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Profiling of Carotenoids in Tomato Juice by One- and Two-Dimensional NMR

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Epidemiological data have shown a link between dietary intake of tomatoes and tomato products (rich in carotenoids) and a decreased risk of chronic diseases. The carotenoid profile in tomato products depends on tomato variety as well as the thermal conditions used in processing. The final carotenoid profile may affect the bioaccessibility and bioavailability of these biomolecules. Therefore, nondestructive, reliable methods are needed to characterize the structural and stereochemical variation of carotenoids. $CDCl_3$ rapid extraction was used to extract carotenoids from tomato juice as an alternative rapid procedure that minimizes solvents and time consumption prior to NMR analysis. The profile of these biomolecules was characterized by application of high-resolution multidimensional NMR techniques using a cryogenic probe. The combination of homonuclear and heteronuclear two-dimensional NMR techniques served to identify (*all-E*)-, (5*Z*)-, (9*Z*)-, and (13*Z*)-lycopene isomers and other carotenoids such as (*all-E*)-β-carotene and (15*Z*)-phytoene dissolved in the extracted lipid mixture. The use of one-dimensional NMR enabled the rapid identification of lycopene isomers, thereby minimizing further isomerization of (*all-E*)-lycopene as compared to HPLC data. On the basis of the assignments accomplished, the carotenoid profile of typical tomato juice was successfully determined with minimal purification procedures.

KEYWORDS: 1D and 2D NMR; HPLC; UV−visible; carotenoids; lycopene isomers; lipid mixture

INTRODUCTION

A positive relationship between the consumption of tomato and tomato-based products and a decreased risk of chronic diseases is in part attributed to the antioxidant activity of carotenoids (*1*). Lycopene ($C_{40}H_{56}$, molecular weight 536.85) (*2*), an acyclic compound with 11 conjugated and 2 unconjugated double bonds, is the predominant carotenoid in tomatoes followed by minor compounds such as phytoene and β-carotene (**Figure 1**) (*3*). In raw ripe tomatoes, lycopene and other carotenoids are prevalently present in the (*all-E*)-configuration.

During thermal processing, the concentration of lycopene isomers in the (*Z*)-configuration may increase due to solubilization of lycopene into the lipid phase followed by heat-induced isomerization (*4*). Thermal treatments of foods rich in lycopene have been shown to improve the bioavailability of this biomolecule due to the breakdown of the tomato matrix, resulting in increased release of carotenoids (*5*, *6*). Several hypotheses are still under investigation as the role of processing in affecting the higher bioavailability of (*Z*)-isomers of lycopene (*1*, *4*, *6*, *7*). Additionally, the carotenoid composition in tomato is likely affected by factors such as cultivar, variety, maturity, geographical site of production, and storage after the processing (*8*−*11*). Therefore, nondestructive, reliable methods are needed to characterize these compounds.

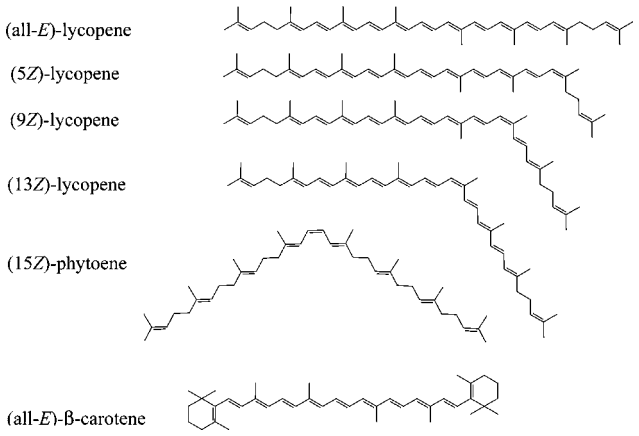

Figure 1. Main carotenoid structures present in tomato products.

