Chapter

Carotenoids in Thermal Adaptation of Plants and Animals

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Abstract

The support of carotenoids in photosynthesis is well documented. However, what is their role in parts of plants where there is no photosynthesis such as in fruits or stems or even in parts which are not exposed to the light at all, such as seeds or roots? Why are carotenoids essential for all animals and humans and present in almost every tissue in their body? The answer is that carotenoids can make complexes with lipids, which results in an increase of lipid thermal energy absorption and a reduction of viscosity. These changes help to expand the temperature range for the functionality of lipid structures, improve the capacity of thermal homeostasis and support adaptation and survival of living species to environmental stress and in particular to temperature variations. Working as "thermal antennas" carotenoids can increase lipid thermal energy conductivity, heat storage and heat retaining capacity. This, on the one hand, can reduce the freezing/melting points of plant and animal lipids and makes carotenoids work as antifreezers in microorganisms, plants or ectothermic animals. On the other hand, the thermal antennas can help absorb, transmit and accumulate external thermal energy essential to activate and support cellular metabolism. In addition, we describe how these properties of carotenoids can affect lipid parameters in nutrition, physiology and pathology.

Keywords: carotenoids, complex with lipids, thermal adaptation, heat storage and conductivity, lipid viscosity, cholesterol crystals, atherosclerosis, calorie intake

1. Introduction

Carotenoids are tetraterpenoid molecules, which play an important role in the photosynthesis of bacteria, algae, fungi and plants. However, these molecules may have other not always clear roles because they could be synthesised in fruit chromoplasts or in plant roots, which are not involved in light-dependent energy conversion. In animals and humans, who can get carotenoids only with food, these molecules have an additional functionality, for example, in the retina they facilitate filtration of light with different wavelengths.

Apart from light-harvesting and antioxidant activities, carotenoids can modulate lipid phase transition in cellular membranes, which affects their fluidity, polarity and, consequently, their functional properties [1, 2]. In contrast to membrane biology,

the role of carotenoids in non-membrane lipid structures, such as lipid droplets or circulating lipoproteins, remains unclear. Only one property of these molecules is well documented, that they are hydrophobic antioxidants and can effectively protect lipoproteins and other lipids from free radical oxidation, which typically occurs in different pathological conditions [3, 4].

In this paper, based on our observations, we suggest that carotenoids may have another biological role. By creating thermodynamically favourable complexes with lipids, they can work as "thermal antennas" facilitating thermal energy absorption and conductivity, increase heat storage and heat retaining capacity and distribution within the lipid matrix of cellular and tissue structures. The reduction of lipid viscosity by carotenoids not only improves the control of thermogenesis but also increases oxygen solubility in lipid extra- and intra-cellular structures and boosts plasma lipoprotein oxygen transport, activation of mitochondria and tissue respiration.

2. Materials and methods

In this paper, we present the results of our experiments on the interaction of different carotenoids and lipids *in vitro*, *ex vivo* and *in vivo.* In these experiments, we tested the representatives of two main groups of carotenoids: highly hydrophobic carotenes—lycopene and β-carotene, and less hydrophobic ones, containing oxygen xanthophylls—lutein, zeaxanthin and astaxanthin. Since in most of the experiments interaction of different carotenoids and different lipids led, in principle, to similar patterns or trends, and due to the limitation of the space in this paper, we present here as an example only representative data from each set of undertaken experiments.

3. Product preparation

3.1 Saturated predominantly long-chain fatty acids—dairy butter

546 mg of lycopene (Lyc-O-Mato, LycoRed) was blended into molten 2340 g of unsalted dairy butter (President, France) at 45°C. The blend was stirred thoroughly for 10 min to ensure an even mixture, which was afterwards dispensed into 30 g individual moulds/containers by pouring. Each 30-g butter sample contained 7-mg lycopene. Storage in sealed containers was at –20°C up to 3 months and at +4–8°C for no more than 1 month.

3.2 Monounsaturated and polyunsaturated fatty acids—vegetable oils

70 mg of lycopene was blended into a 50 mL volume of olive oil (Extra Virgin, Napolitana) or sunflower oil (Flora). Once dispersed, carotenoid oil samples were stored at the ambient temperature of 20–21°C away from light.

3.3 Lycopene supplement formulation for clinical trial

Preparation of highly bioavailable GA lycopene was described in our earlier publication [5].

4. Clinical trial

4.1 Study design

The studies were conducted at the Institute of Cardiology, the Ministry of Health of the Russian Federation (Saratov, RF). The protocol was approved by the local ethics committee. All patients were informed about the purpose of the study and had given written consent regarding their participation in the study.

Two-group studies were executed in a parallel, double-blind, controlled fashion for 4–8 weeks. Subjects visited the clinic for anthropometric and blood pressure measurements and for drawing blood samples. During the first visit to the clinic, subjects received an aliquot of study product sufficient for the first two study weeks. Then, subjects were provided with additional 14-day aliquots of study product during visits to the clinic after every two follow-up weeks.

Of the three-group or multi-group studies, two intervention groups were run at the same time, followed by the other group(s), also two at the same time.

4.2 Selection criteria

Study subjects were selected from the pool of patients based on the criteria for oxidative stress and having low-to-moderate hyperlipidemia.

The criterion for hyperlipidemia would be applied in most studies to facilitate the study of subjects with increased cardiovascular risk. Subjects were, nevertheless, not under medical treatment for the management of cardiovascular risk factors.

4.2.1 Inclusion criteria

- Caucasian male or female subjects 47–69 years old
- C-reactive protein (CRP) > 6 μg/mL (*if applicable)*
- Malondialdehyde > 40 μM/mL (*if applicable)*
- Untreated low-to-moderate hyperlipidemia (total cholesterol (TC) ≥ 250 mg/dL and low-density cholesterol (LDL) ≥ 150 mg/dL.)
- Willing to sign to informed consent
- Non-smokers or moderate smokers (≤ 10 cigarettes daily)
- No anti-hypertensive, hypoglycemic, lipid-lowering or any other cardiovascular drugs
- Willingness and ability to comply with the protocol for the duration of the trial.

4.2.2 Exclusion criteria

- Unwillingness to sign to informed consent
- Unable to comply with the protocol for the duration of the trial

- Significant medical condition that would impact safety considerations
- Significantly elevated liver-specific enzymes, hepatitis, severe dermatitis, uncontrolled diabetes, cancer, severe gastrointestinal disease, fibromyalgia, renal failure, recent cerebrovascular accident (CVA), pancreatitis, respiratory diseases and epilepsy
- Compulsive alcohol abuse (> 10 drinks weekly), or regular exposure to other substances of abuse
- Participation in other nutritional or pharmaceutical studies
- Positive test for tuberculosis, human immunodeficiency virus (HIV) or Hepatitis B.

