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Liquid Chromatography−Mass Spectrometry of *cis*- and all-*trans*-Lycopene in Human Serum and Prostate Tissue after Dietary Supplementation with Tomato Sauce

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Several epidemiological studies suggest a lower incidence of prostate cancer in men who routinely consume tomato products. Tomatoes are the primary dietary source of lycopene, which is among the most potent antioxidants of the carotenoids. Men with clinical stage T1 or T2 prostate adenocarcinoma were recruited ($n = 32$) and consumed tomato sauce based pasta dishes for 3 weeks (equivalent to 30 mg of lycopene per day) before radical prostectomy. Prostate tissue from needle biopsy just before intervention and prostectomy after supplementation from a subset of 11 subjects was evaluated for both total lycopene and lycopene geometrical isomer ratios. A gradient HPLC system using a C_{18} column with UV−vis absorbance detection was used to measure total lycopene. Because the absorbance detector was insufficiently sensitive, HPLC with a C_{30} column and positive ion atmospheric pressure chemical ionization mass spectrometric (LC-MS) detection was developed as a new assay to measure the ratio of lycopene *cis/trans* isomers in these samples. The limit of detection of the LC-MS method was determined to be 0.93 pmol of lycopene on-column, and a linear response was obtained over 3 orders of magnitude. Total lycopene in serum increased 2.0-fold from 35.6 to 69.9 μg/dL (from 0.664 to 1.30 μM) as a result of dietary supplementation with tomato sauce, whereas total lycopene in prostate tissue increased 3.0-fold from 0.196 to 0.582 ng/mg of tissue (from 0.365 to 1.09 pmol/mg). all-*trans*-Lycopene and at least 14 *cis*-isomer peaks were detected in prostate tissue and serum. The mean proportion of all-*trans*-lycopene in prostate tissue was ~12.4% of total lycopene before supplementation but increased to 22.7% after dietary intervention with tomato sauce. In serum there was only a 2.8% but statistically significant increase in the proportion of all-*trans*-lycopene after intervention. These results indicate that short-term supplementation with tomato sauce containing primarily all-*trans*-lycopene (83% of total lycopene) results in substantial increases in total lycopene in serum and prostate and a substantial increase in all-*trans*-lycopene in prostate but relatively less in serum.

KEYWORDS: ***cis*- and all-*trans*-lycopene; LC-MS; APCI; prostate cancer; tomato sauce; dietary supplement**

INTRODUCTION

Lycopene, the red pigment of the tomato, is among the most efficient singlet oxygen quenchers of the natural carotenoids (*1*, *2*). Although twice as efficient as β-carotene at quenching singlet oxygen, lycopene has no provitamin A activity, so that its potential pharmacological effects cannot be associated with vitamin A. Giovannuci et al. (*3*, *4*) and Gann et al. (*5*) have reported a correlation between consumption of tomato products and reduced incidence of prostate cancer, which is the second leading cause of cancer death among American men over age 65 (*6*). In addition, lycopene has been suggested to reduce the risk of bladder cancer (*7*) and protect against radiation damage (*8*).

Lycopene occurs in food primarily as an all-*trans* isomer, the form that is biosynthesized by plants and found in fresh

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tomatoes (*9*). Exposure of lycopene to heat and light during cooking or food processing will produce a variable mixture of all-*trans*-lycopene and *cis* isomers. Recently, Boileau et al. (*10*) reported that *cis* isomers of lycopene were more bioavailable than all-*trans*-lycopene in the ferret. This information, in combination with measurements showing that *cis*-lycopene isomers constitute often >50% of total lycopene in human blood and tissues (*11*, *12*), has prompted speculation that *cis*-lycopenes might be more readily absorbed by humans. However, data on human bioavailability of lycopene and its isomers are scarce. Human tissue specimens are often limited to biopsies consisting of <10 mg each, so a reliable and sensitive method is needed to simultaneously measure *cis*- and *trans*-lycopene in these samples.

