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Isomerization of Dietary Lycopene during Assimilation and Transport in Plasma

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Diets of individuals were supplemented with tomatoes, either cooked or as tomato purée in order to compare uptake of lycopene from intact and homogenized fruit tissue matrices. Following a diet containing cooked tomatoes over three consecutive 7-day periods, little change in the carotenoid levels in plasma lipoproteins occurred. In contrast, a diet supplemented with concentrated tomato purée, over a 2 week period, caused a significant ($p < 0.05$) increase in lycopene levels in plasma, showing that the lycopene within intact cells is less bioavailable than that from processed tissue. The isomeric composition of plasma lycopene was significantly different to that of the ingested purée. A number of *cis*-isomers (predominantly 5-*cis*, 13-*cis* and 9-*cis*-) were detected in plasma, that are not present in the lycopene from purée. The significance of the increase in lycopene following dietary supplementation with respect to bioavailability and the causes of isomerization are discussed.

Keywords: Carotenoid, lycopene, bioavailability, *cis–trans* isomers, tomato

INTRODUCTION

Epidemiological data provide convincing evidence that consumption of a diet rich in fruit and vegetables is associated with a reduction in the incidence of epithelial cancers and coronary heart disease.[1] Among the constituents of fruit and vegetables under investigation as protective agents are the carotenoids, a large group of lipophilic isoprenoids.[2] Of the 600 or so carotenoids identified in nature, 34 (including 13 geometrical isomers and 8 metabolites) have been identified in human blood.[3,4] Of these, lycopene (ψ,ψ-carotene), the red pigment of tomato, watermelon and pink grapefruit, is currently receiving much attention (reviewed in[5,6]). Epidemiological studies concerned with tomato-based diets have reported that tomato consumption is inversely correlated to the risk of certain digestive tract cancers,[7] and that intake of lycopene, but not other carotenoids, is inversely correlated with the

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risk of prostate cancer[8] and myocardial infarction.[9] There is evidence that serum lycopene is significantly lower in individuals who develop pancreatic cancer,[10] bladder cancer[11] or cervical intraepithelial neoplasia.[12]

The mechanisms by which lycopene and other carotenoids could exert these effects are not fully understood. Carotenoids have intrinsic antioxidant properties;[13] amongst dietary carotenoids, lycopene quenches singlet oxygen with the highest efficiency[14] and, in antioxidant screening studies, has the highest capacity to quench the $ABTS^{\bullet+}$ radical, a chromogenic indicator of antioxidant activity.[15] Carotenoids enhance gap-junctional communication by stimulating expression of the gene encoding connexin 43.[16] The effects on gap-junctional communication are independent of provitamin A and antioxidant activity, demonstrating that multiple modes of action must be considered. In addition, lycopene has been shown to inhibit proliferation of cultured cells when they are stimulated by autocrine mitogens.[17]

After ingestion, absorbed carotenoids are incorporated by the intestinal mucosa into chylomicrons and released via the lymphatic system into the bloodstream.[18] Later, carotenoids become distributed amongst other lipoproteins, with the majority of plasma carotenoids being carried in the low density lipoprotein (LDL) fraction. When compared with other dietary carotenoids, the concentration of lycopene in plasma is high in most Western populations.[19] Lycopene accumulates in tissues rich in LDL receptors such as liver, adrenals and testes.[20] However, further details of the nature and location of lycopene metabolism *in vivo* are lacking and it is unclear to what extent lycopene metabolites may be involved in any protective mechanisms. One noted difference between ingested lycopene and that found in human tissues is its isomeric composition.[20–23] Commonly, the majority (> 70%) of the lycopene present in fresh and industrially processed tomatoes is in the all-*trans* form,[22] yet in plasma it accounts for only *ca.* 45% of total lycopene and in prostate tissue, just 17%.[21] Isomerization occurring *in vivo* may have significant consequences since the large three-dimensional differences between these geometric isomers could influence their pharmacological properties.

Most consistent epidemiological evidence supporting the potentially protective effect of tomato consumption comes from studies where an association with plasma lycopene concentration has been demonstrated. Therefore, we have used supplementation studies with tomato products to estimate the sensitivity of circulating lycopene levels to dietary manipulation, to compare bioavailablity from different matrices and to measure the isomerization of lycopene *in vivo*.

