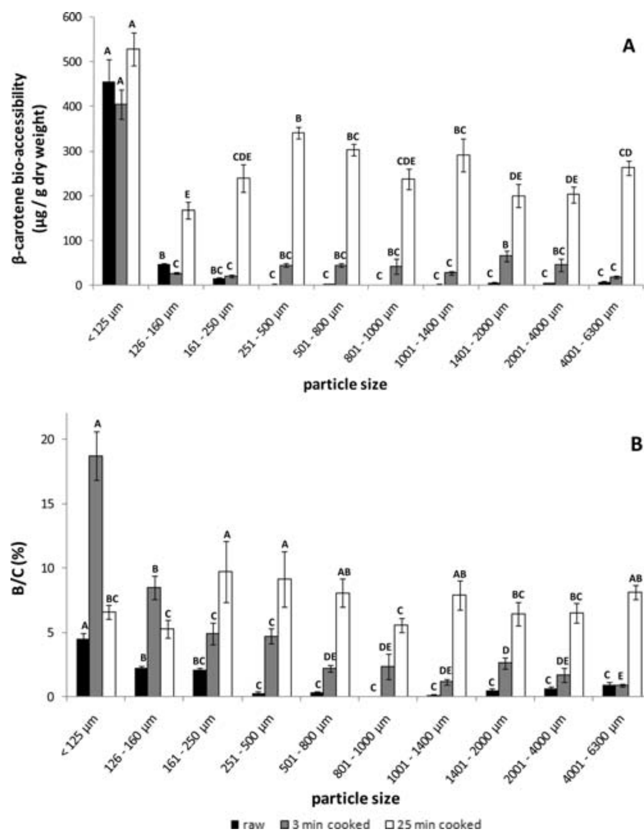

Figure 1. (**A**) Absolute *all-E-β*-carotene bioaccessibility (± standard deviation) (μg/g dry weight) for different carrot tissue particle size fractions. (**B**) *all-E-β*-Carotene bioaccessibility as a percentage of the *all-E-β*-carotene concentration (B/C ± standard deviation) for different carrot tissue particle size fractions. Particle size fractions were generated by (cooking and) mechanical processing followed by wet sieving. Absolute and relative bioaccessibility means of different carrot tissue particle size fractions for each sample type (raw, gently cooked and intensely cooked) indicated with a particular letter (A, B, C, D, E) are not significantly different.

particle size. However, this dependency was found to be more pronounced as the thermal treatment was less intense: in **Figure 1A**, it can be observed that, for raw carrots, particles larger than 160 μm only released very limited amounts of *all-E-β*-carotene during the *in vitro* digestion procedure. On the contrary, when the dimensions of the particles became smaller, a significant increase in the *all-E-β*-carotene bioaccessibility was detected, with a maximal, significantly higher value for particles between 41 and 80 μm. As the measured *all-E-β*-carotene bioaccessibility might be dependent on the *all-E-β*-carotene concentration in the carrot particles, the absolute bioaccessibility values were converted to relative bioaccessibility values (ratio of bioaccessibility to concentration (%), **Figure 1B**), and for the raw carrots, a significant decrease in the *all-E-β*-carotene bioaccessibility could be observed when the particle size increased.

For 3 min cooked carrots, a similar trend can be observed, i.e. a significantly higher *all-E-β*-carotene bioaccessibility for the smallest particles (41−80 μm) compared to larger particles, although slightly higher amounts of *all-E-β*-carotene were released from the larger particles compared to raw carrots (**Figure 1A**). If the relative bioaccessibility (ratio of bioaccessibility to concentration (%), **Figure 1B**) is considered, the same trend, but higher values, were detected compared to raw carrots: the smaller the particle size, the higher the *all-E-β*-carotene bioaccessibility. Remarkably, the fraction containing particles smaller than 125 μm showed a very high relative *all-E-β*-carotene bioaccessibility compared to the raw or intensely treated carrots.

