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Detection of Key Factors Affecting Lycopene *in Vitro* Accessibility

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ABSTRACT: On the basis of a Plackett−Burman experimental design for a resolution IV level obtained via a *foldover* strategy, the effect of 11 factors on lycopene *in vitro* accessibility was investigated. The selected factors were thermal treatment (X_1), olive oil addition (X_2), gastric pH (X_3), gastric digestion time (X_4), pepsin concentration (X_5), intestinal pH (X_6), pancreatin concentration (X_7), bile salts concentration (X_8), colipase addition (X_9), intestinal digestion time (X_{10}), and intestinal digestion speed (X_{11}). Tomato passata was used as a natural source of lycopene. Samples were collected after gastric and intestinal digestion, and from the micellar phase, to quantify the (*all-E*)-lycopene and its (*Z*)-isomers by HPLC. Except for X_3, X_6, X_7, and X_{11}, the other factors studied explained lycopene *in vitro* accessibility, mainly regarding intestinal digestion, with R^2 values ≥ 0.60. Our results showed that the accessibility of lycopene is influenced by the conditions applied during *in vitro* intestinal digestion.

KEYWORDS: *(all-E)-lycopene, (Z)-lycopene, accessibility, isomerization, in vitro digestion, Plackett−Burman experimental design*

INTRODUCTION

Lycopene is an acyclic C_{40} nonpolar straight chain, containing 11 conjugated and two unconjugated double bonds, and is an effective singlet oxygen quencher that is 2 times greater than *β*-carotene and 10 times greater than vitamin E.[1] Lycopene is a carotenoid whose biological activities and protective effects on cardiovascular diseases and different types of cancer are recognized in the scientific literature,[2,3] due to the interest in bioactive compounds of plant foods for human nutrition.

Lycopene is present in its natural (*all-E*)-isomeric form in tomatoes[4] and in other red fruits, such as watermelon, pink grapefruit, guava, and apricot.[5] In contrast to foods, in the human body (serum and tissues) lycopene is found mainly as (*Z*)-isomers, accounting for more than 50% of total lycopene.[6−9] Various external conditions (temperature, light exposure, and contact with chemical agents) induce transformation to *cis*-lycopene isomers in tomato and other foods, but the major isomerization reactions occur *in vivo* during digestion or within the intestinal cells.[10,11] In addition, (*Z*)-isomers of lycopene could be more efficiently solubilized in the micelles before absorption and can pass better through the cell membrane, increasing its bioavailability.[6,12]

Many factors determine the bioaccessibility and bioavailability of carotenoids. The physical state and history of a food item clearly have a very marked effect on the availability of lycopene for absorption in the human body. This indicates that disruption of the food matrix and thermal history, via a processing technique, could be one of the most important factors affecting lycopene bioaccessibility.[10,13] Moreover, other factors are also critical for increasing the bioavailability and hence the absorption of lycopene and other carotenoids in the intestine, which are described. First, lycopene must be released from the food matrix, which is facilitated by the thermal treatment of food, since industrial processing leads to a disruption of vegetal tissues, breaking down the complex carotenoid−protein and weakening the interaction with the cell membrane. These facts improve the efficiency of the mastication and the effect of stomach and intestine digestive enzymes on the availability. Second, during intestinal digestion, carotenoids must be solubilized into micelles, a phenomenon favored by the intestinal pH, the carotenoids' hydrophobicity, the amount of bile salts secreted during digestion, and the presence of oil or fat in a carotenoid-rich diet.[14−16] After solubilization into micelles, they are taken up into enterocytes by passive diffusion or by active transport associated with a lack of membrane transport proteins, such as SR-BI, NPC1L1, ABCA1, and CD36,[17] and then are packaged into triacylglycerol-rich chylomicrons and secreted into the lymph for delivery to the bloodstream, where they are rapidly degraded by lipoprotein lipase.[18] The resulting chylomicrons remnants are rapidly taken up by the liver, which secretes lycopene and other carotenoids associated with hepatic lipoprotein.[11,18,19] However, the absorption efficiency of lycopene is variable, since many factors affect uptake in the enterocyte, and in the population there are low and high lycopene absorbers.

In the absence of a functional marker of the biological activity of carotenoids without vitamin A activity, the bioavailability of these compounds has largely been taken to mean assimilation efficiency, or the ability to accumulate them in some defined body pools.[17] Although methods involving human subjects are probably of greatest value, the interindividual variability is likely to be significant. Thus, the *in vitro* method is an alternative to human intervention studies and is useful for determining the *in vitro* accessibility of carotenoids in a food matrix, measuring the fraction available for absorption in the small intestine, and hence to estimate the bioavailability. *In vitro* methods have been developed to quantify the bioaccesibility and bioavailability of

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several nutrients of foods, and they are becoming increasingly popular for analyzing the availability of carotenoid-rich foods.[15,20−24] All methods are based on gastric digestion, followed by intestinal digestion and the separation of the micellar fraction, but in general, all use similar conditions and only in the procedure described by Reboul et al.[15] have some parameters of the *in vitro* model been optimized to look more like human physiology.