MATERIALS AND METHODS

Materials

The all-*trans* crystalline reference carotenoids lycopene, β-carotene, β-cryptoxanthin, canthaxanthin and ethyl-β-*apo*-8′-carotenoate were obtained from Hoffman-La Roche (Basel, Switzerland); lutein was supplied by Kemin Industries (Des Moines, IA, USA). All standards were at least 97% pure. Ammonium acetate and all solvents were of HPLC grade and were obtained from Merck (Lutterworth, Leics., UK). All other chemicals were of analytical grade and were also obtained from Merck. Fresh tomatoes and pizza bases were purchased from a local supermarket; double-concentrated tomato purée was the gift of Safeway Stores plc, Hayes, Middlesex, UK.

Dietary Supplementation Studies

(a) Supplementation with cooked tomatoes Three healthy volunteers (2 female, 1 male, age range 25–32 years) took part in the study, which consisted of three consecutive 7-day periods. The first period involved a self-selected diet; the second, a diet devoid of tomato products, whilst in the third 5 cooked tomatoes were eaten per day. Fresh tomatoes were chopped and heated at

about 180°C for 5 min with 5 mL olive oil before incorporation into a meal. After an overnight fast, a blood sample (20 mL) was taken by venepuncture from the antecubital vein from each volunteer at the beginning and the end of each 7-day period. LDL was isolated from each plasma sample and analysed for carotenoid and protein contents.

(b) Supplementation with tomato purée The same healthy volunteers (2 female, 1 male, age range 25–32 years) took part in this study, which consisted of 2 periods. During the first period (7 days), a self-selected diet was consumed; during the second (14 days), volunteers ingested double-concentrated tomato purée. Double-concentrated refers to the reduction of liquid content of the purée by the manufacturer prior to canning. On each day during the supplementation period, 35 g tomato purée was heated for 5 min with 5 mL olive oil, spread over a supermarket cheese and tomato pizza base, oven-baked at 200°C for 15 min, and consumed when desired. At the beginning and the end of each study period, a fasting blood sample (20 mL) was taken from each volunteer by venepuncture. From these samples, plasma was separated, LDL isolated and both were analysed for carotenoid and protein contents.

(c) Analysis of lycopene isomers in blood Three healthy male volunteers (age range 30–55 years) consumed a high-fat, lycopene-free breakfast comprising 4 slices buttered toast, 2 rashers bacon, 1 sausage and 2 cups milky tea (63 g fat in total). No tomato products had been consumed during the preceding 24 h. Five and a half hours post prandium, a blood sample (20 mL) was collected from each volunteer by venepuncture. Blood was fractionated to yield plasma and its lipoprotein constituents, each of which was analysed for carotenoids.

Plasma and Lipoprotein Preparation

Blood was collected by venepuncture into vials containing acid citrate dextrose and 100 μM EDTA. LDL was isolated by using a modified discontinuous ultracentrifugation method.[25] Isolated LDL was then sterilized by passing it through a 0.2 μm filter (Flowpore, ICN Pharmaceuticals, CA, USA). Plasma and lipoprotein samples were stored at −70°C and in the dark until analysis.

Extraction and Identification of Carotenoids

All manipulations were carried out on ice, shielded from strong light. Solvents contained 0.025% (w/v) BHT as antioxidant. Extractions were carried out in duplicate and to each replicate, an appropriate quantity of acetone-solubilized internal standard was added (for C_{18} HPLC separations, this was ethyl-*β-apo-8′*-carotenoate; for C_{30} HPLC separations, canthaxanthin). Tomato products were homogenized in 4–8 vol methanol using an Ultra-Turrax T25 homogenizer (Janke & Kunkel, Staufen, Germany). For plasma and lipoprotein samples (typically 0.5–1 mL), 1 vol methanol was added and homogenization was achieved by vigorously vortexing for 30 s. Thereafter, carotenoids were extracted from all samples by the following method: 3 vol hexane/dichloromethane (5 : 1 v/v) was added and the mixture was vortexed vigorously for 30 s. The phases were separated by centrifugation at 200 g for 5 min and the supernatant was collected by glass pipette. With tomato-derived samples, good phase separation sometimes required the addition of 1 vol Garbus wash (2 M KCl, 0.5 M potassium phosphate, pH 7.4).[26] Extraction was repeated and the extracts were combined before evaporation under a stream of nitrogen gas. The carotenoid-containing residue was then dissolved in 40–100 μL ethanol/1,2-dichloroethane (2 : 1 v/v) and 20 μL was injected on to the HPLC column. Recovery of the internal standard was greater than 90% in all cases.