4.3 Experimental procedure

Recruited study subjects were non-randomised into two intervention groups: a lycopene group (n=10) and a placebo group (n=10). This non-randomised method was chosen to ascertain the similarity of subject characteristics between study groups with respect to age, gender distribution and total cholesterol. Because the experimental groups were small (n=8–10), randomisation would likely increase the risk of variable group composition.

The Principal Investigator was provided with coded study product, according to the number of subjects in intervention(s) and control, and allocated an equal number of study subjects to either study product such that each group (corresponding to the same product code) matches the other group in numbers and demographic and clinical variables (as explained above). The Principal Investigator was blinded to which codes represented active or control product.

Prior to the start of the study, subjects were instructed to refrain from the consumption of dairy butter and tomato-based products for 10 days before beginning the trial and for the duration of the study. At the start of the study, following the end of the 10-day run-in period, blood samples were drawn and subjects were checked for meeting inclusion criteria. Then, subjects were distributed with a 2-week supply of the study product. Anthropometric measurements and blood pressure were taken and blood samples drawn.

All volunteers were instructed to ingest one sample of dairy butter once a day with the main meal.

All blood parameters were measured in the morning between 8 and 10 am.

Blood was collected in the morning after an overnight fast from arm veins of the volunteers. Serum was separated from the clotted mass by centrifugation and aliquots were stored at –80°C prior to analysis.

4.4 Compliance

All volunteers were instructed to ingest dairy butter sample once a day with the main meal. All volunteers were instructed to keep their butter packaging and bring it to the following clinical visit. After the verification of compliance, the packaging was exchanged for a fresh 14-day supply of products.

5. Methods

5.1 Microscopy—product samples

Microscopy was used to measure the size of lipid droplets and fat globules. For the visualisation of lipid droplets and fat globules and the quantification of their size, a binocular microscope Olympus BX41 was used with Cell^B software for morphometric analysis. All the parameters were collected from 10 randomly selected microscopic fields of 800 μ^2 at ×1000).

5.2 Microscopy—clinical samples

Typical skin smear samples were collected before the supplementation and 4 weeks after [5]. The duplicates of the specimens were fixed, stained and analysed: the first one for lycopene with fluorescein isothiocyanate conjugated monoclonal mouse antibodies, at 200× magnification, and the second for lipid droplets of the sebum with Oil Red O, at 1000× magnification, as described previously [6].

5.3 Melting and defrosting time

Approximately 20 mg of butter, or pork fat, or beef fat, or cocoa butter with or without certain concentrations of carotenoids, were placed on the surface of the laboratory slides and incubated at 37°C in a laboratory incubator (TLK39) until they melted.

To determine defrosting time, frozen oil aliquots of 200 mL were incubated at an ambient temperature of 20±2°C until they were completely melted. The period of time required for the tested sample to be melted or defrosted was measured with a laboratory timer (QUANTUM).

5.4 Cooking chicken breast

Chicken breast samples of 150 g were used. Before cooking, each sample was doused either in the pure olive oil (control) or in oil with a carotenoid. The oven was pre-heated to 275°C. A probe thermometer with a digital reading was placed in the thickest point of the sample. The sample was then placed in the oven and a reading taken and recorded every 300 sec for the 900-sec duration of the cooking.

5.5 Cooking salmon

Wild pacific Keta salmon fillets, 115 g each, were oven-cooked in individual small aluminium foil containers. The following preparation conditions were applied before cooking: either addition of 25 mL of the olive oil containing Lycopene 0.23 mg/mL) or control—addition of 25 mL of pure olive oil. Salmon fillets were turned several times making sure that they are completely covered with the added oil. After that, 500 mg of salt and 5 mL of fresh lemon juice were applied on the surface of each portion of fish. During the cooking process, the measurement of the internal (doneness) temperature of the fish was performed using a digital thermometer at the following time points: 8 min, 12 min, 16 min, and 20 min. All the containers had to be taken out of the oven for temperature measurements; hence, only the time when the fish was in the oven was counted as the cooking time. Once the internal temperature of the fish

reached 62°C (doneness temperature for salmon), a small (about 1 g) fragment of fish was immediately taken from the relevant piece (using a scalpel blade and thumb forceps) and placed in a 15-mL laboratory tube containing 1 mL of distilled water. An additional sample of fish was also taken before cooking.

5.6 Salmon sample preparation

Samples were transferred to the laboratory and weighed using analytical scales (Discovery DV114C, OHAUS Corp.). Distilled water was added to each sample apart from the 'juice' and 'sauce' samples to provide the ratio of 9 mL of water per 1 g of sample (these samples were regarded as 1/10 dilutions). 1 mL of distilled water was added to the 'juice' and 'sauce' samples to produce 1/2 dilutions. Following this step, all the samples were homogenised using IKA T10 basic Ultra-Turrax homogeniser system at maximum speed (30,000 RPM). After the homogenisation of each sample, the homogeniser was disassembled, and both its rotor and stator were carefully cleaned in order to prevent sample cross-contamination. Following homogenisation, second dilutions with four volumes of distilled water (1-mL homogenate + 4 mL of water) were prepared from the homogenates of the raw fish, fish cooked with water and fish cooked with either pure olive oil, or containing lycopene, or astaxanthin (resulting in 50× dilutions). Similar dilutions were made from salmon 'juice' and 'sauce' samples described above (resulting in 10× dilutions). Finally, additional fivefold dilutions were made from the 50× dilutions of homogenates prepared from all salmon samples (resulting in 250× dilutions).

5.7 Vitamin B12 concentration determination

All samples were analysed within 48 hours following cooking experiments. Vitamin B12 BioAssay™ ELISA Kit (US Biological) was used for Vitamin B12 concentration determination. Vitamin B12 concentrations in the samples from the cooking experiment were determined in both undiluted supernatants (1 g of liver + 19 mL of buffer) and dilutions 1/2, 1/4 and 1/8 (the latter only for the samples cooked without lemon juice). The dilutions were prepared using sample dilution buffer (phosphate-buffered saline—PBS) supplied with the kit. B12 concentration evaluation was performed in 50 μL of solution according to the protocol provided with the kit.

Vitamin B12 concentration determination was performed using Multiscan FC microplate photometer (Thermo Fisher Scientific) by measuring optical light absorbance at 450 nm (reference wavelength 620 nm) as recommended by the kit manufacturer. All the calibration standards were measured in duplicates. Measurement results were analysed using SkanIt software for Multiscan FC system (a four-parameter logistic algorithm was applied). Vitamin B12 concentrations in the original samples were obtained by re-calculation taking into account sample dilutions during material processing. Once all the measurements were completed, the results of the two cooking experiments were combined by taking average concentration value for each set of conditions.