The aims of the present study were to identify the critical factors affecting the *in vitro* availability of lycopene from tomato passata, during gastric and intestinal digestion, with special attention to the isomerization reaction, since (*Z*)-isomers are better absorbed and thus show higher bioavailability in the human body.[6,12]

MATERIAL AND METHODS

Samples. Commercial tomato passata provided by Juver Alimentación S.L.U. (Cabezo de Torres, Murcia, Spain) was used in this study as a natural dietary source of lycopene. Passata was bottled in a 750 mL glass container. All samples were from the same batch and were refrigerated until the analysis was conducted.

Reagents. The enzymes pepsine (P-7000, 370 units/mg solid, from porcine stomach mucosa), pancreatin (P-1750, from porcine pancreas), and colipase (C-3028, colipase from porcine pancreas) and bile salt (B-8756) were obtained from Sigma Chemical (Madrid, Spain). The standard of carotenoids, *trans*-β-apo-8′-carotenal, used as the internal standard solution, was obtained from Fluka (Buchs, Switzerland), whereas *all-trans*-lycopene came from CaroteNature (Lupsingen, Switzerland). The present study included a sample obtained from the Community Bureau of Reference (BCR) such as BCR-485 (mixed vegetables), which was used as the reference material for the quality control of the HPLC analysis of lycopene. The role of certified reference materials (CRMs) in analytical chemistry is now well established, particularly for calibrating and verifying the accuracy of analytical methods.[25] All other chemicals were of analytical grade.

Experimental Design. After a review of the scientific literature and our own experience, 11 factors were identified as apparent critical factors (ACFs). Table 1 shows the factors and levels included in the experimental design. In this way, the main items in this research are (1) the aim of paper, which is determining how ACFs affect *in vitro* lycopene availability, and (2) at the same time, because the ACF number is high and, thus, screening the full factorial experimental design includes a very high run number (2^{11} = 2.048), an adequate experimental design such as a fractional factorial experimental design must be used to identify and separate out those factors that demand further investigation at the optimization phase.

Table 1. Eleven Apparent Critical Factors Considered for the Two-Level Foldover Experimental Design at the Resolution IV Level and Experimental Ranges Expressed in Coded and Actual Units

	symbol		levels	
ACF	code	actual	−1	+1
thermal treatment (min)	X_1	T_t	0	15
olive oil addition (%)	X_2	oil_A	0	5
gastric pH	X_3	pH_G	2	4
gastric digestion time (min)	X_4	$time_G$	15	60
pepsin concentration (mg/100 g)	X_5	C_{PEP}	500	800
intestinal pH	X_6	pH_I	6	7.5
pancreatin concentration (mg/100 g)	X_7	C_{PAN}	100	180
bile salts (mg/100 g)	X_8	C_{BIL}	100	1000
colipase addition (μg/100 g)	X_9	C_{COLIP}	0	125
intestinal digestion time (min)	X_{10}	$time_I$	60	120
intestinal digestion speed (rpm)	X_{11}	S_{ID}	40	80

The Plackett−Burman (PB) experimental designs[26] are based on Hadamard matrices in which the number of experimental runs is a multiple of 4. For example, a 12-run PB experimental design is generally used for studying 11 factors at the resolution III level. In our study, we used a resolution IV level; thus, the main effects are not confounded by two-factor interactions.[27] This resolution level was attained via a *foldover* strategy following Box and Hunter's criteria;[28] thus, a 24-run PB experimental design was used. The obvious advantage of this specific experimental design is the limited number of runs for evaluating a large number of factors,[29] such as 24 experiments versus 2048 to screen 11 factors. In this way, we identify the influencing factors (IFs) affecting responses for the ACF group considered (Table 1) with the least expenditure of time and resources.