Carotenoids are sensitive to light, heat, air, and metal surfaces; as a result, extractions of these compounds and subsequent analytical identification and quantification are most probably affected by physicochemical reactions such as degradation and

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isomerization (*12*−*14*). It has been recognized that a need exists for methods that minimize losses during sample preparation or physicochemical reactions of these compounds during analysis (*15*−*17*). In addition, positive identification of carotenoids in a mixture utilizing standards has proven to be difficult due to the inherent instability of these biomolecules (*14*).

A variety of techniques have been used to identify and quantify the different carotenoids and their isomers. High-performance liquid chromatography (HPLC) equipped with a photodiode array (PDA) system is the most common method (*14*, *18*); however, because the identification is based only on UV−visible spectra, it is insufficient to obtain complete structural and stereochemical information of carotenoids. Mass spectrometry (MS) enables the quantification and elucidation of carotenoid structure on the basis of the molecular mass and fragmentation; however, it does not distinguish stereoisomers (*17*).

High-resolution nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for the identification of purified organic compounds. The current availability of higher magnetic field strength (800−900 MHz) and the application of two-dimensional (2D) pulse sequences have facilitated the identification of specific compounds in complex mixtures without purification or severe and time-consuming extractions that can result in qualitative and quantitative modifications (*19*). The combination of 2D homonuclear and heteronuclear correlated techniques using pulsed field gradients makes NMR an indispensable analytical tool for structural investigations (*20*). In addition, the development of cryogenic probe technologies has significantly increased the sensitivity of the system, affecting experimental time and/or concentration of compounds needed to obtain adequate spectra (*21*). In consideration of all these recent advances, NMR may prove to be an indispensable tool for metabolomic and metabonomic studies (*22*−*26*).

Capitalizing on these NMR advances, the principal aim of this study was to profile carotenoids in a lipophilic mixture extracted from typical tomato juice.

MATERIALS AND METHODS

Carotenoid Extractions. Tomato juice was first centrifuged to separate pulp from serum. Two different procedures, denoted conventional and rapid extractions, were performed to determine the optimal conditions for NMR analysis of carotenoids. The extraction procedures were applied to the tomato pulp under subdued red light to limit isomerization and photodegradation. All of the samples after every extraction were flushed with argon to minimize oxidative reactions.

Conventional Extraction. Approximately 5 g of tomato pulp, 1 g of calcium carbonate, and 4 g of Celite as filtering aid were dissolved in 50 mL of methanol before the mixture was homogenized using a Tissumizer (Tekmar SDT 1810; Cincinnati, OH) for 1 min. The mixture was extracted in 50 mL of methanol and filtered under vacuum using a combination of Whatman filter papers no. 1 and 42. The filter cake was resuspended in 50 mL of 1:1 acetone/hexane, homogenized for 1 min, and filtered with the same filter paper combination. The acetone/hexane extraction was repeated until the filter cake was devoid of color. The filtrates obtained by vacuum filtration were combined in a separator funnel. Approximately 10 mL of deionized water was added to hasten the phase separation of the hexane layer water, which was saved; this step was repeated three times. The purified extracts were passed through anhydrous sodium sulfate to remove any water contamination. Aliquots of 3 mL were removed and dried under a stream of nitrogen at ambient temperature. The dried samples were stored in the dark at −20 °C and then dissolved in $CDCl_3$ (99.8% deuterated; Wilmad, Buena, NJ) prior to NMR analysis and in methanol/methyl *tert*-butyl ether (MTBE) (1:1) for HPLC separation.

Rapid Extraction. Two milliliters of $CDCl_3$ was added to 5 g of tomato pulp. The mixture was agitated in the dark with a Touch Mixer model 231 (Fisher Scientific, Chicago, IL) and finally centrifuged for 5 min to promote phase separation. The red hydrophobic liquid at the bottom of centrifuge tubes was pipetted, then filtered through hydrophobic syringe filters PTFE 25 mm (ultraresistant solvent, Fisher Scientific), and finally analyzed by NMR immediately after spectrometer setup procedures.