5.8 Formation of cholesterol crystals

A solution of 198 mL of 1 g of cholesterol (Sigma) in 99% ethanol was divided in two equal parts. In one part, 1 mL of ethanol containing 100 μg of the dissolved lycopene was added. This provided a ratio of lycopene to cholesterol as $1:10^6$.

Into the other part, the control, 1 mL of the ethanol itself was added.

Then, both the samples were left in a dark room for evaporation under 20–22°C.

Recording of the status of both the samples was made at least daily or at even shorter intervals.

5.9 Disassembly of cholesterol crystals

20 mL of ethanol solutions with different concentrations of lycopene were added to 100 mg of crystallised cholesterol. As a control, 20 mL of the same ethanol was used but without any lycopene. After gentle stirring for a couple of minutes, at the room temperature of about 20–22°C, recording of the results was made.

5.10 Ex vivo experiment with cholesterol crystals in the arterial wall

It was important to try to check whether carotenoids, and lycopene in particular, could affect the folding of cholesterol crystals, which are not just synthesised by a manufacturer, but produced naturally, and particularly those, which are developed during pathological process in human. For this purpose, we used pieces of atherosclerotic abdominal aorta, which were obtained, during a combined vascular graft and bypass surgery.

Comparable types of atherosclerotic lesions were collected. This was in terms of their stages of development, with prominently featured cholesterol crystals of similar size, embedded into the atheromatous tissues of the aorta wall. As a control material, we collected the pieces of atherosclerotic abdominal aorta containing calcium phosphate crystals.

First, an ethanol solution 1 μg/mL of lycopene was prepared. It was then diluted by PBS 10-fold. As a control, solution was made with the same ratio ethanol to PBS but without lycopene. Then, the pieces of aorta were incubated, in light-protected containers at a room temperature of about 20–22°C for 13 days.

5.11 Clinical parameters

The body mass index (BMI) of the participants was measured in the morning and calculated in kg/m^2. Pulse rate and systolic and diastolic blood pressure were measured three times in the left arm of the seated patient after 15 min of rest. The time between measurements was no less than 2 min. The mean number for each parameter was calculated.

All the body and vascular parameters were measured in the morning between 8 and 10 am.

5.12 Blood biochemistry

Biochemistry and inflammatory markers, glucose, total cholesterol (TC), triglycerides (TG), high-density cholesterol (HDL), low-density cholesterol (LDL), C-reactive protein (CRP) and oxidised LDL (LDL-Px) were measured using commercially available analytical kits according to manufacturers' instructions (BioSystems, Medac, R&D Systems). Inflammatory oxidative damage (IOD—malondialdehyde) was measured applying a colorimetric method [7].

5.13 Statistics

For the assessment of normally distributed parameters, the Shapiro-Wilkinson method was used. Student's *t*-test was then applied for both the paired and unpaired samples.

In cases where parameters were not normally distributed, the Mann-Whitney U test and the Kruskal-Wallis test were used.

The analysis of variance (ANOVA) and the analysis of covariance (ANCOVA) were used with post hoc analysis (Statistica9 suit, StatSoft; Inc.). Statistical significance between two-tailed parameters was considered to be $p<0.05$.

6. Results

6.1 Carotenoid-lipid interactions

The integration of carotenoids and lipids resulted in a number of changes in the light absorption spectrum of the former, subject to the specific nature of the interacting molecules. For example, there was a red shift in the visible part of absorption of lycopene when it was added into the sunflower oil (**Figure 1a**) or a hyperchromicity in the spectrum of lutein when it was added to the cocoa butter (**Figure 1b**). These changes indicate that the carotenoids were able to interact with the lipid molecules and created physical complexes with them, which would be thermodynamically more favourable than when all these molecules were present in free forms.