For each ACF, we generated a X_i coded independent variable (i = 1 to 11), because it is advisable to transform natural variables into coded variables, and these coded variables are usually defined as dimensionless with zero mean and the same spread or standard deviation.[30] Each ACF (independent variables or factors) was coded according to the equation $X_i = (x_i - x_i^*)/\Delta x_i$, where X_i is the coded value of the ith independent variable, x_i is the uncoded value of the ith independent variable, x_i^* is the uncoded value of the ith independent variable at the central point, and Δx_i is the step change value.[30]

We investigated the effect of 11 ACFs on the availability of carotenoids according to the scientific literature review.[12−16,20−24] The selected factors were thermal treatment (X_1), olive oil addition (X_2), gastric pH (X_3), gastric digestion time (X_4), pepsin concentration (X_5), intestinal pH (X_6), pancreatin concentration (X_7), bile salts concentration (X_8), colipase addition (X_9), intestinal digestion time (X_{10}), and intestinal digestion speed (X_{11}). Twenty-four experiments were performed using a random order (trial order), because randomization allows the experimenter to avoid erroneous conclusions due to extraneous sources of variability.[27,31] The PB experimental design was generated, and results were analyzed using the Statistica software package (StatSoft, Inc., Tulsa, OK, USA).

Determination of *in Vitro* Bioavailability. *In vitro* availability of lycopene was determined following the method described by Garret et al.,[20] including several modifications according to the scientific literature,[15,16,22−24] the selected critical factors, and the high and low levels assigned to each factor (Table 1).

About 120 g of tomato passata was used for *in vitro* digestion. In the assays with a high level (+1) for X_2, olive oil was added to the sample to give a final amount of 5%, whereas samples with a low level (−1) were prepared without oil. Then the samples with a high level (+1) of X_1 were heated for 15 min, simulating domestic cooking conditions. After cooking, water was added to samples after reaching 120 g, with the objective to restore the water lost by evaporation. Samples were introduced in brown plastic flasks in which digestion was carried out. For the gastric pH (X_3), the low level (−1) and the high level (+1) had pH values of 2 and 4, respectively, which were adjusted using 6 and 1 M HCl. Samples were tempered for 10 min at 37 °C in a water bath, and then pepsin was added to start the gastric digestion. About 3.125 mL of a pepsin solution (Sigma, P-7000) in 0.1 N HCl at two levels (X_5), giving a final concentration of 500 and 800 mg/100 g, was added to the samples. The gastric digestion (X_4) durations were 15 min in the low level (−1) and 60 min in the high level (+1). Once gastric digestion was completed, 20 g of the digested sample was collected for the analysis of lycopene and its isomers with HPLC, and 40 g was subjected to intestinal digestion. Before the enzymes were added, samples were neutralized with 20% KOH, reaching an intestinal pH (X_6) of 6 and 7.5, according to the low (−1) or the high level (+1) of this factor. Samples were introduced again in a water bath at 37 °C, and after 10 min, 10 mL of a solution containing pancreatin (X_7) (Sigma, P-1750) and bile salt (X_8) (Sigma, B-8756) was added. The low (−1) and high (+1) levels of pancreatin were 100 and 180 mg/100 g, whereas for bile salt, the levels were 100 and 1000 mg/100 g, respectively. These amounts were prepared in four different solutions to be added to the samples according to the run. The addition of porcine colipase (X_9) (Sigma, C-3028) was considered a critical factor, adding in the assays with high level (+1) 50 μg of colipase/mL, dissolved in 0.1 M $NaHCO_3$, giving a final concentration of 125 μg/