Most methods for measuring total lycopene use reversed phase high-performance liquid chromatography (HPLC) separation with a C_{18} column and absorbance detection (*13*, *14*). To resolve multiple *cis* isomers from all-*trans*-lycopene, a C_{30} column has been developed (*15*). Because carotenoids are heat labile, gas chromatographic methods are not used. To increase the sensitivity and selectivity of HPLC-based carotenoid analyses, liquid chromatography−mass spectrometry (LC-MS) methods have been developed using continuous-flow fast atom bombardment (*16*), electrospray (*17*), and atmospheric pressure chemical ionization (APCI) (*18*). Additional selectivity has been obtained by combining HPLC continuous-flow fast atom bombardment with tandem mass spectrometry (*19*), which may be used to distinguish isomeric carotenoids such as lycopene, β-carotene, and α-carotene on the basis of their unique fragmentation patterns. Among these, LC-MS using APCI shows a higher linearity of detector response (exceeding 4 orders of magnitude of carotenoid concentration). Here we report the development and clinical application of a sensitive quantitative method based on C_{30} reversed phase HPLC with APCI mass spectrometric detection.

MATERIALS AND METHODS

Chemicals and Reagents. all-*trans*-Lycopene and pyrogallol were purchased from Sigma Chemical (St. Louis, MO), and butylated hydroxytoluene (BHT) was purchased from Burdick & Jackson (Muskegon, MI). HPLC grade methanol and methyl *tert*-butyl ether were obtained from Fisher Scientific (Fair Lawn, NJ).

Human Subjects. A subset of 11 men (age, 63.7 ± 6.2 years; height, 174.2 ± 11.1 cm; and weight, 87.7 ± 17.7 kg) of 32 overall were recruited from the urology clinic of the Westside Veterans Administration (VA) Hospital (Chicago, IL) and gave informed consent prior to participation in this study as approved by the University of Illinois at Chicago and Westside VA Institutional Review Board. Study candidates were patients at the urology clinic and were scheduled for trans-rectal ultrasound/prostate needle biopsy (TRUS/PNB) for the diagnosis of prostate cancer after showing abnormal digital rectal examinations and/or rising serum prostate specific antigen concentrations. During TRUS/PNB, one extra needle biopsy sample (~7 mg) was obtained for the measurement of lycopene and lycopene isomers. In addition, a blood sample (~7 mL) was obtained for lycopene determination.

Exclusion criteria for participation in this study included allergies to tomatoes or tomato products, simultaneous participation in other research studies, a history of chronic diseases such as inflammatory bowel disease or other cancers, consumption of dietary supplements containing tomato extracts, consumption of more than the RDA for vitamins C, E, A, or β-carotene, or medical problems that would preclude prostatectomy. Smokers were not excluded. Following biopsy, only those candidates whose pathology indicated clinical stage T1 or T2 adenocarcinoma of the prostate and who elected prostatectomy as the course of therapy continued in this study. Within the course of usual care, surgery for these patients would be scheduled to take place 3−5 weeks postdiagnosis. Therefore, the dietary intervention period between diagnosis and surgery was set to 3 weeks.

For 3 weeks before radical prostatectomy, patients consumed 200 g of commercial spaghetti sauce (Hunt-Wesson, Fullerton, CA) in pasta dishes prepared by study cooks, which was equivalent to 30 mg of lycopene per day. In addition to completing diet questionnaires, each patient was contacted six times for 24-h intake recalls. Lycopene values for self-reported dietary intake were calculated on the basis of standard values (*20*). After the 3-week dietary intervention, a 7-mL fasting blood sample and resected prostate tissue (~100−200 mg, wet weight) were obtained.

Preparation of Samples. Serum was prepared immediately from blood samples and frozen at −80 °C until extraction and analysis. Prostate tissue was weighed and homogenized in 0.5 mL of methanol/water (1:1, v/v) containing 1% pyrogallol. Saponification was carried out at 70 °C for 1 h by adding 100 μL of 60% potassium hydroxide. Hexane (2 mL) containing BHT (100 mg/L) was added to the mixture followed by vortex mixing for 1 min. Each sample was centrifuged at 418*g* for 5 min, and the upper hexane layer was removed to another glass tube. The aqueous layer was extracted with another 2 mL of hexane, and the hexane fractions were combined and evaporated to dryness in a vacuum centrifuge. The residue was redissolved in 50 μL of diethyl ether and 150 μL methanol/acetonitrile/tetrahydrofuran (50:45:5, v/v/v) to a total volume of 200 μL. Extracts were stored at 0 °C until analysis on the same day or frozen (−20 °C) overnight. Serum samples were extracted using a procedure identical to that for the prostate tissue except that the saponification step was omitted. Samples were handled in subdued light and stored in the dark. It should be noted that Clinton et al. (*12*) compared saponification and extraction of human prostate tissue to simple methanol extraction and found no differences between the two methods with respect to the *cis/trans* ratios of the extracted lycopene. Also, Ferreira et al. (*21*) compared saponification to no saponification for extraction of lycopene from several tissues and found that saponification resulted in greater total lycopene recovery but no increase in *cis*-lycopene.