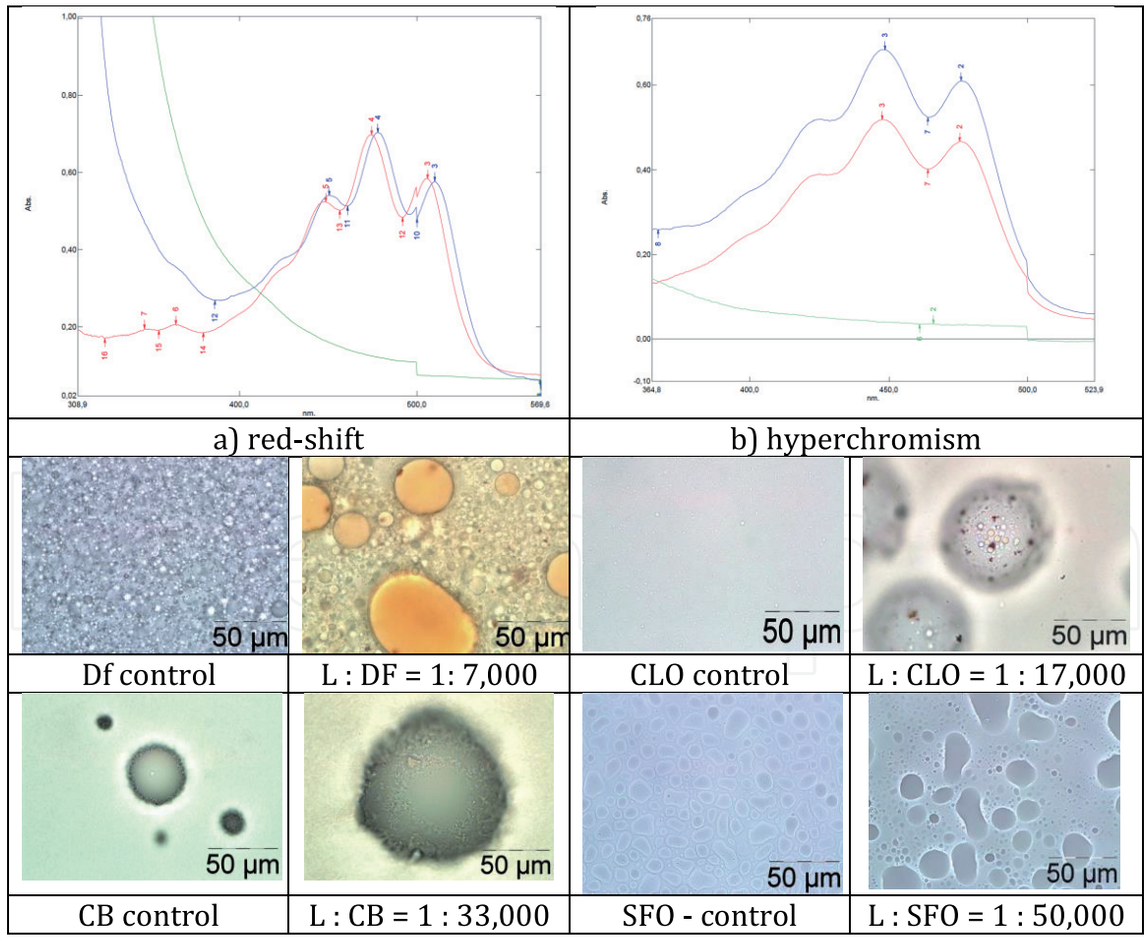


Figure 1.
Formation of thermodynamically favourable complexes between carotenoids and lipids. a) Red shift in light absorption peaks of lycopene embedded into sunflower oil, in 1:330 m/m—blue, control lycopene—red, control sunflower oil—green. b) Hyperchromism in light absorption peaks of lutein embedded into cocoa butter, in 1:330 m/m—blue, control lutein—red, control cocoa butter—green; in all experiments above, ethanol-methylchloride 5:1 w/w was used. Typical microscopy slides at 1000× magnification: DF—dairy fat, CLO—cod liver oil, CB—cocoa butter, SFO—sunflower oil, L—lycopene, m/m.

To visualise how formation of these complexes affects lipids and subsequent changes in their structures, we used light microscopy. It was found that the incorporation of carotenoids into oils or fats matrix may result in a dose-dependent growth of the size of lipid droplets or fat globules. This was observed in a broad range of plant or animal oils and fats. The working concentration at which a particular carotenoid could trigger this microscopy-visual effect was dependent on the structure of carotenoid molecules and on the length and saturation level of fatty acids (FA) in the lipids used. Overall plant lipids with a higher level of unsaturated FA were more sensitive to carotenoid-induced changes than animal lipids. Within the first group, vegetable oils with longer FA were more responsive than nut oils or cocoa butter. Within animal lipids, fish oils with a higher level of unsaturated and longer FA were more responsive than the bird fats, and they, in turn, were more sensitive than mammalian fats (**Figure 1**, microscopy slides).

For example, one molecule of lycopene was able to make noticeable changes in the size of the lipid droplets of the dairy fat/butter starting with a ratio 1:30,000 of molecules of its triglycerides. For the cod liver oil, this threshold was lower at one molecule of lycopene per about 40,000 molecules of the oil, for the cocoa butter, it was at 1 per 80,000 and for the sunflower oil, it was at 1 per about 120,000 molecules of their triglycerides.

6.2 Carotenoids reduce lipid viscosity *in vitro* and *in vivo*

The formation of complexes between carotenoids and lipids resulted in the reduction of their viscosity. Plant oils response was more significant than the animal fats. For example, one molecule of lycopene added to about 67,000 molecules of olive oil

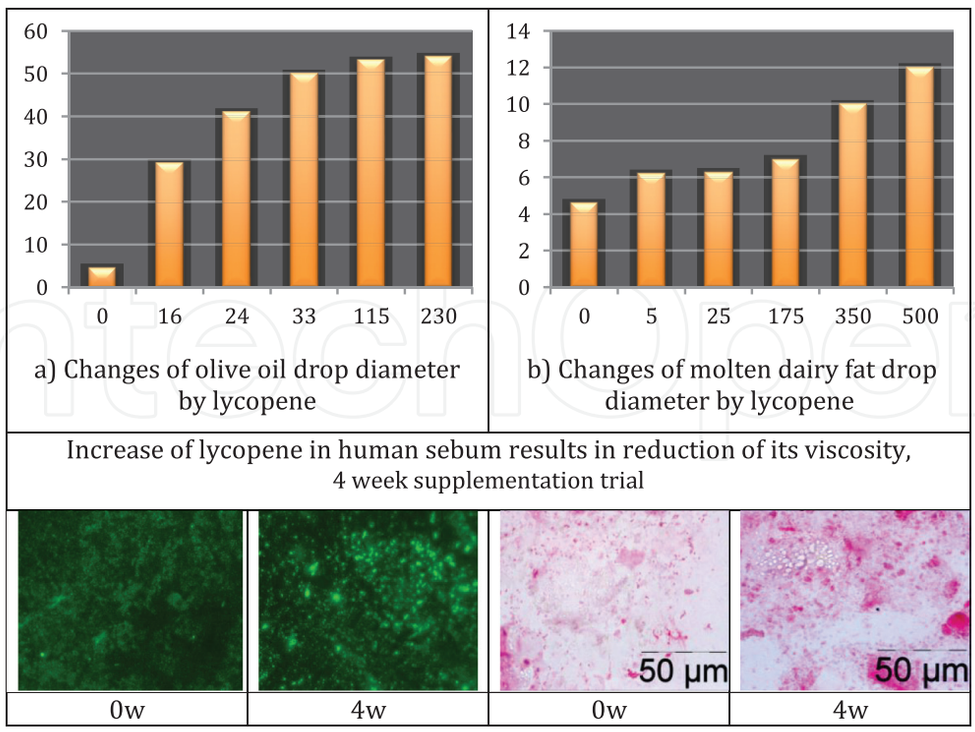


Figure 2.
Carotenoids reduce viscosity of lipids in vitro top slides and in vivo bottom slides. Viscosity was measured as a diameter, y-axis in μm, of a single drop of the oil on the surface of the water (a) or a single drop of the molten butter on the hard surface (b) at 37°C, before the experiment the butter and oil samples were warmed to that temperature too; x-axis—lycopene concentration in μ/g; each column is an average of three independent measurements. The microscopy of typical skin smear samples from 60-year-old clinically healthy man, collected before and 4 weeks after supplementation with 7 mg of highly bioavailable GA lycopene [5].

lipids could reduce viscosity of the oil, in terms of its drops spreadability, by 10-fold, or 1000%; when it was added to about a similar amount of dairy fat lipid molecules, the reduction of the viscosity was only by 50% (**Figure 2a** and **b**).

Carotenoids could change the viscosity of lipids not only when they were incorporated in their matrix *in vitro*, but *in vivo* as well. For example, when middle-aged persons, who were clinically healthy but had age-associated lycopene functional deficiency, were supplemented with this carotenoid for 4 weeks, the viscosity of their sebum, in terms of the diameters of the lipid droplets collected from the surface of the skin, was significantly increased (**Figure 2**, two slides on the bottom-right). The fact that these changes were not just associated with the lycopene intake but caused by its accumulating in these droplets was confirmed by the direct measurement of this carotenoid in the collected sebum (**Figure 2**, two slides on bottom-left).

6.3 Carotenoids increase thermal energy absorption, heat storage capacity, heat retention and thermoconductivity of lipids

The incorporation of carotenoids into plant or animal lipids increases their rate of thermal energy absorption, the amount and the time of this energy storage. For example in **Figure 3**, when the same level of heat was applied, the sunflower oil with carotenoids could start to accumulate this heat faster and become hotter by 5°C, and after the external heat was switched off, the retention of the heat lasted significantly longer.