Table 1. Percentage of Lycopene Isomers Extracted from Tomato Juice Analyzed Using a Reverse-Phase C_{30} HPLC System and a 1D NMR Spectrum[a]

lycopene isomer	retention time (min)	HPLC $R_{extract}$ (% isomers)	HPLC $C_{extract}$ (% isomers)	NMR $R_{extract}$ (% isomers)
(*all-E*)	50.6	89.7 ± 0.91	89.0 ± 0.60	92.4 ± 0.71
(5*Z*)	51.5	4.1 ± 0.40	4.4 ± 0.37	3.8 ± 0.88
(9*Z*)	45.8	2.4 ± 0.10	2.4 ± 0.30	1.2 ± 0.51
(13*Z*)	40.5	3.7 ± 0.56	4.2 ± 0.42	2.6 ± 0.26

[a] $R_{extract}$ denotes rapid extraction using $CDCl_3$ and $C_{extract}$ denotes conventional extraction. All values are expressed as percentage of the total lycopene. Values are mean ± SD.

Extraction parameters (amount of sample, solvent, and time of agitation) for the optimization of the rapid procedure were evaluated by measuring the absorbance in the wavelength range of 200−800 nm by UV−visible spectrophotometer UV-2401 PC (Shimadzu Instruments Inc., Wood Dale, IL). The following parameters were applied for spectrophotometric analysis: slit width, 1 nm; sampling interval, 0.5 nm; scan speed, fast. In the dark, mixtures were agitated for 1, 2.5, 5, and 10 min to optimize extractability of carotenoids and finally centrifuged for 5 min to promote phase separation. Ten microliters of extract was dissolved in 2.5 mL of chloroform and then analyzed by using a UV−visible spectrophotometer.

Nuclear Magnetic Resonance (NMR) Experiments. All NMR experiments were carried out using a Bruker 800 MHz (Bruker Biospin, Rheinstetten, Germany) spectrometer equipped with a 5 mm TXI cryoprobe. The spectra were recorded at ambient temperature (293.15 K). Proton spectra for carotenoid analysis were referenced to the TMS signal ($\delta = 0.00$ ppm). Chemical shifts of lipid extractions were also compared with commercial standards of *all-trans-β*-carotene (≈1 mg dissolved in 0.6 mL of $CDCl_3$) and lycopene (≈0.5 mg dissolved in 0.6 mL of $CDCl_3$) purchased from Sigma Chemical Co. (St. Louis, MO). The following NMR experiments were conducted with the lipophilic mixtures:

1D NMR experiments (19): ^{1}H 30° flip angle of 2.97 μs, 65K data points, 64 scans, spectral width of 11.5 kHz, and relaxation delay of 2 s. 1D experiments were repeated three times for rapid extraction, and the mean values and standard deviations (SD) are reported in **Table 1**.

2D NMR experiments (19). ^{1}H−^{1}H pulse field gradient COSY-45 (correlation spectroscopy) was acquired using a gradient-selected coherence transfer pathway and recorded in magnitude mode with the following parameters: 90° pulse of 8.9 μs, 2K data points, 128 scans, spectral width of 8 kHz, and relaxation delay of 2 s.

^{1}H−^{1}H pulse field gradient TOCSY-MLEV17 (total correlation spectroscopy) was registered in the TPPI phase sensitive mode under the following conditions: 90° pulse of 8.9, 64 scans, 2K data points in f_2 and 512 increments in f_1, spectral width of 8 kHz, 80 ms mixing time, and relaxation delay of 2 s.

The 2D *J*-resolved (J-RES) ^{1}H homonuclear spectrum was recorded using a 90° pulse of 8.9 μs, 48 transients of 4096 complex points, 64 increments, relaxation delay of 2 s, and spectral widths of 4 kHz in the ^{1}H (f_2) (region 4−8 ppm) and 65 Hz in the *J* (f_1) dimensions, respectively.