Preparation of Standards. all-*trans*-Lycopene (1 mg) was dissolved in 2 mL of tetrahydrofuran to obtain a stock solution of 0.5 μg/mL (0.932 μM). The stock solution was diluted with methanol/methyl-*tert*-butyl ether (45:55, v/v) to obtain lycopene standard solutions for quantitative analysis using LC-MS. Isomerization of the all-*trans*-lycopene standard to produce *cis* isomers was carried out in hexane at 4 °C for 2 weeks in a sealed tube in the dark.

HPLC and LC-MS. Reversed phase HPLC with absorbance detection (at 450 nm) based on the method of Stacewicz-Sapuntakis et al. (*14*) was used for the analysis of total lycopene. Because this method was insufficiently sensitive for lycopene isomer quantification in biopsy samples, a method based on LC-MS was developed instead. Gradient HPLC separation with positive ion APCI mass spectrometric detection was used to measure lycopene isomers in serum and prostate tissue samples. HPLC separation was carried out using an Agilent (Palo Alto, CA) 1100 pump system equipped with a photodiode absorbance detector and YMC (Wilmington, NC) C_{30} 3 μm reversed phase carotenoid column, 4.6 × 250 mm. The HPLC solvent system consisted of methanol/methyl-*tert*-butyl ether (55:45, v/v) at 1.0 mL/min for 15 min followed by a linear gradient to methanol/methyl-*tert*-butyl ether (45:55, v/v). An Agilent G1946A LCMSD mass spectrometer equipped with APCI was used for detection of protonated lycopene at *m*/*z* 537. The entire HPLC column effluent was introduced into the APCI LC-MS interface without solvent splitting. Selected ion monitoring (SIM) of *m*/*z* 537 with a dwell time of 1 s was used to monitor the elution of lycopene isomers from the C_{30} column. Other mass spectrometer parameters included a nitrogen nebulizer gas pressure of 50 psi, a nitrogen drying gas temperature of 300 °C, a drying gas flow at 7 L/min, a capillary voltage of 2500 V, a fragmentor voltage of 110 V, a vaporizer voltage of 300 V, and a corona current of 3.0 μA.

RESULTS

HPLC with absorbance detection was used for the analysis of lycopene in the tomato sauce used for the dietary intervention, which was determined to be 83% all-*trans*-lycopene with the

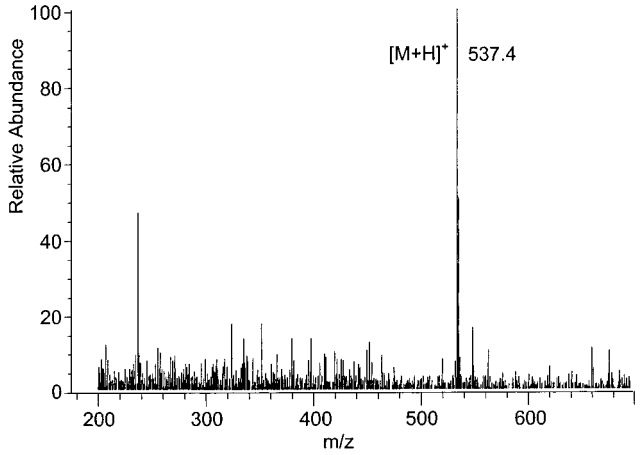

Figure 1. Positive ion APCI mass spectrum of lycopene showing the protonated molecule at *m/z* 537.