The increase in lipid thermoconductivity by carotenoids was demonstrated in another set of experiments. For oils, liquid at room temperature, we froze them first and then measured this parameter in terms of time, which was necessary to defrost these oils. For lipids, solid at room temperature, we assessed thermoconductivity as a time, which was necessary to melt them at +37°C. In these experiments, carotenoid increase in thermoconductivity was more prominent in plant oils than in animal fats.

For example, the same concentration of lycopene, 330 μg/mL, could reduce the defrosting time of the olive oil by 12-fold, but the cod liver oil only by twofold (**Figure 4a** and **b**).

For lipids, solid at room temperature, the same as in above experiments, the concentration of lycopene could increase the melting time of cocoa butter by more than 10-fold, but for dairy fat by only threefold (**Figure 4c** and **d**).

This increase in thermoconductivity could also be observed in the heating not just in lipids but in their emulsions in water too. For example, lycopene in concentration

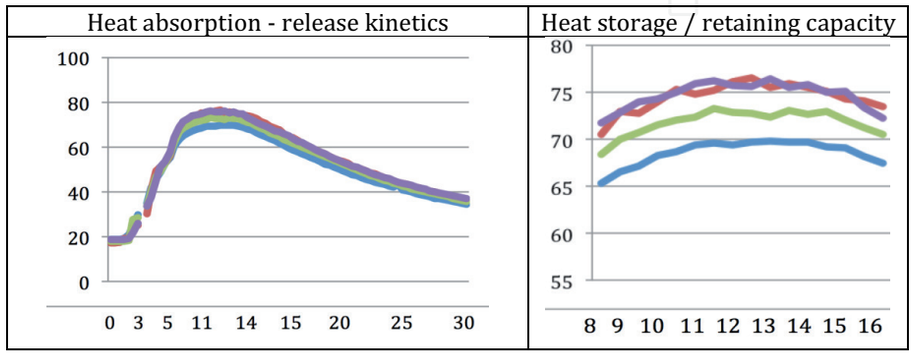


Figure 3.
Carotenoids increase the heat storage capacity of sunflower oil. Vertical axis—temperature t °C/mL, horizontal axis—time of the experiment, in min; blue—control oil, green—with 9.3-μM lycopene, purple—with 9.3-μM β-carotene, red—with18.6-μM astaxanthin.

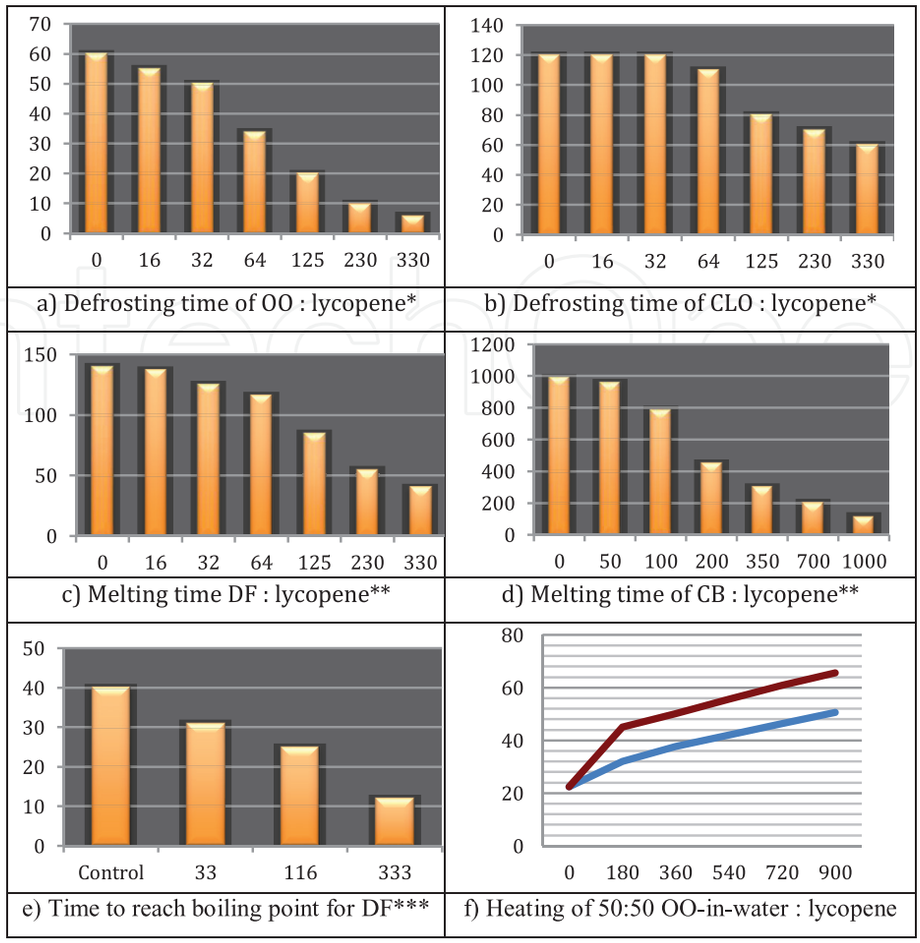


Figure 4.
Carotenoids increase thermal energy conductivity in different lipids and their emulsions. OO—olive oil, other abbreviations as in **Figure 1**. **Defrosting time from –30°C of frozen oils to room temperature, x-axis—lycopene concentration in μg/mL; in kerosene, astaxanthin and lycopene concentrations were 16μg/mL. **Melting time at +37°C to room temperature, x-axis in this and the following experiment—lycopene concentration in μg/g. ***Boling was at +350°C; in all the above experiments, y-axis is time in seconds, and each column is an average of three independent measurements. Kinetics of heating of OO-in-water emulsion, at heated 150°C stove, y-axis—temperature in °C, x-axis—time in seconds, blue line—control, red—with lycopene in 230 μg/mL.*

of 330 μg/g could accelerate the time to reach the boiling point for dairy butter by fourfold (**Figure 4e**). In another experiment, to reach a temperature from 20°C to +45°C for 50:50 olive-oil-in-water emulsion, with the same carotenoid in concentration of 230 μg/mL, took only 3 min, when for the control emulsion, it was 7 min. Moreover, carotenoids were not just able to increase the rate of heating but the maximum level of the temperature the emulsions could reach. In this particular experiment, the increase was by 14°C, from 51°C for the control emulsion to 65°C when lycopene was therein (**Figure 4f**).