2D T-ROESY (transverse rotating frame nuclear Overhauser enhancement spectroscopy (T-ROESY) (*27*) spectra were obtained according to the States-TPPI method using the following parameters: 90° pulse of 8.9 μs, 2K TD, 64 increments, spectral width of 8 kHz, relaxation delay of 2 s, and 200 ms mixing time.

2D NOESY was recorded using a 90° pulse of 8.9 μs for ^{1}H, 2K data points, 64 scans, spectral width of 8 kHz, 250 ms mixing time, and relaxation delay of 2 s.

^{1}H−^{13}C pulse field gradient heteronuclear single quantum correlation spectroscopy (HSQC) was registered in the echo−antiecho phase

selective mode with the following parameters: 90° pulse of 8.9 μs for ^{1}H; 15 μs 90° for ^{13}C with 75 μs for GARP ^{13}C decoupling; relaxation delay, 2 s; 64 scans; 8 and 33 kHz spectral widths in the proton and carbon dimensions, respectively; 1K data points in f_2 and 256 increments in f_1.

The ^{1}H−^{13}C pulse field gradient heteronuclear multiple bond correlation spectroscopy (HMBC) spectrum was recorded in the phase-sensitive mode using States-TPPI with the following parameters: 90° pulse of 8.9 μs for ^{1}H and 15 μs for ^{13}C; 64 scans, 8 and 33 kHz spectral widths in the proton and carbon dimensions, respectively; relaxation delay, 2 s; 2K data points in f_2 and 256 increments in f_1.

NMR data were processed using XWINNMR 3.5 software (Bruker Biospin, Rheinstetten, Germany) running on a Silicon Graphics INDY workstation.

HPLC. A reverse-phase HPLC with PDA system was used to determine the carotenoid profiles in the tomato juices. Separations were achieved using analytical (250 × 4.6 mm i.d.) 3-mm polymeric C30 columns that were prepared at the National Institute of Standards and Technology (Gaithersburg, MD). Guard columns packed with C30 stationary phase were used in-line for all separations (YMC, Inc., Wilmington, NC). Column effluent was monitored via a Waters 996 PDA at 200−800 nm with a scanning rate of 2 scans/s and 1.2 nm spectral resolution. Carotenoid profiles were carried out using a mobile phase of MeOH and MTBE for 60 min at 1 mL/min. An isocratic method was run at 95% MeOH and 5% MTBE for 5 min and changed to gradient run to 70% MTBE for 55 min. The injection volume used in the system was 50 μL. The experiments were repeated six times for each method of extraction, and the mean values and SD are reported in **Table 1**. Chromatographic peak identification was based on comparison to previously reported separations on polymeric C30 columns and UV−visible absorption spectral libraries.

RESULTS AND DISCUSSION

Evaluation of Rapid Extraction Method. The carotenoids of fruits, vegetables, and animal products are mostly lipophilic; therefore, the extraction procedures of nonpolar carotenoids (such as lycopene) with solvents containing chloroform or dichloromethane are usually suggested (*14*). In conventional extraction utilizing several solvents, multiple steps are used to ready the sample for analysis. Every extraction step may result in isomerization and/or degradation (*16*). Therefore, an alternative method was sought that would minimize such steps. Two different procedures, denoted conventional and rapid extractions, were carried out with a goal of minimizing the changes in the carotenoid profile of the original sample. The amount of sample, solvent, and time of agitation were optimized for rapid extraction by measuring the absorbance in the wavelength range of 200−800 nm by UV−visible spectrophotometer. Up to 5 min of extraction time, a significant increase in UV−visible absorption spectra was observed. Little change was detected in the spectra between 5 and 10 min. Therefore, it was determined that the most convenient agitation time prior to centrifugation was 5 min. In addition, $CDCl_3$ and conventional extractions were compared by UV−visible spectra to verify whether the former extraction was able to quickly extract carotenoids in concentration comparable with the conventional procedure. The two methods showed a similar ability to extract carotenoids. Other solvents were studied in the rapid extraction procedure, such as hexane, and showed scarce ability to extract carotenoids from the chloroplasts of the tomato pulp.