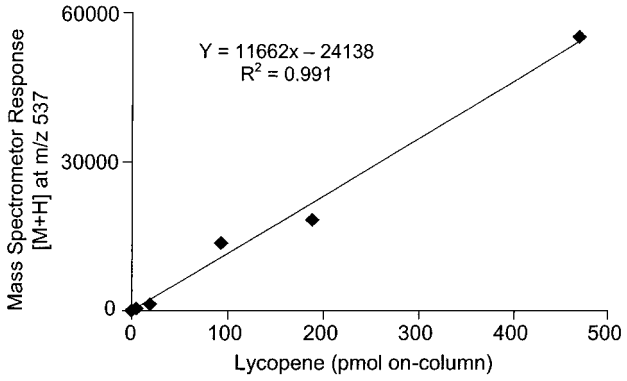

Figure 2. Calibration curve for lycopene obtained using LC-MS with positive ion APCI and SIM of the protonated molecule of *m/z* 537.

remaining 17% consisting of a mixture of *cis* isomers. Although this method was suitable for the measurement of lycopene isomers in tomato sauce or for the measurement of total lycopene as a single chromatographic peak in extracts of serum and prostate tissue, levels of individual lycopene isomers in the extract of a single prostate tissue biopsy were below the limit of quantification using absorbance detection. Therefore, LC-MS was evaluated instead. During LC-MS, the protonated molecule of lycopene at *m/z* 537 was the most abundant ion in the positive ion APCI mass spectrum (**Figure 1**). Consequently, the ion of *m/z* 537 was used for SIM during LC-MS quantitative analysis. The limit of quantitation (defined as an S/N of 3:1) was 0.93 pmol of lycopene injected on-column, and the LC-MS calibration curve for lycopene showed a linear response from 0.93 to 500 pmol with R^2 of 0.991 (**Figure 2**). The within run (same day) reproducibility of the standard lycopene measurements using LC-MS was ±4% ($n = 4$). In addition, the recovery of lycopene from spiked human plasma was 91.0% (SD = 3.64, $n = 3$) and 94.7% (SD = 9.09, $n = 3$) at 200 nM (107 ng/mL) and 500 nM (268 ng/mL), respectively.

The LC-MS SIM mass chromatogram for the isomerized lycopene standard solution is shown in **Figure 3**. The peak for all-*trans*-lycopene eluted at 29.5 min and was identified by comparison to authentic standard. Lycopene *cis* isomers eluted beginning at ~12 min, and the intense peak eluting at 30.5 min was identified as 5-*cis*-lycopene on the basis of a molecular weight of 536.4, the UV−vis absorbance chromatogram, and elution during C_{30} reversed phase chromatography (*12*). The other peaks eluting between 10 and 25 min were identified as other lycopene *cis* isomers on the basis of molecular weight,

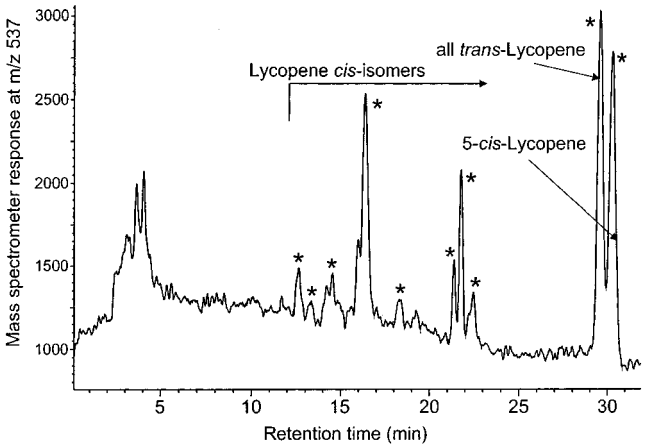

Figure 3. LC-MS positive ion APCI mass chromatogram showing the protonated molecules at *m/z* 537 for all-*trans*- and *cis*-lycopene in an isomerized lycopene standard. Under these HPLC conditions, lycopene isomers eluted after 12 min, whereas β-carotene and α-carotene isomers would elute earlier. Lycopene peaks in this chromatogram that would be detected and integrated by the mass spectrometer data system are marked with an asterisk (*).