Carotenoids were analysed by reverse-phase HPLC on a Waters system (Watford, Herts, UK) consisting of a #616 pump, #996 diode-array

detector and #717 autosampler. Data were collected and analysed using the manufacturer's Millennium software. The major plasma carotenoids all-*trans*-lutein, all-*trans*-β-cryptoxanthin, all-*trans*-β-carotene and all-*trans*-lycopene were quantitated by reference to standard curves constructed using the respective crystalline standards. Throughout the analyses, detection was at 450 nm, flow rate was 1 mL/min and column temperature was maintained at 25°C by a #7955 column oven (Jones Chromatography, Hengoed, Mid- Glamorgan, UK).

For the cooked tomato and tomato purée supplementation studies, 250 × 4.6 mm Nucleosil C_{18} column (Jones Chromatography), coupled to a 10 × 4.6 mm Phase Sep C_{18} guard column (Phase Separations, Deeside, Clwyd, UK) was employed. Solvent A was acetonitrile containing 0.05% (w/v) ammonium acetate, solvent B was methanol and solvent C was isopropanol. The following gradient elution program (45 min in total) was followed: 85A/15B/0C for 13 min, changed linearly to 15A/70B/15C over 1 min, maintained for 18 min, changed linearly to 50A/0B/50C over 1 min, maintained for 11 min, returned linearly to 85A/15B/0C over 1 min. The column was allowed to equilibrate for 15 min between runs in the initial solvent mixture.

Lycopene isomers were analysed using a 250 × 4.6 mm C_{30} HPLC column coupled to a 20 × 4.6 mm C_{30} guard (YMC Inc., Wilmington, NC USA). In these cases, solvent A was methanol, solvent B was water containing 0.04% (w/v) ammonium acetate, and solvent C was *tert*-butyl methyl ether. The method of Yeum *et al.*[27] was modified to give the following gradient elution program (45 min in total): 99A/1B/0C, maintained for 6 min, changed linearly to 84A/1B/15C over 1 min, maintained for 5 min, changed linearly to 34A/1B/65C over 20 min, maintained for 11 min, returned linearly to 99A/1B/0C over 2 min. The column was equilibrated for 15 min between runs. Assignment of isomers was made by reference to the retention times and the absorption spectra captured by the in-line diode array detector in comparison with the spectra of Yeum *et al.*[27] In the HPLC solvent at the time of elution, the main maximum, the *cis*-peak maximum and relative absorption of the *cis*-peak for each of the major lycopene isomers was: all-*trans* (361.5, 471.5 nm, 0.09); 5-*cis*-lycopene (360.5, 467.0 nm, 0.23); 13-*cis*-lycopene (360.5, 407.0, 0.39).

Protein Estimations

The protein content of plasma and lipoproteins was measured using the Markwell assay.[28]

Statistical Analysis

All data are presented as mean ± SEM. Statistical differences between experimental groups were estimated using the paired Student's *t*-test.

RESULTS

Cooked Tomato Supplementation Study

The 4 major carotenoids in LDL were lycopene, β-carotene, lutein and β-cryptoxanthin, with lycopene being the single most abundant carotenoid. The basal concentrations of the carotenoids in the 3 participants and the effects of each experimental period on these concentrations are presented in Table I. The participants showed high inter-individual variability in response to both the depletion and supplementation periods, but it is apparent that neither dietary regime markedly changed their values. The carotenoid content of a representative tomato (mean of 3 fruit samples) was determined, giving an estimated daily lycopene dose of 1.5 mg.

Tomato Purée Supplementation Study

The basal concentrations of the 4 major plasma and LDL carotenoids in the 3 participants and the effects of each experimental period on these concentrations are presented in Table II. The

TABLE I Effect of a low-carotenoid diet followed by dietary supplementation with cooked tomatoes on the concentration of major LDL carotenoids

	Lutein	β-Cryptoxanthin	β-Carotene	Lycopene
Basal value ($n=3$)[a]	0.12 ± 0.03	0.13 ± 0.02	0.62 ± 0.16	1.10 ± 0.15
Change after depletion ($n=3$)	−0.02 ± 0.03	+0.04 ± 0.03	+0.12 ± 0.13	−0.20 ± 0.25
Further change after supplementation ($n=3$)	−0.01 ± 0.01	−0.05 ± 0.03	+0.03 ± 0.22	−0.10 ± 0.04

[a] Basal values are the means of samples taken at each end of the initial self-selected diet period. Values are expressed as nmol/mg LDL protein (mean ± SEM).

