

Optimizing Sampling of Tomato Fruit for Carotenoid Content with Application To Assessing the Impact of Ripening Disorders

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Color defines one aspect of quality for tomato and tomato products. Carotenoid pigments are responsible for the red and orange colors of tomato fruit, and thus color is also of dietary interest. The aims of this study were (1) to determine the relative importance of field sampling and analytical replication when measuring lycopene and β -carotene in tomato fruit and (2) to determine the effect of yellow shoulder disorder (YSD) on the content of lycopene and β -carotene in tomato juice and tissue. Our results show that increasing biological replications is an efficient strategy for reducing the experimental error associated with measurements of lycopene and β -carotene. Analytical replications did not contribute significantly to observed variation, and therefore experimental efficiency will be gained by reducing analytical replications while increasing field replication. We found that YSD significantly reduces lycopene in affected tissue and in juice made from affected fruit. In contrast, β -carotene concentrations were only reduced in affected tissue but were not significantly reduced in juice. With increasing interest in biofortified crops, modulating the carotenoid profile in tomato by minimizing YSD symptoms represents a strategy for improving tomato fruit quality that is currently supported by grower contract structure and processor grades.

KEYWORDS: Lycopene; β -carotene; yellow shoulder disorder; discoloration; nutritional potential

INTRODUCTION

Color and color uniformity of tomato fruit affect grade and appearance of the end product and are therefore important quality attributes in the processing industry. A major quality constraint to producing tomatoes is the presence of yellow shoulder disorder (YSD). YSD is a blotchy ripening disorder that is characterized by discolored regions under the epidermis of mature fruits (1). It is an economic problem because growers receive payment premiums for color quality, and USDA processor grades are largely determined by the amount of off-color tissue in products (2).

The causes of YSD are diverse. The incidence of YSD is influenced by soil fertility, especially potassium nutrition (3) and phosphorus nutrition (4); environmental factors, including low to high temperature fluctuations (5), high pericarp temperature, and high relative humidity (reviewed in ref 6); and genetic background (7). Some varieties appear more or less susceptible, though resistance to YSD has not been explicitly reported (7, 8).

The color of tomato fruit is determined by carotenoid pigments. Ripe fruit contain high levels of lycopene, the pigment that gives tomato its red color. There is considerable interest in the dietary role of lycopene in reducing the risk of certain cancers, including prostate cancer (9–11) and breast cancer (12). Ripe fruit also contains β -carotene, which is synthesized from lycopene. β -Carotene is the carotenoid recognized as a nutrient in tomato fruit due to its pro-vitamin A activity. Each year 750 million people suffer from vitamin A deficiency, and a single serving of tomato products can supply in excess of 30% of recommended daily allowances. Tomato varieties are available that could meet vitamin A dietary requirements with a single serving. With the increasing interest in biofortified crops, modulating the carotenoid profile in tomato is becoming a major focus of germplasm improvement efforts.

A limitation to biofortified tomato products is the lack of economic incentives within current contract and pricing structures. Picha (6) reports a subjective deficiency in lycopene in yellow shoulder tissue based on discoloration and a reduction in total carotenoids in tomato fruit from plants that received low K fertility. However, this report does not provide evidence of the effect and variability of YSD on carotenoid content nor the potential effects on the nutritional value of affected fruit. Because YSD affects grower premiums and processor grades, it may be economically advantageous to quantify how a common

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ripening disorder, YSD, affects the content of carotenoids in tomato products. The aims of the present study were (1) to optimize sampling for lycopene and β -carotene by addressing the relative importance of biological and technical replications and (2) to determine the effect of YSD on the content of lycopene and β -carotene.

MATERIALS AND METHODS

Plant Material. Five processing tomato varieties were used in the study: OH8245, PS696, FG99-36, FG00-118, and FG00-124. PS696 is a commercial hybrid, which is susceptible to YSD. OH8245 is an open pollinated variety (13) and is also susceptible to YSD. FG99-36, FG00-118, and FG00-124 are experimental varieties with above average agronomic performance. A complete randomized experimental design consisting of two replications each year was grown at the Ohio Agricultural Research and Development Center (OARDC) North Central Agricultural Experimental Station in Fremont, OH, in 2003 and 2006. Each plot consisted of 20 plants per genotype spaced 30 cm apart, with plots spaced 150 cm apart. All field plots were planted and maintained following conventional practices (14).