Table 1. Overview of the Effects of Thermal and Mechanical Processing on Particular Structural Characteristics of Carrots (Based on Literature Data) and Their Link to the *all-E-β*-Carotene Bioaccessibility for Raw and Cooked Carrot Samples (Experimental Data of This Study)

	thermal processing			mechanical processing[a]	*all-E-β*-carotene bioaccessibility	
					<carrot cell	carrot cell clusters
raw	no effect on cell and organelle membranes	no effect on carotene−protein complexes	no effect on pectin	cell breakage	high	very low
gently cooked	cell and organelle membrane damage	carotene−protein complexes affected	limited pectin solubilization	cell breakage	very high	low
intensely cooked	cell and organelle membrane damage	carotene−protein complexes affected	pronounced pectin solubilization	cell separation	high	high

[a] Main mode of particle size reduction.

For the 25 min cooked carrots, the relation between the particle size and the *all-E-β*-carotene bioaccessibility differed from what was observed for the raw and gently cooked carrots. A high *all-E-β*-carotene bioaccessibility was detected for all carrot tissue particle sizes, and hence no specific dependency of the *β*-carotene bioaccessibility on the particle size was observed (**Figure 1**). The relative bioaccessibility values (ratio of bioaccessibility to concentration (%), **Figure 1B**) showed the same trend.

It is known that both the location and the physical state of carotenoids in the cell considerably determine their bioaccessibility (*5*). In the case of carrots, *β*-carotene can be present as "carotene crystals" (free crystals from the carrot chromoplasts) and "carotene bodies" (the native membrane-bound carotene structure in carrot chromoplasts). The chromoplast itself is the organelle containing the crystalline *β*-carotene, which can form complexes with proteins (*24*, *25*). Furthermore, it has been indicated several times that plant cell walls are limiting factors for nutrient or lipid bioaccessibility (*21*−*23*). Hence, structural changes occurring during thermal and mechanical processing of carrots (**Table 1**) can explain the observed changes in *all-E-β*-carotene bioaccessibility.

For raw carrots, mixing/blending causes cell breakage (*26*), resulting in a good release of intracellular located *β*-carotene for the fraction containing particles smaller than a carrot cell. Cell breakage is thus a prerequisite for a high *β*-carotene bioaccessibility. However, in raw, ruptured carrot cell fragments, *β*-carotene might still be encapsulated in the carrot chromoplasts. For raw carrot cell clusters, digestive enzymes can only access *β*-carotene in broken carrot cells at the outside of a cluster, explaining the low *β*-carotene bioaccessibility observed for the large fractions.

For the gently cooked carrots, it is assumed that the extent of cell breakage still dominates on the extent of cell separation upon mixing/blending. However, mild heating will affect cell and organelle membranes (*27*), leading to a better interaction between digestive enzymes and *β*-carotene containing structures compared to the raw samples. Moreover, the carotene−protein interactions can be disrupted, which favors the release of *β*-carotene (*28*). These factors may explain the very high relative *all-E-β*-carotene bioaccessibility for the fraction containing particles smaller than a carrot cell. For particles larger than a cell, the *all-E-β*-carotene bioaccessibility is low, but higher than for the raw carrots due to the additional effect of the mild heat treatment on cell and organelle membranes and on carotene−protein complexes.

Intense heating causes cell separation (*23*). As a consequence, only limited cell breakage will happen upon mixing/blending of intensely cooked carrot samples, which might suggest that the release of *β*-carotene will be obstructed (*22*). However, due to pectin solubilization as a result of *β*-eliminative pectin degradation during intense heating (*29*, *30*), the porosity of the cell wall is strongly affected (*23*), providing good contact between digestive