TABLE II Effect of dietary supplementation with tomato purée on the concentration of major plasma and LDL carotenoids

	Plasma[a]				LDL[b]			
	Lutein	β-Cryptoxanthin	β-Carotene	Lycopene	Lutein	β-Cryptoxanthin	β-Carotene	Lycopene
Basal value[c] ($n=3$)	0.18 ± 0.02	0.12 ± 0.01	0.31 ± 0.07	0.44 ± 0.08	0.11 ± 0.01	0.15 ± 0.02	0.61 ± 0.17	0.89 ± 0.11
Change after supplementation ($n=3$)	−0.01 ± 0.01	−0.02 ± 0.01	+0.02 ± 0.02	+0.15 ± 0.03[d]	−0.01 ± 0.02	−0.02 ± 0.01	+0.10 ± 0.07	+0.22 ± 0.19

[a]Plasma values are expressed as μM (mean ± SEM). [b]LDL values are expressed as nmol/mg LDL protein (mean ± SEM).[c]Basal values are the means of samples taken at each end of the initial self-selected diet period. [d]Statistically significant increase ($p < 0.05$).

major carotenoids were the same as in the previous study; before supplementation, lycopene was again the predominant carotenoid. At the end of the supplementation period, plasma lutein, β-cryptoxanthin and β-carotene remained unchanged but plasma lycopene showed a significant ($p < 0.05$) increase. Changes in the same carotenoids in LDL showed higher inter-individual variation and no clear trends were discernible. The carotenoid content of a single, representative pizza topping (duplicate analysis) was determined, giving an estimated daily lycopene dose of 21 mg.

The isomeric composition of plasma lycopene in supplemented individuals was significantly different from that of the ingested tomato purée (Figure 1). The major lycopene isomers were well resolved from the other carotenoids, but some *cis*-isomers of lycopene were incompletely resolved from each other, preventing the quantitation of individual *cis*-isomers. Therefore, the total amounts of all *cis*-isomers have been summed. Assuming that all lycopene isomers have equal extinction coefficients at 450 nm, the lycopene in the cooked purée was 84% all-*trans*, whereas plasma lycopene in supplemented individuals was only 40–45% in the all-*trans* form. The major *cis*-isomers in plasma were, in decreasing order of abundance, 5-*cis*-, 13-*cis*- and 9-*cis*-isomers. Less abundant *cis*-isomers were also found, as shown in Figure 1.

Lycopene Isomers in Lipoproteins

The isomeric composition of lycopene in plasma and each of its constituent lipoprotein fractions is presented in Figure 2. The proportion of lycopene in the all-*trans* form was similar in plasma, chylomicrons, VLDL and LDL at 43–45%, but in HDL, it was slightly lower at *ca.* 37%.

DISCUSSION

For each of the participants in these experiments, lycopene, β-carotene, lutein and β-cryptoxanthin were the major baseline plasma carotenoids, with lycopene being the single most abundant carotenoid in all but one individual, where β-carotene

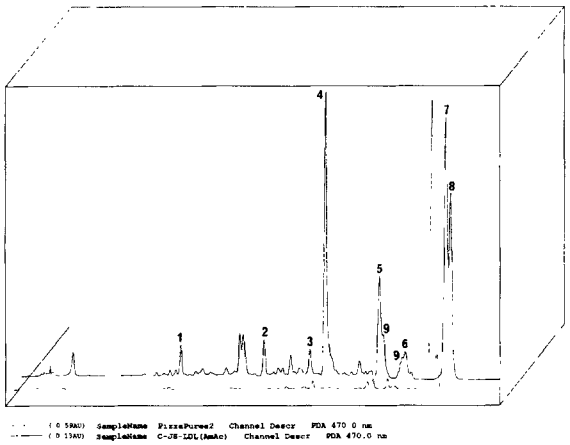

FIGURE 1 Representative HPLC chromatograms from the tomato purée supplementation study. Overlaid are carotenoids present in cooked tomato purée as consumed during the experimental supplementation period (*lower trace*) and blood plasma from a supplemented individual after 14 days (*upper trace*). Carotenoids were separated on a C_{30} reverse-phase column and detection was at 450 nm. Labelled peaks are 1, all-*trans*-lutein; 2, all-*trans*-β-cryptoxanthin; 3, all-*trans*-α-carotene; 4, all-*trans*-β-carotene; 5, 13-*cis*-lycopene; 6, 9-*cis*-lycopene; 7, all-*trans*-lycopene; 8, 5-*cis*-lycopene; 9, other *cis*-isomers of lycopene.