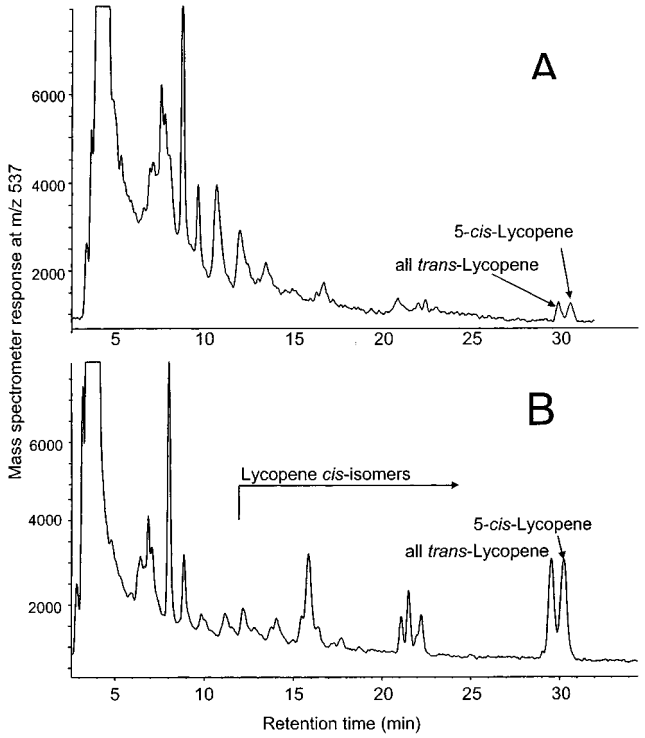

Figure 4. Positive ion LC-MS analysis of lycopene isomers in extracts of (A) a biopsy of human prostate before supplementation and (B) resected prostate tissue from the same patient after 3 weeks of dietary supplementation with tomato sauce containing 30 mg of lycopene per day.

UV−vis absorbances, and HPLC retention times, but the specific structures of these *cis* isomers were not determined.

On the basis of the retention times, molecular weights, and UV−vis absorbances of the lycopene isomers observed in the standard mixture (**Figure 3**), lycopene isomers were identified in the LC-MS chromatograms of extracts of human prostate tissue and serum. Examples of LC-MS chromatograms of human prostate tissue from the same subject before and after 3 weeks of dietary intervention with tomato sauce containing lycopene at 30 mg per day are shown in **Figure 4**. In addition, LC-MS

Table 1. Lycopene Isomers in Human Prostate Tissue before and After Tomato Sauce Dietary Intervention

	prostate biopsy tissue before intervention				prostate tissue resected after intervention			
patient	total lycopene (ng/mg of tissue)	ratio (all-*trans*/*cis*)	% (all-*trans*/total)	all-*trans*-lycopene (ng/mg of tissue)	total lycopene (ng/mg of tissue)	ratio (all-*trans*/*cis*)	% (all-*trans*/total)	all-*trans*-lycopene (ng/mg of tissue)
104	0.13	0.170	14.53	0.02	0.11	0.074	6.85	0.01
116	0.14	0.141	12.36	0.02	0.56	0.231	18.73	0.10
117	0.38	0.226	18.40	0.07	0.58	0.223	18.23	0.11
118	0.07	0.091	8.33	0.01	0.33	0.298	22.95	0.08
119	0.54	0.215	17.70	0.10	1.68	0.333	25.03	0.42
120	0.21	0.290	22.50	0.05	0.28	0.284	22.13	0.06
121	0.07	0.046	4.35	0.003	0.76	0.282	22.00	0.17
123	0.08	0.081	7.23	0.01	0.66	0.232	18.73	0.12
125	0.17	0.083	7.68	0.01	0.58	0.369	26.95	0.16
128	*a*	*a*	*a*	*a*	0.60	0.656	39.63	0.24
129	0.17	0.124	11.05	0.02	0.26	0.392	28.18	0.07
av ± SD	0.196 ± 0.151	0.147 ± 0.078	12.4 ± 5.80	0.031 + 0.032	0.582 ± 0.415	0.295 ± 0.144	22.67 ± 8.02	0.140 + 0.112

[a] Samples were damaged.