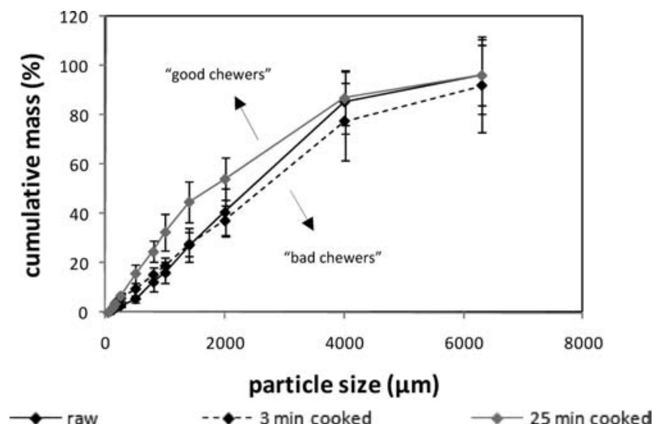

Figure 2. Average cumulative mass of particle size fractions (± standard deviation) for the three carrot samples (raw, gently cooked and intensely cooked) after human mastication. The technique of wet sieving was used to separate the different fractions. Based on the study of Fontijn-Tekamp et al. (*33*), regions where the mastication curves for "good chewers" and "bad chewers" can be found are indicated.

enzymes and *β*-carotene containing structures, both for larger and smaller particles. Hence, in the case of intensely cooked carrots, it can be stated that carrot tissue particle size is not a key factor determining the *all-E-β*-carotene bioaccessibility. It should be noted however that pronounced leaching of pectin during intense heating may enhance the inhibitory effect of fiber as explained by Rich et al. (*25*). Our hypothesis is that this justifies why the relative *all-E-β*-carotene bioaccessibility was detected to be only limited higher than the relative *all-E-β*-carotene bioaccessibility in the raw carrots and much lower than the relative *all-E-β*-carotene bioaccessibility in the 3 min cooked carrots for the fraction containing particles smaller than 125 *μ*m (**Figure 1B**).

Summarizing, it can be concluded that the extent to which the carrot pieces are thermally processed affects the importance of the carrot tissue particle size in determining the *all-E-β*-carotene bioaccessibility. Moreover, as highlighted by Stahl et al. (*5*), the release of nutrients was shown to be dependent on the mode of particle size reduction (cell separation or cell breakage).

Part 2. Particle Size Reduction by Human Mastication and How To Mimic This Step during *in Vitro* Digestion. In **Figure 2**, the average particle size distribution curves for the three carrot samples (raw, gently cooked and intensely cooked carrots) chewed by humans (mastication curves) are shown. It can be observed that the average mastication curves are very similar for carrot samples having different textural/structural characteristics. This suggests that the average chewing behavior of humans is only very limited influenced by the textural/structural quality of carrots, which is somehow in contrast with the conclusion of