The plots were harvested when 80% of the fruits were ripe. One hundred ripe fruits were randomly collected from each plot, and the proximal end of each fruit was cut. The fruits were then categorized as either not affected by YSD (non-YSD) or affected by YSD (YSD). In 2003, flesh at the proximal end of non-YSD and YSD fruit was dissected and saved in a tube as tissue. In both 2003 and 2006, juice was processed from each plot and each category within a plot using a commercial blender. For each sample, two separate 50 mL juice samples (100 mL) were kept for further analysis. In total, four juice samples were collected from each plot, two from each phenotype (YSD and non-YSD). The samples were stored at $-20\text{ }^{\circ}\text{C}$ until carotenoid extraction.

Trait Evaluation: Carotenoid Quantification. Carotenoid extraction was carried out under red light following a hexane/acetone-based protocol modified from Ferruzzi et al. (15). Juice samples were thawed to room temperature, and 5.0 g of sample was homogenized in 50 mL of methanol with 1.0 g of CaCO_3 and 4.0 g of Celite. The methanol extract was filtered as described by Nguyen et al. (16). The filtrate was suspended in 50 mL of 1:1 acetone/hexane and allowed to stand for 1 min prior to homogenization. Repeated acetone/hexane extractions (up to three times) were required to recover the majority of carotenoids. Three milliliters of extract were collected in five 12 mL glass vials and dried under nitrogen. The vials were then wrapped in aluminum foil and stored at $-20\text{ }^{\circ}\text{C}$ until further use.

We used a Waters 2690 reverse-phase HPLC system equipped with a photodiode array detector for analysis. The carotenoid extracts were reconstituted with appropriate volumes of MTBE, depending on the volume of the dried extracts and on the desired concentration range of the analysis. An absorbance reading using a UV spectrophotometer was recorded for each carotenoid extract at 471 nm wavelength; a dilution of the solution followed if the absorbance reading was greater than 1.0. Separations were achieved using a C_{18} column (Vydac 201TP54; 4.6 mm \times 250 mm). The separation of total carotenoids was carried out at a flow rate of 1.0 mL/min using a multistep linear gradient of 80–100% MTBE in 98% methanol:2% 1 M ammonium acetate for 30 min. Standards of β -carotene and lycopene were purchased from Sigma Chemical Co. The peak identification and the subsequent quantification of β -carotene and lycopene were achieved using the standard curve for each compound and their molar absorptivity coefficients (reviewed in ref 16). The carotenoid content was reported in units of milligrams per 100 g of sample on a fresh weight basis.

Digital Phenotyping for YSD. We employed the software Tomato Analyzer (TA) to collect objective measurements of color. TA is an image processing software application that recognizes and collects data from JPEG files (17). We used a flatbed scanner to scan the cut surface of the proximal end for 12 fruits within each category/plot and saved a JPEG image. The color function in TA records RGB values of each pixel of the selected object on the image and translates these values into average $L^*a^*b^*$ values, which are then used to calculate hue, $180/\pi \cdot \arccos(a/(a^2 + b^2)^{1/2})$, and chroma, $(a^2 + b^2)^{1/2}$. The percent YSD

tissue was calculated from the proportion of pixels that fall into a defined range of hue values. YSD symptoms are discolorations in the yellow to green region of the hue color wheel. To capture the maximum discoloration due to YSD, we tested different ranges of hue values: 50–120, 55–120, 60–120, 65–120, 70–120, and 65–180. The percentage of discolored tissue (% YSD) was collected for all five genotypes evaluated in 2006 and for both categories within each genotype (YSD and non-YSD).

We tested multiple parameters to estimate % YSD. The measurement a/b is widely used in the tomato industry as an indication of red color. However, its use is based on an error in trigonometric calculations and the fortunate fact that a/b proves linear through the narrow range of red found in tomato fruit (18). Chroma, which is calculated using a^* and b^* , is a measure of color intensity. However, chroma is a poor measure of tomato color quality because high chroma due to high b^* values would constitute poor quality while high chroma due to high a^* values would constitute good quality. Previous indices to measure fruit affected by YSD incorporated information on the difference between two measurements of L^* and hue and the absolute value of L^* and hue (4). The ability to measure color on a pixel by pixel basis using TACT has improved our estimates (19). Hue, also calculated from a^* and b^* , represents the best estimate of red as humans perceive color and therefore is an appropriate measurement.