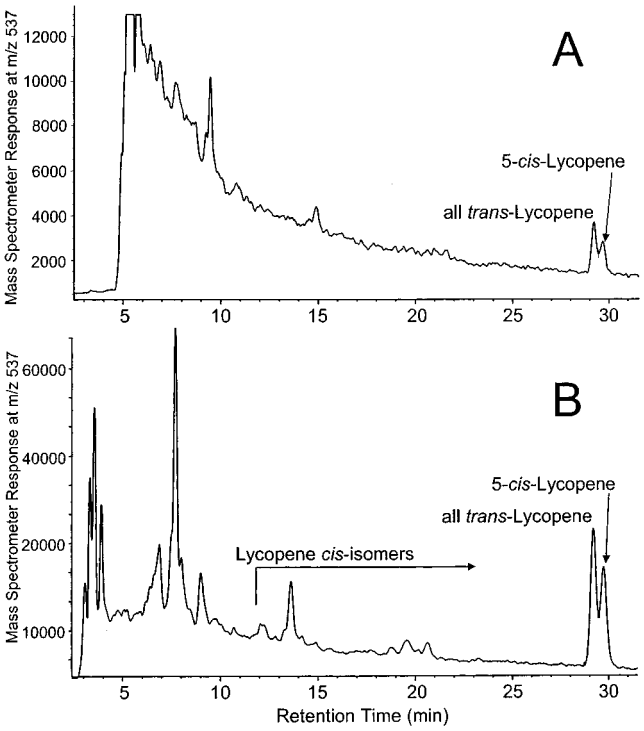

Figure 5. Positive ion LC-MS analysis of lycopene isomers in extracts of (A) human serum prior to dietary intervention and (B) human serum from the same subject after 3 weeks of dietary intervention with tomato sauce containing 30 mg of lycopene per day.

chromatograms of serum extracts from the same individual before and after dietary intervention are shown in **Figure 5**. In these figures, β-carotene and α-carotene eluted before 10 min, whereas lycopene eluted after 12 min.

After 3 weeks of dietary intervention, the total lycopene levels in prostate tissue increased 3.0-fold (0.196 ± 0.151 vs 0.582 ± 0.415 nmol/g of tissue) (**Table 1**), and total lycopene concentrations increased 2.0-fold (35.6 ± 23.9 vs 69.9 ± 38.9 μg/dL) in serum (**Table 2**). Although these mean values show large interindividual variation, they are consistent within individuals, each of whom served as his own control. The matched pair *t* test *p* values for the change in total levels of lycopene were statistically significant at $p = 0.003$ and 0.007 for serum and prostate tissue levels, respectively. In addition, the correlation coefficients between the total lycopene values before and after intervention were 0.667 for serum and 0.694 for prostate tissue ($p < 0.05$).

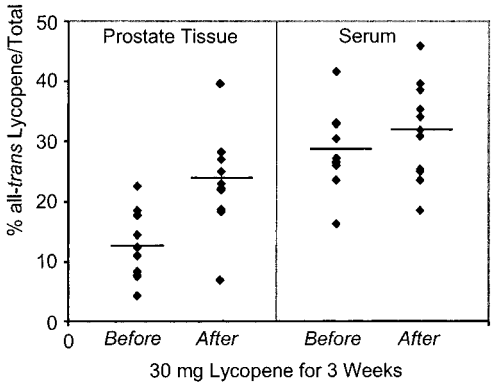

Figure 6. Levels of lycopene *cis/trans* isomers in human serum and prostate tissue before and after dietary intervention with tomato sauce containing lycopene.

The mean percentage of all-*trans*-lycopene in prostate tissue was 12.4 ± 5.8% at the start of the study and increased to 22.7 ± 8.0% after dietary intervention (**Figure 6**; **Table 1**). The matched pair *t* test *p* value for the change in tissue all-*trans*-lycopene was statistically significant at $p = 0.015$. Although serum concentrations of total lycopene increased 2.0-fold as a result of dietary supplementation, the percentage of all-*trans*-lycopene increased only slightly from 28.9 to 31.7%. This increase was statistically significant at the 0.087 level.

DISCUSSION

Supplementation with 30 mg of dietary lycopene in tomato sauce increased levels of total lycopene in serum and prostate tissue an average of 2.0- and 3.0-fold, respectively. These results are important because they show that dietary supplementation with food containing lycopene can produce elevated lycopene levels in the prostate. In addition, these data suggest that serum levels are an imperfect indicator of prostate levels. Although both serum and prostate levels of lycopene increased as a result of dietary supplementation, prostate levels increased to a greater extent than did serum. Furthermore, the percentage of all-*trans*-lycopene increased much more in the prostate than in serum. Although the lycopene in tomato sauce used in this study was 83% all-*trans*-lycopene, the percentage of the all-*trans* isomer increased slightly in serum, whereas it increased 2.0-fold in prostate tissue. This is a surprising result and another example of how serum is an imperfect surrogate for prostate levels of lycopene. Whether this difference is due to some physiological process is unknown.