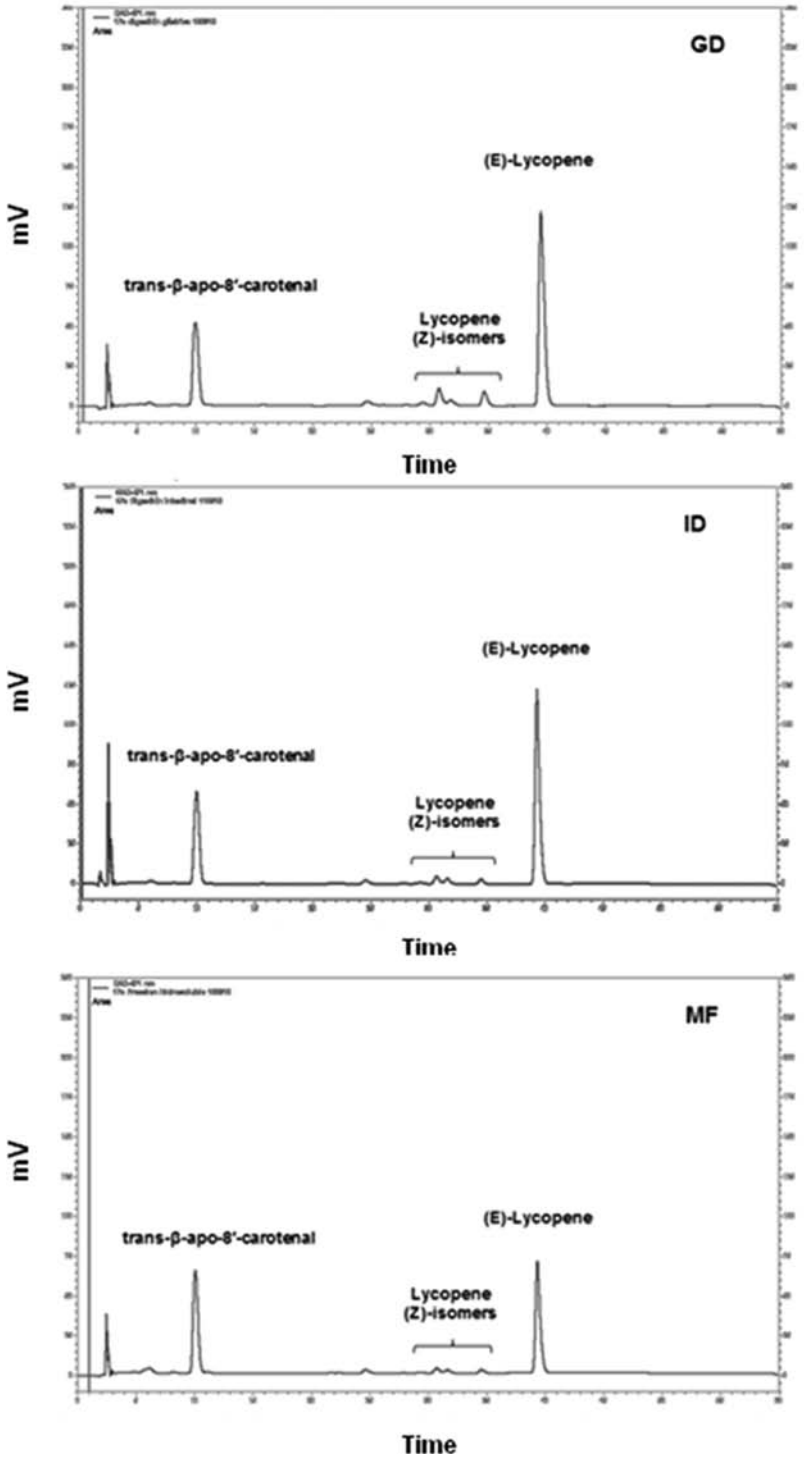

Figure 1. Representative chromatograms showing (*all-E*)-lycopene and (*Z*)-lycopene isomer (5*Z*, 9*Z*, 13*Z*, and 15*Z*) profiles in gastric digestion (GD), intestinal digestion (ID), and micellar fraction (MF) samples. Chromatograms are obtained from trial 17 (see Tables 2, 3, and 4).

100 g of the sample. The intestinal digestion (X_{10}) durations were 60 and 120 min, whereas the speed of agitation (X_{11}) during intestinal digestion was 40 and 80 rpm for low (−1) and high (+1) levels of these factors. After intestinal digestion, 20 g of the digested sample was collected to determine the lycopene content and its isomers with HPLC, and the other 10 mL of the sample was used to separate the phase containing the micelles after decantation for 16 h at room temperature and low-speed centrifugation (5000 rpm for 20 min) according to the procedure described by Granado-Lorencio et al.[24] An aliquot of the micellar fraction was also used to quantify the lycopene and its isomers. Thus, calculations of transfer from the duodenal digesta to the aqueous micellar phase (micellization) were estimated using the lycopene proportion in the supernatants. Each trial was carried out in triplicate, obtaining three samples of each one of the analyzed fractions, gastric digestion (GD), intestinal digestion (ID), and micellar phase (MF).

Quantitative Determination of Lycopene. Lycopene was analyzed using HPLC with diode array detection according to Böhm et al.[32] after three extractions with methanol/tetrahydrofuran (1:1, v/v) containing 0.1% butylated hydroxytoluene. Briefly, 400 mg of MgO,

Table 2. Content of (*all-E*)-Lycopene (R_1) and (*Z*)-Lycopene (R_2), Expressed in mg/kg, and Percentage of Isomerization (R_3) after Gastric Digestion (GD Samples)[a]

run	trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	R_1	R_2	R_3
13	1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	15.50	1.48	8.74
22	2	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	10.01	3.78	27.42
2	3	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	7.47	3.51	31.97
5	4	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	10.47	3.18	23.27
28	5	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	7.98	2.33	22.60
4	6	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	8.67	1.25	12.59
12	7	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	8.81	1.79	16.86
20	8	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	10.18	1.20	10.53
29	9	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	9.26	0.94	9.25
32	10	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	9.27	1.27	12.01
17	11	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	8.46	1.48	14.92
9	12	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	7.61	3.23	29.77
3	13	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	8.27	2.96	26.36
15	14	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	9.94	0.95	8.70
1	15	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	10.82	1.43	11.69
7	16	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	9.84	1.37	12.21
21	17	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	11.53	2.14	15.62
25	18	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	12.07	1.33	9.90
30	19	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	8.49	1.24	12.77
6	20	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	11.02	1.12	9.24
16	21	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	13.24	1.00	6.70
23	22	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	12.08	1.62	11.80
27	23	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	12.04	1.16	8.76
31	24	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	11.26	1.18	9.49

[a]Mean values of three measurements.