Statistical Analysis. All statistical analyses were performed using SAS software (version 9.1; SAS Institute Inc., Cary, NC). The following model was used to test the sampling effect on lycopene and β -carotene: $Y_{ijklm} = \mu + P_i + R_{j(i)} + E_{k(ij)} + I_{l(ijk)} + \epsilon_{ijklm}$, where Y_{ijklm} was the trait measured (lycopene or β -carotene), μ was the overall mean, P_i was the effect due to the i th plot, $R_{j(i)}$ was the effect due to the j th sample within-plot, $E_{k(ij)}$ was the effect due to the k th extraction, $I_{l(ijk)}$ was the effect due to HPLC injection, and ϵ_{ijklm} was the experimental error. In this nested design, all effects were random, and the approximate F -tests were adjusted for the corresponding error terms.

The model designed to test the effect of YSD on lycopene and β -carotene included the main effects YSD phenotype, genotype nested within YSD phenotype, year, and block. The within-plot variation represented the juice sampling effect and was nested within block, genotype, and YSD phenotype. Year, block, and within-plot variation were considered random factors. The approximate F -tests were adjusted for their corresponding error term. The model was $Y_{ijklmn} = \mu + P_i + G_{j(i)} + Y_k + B_{l(k)} + R_{m(ijkl)} + \epsilon_{ijklmn}$, where P_i was the effect due to YSD phenotype, $G_{j(i)}$ was the effect due to genotype, Y_k was the year effect, $B_{l(k)}$ was the block effect, $R_{m(ijkl)}$ was the within-plot effect, and ϵ_{ijklmn} was the experimental error.

For each model, the estimates of variance were obtained using the restricted maximum likelihood (REML) method with the MIXED procedure of SAS. The percent total variance was reported to represent the proportion of variance explained by each factor and nested factor.

Correlations between the extent of % YSD in tomato fruit and lycopene content in corresponding juice samples were determined using linear and nonlinear regressions. For the latter, we tested the exponential, logarithmic, polynomial, and power regressions to determine the best fit for the relationship. The relationship was derived from the 2006 TACT data for % YSD of fruits and lycopene content in the corresponding juice samples for each genotype–phenotype combination ($N = 20$).

RESULTS

Variance in Lycopene and β -Carotene. Total phenotypic variation for lycopene and β -carotene was partitioned to ascertain the relative importance of field and analytical sampling (Table 1). In the field sampling, there were significant differences among and within plots for both lycopene and β -carotene. The most significant field variation was observed between plots for lycopene (49.83% of total variation) and β -carotene (52.52% of total variation). The proportion of within-plot variation was 7.3% and 3.0% for lycopene and β -carotene, respectively. In total, experimentally controlled aspects of field sampling accounted for 57% and 55% of the total variation for lycopene

Table 1. Optimizing Sampling for Lycopene and β -Carotene Content in Juice Samples of Tomato

sources of variation	term in model	DF	trait			
			lycopene		β -carotene	
			mean squares	% total variance	mean squares	% total variance
plot	P_i	1	38.3 ^a	49.8	0.990 ^a	52.5
rep(plot)	$R_{(i)}$	2	4.32 ^b	7.31	0.0745 ^a	2.97
extraction	$E_{k(j)}$	4	0.516 ^c	0.00	0.0191 ^c	0.00
HPLC injection	$I_{k(jkl)}$	8	1.65 ^c	0.00	0.0183 ^c	0.00
exptl error	ϵ_{ijklm}	16	2.24	42.9	0.0709	44.5

^a Significant at $\alpha = 0.10$. ^b Significant at $\alpha = 0.05$. ^c Not significant.