6.4 Carotenoid increase in heat absorption of oils accelerates the cooking process and renders it healthier

Increase in thermoconductivity of oil doped by carotenoids may result in an accelerated cooking process. For example, to reach the 84°C temperature of completed cooking for a piece of chicken breast, coated in olive oil at an oven temperature of 180°C, took 15 min. When this oil contained lycopene or astaxanthin, in concentration 0.23 mg/mL, cooking took 13 and 10 min, respectively (**Figure 5**). For a fillet of wild salmon to

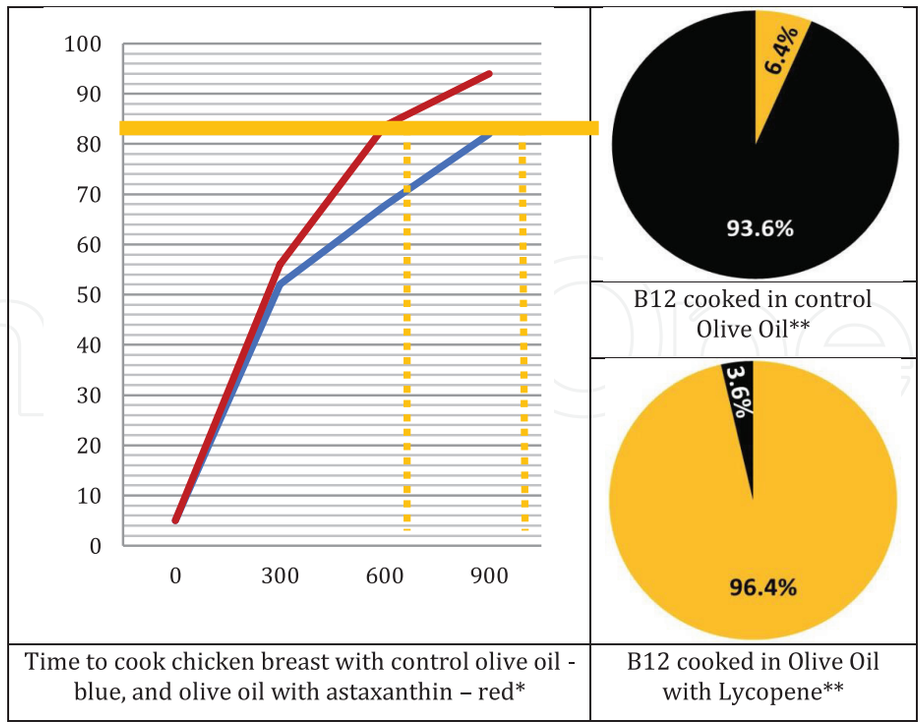


Figure 5.
*Carotenoids in olive oil accelerate cooking time of chicken breast and preserve vitamin B12 in cooked salmon. *Astaxanthin concentration was 0.23 mg/mL in olive oil; y-axis—temperature in °C, x-axis—time in seconds. **Yellow—content of preserved intact vitamins, black—lost vitamins.*

reach the temperature of completed cooking at 62°C, it took 9 min for the samples in the control oil and 7 or even 6 min for the oil with lycopene or astaxanthin, respectively.

The observed acceleration of the cooking process and, consequently, reduction in the cooking time may help in preservation in the cooked food of important thermosensitive macronutrients or vitamins. For example, in the above experiment, the concentration of vitamin D3 in the baked salmon was only about one-third of its precooked level. However, when oil contained astaxanthin or lycopene, the remaining level of this vitamin was significantly higher, more than 55 or 82%, respectively. In the same fish, samples of Vitamin B12 were more sensitive to the cooking temperature; by the end of the experiment, only about 6% of these molecules were detected there. Using the oil with astaxanthin did not make much difference, but with lycopene saved more than 96% of this vitamin in the cooked fish (**Figure 5**).

6.5 Carotenoids reduce the digestion rate of lipids and their absorption level

The industrial process of refining oil production removes all its ingredients, including carotenoids, which are originally present in freshly pressed plant oils and fats. As a result of this, the lipid droplets of these oils have higher viscosity, are significantly smaller, and have a faster digestibility rate leading to an increase in calorie release and absorption. As a result of this, refined vegetable oils alongside refined sugars are the main food sources of easily released calories, the main dietary factor contributing to the development of the global obesity pandemic.

The reintroduction of carotenoids to plant oils and fats increases the size of their droplets or globules (**Figure 1**). Consequently, the time of digestions of these lipids will increase, and subject to the ingested lipid volume, not all lipids would be digested and absorbed. This would lead to a reduction in the amount of the absorbed lipid in the postprandial blood.

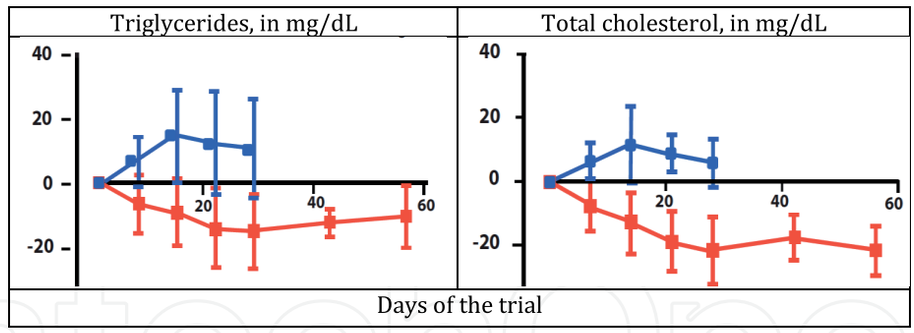


Figure 6.
Effect of daily ingesting of dairy butter with or without lycopene on the level of serum lipids in the fasting blood of volunteers. Blue—30 g of control dairy butter, red—30 g of the butter with 7-mg lycopene.

This was confirmed in a crossover clinical trial on healthy volunteers, who were asked to ingest different unmodified fat or fat-rich food products and, after one week's rest, ingest the same products but modified by carotenoids. Postprandial blood was analysed to assess the level and kinetics of the absorbed lipids. For example, after ingestion of 50 g of control dairy butter, area under the curve (AUC) for the first 4 hours for serum total cholesterol was 33±3.9 mg/dL and for triglycerides 25±2.7 mg/dL (n=10). However, the AUC after ingestion of the same amount of butter with 7 mg of lycopene and these parameters were reduced for cholesterol significantly to 22±2.5 mg/dL ($p<0.05$) and for triglycerides only as a trend to 19±2.2 mg/dL ($p>0.5$).

After repeating this experiment with 50 mL sunflower oil, with a slice of white bread, the effect of ingestion of lycopene-modified fat was even stronger. In the control experiment, the AUC for serum total cholesterol was 75±9.2 mg/dL and for triglycerides 56±6.8 mg/dL (n=10). The ingestion of the same amount of this oil but with 7 mg of lycopene resulted in a significant reduction of these parameters to 32±4.3 mg/dL ($p<0.01$) and 12±3.9 mg/dL ($p<0.001$).