Table 2. Lycopene Isomers in Human Serum before and after Tomato Sauce Dietary Intervention

	before intervention				after intervention			
patient	total lycopene (μg/dL)	ratio (all-*trans*/*cis*)	% (all-*trans*/total)	all-*trans*-lycopene (μg/dL)	total lycopene (μg/dL)	ratio (all-*trans*/*cis*)	% (all-*trans*/total)	all-*trans*-lycopene (μg/dL)
104	19.8	0.437	30.40	6.02	21.7	0.519	34.15	7.41
116	26.1	0.308	23.53	6.14	124.0	0.308	23.53	29.18
117	104.5	0.194	16.23	16.96	146.9	0.228	18.57	27.28
118	17.9	0.363	26.63	4.77	59.3	0.332	24.90	14.8
119	31.2	0.437	30.41	9.49	105.0	0.658	39.69	41.67
120	28.4	0.358	29.83	8.47	46.2	0.339	25.33	11.7
121	23.2	0.373	27.15	6.30	43.4	0.446	30.86	13.4
123	39.3	0.351	26.00	10.2	45.5	0.631	38.70	17.6
125	38.1	0.492	33.00	12.6	61.4	0.470	31.96	19.6
128	36.3	0.493	33.03	12.0	71.0	0.546	35.32	25.1
129	26.3	0.717	41.75	11.0	44.2	0.846	45.84	20.3
av ± SD	35.6 ± 23.9	0.411 ± 0.133	28.90 ± 6.41	9.45 ± 3.62	69.9 ± 38.9	0.484 ± 0.182	31.71 ± 8.11	20.7 ± 9.65

On the basis of studies with ferrets, Boileau et al. (*10*) hypothesized that all-*trans*-lycopene is absorbed less efficiently from the gut than are *cis* isomers. An alternative explanation might be that during solubilization and absorption in the intestine, all-*trans*-lycopene is released from its stabilizing food matrix and begins to equilibrate to a thermodynamic mixture of isomers. The LC-MS analysis of an equilibrated mixture of lycopene isomers is shown in **Figure 3**. These isomers were formed by storing a hexane solution of all-*trans*-lycopene in the dark for 2 weeks at 4 °C in the absence of oxygen. In this standard solution, the amount of all-*trans*-lycopene was 30.6%, which is similar to the levels measured in human serum before and after dietary intervention. These data are also consistent with those of Clinton et al. (*12*), who reported serum levels of all-*trans*-lycopene in men to be from 27 to 42%. Together, these results support the hypothesis that serum levels of lycopene isomers reflect thermodynamic equilibration instead of selective absorption of specific isomers (*22*).

Lycopene levels in the prostate increased more than serum concentrations, and these results are consistent with those of Clinton et al. (*12*), who reported that lycopene accumulates in prostate tissue. Furthermore, we found that the proportion of *cis*-lycopene in human prostate tissue was greater than that in human serum. Specifically, prostate tissue lycopene was 12.4% all-*trans* and 22.7% all-*trans* before and after dietary intervention, respectively. For comparison, Clinton et al. reported 12−21% all-*trans*-lycopene in the human prostate, which is identical to our findings. Although our data confirm that *cis*-lycopene accumulates in the human prostate, the mechanism for this process and the reasons for different ratios of *cis* and all-*trans* isomers in prostate compared to serum remain unknown. Because the ratio of *cis/trans*-lycopene in serum was different from that in prostate tissue, there appears to be either selective absorption of *cis*-lycopene isomers by the prostate, selective stabilization of *cis* isomers, or selective metabolism and degradation of all-*trans*-lycopene in the prostate.

It is interesting to note that total levels of all-*trans*-lycopene in the prostate increased ~6-fold (12.4% × 0.196 vs 22.7% × 0.582; **Table 1**), which is more than *cis*-lycopene levels increased in the prostate as a result of dietary intervention. Therefore, an alternative explanation for these results might be that all-*trans*-lycopene is taken up preferentially by the prostate and then partially degraded or isomerized to *cis* forms. The elucidation of the correct explanation for these results will require additional investigation.

Finally, APCI-LC-MS provides a sensitive method for the quantitative analysis of lycopene isomers in human serum and prostate tissue. Serum preparation requires only simple hexane extraction, whereas prostate tissues require saponification to release lycopene followed by hexane extraction. Because lycopene and its isomers are light, heat, and air sensitive (*23*), a minimum of sample handling is preferred to ensure sample stability and to prevent the formation of artifacts.

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