Table 3. Content of (*all-E*)-Lycopene (R_1) and (*Z*)-Lycopene (R_2), Expressed in mg/kg, and Percentage of Isomerization (R_3) after Intestinal Digestion (ID Samples)[a]

run	trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	R_1	R_2	R_3
13	1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	14.83	0.72	4.64
22	2	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	9.77	0.56	5.38
2	3	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	15.71	0.65	3.97
5	4	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	13.13	1.45	9.97
28	5	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	7.26	1.30	15.17
4	6	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	9.05	0.80	8.09
12	7	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	10.07	0.52	4.87
20	8	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	12.60	0.67	5.01
29	9	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	11.22	0.68	5.75
32	10	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	11.61	0.63	5.15
17	11	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	10.19	0.63	5.81
9	12	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	9.53	1.48	13.43
3	13	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	9.53	0.91	8.73
15	14	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	10.10	1.72	14.57
1	15	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	11.03	1.67	13.16
7	16	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	11.58	0.94	7.52
21	17	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	10.63	0.90	7.81
25	18	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	12.94	1.09	7.74
30	19	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	12.23	1.26	9.33
6	20	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	11.75	0.87	6.89
16	21	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	12.79	1.07	7.69
23	22	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	11.17	1.31	10.51
27	23	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	12.72	0.96	7.04
31	24	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	11.47	0.91	7.37

[a]Mean values of three measurements.

200 μL of *trans*-β-apo-8′-carotenal (internal standard solution), and 35 mL of methanol/tetrahydrofuran were added to 0.6 g of the sample and homogenized for 5 min using a blender. The resulting solution was vacuum filtered through no. 5 Whatman paper. The extraction was repeated twice (until the residue was colorless), and the combined extracts were dried under vacuum at 30 °C in a rotary evaporator. The

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Table 4. Content of (*all-E*)-Lycopene (R_1) and (*Z*)-Lycopene (R_2), Expressed in mg/kg, and Percentage of Isomerization (R_3) in the Micellar Fraction (MF Samples)[a]

run	trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	R_1	R_2	R_3
13	1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	1.18	0.04	3.13
22	2	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	0.75	0.01	1.84
2	3	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	0.62	0.02	3.57
5	4	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	0.52	0.02	4.45
28	5	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	1.38	0.09	6.26
4	6	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	0.35	0	0
12	7	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	0.52	0.04	7.33
20	8	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	1.36	0.10	7.12
29	9	+1	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	0.36	0.04	9.93
32	10	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	−1	0.23	0.03	11.67
17	11	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	1.58	0.15	8.67
9	12	+1	+1	−1	−1	−1	+1	−1	−1	+1	−1	+1	0.24	0.07	21.24
3	13	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	1.35	0.15	9.81
15	14	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	0.47	0.09	16.21
1	15	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	0.31	0.04	11.05
7	16	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	1.98	0.30	13.11
21	17	−1	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	1.92	0.20	9.44
25	18	−1	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	1.03	0.12	10.47
30	19	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	0.53	0.14	21.38
6	20	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	0.50	0.07	11.61
16	21	−1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	0.25	0.04	12.67
23	22	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	0.06	0	0
027	23	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	0.09	0	0
31	24	−1	−1	−1	+1	−1	−1	+1	−1	+1	+1	+1	0.67	0.10	13.07

[a]Mean values of three measurements.

residue was redissolved in methanol/methyl *tert*-butyl ether (1:1, v/v) until the solution reached the defined volume of 10 mL. The solution was centrifuged at 11 000 rpm for 10 min and then used for HPLC analysis, which was performed with 1.3 mL/min methanol (solvent A) and methyl *tert*-butyl ether (solvent B) by using a gradient procedure on a C_{30} column (250 × 4.6 mm, 5 μm, Trentec, Gerlingen, Germany). The injection volume was 90 μL, and the gradient elution started with 90% A and 10% B to reach 55% A at 35 min, 40% at 40 min, then isocratic for 10 min, and finally 90% A at 60 min. Lycopene and its *cis*-isomers were quantified at 472 nm, and (*all-E*)-lycopene was identified by chromatographic comparison with the pure (*all-E*)-lycopene standard. However, since standards for lycopene (*Z*)-isomers were not commercially available, they were tentatively identified on the basis of the retention times and absorption spectrum characteristics described in the literature[8] and as it is shown in Figure 1. All extractions were carried out under subdued light and were performed in triplicate for each sample. The analytical method was validated for total lycopene using certificate reference material BCR-485 (mixed vegetables). The indicative value for (*all-E*)-lycopene in the reference material is 14.2 mg/kg, and this study obtained a mean value of 15.0 ± 0.6 mg/kg after five samples were analyzed. Lycopene content was analyzed in duplicate in each samples obtained from the *in vitro* digestion (GD, ID, and MF).