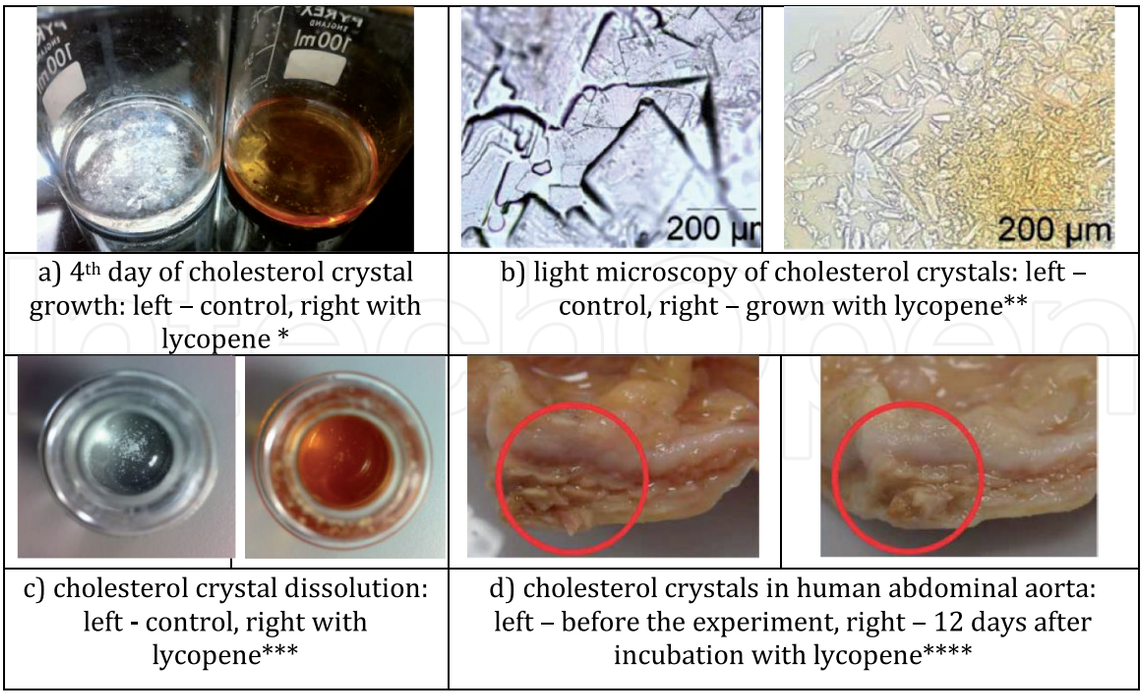
In the next set of experiments on clinically healthy persons, with borderline hyperlipidaemia, we demonstrated that regular, daily intake of dairy butter with incorporated lycopene could reduce serum triglycerides, total cholesterol and LDL (**Figure 6**).

The butter trial was planned for 2 months. However, its control group was terminated earlier on ethical grounds because there was a significant rise in both blood lipids. In the lycopene butter group, at the end of the trial, the reduction of triglycerides was by 10 mg/dL and total cholesterol by about 20 mg/mL.

The daily ingestion of lycopene chocolate resulted in a significant reduction of both lipids, while in the groups which ingested either the same amount of control chocolate or lycopene in a capsule, there were no changes in these parameters.

6.6 Carotenoids reduce the rate of formation cholesterol crystals and facilitate their dissolutions

The ability of carotenoids to create thermodynamically favourable complexes with lipids, which changes their crystalline properties, can also be observed on their interactions with cholesterol. It was observed that the addition of carotenoids could significantly reduce the rate of cholesterol crystallisation. For example, in the experiment described in the legend to **Figure 7a**, visible cholesterol crystals in the control solution started to appear in 24 hours from the start of the evaporation of the solvent. When lycopene was introduced in a ratio of 1:1000 molecules of cholesterol, it took five times longer before these crystals started to be observed.



a) 4th day of cholesterol crystal growth: left – control, right with lycopene *

b) light microscopy of cholesterol crystals: left – control, right – grown with lycopene**

c) cholesterol crystal dissolution: left - control, right with lycopene***

d) cholesterol crystals in human abdominal aorta: left – before the experiment, right – 12 days after incubation with lycopene****

Figure 7.
*Lycopene and cholesterol crystals: reduction of their growth rate (a), disruption of their folding (b) and facilitation of their dissolution in vitro (c) and ex vivo (d). *2.5 mM of cholesterol in ethanol without or with 2.5-μM lycopene were evaporate at room temperature in a dark room. **Molecular ratio lycopene to cholesterol 1:4000; ***5 mM of cholesterol in ethanol without or with 2.5-μM lycopene. ****Incubation at room temperature in a dark room in the PBS solution with pre-diluted in ethanol lycopene in concentration 0.1 μg/mL, with NaN_3 to prevent microbial growth.*

It was interesting that it was not just the reduction in the rate of crystallisation we observed but also a new type of crystals emerged: they were significantly smaller, and some had needle forms in contrast to the much bigger slab-shaped crystals of unmodified cholesterol (**Figure 7b**). These observations confirmed that that lycopene, like other carotenoids, could create physical complexes with this type of lipids, disrupt their folding, clusterisation and affect their crystal structures.

This ability of carotenoids to disrupt folding in already existing cholesterol crystals was observed in our *in vitro* and *ex vivo* experiments. For example, lycopene could dissolve or help ethanol to dissolve cholesterol crystals in a mole ratio of 1:4000 or below (**Figure 6c**). Lutein was able to do the same, although in a lower concentration range (data not presented).

In the *ex vivo* experiment, a piece of abdominal aorta with massive atherosclerotic lesions and a combination of cholesterol and calcium phosphate crystals were incubated in phosphate-buffered saline containing lycopene pre-dissolved in ethanol. After 12 days of this incubation, not only a substantial amount of fat deposits of atherosclerotic plaques was dissolved, but also the number and size of cholesterol crystals were significantly reduced (**Figure 7d**).

At the same time, this incubation did not affect either the number or the size of calcium crystals in this piece of aorta or cholesterol crystals from a similar type of atherosclerotic lesion (data not presented).

7. Discussion

Our experiments demonstrated that the interaction of carotenoids with lipids could result in the formation of their complexes, which were thermodynamically

more favourable than when these two groups of molecules were separate. As a result of this, carotenoids were better able to absorb the light energy in the longer wavelength of the red part of the spectrum.