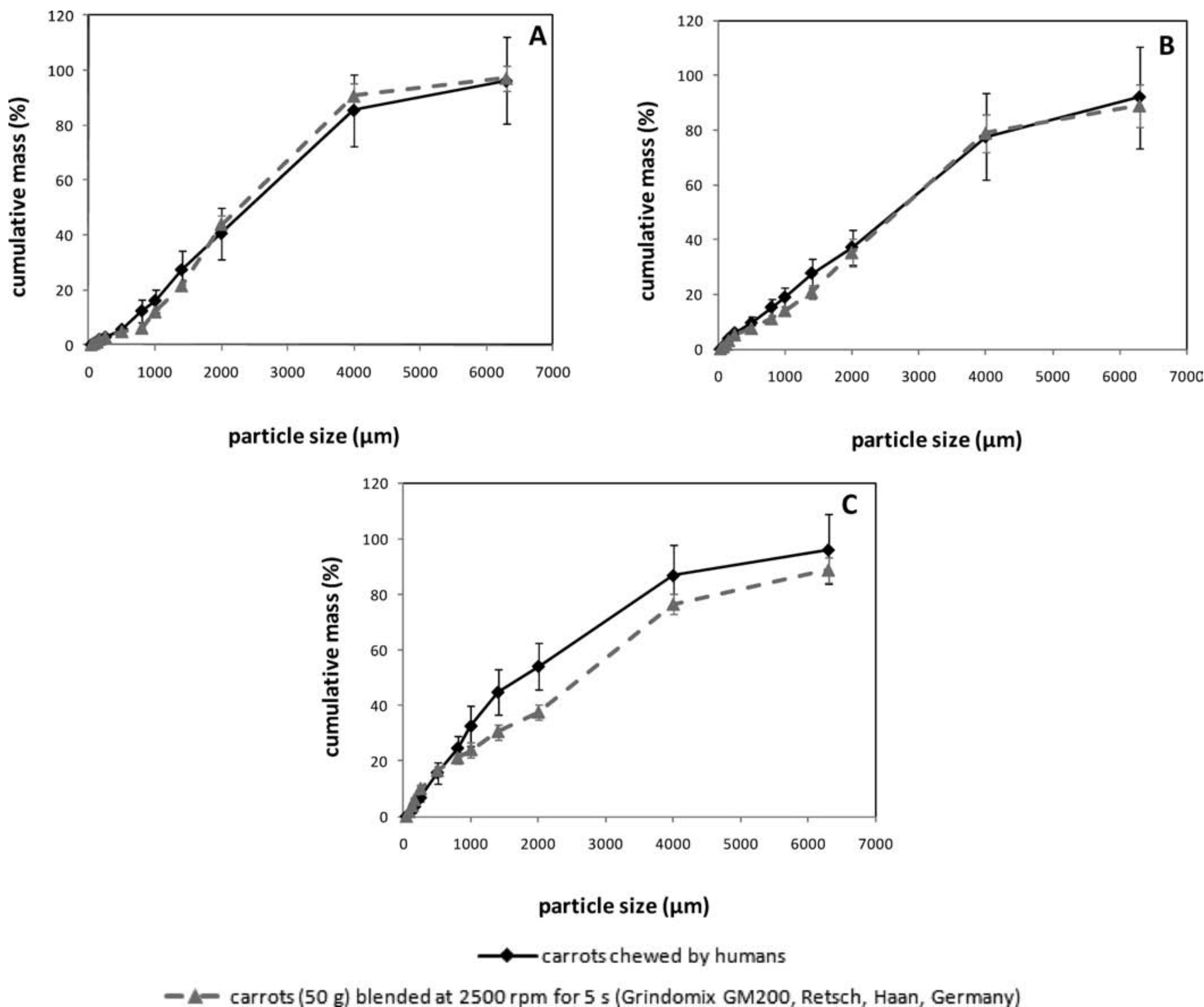

Figure 3. Average cumulative mass of particle size fractions (± standard deviation) for the three carrot samples after human mastication and after *in vitro* mastication (= blended at 2500 rpm for 5 s using a Grindomix GM200). The technique of wet sieving was used to separate the different fractions. **A** = raw, **B** = 3 min cooked, **C** = 25 min cooked.

Hoebler et al. (*31*), stating that mastication strongly depends on food texture. However, the latter study compared the mastication curves of different food products (bread, spaghetti and tortiglioni) having a similar chemical composition but a different texture. Comparing mastication curves of different food products, although having the same chemical composition, might explain why texture is indicated as being a key factor in their study, while our results showed only limited dependence of the chewing behavior on the texture for a particular food matrix.

For most subjects, the particle size distribution curves had the same shape, which is in agreement with the results of Jalabert-Malbos et al. (*32*). Nevertheless, an intersubject variability could be observed: the mastication curves of some subjects were clearly below/above the average mastication curve, referring to respectively "bad chewers" and "good chewers" (*33*) (**Figure 2**), while the chewing pattern of other subjects was clearly "on average". For most consumers, a personal chewing behavior (i.e., "good chewer", "bad chewer" or "average chewer") could be recognized that was independent of the sample texture.

In order to identify a blending technique that could imitate the particle size reduction during human mastication, the particle size distribution curves of chewed and mixed/blended carrot samples were compared and a suitable technique could be identified. In **Figure 3**, the average mastication curves and the average particle size distribution curves obtained when blending 50 g of carrot pieces at 2500 rpm for 5 s (Grindomix GM200, Retsch, Haan, Germany) are compared. For the raw (**Figure 3A**) and the 3 min cooked carrots (**Figure 3B**), this blending technique was found to result in a good correspondence between actual and simulated chewing based on the particle size distribution. For the 25 min cooked carrots (**Figure 3C**), blending the carrot pieces with the selected blending technique resulted in somewhat larger particles compared to the particles obtained by actual human chewing. However, in the first part of this work, it was shown that the influence of particle size on the *all-E-β*-carotene bioaccessibility is rather limited for intensely cooked carrots (**Figure 1**). Hence, it can be expected that small differences in particle size distribution between actual and simulated chewing will only lead to limited or no differences in the overall *all-E-β*-carotene bioaccessibility. Therefore, it is suggested that the selected blending technique (50 g carrot pieces, 5 s, 2500 rpm, Grindomix) is suitable to mimic human mastication *in vitro* in an accurate way for this case study on carrots.