Table 2. Effect of Yellow Shoulder Disorder (YSD) on Lycopene and β -Carotene Content in Juice Samples of Tomato over 2 Years

sources of variation	term in model	DF	trait			
			lycopene		β -carotene	
			mean squares	% total variance	mean squares	% total variance
YSD phenotype	P_i	1	44.9 ^c	6.40	0.0331 ^d	0.00
geno (YSD pheno)	$G_{(i)}$	8	6.57 ^d	2.39	0.150 ^a	1.44
year	Y_k	1	299 ^c	83.0	21.1 ^c	94.5
block	$B_{(k)}$	2	3.64 ^d	0.698	0.00 ^d	0.00
rep(plot)	$R_{m(jkl)}$	40	3.44 ^c	9.44	0.0624 ^b	2.13
exptl error	ϵ_{ijklmn}	39	1.14	7.50	0.0234	1.91

^a Significant at $\alpha = 0.05$. ^b Significant at $\alpha = 0.01$. ^c Significant at $\alpha = 0.001$. ^d Not significant.

and β -carotene, respectively. Uncontrolled error accounted for 43% and 45% of variation for lycopene and β -carotene, respectively. In contrast, there was no significant variance due to analytical sampling for either carotenoid. Neither replicated extraction nor replicated HPLC injection contributed to the total phenotypic variation for either lycopene or β -carotene.

Effect of YSD on Lycopene and β -Carotene. We sought to address the effect of YSD on lycopene and β -carotene content (Table 2). There were significant differences in the lycopene content of juice made from YSD and non-YSD fruit over 2 years. In contrast, juice made from YSD or non-YSD fruit did not differ significantly for β -carotene content. The variation due to YSD phenotype accounted for 4.15% of the total phenotypic variation for lycopene but none for β -carotene. YSD reduced lycopene content by 14% in the first year and 24% in the second year, and these reductions were highly significant (Table 3). Although there was a trend toward reduced β -carotene in juice made from YSD-affected fruit in 2003, the variety FG99-36 had more β -carotene in the juice made from affected fruit. In 2006, varieties FG00-0124 and FG00-118 had more β -carotene in juice made from YSD-affected fruit, FG99-36 had equal amounts in affected and unaffected fruit, and PS696 and OH8245 had more β -carotene in juice from nonaffected fruit (Table 3). We also compared carotenoid concentration in YSD tissue versus non-YSD tissue. There was a highly significant difference between tissue types, with a reduction in both lycopene and β -carotene in YSD tissue ($P < 0.0001$; data not shown). Compared to non-YSD tissue, YSD tissue showed a reduction of 61.5% and 71.5% for lycopene and β -carotene, respectively.

Genotypes were tested within each level of the YSD phenotype. There was no significant difference among genotypes when concentrations were averaged across 2 years for lycopene; however, significant differences were observed for β -carotene (Table 2). In 2003, PS696 had the highest β -carotene content

(1.67 mg/100 g fw) whereas FG00-124 had the lowest (1.27 mg/100 g fw). In 2006, similar trends were observed. OH8245 had the highest β -carotene content (0.472 mg/100 g fw), followed by PS696 (0.354 mg/100 g fw). FG00-124 again had the lowest β -carotene content (0.137 mg/100 g fw).

The year effect was highly significant for both lycopene and β -carotene (Table 2). Year to year differences explained 78.4% of the total phenotypic variation for lycopene and 94% for β -carotene. In the combined analysis, we did not detect significant block effects for either carotenoid. However, sampling within a plot was significant for both carotenoids, explaining 3.99% and 2.48% of the total phenotypic variation for lycopene and β -carotene, respectively. Less than 2% of the variation was left unexplained by the model for both traits. The significant year effect was evident in lycopene content with a 1.7-fold increase in the YSD samples and a 1.9-fold increase in the non-YSD samples from 2003 to 2006 evaluations. As for β -carotene, there was a 5.2-fold and 5.3-fold decrease between years in the YSD and non-YSD samples, respectively (Table 3).

Relationship between Extent of YSD and Lycopene Content. We investigated several hue ranges to determine the relationship between tissue color and carotenoid content. As we broadened the hue range by decreasing the lower boundary values (70–120, 65–120, 60–120, 55–120, 50–120), the correlation decreased and its corresponding p -value increased ($r^2 = 0.2837$, $P = 0.0189$; $r^2 = 0.2817$, $P = 0.0194$; $r^2 = 0.2760$, $P = 0.0209$; $r^2 = 0.2296$, $P = 0.0379$; $r^2 = 0.1206$, $P = 0.1452$, respectively, for each hue range). For the 70–120, 65–120, 60–120, and 55–120 hue windows, the linear correlation was significant, suggesting a decrease in lycopene content in juice with increasing % YSD in raw tomato fruits used to produce the juice (Figure 1). The linear regression represented the best fit for the relationship between the extent of % YSD in fruits and lycopene in the corresponding juice samples when compared to nonlinear models (data not shown). Increasing the upper boundary values to hue = 180 did not improve the precision of correlations. The correlation with β -carotene was not significant.