The physical properties of lipids in these complexes were also changed due to a possible reduction in their cohesiveness between these molecules. For oil droplets or fat globules, this resulted in a reduction of their surface tension and, consequently, facilitated their fusion or enlargement. The larger the lipid droplets are, the less energy they have. Therefore, carotenoids can create complexes with lipids, which trigger their transition into a thermodynamically more favourable and stable phase. The fact that a single molecule of the carotenoid can affect the behaviour of 10,000 or 100,000 molecules of lipids may imply the possibility that the released energy, after formation of this type of complex, can dissipate beyond its physical location and cause a long-range transition of the lipid matrix, with consequent physical changes of its properties.

Lipid droplets with increased size would have less friction between each other, and the oils and fats became less viscous. Whether the reduction in the viscosity was a result of blending carotenoids in existing oil or fat products, or their incorporation into *de novo* assembled lipid structures, the effect in principle was the same. This was confirmed in the clinical trial, when the increase in lycopene concentration in the skin sebum resulted in the reduction of the viscosity of its droplets (**Figure 2**). These changes in sebum quality were similar to the observations on the supplementation of volunteers with other carotenoids such as lutein (results not presented here) and astaxanthin [6].

A significantly higher response in changes of the viscosity of plant lipids, over animal ones, could probably be either a result of their stronger interaction with carotenoids or easier spreadability of conformational changes in the plant lipids than in the animal fats. Or this can be a combination of both of these factors. Animals cannot synthesise carotenoids but plants can, and this is probably why the affinity to these molecules to other plant molecules, lipids, is higher than to animal ones.

The increased surface area of enlarged oil droplets or fat globules would have more surface-to-surface contacts with each other, which would facilitate the transfer of changing temperature between these particles. Whether there is a thawing of frozen liquid hydrocarbons, or melting of solid lipids, or their heating, the incorporation of carotenoids into these molecules accelerated temperature energy transfer within them.

Since one molecule of a carotenoid was able to facilitate temperature changes in 10,000, or 100,000 or more molecules of lipids, it is unclear whether this was due to changes in lipid molecule thermoconductivity, or carotenoids, in their complex with lipids, may work as 'thermal antennas', which could dissipate and distribute thermal radiation/energy far beyond the physical location of carotenoid-lipid complex.

The ability of carotenoids to increase the accumulation of thermal energy and its distribution was significantly higher for the plant lipids than for the fish oil, which, in turn, responded better than the mammalian fats. The reason for this effect of carotenoids on heat absorption gradient in different lipids could be the fact that plants are exposed to much broader variations in environmental temperature changes than ectotherms/poikilotherms like fish, when the body temperature of endotherms like mammals is constant.

The viscosity of lipids is the essential parameter, which controls cellular membrane permeability to electrolytes and nutrients, energy synthesis, cell growth and proliferation. One of the main factors determining the viscosity of lipids, and their ability to conduct the heat or the cool, is a lipid composition, a ratio of triglyceride saturated and unsaturated fatty acids, their length, other incorporated lipids, etc.

Our experiments indicate that carotenoids could have a new biological role not only to control viscosity of lipids but their thermal energy absorption, retention and conductivity too. If this is the case, this could be a much more efficient pathway to control these parameters. For a plant cell, to synthesise one molecule of a carotenoid, which can change the viscosity of 10,000 or even 100,000 molecules of lipids, would be much faster and more economic than to activate a lipid replacement process, which would involve a few hundred or thousand more new lipid molecules to be synthesised.

This possible new role of carotenoids as a factor facilitating adaptation to environmental, and in particular, temperature variation stresses, may explain a number of observations, which do not have clear explanations. What is the role of carotenoids, which are not involved in photosynthesis whether in a plant or in a light harvesting microorganism? What is the role of these molecules in parts of the plant where photosynthesis is not happening at all, like fruits or roots?

Since plants, or microorganisms such as algae, have exposure to much higher day-night, seasonal or other environmental temperature variability than animals, it is not surprising that the level of carotenoids in their tissue is 10^3–10^6 higher than in animals [8, 9]. Within animals, ectotherms, which do not have their own mechanism to control their temperature, rely more on the accumulation of ingested carotenoids than endotherms, which can maintain their thermal homeostasis. It is not surprising that in tissues of fish or reptiles, carotenoid concentration could be from 10- to 100-fold higher than in mammalians [10, 11].

The ability of carotenoids to work as antennas facilitating transmission and distribution of the thermal energy within lipid matrixes may find its practical applications in different industries. This carotenoid property may improve the performance of lipids, and possibly other hydrocarbons, when they are used for the production of greases, lubricants, liquid crystal devices, nanotubes, thermal energy storage, biodiesel and some other products, oils and fuels.

For example, carotenoids can accelerate the heating of oil or lipid-in-oil emulsions in general (**Figure 4f**), or when they used for cooking purposes in particular. This can shorten the time of the cooking process, save fuel, which is used to generate heating energy, and preserve more thermo-sensitive vitamins and micronutrients in the finished cooked meal (**Figure 5**). The correlation between the rate of acceleration of the heating of the cooking meal, in oils with different carotenoids, and the level of preserved thermo-sensitive vitamins was not always observed. This was probably due to additional antioxidant properties of carotenoids, which could contribute to preservation of these vitamins in the cooking process.

Another useful application of the ability of carotenoids to disrupt lipid folding would be to increase the size and reduce the viscosity of oil droplets and fat globules in food products. We demonstrated that the ingestion of dairy butter, vegetable oils and chocolate with enlarged lipid particles resulted not only in the reduction of the postprandial lipidaemia but also, if these products were regularly consumed, in the reduction of elevated fasting blood lipids. This means than carotenoids can be used to convert edible oils and fats into lipid lowering and weight management food products.

Perhaps, the culinary practice of cooking in oils/fats with lycopene-rich tomato sauce is a contributing factor as to why Italians are one of the slimiest nations in Europe and the USA [12, 13].

In our *in vitro* and *ex vivo* experiments, on the effect of lycopene and lutein of formation and dissolution of cholesterol crystals, we used a range of the ratio

between these molecules the same as is present in humans from 1:4000 to 1:1000. The observed ability of carotenoids to interact with cholesterol molecules, to reduce the rate of its crystallisation, may be used for the prevention or control of the growth of cholesterol crystals and/or for the treatment by facilitating the disassembly of the already formed crystals, which are responsible for the rupture of atherosclerotic plaques leading to heart attack or ischaemic stroke [14, 15].

To assess the industrial, nutritional and medical applicability of using carotenoids to affect lipid properties, to create new materials, food and health care products would require more work and expertise in different fields.

In conclusion, it should be said that the main objective of the presentation of the data in this paper is to illustrate the new phenomenon, its potential biological role and practical applicability. The main body of the backing/supporting experiments on different carotenoids, lipids, doses, conditions, products, clinical trial participants, etc., would be a subject of future separate publications.

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Conflict of interest

The author is a founder and director of Lycotec Ltd, the company that research and develop carotenoid based technologies.

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