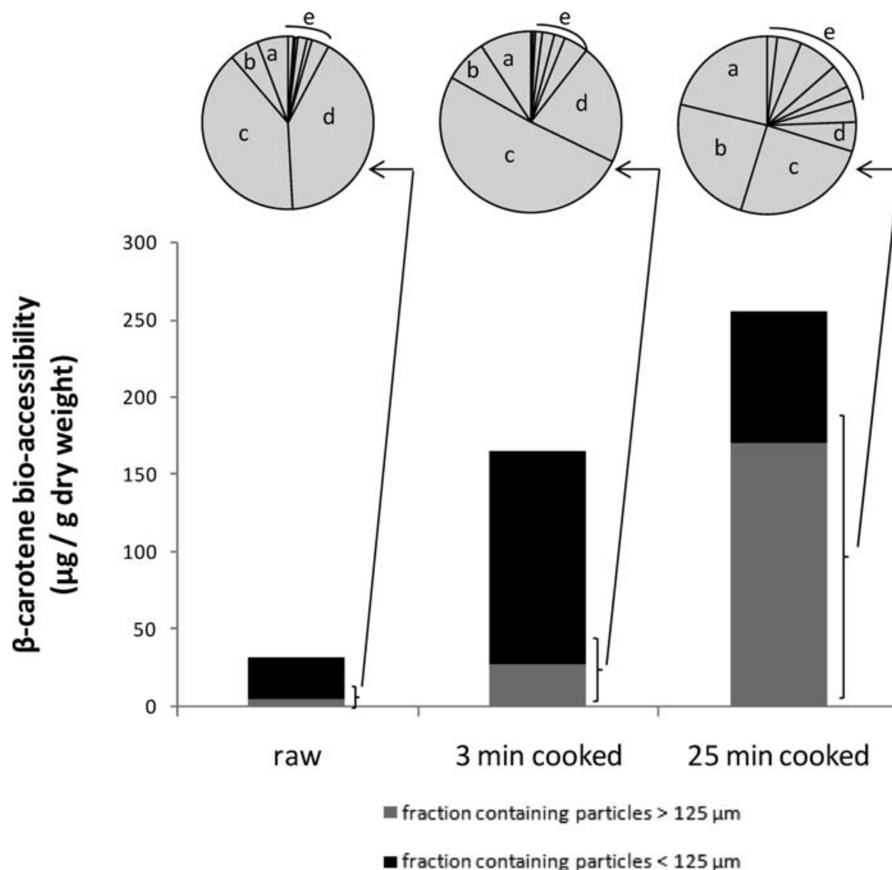

Figure 4. Contribution of the fractions with different particle sizes to the overall *all-E-β*-carotene bioaccessibility (μg/g dry weight) for raw carrots, 3 min cooked carrots and 25 min cooked carrots (with $a > 6301$ μm; 4001 μm $< b <$ 6300 μm; 2001 μm $< c <$ 4000 μm; 1401 μm $< d <$ 2000 μm; 126 μm $< e <$ 1400 μm). Contributions are predictions based on (i) the amount of the different fractions obtained after blending (*in vitro* masticating) raw, gently and intensely cooked carrots and (ii) the relation between the β-carotene bioaccessibility of each fraction and the carrot tissue particle size as determined during the first part of this study (see **Figure 1**).