DISCUSSION

Measurement of carotenoids using HPLC is labor intensive and expensive. Therefore, sampling strategies that optimize the balance between biological replication (either field or genotype) and technical replication (extraction and injection) maximize resources and permit more precise measurements of variation. Our results demonstrated that biological replications should be increased relative to technical replications to reduce the error associated with quantifying lycopene and β -carotene. The detection of significant plot to plot variation emphasizes the need to measure replicates within a location. Our results reveal that variation in field sampling is as high as 58% for lycopene and 55% for β -carotene. The variation due to analytical sampling, which represents carotenoid extraction and HPLC injection, was negligible among replicates. Minimizing replication for analytical sampling does not have a negative effect on the precision of the carotenoid estimates.

Value-added whole and diced tomato products require tomatoes with high color quality. Both color and color uniformity are affected by yellow shoulder disorder (YSD), a ripening disorder that results in discoloration of the proximal end tissues of the fruit, thus reducing appearance and value. This study indicates that YSD also affects the value of tomato products from the perspective of a health-conscious consumer. We demonstrated a significant negative effect of YSD on lycopene

Table 3. Means of Lycopene and β -Carotene Content in YSD and Non-YSD Juice Samples of Tomato

genotype	phenotype	2003		2006	
		lycopene (mg/100 g fw) ^y	β -carotene (mg/100 g fw)	lycopene (mg/100 g fw)	β -carotene (mg/100 g fw)
FG00-118	non-YSD	5.85 ^a	1.46	12.9 ^a	0.192
FG00-118	YSD	5.04 ^b	1.39	9.20 ^b	0.212
FG00-124	non-YSD	5.51 ^a	1.37 ^a	8.08 ^a	0.138 ^b
FG00-124	YSD	4.46 ^b	1.27 ^b	6.87 ^b	0.239 ^a
FG99-36	non-YSD	5.97 ^a	1.53 ^b	11.6 ^a	0.296
FG99-36	YSD	5.03 ^b	1.70 ^a	8.83 ^b	0.289
OH8245	non-YSD	5.70 ^a	1.64 ^a	9.35 ^a	0.472 ^a
OH8245	YSD	5.01 ^b	1.53 ^b	9.39 ^b	0.399 ^b
PS696	non-YSD	6.30 ^a	1.68 ^a	14.0 ^a	0.354 ^a
PS696	YSD	5.78 ^b	1.58 ^b	8.36 ^b	0.301 ^b
mean non-YSD		5.87	1.54	11.2	0.290
mean YSD		5.06	1.49	8.53	0.288
LSD ($\alpha = 0.05$) ^z		0.498	0.0674	1.46	0.0212
P-value		0.0004	0.7687	0.0023	0.8155
% difference		13.7	2.73	23.7	0.826

^y Significant within-genotype differences for YSD and non-YSD juice samples are indicated by letters. ^z Least significant difference (LSD), P-value, and % differences based on mean separations for differences between YSD and non-YSD juice samples across all genotypes.

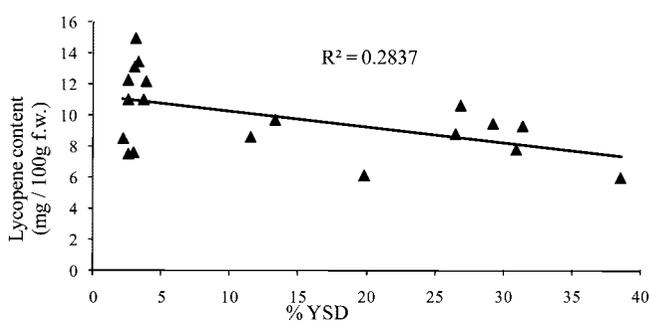


Figure 1. Relationship between extent of yellow shoulder disorder (YSD) in tomato fruit and lycopene content in corresponding tomato juice samples.