RESULTS AND DISCUSSION

The mean value of total lycopene in tomato passata was 17.32 mg/kg, with 5.9% *cis*-isomers (data not shown). The stability of lycopene during *in vitro* digestion was calculated as a function of the total lycopene quantified on the aliquot fractions obtained after gastric digestion and intestinal digestion. Results are shown in Figure 2, and the highest stability was observed after gastric digestion rather than intestinal digestion. In general, around 99% and 60% of the total lycopene of the tomato passata were recovered in GD and in the chyme fraction (ID); these values ranged from 97% to 50%. These values are in agreement with those reported in the scientific literature by different authors,[12,24] who described that the stability of carotenoids during *in vitro* digestion is higher than 60%.

Table 2 and Table 3 show the content of (*all-E*)-lycopene and its (*Z*)-isomers and the percentage of isomerization in the GD and ID samples of tomato passata, respectively. Although several authors[11,23] reported that the (*Z*)/(*all-E*) ratio of processed food was apparently unaffected during the gastric and duodenal phases, we observed a contrary behavior. In the GD fraction (Table 2), the content of (*all-E*)-lycopene ranged from 7.61 to 15.50 mg/100 g, whereas the amount of (*Z*)-isomer ranged from to 0.94 to 3.78 mg/kg. In some trials, the percentages of (*Z*)-isomers were significantly high, reaching values close to 30%, which could be related to the pH. The presence of acid medium induces the isomerization reaction of lycopene in food[33] and under conditions of simulated gastric digestion.[34,35] Thus, we agree with these authors[34,35] that pH-driven isomerization is responsible at least partially for the high (*Z*)-lycopene proportion found in the human body, since (*Z*)-isomers can be generated in the stomach. After ID (Table 3), (*all-E*)-lycopene contents ranged from 7.26 to 15.71 mg/kg, showing a lower content of (*Z*)-isomers than those described in GD. Moreover, the lower proportion of the (*Z*)-isomers in the chyme samples compared with the GD samples showed that the pH-driven isomerization reaction is reversible,[35] and in chyme, when reaching more neutral pH levels, a change from *cis*- to *all-trans*-isomers takes place. Table 4 and Figure 1 show the total lycopene incorporated into micelles (MF), which is a small proportion of the total lycopene contained in the tomato passata (ranging from 2% to 12%). The amount of (*all-E*)-lycopene ranged from 0.06 to 1.98 mg/kg, whereas for the (*Z*)-isomers all values were lower than 0.30 mg/kg (Table 4). Different authors have reported that lycopene from tomato

Table 5. Effects of Coded Factors on Each Response in Terms of Lycopene (mg/g) Extracted and Determined after Gastric Digestion (GD), after Intestinal Digestion (ID), and in the Micellar Fraction (MF) of (*all-E*)-Lycopene and (*Z*)-Isomers and Factors Affecting *cis* Isomerization (%)[a]

	responses								
	GD			ID			MF		
factor	*E*	*Z*	%	*E*	*Z*	%	*E*	*Z*	%
X_1	−1.71			−1.74	0.39	4.04			
X_2	1.03			1.18	0.28		−0.41	−0.05	
X_3									
X_4				−1.04		1.47			4.10
X_5							0.46		
X_6									
X_7									
X_8					−0.17				−3.87
X_9				−0.99					
X_{10}				0.91					3.13
X_{11}									
R^2	0.32	0.14	0.10	0.60	0.65	0.62	0.44	0.37	0.51

[a]A strong effect was considered when $p < 0.200$, and then the factor was considered an influencing factor (IF).

puree is the least bioaccessible carotenoid, compared with lutein from spinach and β-carotene from carrots,[15] and the proportion of lycopene incorporated in micelles depends on the presence of oil and the chain length and saturation degree of the fatty acids.[16] The isomerization percentage in MF reached values around 20%, showing that (*Z*)-isomers are better incorporated into micelles than (*all-E*)-lycopene.[6,12]