To validate the usefulness of the identified blending technique, the overall *all-E-β*-carotene bioaccessibility was determined for the three carrot samples using the *in vitro* digestion procedure including the selected blending technique as an imitation of the chewing step. Respectively 34.9 (±2.1), 175.6 (±17.9) and 179.7 (±53.6) μg of *all-E-β*-carotene per g of dry matter was bioaccessible in the raw, gently cooked and intensely cooked carrots (**Figure 4**). The results obtained were compared with values predicted based on the particle size distribution after blending (i.e., *in vitro* mastication) and the link between the carrot tissue particle size and the *all-E-β*-carotene bioaccessibility (results obtained in the first part of the current study, **Figure 1**). In this way, *all-E-β*-carotene bioaccessibility was predicted to be respectively 31.5, 163.8, and 254.7 μg per g of dry matter for the raw, gently cooked and intensely cooked carrot samples. The experimentally determined *all-E-β*-carotene bioaccessibility data are thus in good agreement with the *all-E-β*-carotene bioaccessibility values predicted based on the particle size distribution of the blended samples. Generally, the results have proven that the approach suggested in this investigation, i.e. (i) relating the carrot tissue particle size to the *all-E-β*-carotene bioaccessibility, (ii) identifying the average particle size distribution after human chewing, (iii) simulating the average mastication curve *in vitro* by a blending technique and finally (iiii) including the selected blending technique in the *in vitro* digestion procedure, is a good starting point for standardizing the imitation of the chewing step *in vitro*.

The *all-E-β*-carotene bioaccessibility values predicted based on the particle size distribution of the blended samples and the relation between particle size and bioaccessibility also allow evaluating the role of both thermal and mechanical processing (**Figure 4**). Thermal processing has a large, increasing effect on the overall *all-E-β*-carotene bioaccessibility, but surprisingly, *all-E-β*-carotene bioaccessibility values for gently and intensely cooked carrots were quite similar. Although changing the intensity of the thermal process did not markedly affect overall bioaccessibility, it clearly affected the contribution of different particle size fractions to the overall bioaccessibility. Whereas carrot tissue fragments smaller than a cell contributed to a large extent to the overall bioaccessibility for gently cooked carrots (this was also the case for raw carrots), the fractions containing cell clusters fulfilled a more important role in the bioaccessibility of intensely cooked carrots. Hereby, the contribution of specific particle size fractions to the overall *all-E-β*-carotene bioaccessibility is mainly dependent on two factors: on the one hand, the amount of a fraction with a particular particle size is important, while on the other hand, the absolute bioaccessibility in particles of that particular fraction (as determined in the first experimental part, **Figure 1**) should be considered. The high *all-E-β*-carotene bioaccessibility in some subfractions of both the raw and gently cooked carrots (fractions containing particles smaller than a carrot cell) is the main reason for the high contribution of these small fractions to the overall bioaccessibility. On the contrary, the high amount of particles larger than 1400 μm (having a moderate bioaccessibility) after blending intensely cooked carrots is the main reason why particles larger than 125 μm fulfilled a more important role in determining the overall bioaccessibility of intensely cooked carrots.

In conclusion, this case study on carrots clearly emphasized the importance of both mechanical and thermal processing of carrots in determining the *all-E-β*-carotene bioaccessibility. The results also proved the importance of (*in vitro*) mastication as a particle size reduction technique and allowed identifying a mixing technique to mimic the mastication step as part of *in vitro* digestion of carrot samples. In order to fine-tune the oral phase of *in vitro* digestion of other solid food products based on scientific knowledge, the approach implemented in this work is proven to be a good starting point.

The strong dependency of the *all-E-β*-carotene bioaccessibility on the particle size for raw and gently cooked carrots clearly showed the importance of cell wall rupture to release a substantial amount of lipophilic nutrients from unprocessed and gently processed plant material. Results of the present work also demonstrated that human mastication could not compensate for the low *all-E-β*-carotene bioaccessibility in raw carrot pieces. Mechanical unit operations such as juicing and blending, disrupting the tissue to fractions smaller than a carrot cell, are thus essential in this context. After intense thermal treatment, a considerable amount of *all-E-β*-carotene could be released from all carrot particles including cell fragments smaller than a carrot cell as well as small and large cell clusters. The contribution of mastication to the high *all-E-β*-carotene bioaccessibility of intensely cooked carrots is thus less essential.

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