and β -carotene content in tissue. Only lycopene content was significantly reduced when affected fruit were used to make juice. In the first year of evaluation, the mean lycopene content in juice made from YSD-affected fruits was reduced by nearly 14%; the reduction was 24% in the second year. With the potential health benefits of lycopene (reviewed in ref 9), modulating the lycopene profile of tomato products by reducing the incidence of YSD is of potential interest. Currently, the market for processing tomatoes does not offer incentives for increasing bioactive carotenoids. However, grower contracts often reward or penalize color quality, and processor grades of peeled products are also affected by color uniformity (20). Fruit affected by YSD often are processed into sauces and cover juice where appearance is less of an issue. Our results suggest that this approach does not significantly affect the nutritional status of the product as defined by β -carotene content. It is unclear what will be the consequences of lower lycopene levels in sauces and juice made from YSD-affected fruit. Studies aimed at assessing human absorption of lycopene suggest that the *trans*-lycopene isomer is inefficiently absorbed and that high levels of lycopene do not result in increased absorption (21). Therefore, despite reduced lycopene, it is unclear that juice made from YSD-affected fruit is of lower value from a dietary perspective.

Reduced lycopene levels and current contract and grading practices suggest that breeding varieties for resistance to YSD might be an effective strategy for increasing the value of tomatoes for growers, processors, and possibly consumers. The effect of genotype nested within each level of phenotype, genotype (YSD) and genotype (non-YSD), contributed signifi-

cantly to the total phenotypic variation, though the contribution to total variation was low. This study was not designed to select for genotypes with resistance to YSD, but our results suggest that a sampling strategy for genetic variation should increase biological replications and reduce technical replications in order to sample a large number of genotypes (varieties). Our results also suggest that attention should be paid to specific genes that are currently in use in breeding programs. For example, the old gold crimson (*og^c*) gene is a naturally occurring mutation of the fruit-specific β -cyclase that results in enhanced red color with increased lycopene and reduced β -carotene (22, 23). FG00-124, a hybrid homozygous for *og^c*, had lower β -carotene in both 2003 and 2006. This is not a surprising result given the function of the *og^c* protein (23). These results are also consistent with those of Sacks and Francis (7), who reported that *og^c* explained 18–27% of the total phenotypic variation for color. In 2006, several hybrids had more β -carotene in the YSD juice samples than in the non-YSD samples. It is intriguing that these hybrids were either homozygous or heterozygous for *og^c*, while varieties that contained more β -carotene in non-YSD juice were wild type. More genotypes need to be evaluated to determine if the contrasting results for β -carotene content are supported.

The year to year and within field environmental effects were also significant and explained a high proportion of phenotypic variation for lycopene and β -carotene. Although the reduction in lycopene due to YSD was consistent from one year to the other, there was a marked increase in the overall mean of lycopene the second year compared to the first. Environmental influences such as weather, soil fertility management practices, and/or soil properties have been reported to affect the severity of YSD (reviewed in refs 3 and 4). In Fremont, OH, there was twice as much precipitation in June 2006 compared to June 2003. The precipitation in July was similar both years, but August 2003 had 1.3 times more precipitation than 2006. Overall, the average maximum air temperature for the summer months in 2006 was 2 °F higher than in 2003. Several reports suggest that temperatures above 90 °F (32 °C) reduced lycopene biosynthesis (24, 25). During the fruit maturing stage (approximated to the period between 20 July and 20 August), there were 5 days at 90 °F or above in 2006 and none in 2003. We observed higher lycopene content in 2006 despite higher average air temperatures. Fertility management practices were very similar both years, as conventional practices were followed (14).

Thus year-to-year environmental fluctuations appear to be important in determining concentrations of carotenoids, though our experiments were not designed to detect cause and effect.

We obtained color data from the same fruits used to make up the juice for each sample. Analysis of the digital images with the Tomato Analyzer (17) allowed us to determine the degree of tissue affected by YSD at the proximal end of the tomato fruit. The correlation between the proportion of YSD tissue and lycopene content can be utilized as a prediction tool. Results from 2006 suggest that for every 10% increase in the area of raw fruit affected by YSD, there is a decrease in lycopene content in juice of 1.03 mg/100 g fw.

In conclusion, quantification of lycopene and β -carotene concentration in tomato juice samples can be more precise by increasing biological replications while minimizing analytical replications. YSD affects the health-beneficial carotenoids predominantly by reducing lycopene content.

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