The different amounts of lycopene isomers quantified in the 24 trials after DG, DI, and MF are related to the different conditions used in the assays, in which we screened the factors that are critical for determining the *in vitro* availability of lycopene. Results obtained in the screening phase may be discussed on the basis of three empirical principles, i.e., the sparsity principle (only a small number of the candidate factors are critical), the hierarchy principle (primary effects are more likely to be critical than secondary effects from two-factor interactions, which in turn are more likely to be critical than three-factor interaction, and so forth), and the heredity principle (it is unusual for an interaction to be critical unless at least one of the factors involved has an active primary critical effect). On the basis of these principles, clearly set out by Miller and Sitter,[36] an error type I value of 0.20 was considered to identify the most important primary effects and thus to identify the IF. Because at the resolution IV level secondary effects are confused, we deliberately discarded information on second-order interactions provided by the fractional factorial design used. Table 5 shows the effect of the 11 coded factors on the measured responses (mg of (*all-E*) and (*Z*)-lycopene) after DG, DI, and MF to determine the factors that influenced the *in vitro* lycopene availability. A strong effect was considered when $p < 0.20$, as described above, and then the apparent critical factor (Table 1) was considered an IF.

For the GD only, the effect of the factors coded from X_1 to X_5 can be considered, since the other factors (X_6 to X_{11}) are related to the conditions of ID. Thus, after GD only two factors can be considered IFs, but with a contrasting effect. The thermal treatment (X_1) applied to the tomato passata before the analysis had a negative effect on the content of (*all-E*)-lycopene. In general, the changes in the carotenoids could be isomerization and oxidation,[37] and in tomato products cooked at temperatures above 100 °C, degradation occurred faster than isomerization, increasing as a function of cooking time.[38] Since the oxidation products of lycopene were not measured in this study, the negative effect of thermal treatment could lead to a reduction in (*all-E*)-lycopene associated with the oxidation reactions. The other significant factor was the addition of olive oil (X_2), which can be considered an IF with a positive effect on the total content of (*all-E*)-lycopene. This effect could be related to the fact that the presence of oil or fat facilitates the solubilization of lycopene[14] when it is separated from the protein complexes during gastric digestion.[21] Although a higher proportion of (*Z*)-isomers was quantified in GD samples, no factor was considered an IF for the amount of these isomers, even the gastric pH (X_3). In this study we selected two values for X_3 according to the different physiological conditions, fasting state and after the ingestion of a meal. Thus, a gastric pH around 1.5−2[34,35] simulates the fasting state *in vivo*, whereas higher values simulate the physiological conditions of the stomach after meal intake, since after vegetable-rich meals pH ranges between 5.8 after meal intake and 3, 3 h later.[15,39] Whereas in the former *in vitro* models pH-driven isomerization has been described,[34,35] in the later *in vitro* models, isomerization of lycopene associated with pH was not observed.[11,15]

For the ID and MF samples, seven of the 11 key factors were considered IFs for the content of (*all-E*)- and (*Z*)-lycopene, which are described together since both fractions are obtained after intestinal digestion. Thermal treatment (X_1) was considered an IF with a negative effect for the content of the (*all-E*)-isomer and with a positive effect for the content of (*Z*)-isomers detected in the ID. Thermal treatment, followed by gastric digestion, leads to (*Z*)-isomerization, reducing the content of (*all-E*)-lycopene and increasing its (*Z*)-isomers.[35] Although industrial pasteurization applied in the commercial sterilization of some tomato products did not increase the percentage of isomerization,[40] domestic cooking could induce isomerization, because the tomato is cooking at a higher temperature and in the presence of oxygen.[41] Gastric digestion (X_4) duration is an IF, with a negative effect on the content of (*all-E*)-lycopene and a positive effect on the amount of (*Z*)-isomers that reaches the small intestine and is incorporated into micelles. In addition, the percentage of isomerization in ID and the MF increased according to the gastric digestion time. This

effect is related, as has been described above, to the pH-driven isomerization.[34,35] The time that food is in the stomach during digestion is related to the composition of the food. Protein and fat produce major hydrochloric acid secretion in the stomach mucosa, providing a longer time for stomach emptying. We consider in this study two durations for gastric digestion, 15 and 60 min, in contrast with the 30 min considered by other authors.[15] Fifteen minutes represents the time that tomato passata alone will be in the stomach, whereas 60 min is the duration when tomato passata is ingested with other foods, increasing the time of digestion. According to our results in this *in vitro* model, when tomato passata is under gastric digestion conditions for 60 min there is a higher percentage of (*Z*)-

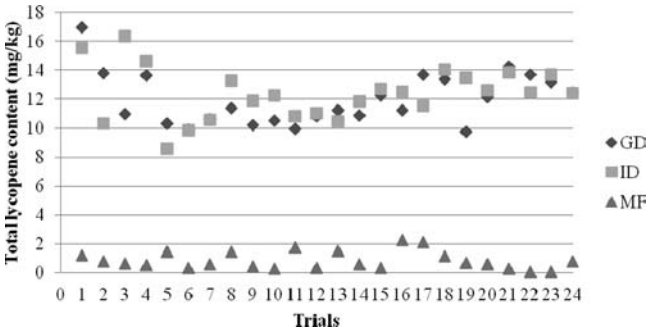

Figure 2. Stability of total lycopene after gastric digestion (GD) and intestinal digestion (ID) and the amount of total lycopene solubilized into the micellar fraction (MF). Data are expressed as the mean values of three determinations.

lycopene in the chyme and micelles, increasing the bioaccessibility.