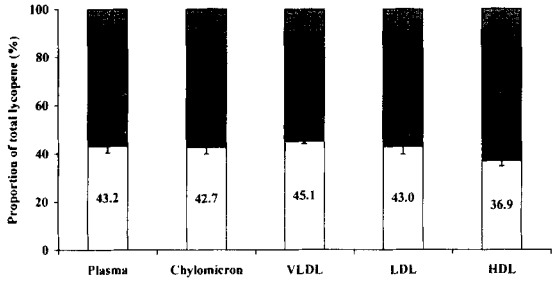

FIGURE 2 Isomeric composition of lycopene in plasma and its lipoprotein fractions. Five and a half hours after consumption of a high-fat, lycopene-free meal, plasma from 3 individuals was fractionated to yield the lipoprotein subclasses shown. Isomeric composition was estimated by HPLC using a C_{30} reverse-phase column. White bars with an enclosed percentage represent all-*trans*-lycopene; filled bars represent total lycopene *cis*-isomers. All values are mean ± SEM ($n = 3$). SEM values are 2.7, 2.9, 1.0, 3.5 and 2.3 for plasma, chylomicrons, VLDL, LDL and HDL, respectively.

was most abundant. Similar distributions have been described previously.[19,27]

It has previously been shown that increases in LDL β-carotene closely mirror increases in plasma β-carotene after a single oral dose of encapsulated β-carotene,[29] with the peak change occurring 2–3 days after ingestion. In our cooked tomato supplementation study, LDL, the major carrier of carotenoids in plasma, was analysed for its response to both a low-carotenoid diet and a self-selected diet supplemented with cooked tomatoes. Adherence to a low-carotenoid diet for 7 days had variable effects on the LDL content of the major carotenoids that precluded the identification of any trends in such a small population. Recent estimates of the half-life of lycopene in the plasma of regular tomato-consumers have been 11–14 days[30] and 12–33 days,[31] so the lack of significant change within 7 days was not entirely unexpected. After this normalization period, a 7-day supplementation period where 1.5 mg/day lycopene was provided from cooked tomatoes did not significantly alter the LDL concentration of lycopene or any other major carotenoid. This supports previous findings[8] that high consumption of fresh tomatoes is not a strong predictor of high plasma lycopene concentration and that concentrated, processed tomato products are more effective sources of plasma lycopene.

Therefore, the second supplementation study used tomato purée as a source of lycopene. The manufacture of this product involves substantial maceration of tomatoes and double-concentration of the solids. Yet, after 14 days' supplementation with 21 mg/day lycopene from this source, only a one-third increase in plasma lycopene was observed. The quantity of tomato purée present in the experimental meal was far in excess of that present in a normal pizza. The LDL response, when expressed as nmol carotenoid per mg LDL protein, showed much higher inter-individual variation than the plasma response.

Other reported studies attempting to increase plasma lycopene concentration by chronic ingestion of tomato juice or tomato sauce over longer periods have been less successful than similar efforts using, for example, carrots or broccoli as sources of β-carotene or lutein, and much less successful than the use of capsules to boost plasma β-carotene.[27,30] Since the ready bioavailability of

lycopene from tomato paste has been demonstrated,[23] it appears that the removal of lycopene from plasma, by tissue uptake or by chemical/enzymic modification, may be more rapid than the corresponding process with β-carotene or lutein.

Other investigations dealing quantitatively with the effect of chronic tomato product consumption on plasma lycopene concentration are summarized and compared to the present study in Table III. It is noticeable that the larger increases in plasma lycopene are associated with individuals whose initial plasma lycopene concentrations were relatively low. This suggests that the upper limit of plasma lycopene may not be greatly higher than that currently found in the highest tomato-consuming populations. One study employing mixed fruit and vegetable extracts,[33] however, argues against this hypothesis. In that investigation, a relatively low dose of lycopene (0.9 mg/day) in the form of encapsulated dried powder produced a substantial (*ca.* 2000%) increase in plasma lycopene. There are presently no similar claims for other lycopene supplements in the literature.