Many factors during digestion of carotenoids in the large bowel are involved in its bioavailability. According to Tyssander et al.,[14] intestinal pH values of around 6−7, the presence of oil, and the concentration of bile salts are the main factors that determine the solubilization of carotenoids before absorption. Huo et al.[16] described that 0.5−1% of oil or fat in food allows the solubilization of carotenoids in the micelles. The addition of olive oil (X_2) is considered an IF during ID and in the MF, since olive oil facilitates the solubilization of lycopene, giving a positive effect in quantifying both isomers in the ID, but with a significant negative effect for the amount of this carotenoid in the MF. The amount of oil in the samples with a high level of this factor was higher than the recommended percentage,[16] resulting in a positive effect in the quantified amount of lycopene during ID, but diminishing the solubilization of lycopene into the micelles. Although the effect in the MF was very low, this different behavior could be explained because the higher oil content requires more bile salts to form the micelles; so in trials with high oil levels the fat could be incompletely digested, solubilizing the lycopene and making its incorporation into the micellar phase more difficult. Another factor considered an IF for the (*all-E*)-lycopene quantified in the MF is the addition of pepsin (X_5). The high amount of pepsin during gastric digestion facilitates the breakdown of the lycopene−protein complexes, resulting in the greatest quantification of native lycopene present in foods. During *in vitro* intestinal digestion, the concentration of bile salt (X_8) showed a significant negative, but very low, effect on the content of (*Z*)-isomers of lycopene and in the isomerization percentage of the MF. Although bile salts favor the *in vitro* availability of carotenoids,[15] by increasing the solubilization in the micellar phase, the concentration of bile salts had no significant influence on (*all-E*)-lycopene in both analyzed fractions (ID and MF). Although a higher percentage of (*Z*)-isomers has been transferred to micelles, compared with the ID samples (Tables 3 and 4), the high level of bile salt did not facilitate this phenomena, and the solubilization could be determined only by the chemical forms, since a negative effect was observed between the percentage of isomerization and the concentration of bile salts. The addition of colipase (X_9) had a negative effect on the amount of (*all-E*)-lycopene after the ID, because this protein cofactor activates pancreatic lipase[43] and facilitates the hydrolysis of fat, which could reduce the solubilization of lycopene, showing a contradictory effect to factor X_2. However, we have not determined if the added oil was completely digested during intestinal digestion. The addition of colipase is not usual in the *in vitro* model, but according to the negative effect did not appear to be considered an important factor during digestion. The time of the ID is also considered an IF for the total content of (*all-E*)-lycopene in the ID and in the percentage of isomerization of the MF, since the longer period during digestion increases this percentage in the micelles.[14] However, the speed (X_{11}) applied during *in vitro* digestion had no effect on the measured responses, in either ID or MF.

Explained variance (R^2) showed the percentage of variability explained by the influence factors for each response and in the three fractions (GD, ID, and MF). Taking into account the R^2 value (Table 5), during GD the factors considered IFs (X_1 and X_2) had a low effect on the measured responses, with R^2 values of 0.32, 0.14, and 0.10 for (*all-E*)-lycopene, (*Z*)-isomer, and percentage of isomerization, respectively, so the variability of the GD results obtained may be explained by other factors different from those included in the present study (Table 1). However, the IF of the ID (X_1, X_2, X_4, X_8, X_9, and X_{10}) explained the variation in 60% of (*all-E*)-lycopene, 65% of (*Z*)-lycopene, and 62% of the percentage of isomerization. For the lycopene content in the MF, the R^2 values were 0.44, 0.37, and 0.51 for (*all-E*)- and (*Z*)-lycopene and for the percentage of isomerization. These data showed that the key factors that affect the availability of lycopene during *in vitro* digestion are related to intestinal digestion, as has been described in other *in vitro* studies.[11−15]

We conclude that many factors related to intestinal digestion affect the *in vitro* availability of lycopene, including the main factors that have been described under physiological conditions. The *in vitro* model could be used as a method for estimating the accessibility and availability of lycopene to be absorbed taking into consideration the isomerization reaction, but the conditions must be standardized to achieve comparable results among different studies. This study represents the first step to optimize this *in vitro* method, in order to standardize the procedure.

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Notes

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