In common with previous reports,[21–23] we found that the proportion of lycopene existing in the all-*trans* conformation is much lower in plasma than in common dietary tomato products. After 14 days' supplementation with lycopene from cooked tomato purée (84% all-*trans*), plasma lycopene was 40–45% in the all-*trans* conformation Figure 1. The agent(s) responsible have not been clearly elucidated, but isomerization of lycopene *in vivo* is attributable in part simply to the change of state that occurs during digestion in the gut. In tomato fruit, all-*trans* lycopene is deposited within chromoplasts in crystalline form,[32] a state in which its geometrical configuration is known to be stable.[35] Similar crystals are probably found in the purée, despite disruption of the cell walls. Under the action of bile salts in the gut, carotenoid crystals are disrupted and become solubilized in micelles before uptake by the intestinal mucosa.[18] After solvation, the loss of conformational crystal packing constraints leaves carotenoids susceptible to thermally-induced *cis-trans*-isomerization, a process which proceeds spontaneously, but slowly at ambient temperature (and presumably faster at 37°C), without the need for any catalyst.[35] In this reaction, the interconversion of geometrical isomers is readily reversible, leading to a pseudo-equilibrium mixture with some irreversible loss of intact carotenoid. Importantly, the thermally-induced isomerization of lycopene is known to be significantly quicker and to occur to a greater extent than that of β-carotene under equivalent conditions (for example, 45 : 55 all-*trans*: *cis*-lycopene cf. 86 : 14 all-*trans*: *cis* β-carotene was formed after refluxing in petroleum ether).[35] This difference is reflected in the enthalpy of activation for the reaction, a parameter that is predicted to be some 2 kcal/mol lower for lycopene than for β-carotene.[34]

However, in the presence of an appropriate catalyst, *cis-trans* isomerization of carotenoids can be accelerated markedly. Although there are no known enzymes capable of catalyzing the process in the human body, characterized non-enzymic catalysts include H^+, one-electron oxidants such as Fe^{3+}, iodine, other paramagnetic substances including O_2 and $NO^{\bullet}$, and various active surfaces.[35,36] Therefore, the isomerization of lycopene and an equilibrium that more strongly favours *cis*-isomers may account for the sizeable proportion of *cis*-lycopenes found in plasma. The presence of acid in the stomach is perhaps the most plausible cause of *cis*-isomer formation, but this does not explain the diversity in the distribution of geometrical carotenoid isomers found in different organs of the body.[21] The possibility of different catalysts operating in specific parts of the body thus remains to be clarified, and further studies are underway in our laboratory.

To obtain further information on the progression of lycopene isomerization *in vivo*, we fractionated plasma into lipoprotein subclasses after a high-fat, lycopene-free meal and determined the ratio of all-*trans*- to *cis*-lycopene in each subclass. There was no significant difference in this

TABLE III Effect of chronic supplementation with various tomato products on plasma lycopene concentration

Source of lycopene (daily consumption)	Daily lycopene intake for a 70 Kg person (mg)	Supplementation period (days)	Study group composition	Initial plasma lycopene concentration (nM)	Mean change in plasma lycopene concentration (nM)	Reference
Tomato purée (35 g)	21	14	1M + 2F (25–32 years)	440 ± 80	150 ± 30	This study
Tomato juice (180 g)	12	42	5M (20–45 years)	890 ± 340	34 ± 310	[30]
Tomato juice boiled with corn oil (1%) for 1 h	13	4	1 subject	210	320	[28]
Tomato juice (390 g)	36	28	25F (students)	240 ± 160	550 ± 190	[24]
Tomato juice (160 g)	17	19	1M (26 years)	50	550	[38]
Tomato sauce (33 g)	3.3	15	9M (20–40 years)	380 ± 50	≈180	[27]
'Juice Plus +' mixed fruit and vegetable extracts	0.9	28	5M + 10F (18–53 years)	260 ± 310	≈5200	[33]

parameter between chylomicrons, VLDL, LDL and HDL indicating that isomerization probably occurs to a large extent in the intestine and/or lymphatic system. Gärtner *et al.*[23] noted that all-*trans* lycopene still accounted for *ca.* 65% of newly-absorbed lycopene in chylomicrons 6 h after ingestion of fresh tomatoes, suggesting that *cis*-isomers are still accumulating at this time point. The individuals in our experiment had consumed no tomato products in the previous 24 h and hence, lycopene present in chylomicrons could have been the result of delayed release from the intestinal mucosa. Such a delayed release has been noted before.[29,36]

The proportion of lycopene *cis*-isomers was slightly, but not significantly, lower in the HDL subclass. HDL is secreted by the liver and participates in 'reverse cholesterol transport', acquiring cholesterol esters from tissue stores and delivering them to the liver.[37] If lycopene also follows this route, one might predict that the isomeric composition of HDL lycopene be more representative of tissue stores than that in other lipoprotein subclasses. Since tissue-specific proportions of all-*trans* lycopene can be as low as 17%,[21] this may account for the lower proportion of this isomer in HDL.

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