The percentages of lycopene isomers extracted were compared for the two extraction procedures utilizing HPLC analysis (**Figure 1**), and the two methods showed similar results (**Table 1**). However, the rapid procedure significantly reduced the solvent and time consumption (5 min of rapid procedure versus at least 1 h of conventional one), and therefore it was designated the preferred method and utilized in all NMR analyses discussed below.

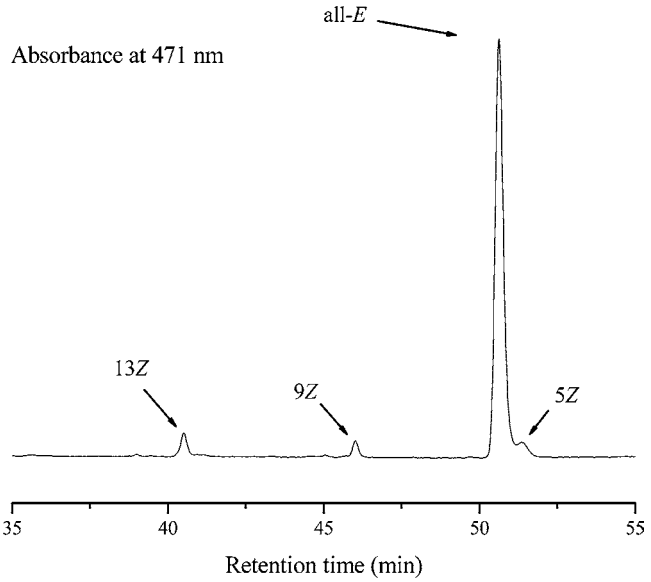

Figure 2. Representative HPLC chromatogram of lycopene isomers extracted from tomato juice by rapid procedure (details under Materials and Methods).

Carotenoid Characterization. In this work, the assignment of carotenoids was accomplished in the lipophilic mixture by combination of homonuclear and heteronuclear 2D NMR techniques. Literature chemical shift data of these specific biomolecules were used as guidelines for many compound assignments (*9*, *28*). Results were also compared to data obtained by reverse-phase C-30 HPLC with a PDA system (**Figure 2**; **Table 1**).

Figure 3 shows ^{1}H−^{1}H COSY-45 spectra comparing the chemical shifts of scalar coupled nuclei of a commercial (*all-E*)-lycopene standard (**Figure 3a**) and the lipophilic carotenoid extract from tomato juice (**Figure 3b**). The spectra are concentrated in the olefinic region related to the resonance of conjugated double bonds (5.8−7 ppm), because this is the key area for the identification of most of these carotenoids by NMR. COSY spectra were compared to the other homonuclear and heteronuclear 2D NMR spectra, and the combination of all 2D NMR techniques revealed that (*all-E*)-lycopene was the predominant carotenoid in tomato juice; the assignments of ^{1}H and ^{13}C signals are reported in **Table 2**. Coupling constants were determined by 1D NMR and 2D ^{1}H J-RES spectra (**Tables 2** and **3**) and were found to be in good agreement with chemical shifts reported in the literature (*9*, *28*, *29*). In addition to the (*all-E*)-lycopene (**Table 2**), HPLC (**Figure 2**) and 1D and COSY spectra **(Figure 3)** showed the presence of other isomers attributable to the presence of small quantities of (*Z*)-lycopene isomers in both commercial and extracted samples (*11*). Because carotenoids have many peaks in common with (*all-E*)-lycopene, other 2D NMR techniques were needed to identify these isomers and other carotenoids. The uniqueness of some proton and carbon chemical shift signals enabled the identification of various carotenoids present in the same hydrophobic mixture; therefore, several homonuclear and heteronuclear techniques were used in combination to elucidate each carotenoid (*9*). **Table 3** lists ^{1}H and ^{13}C chemical shifts for all other carotenoids identified in the mixture as detailed below.

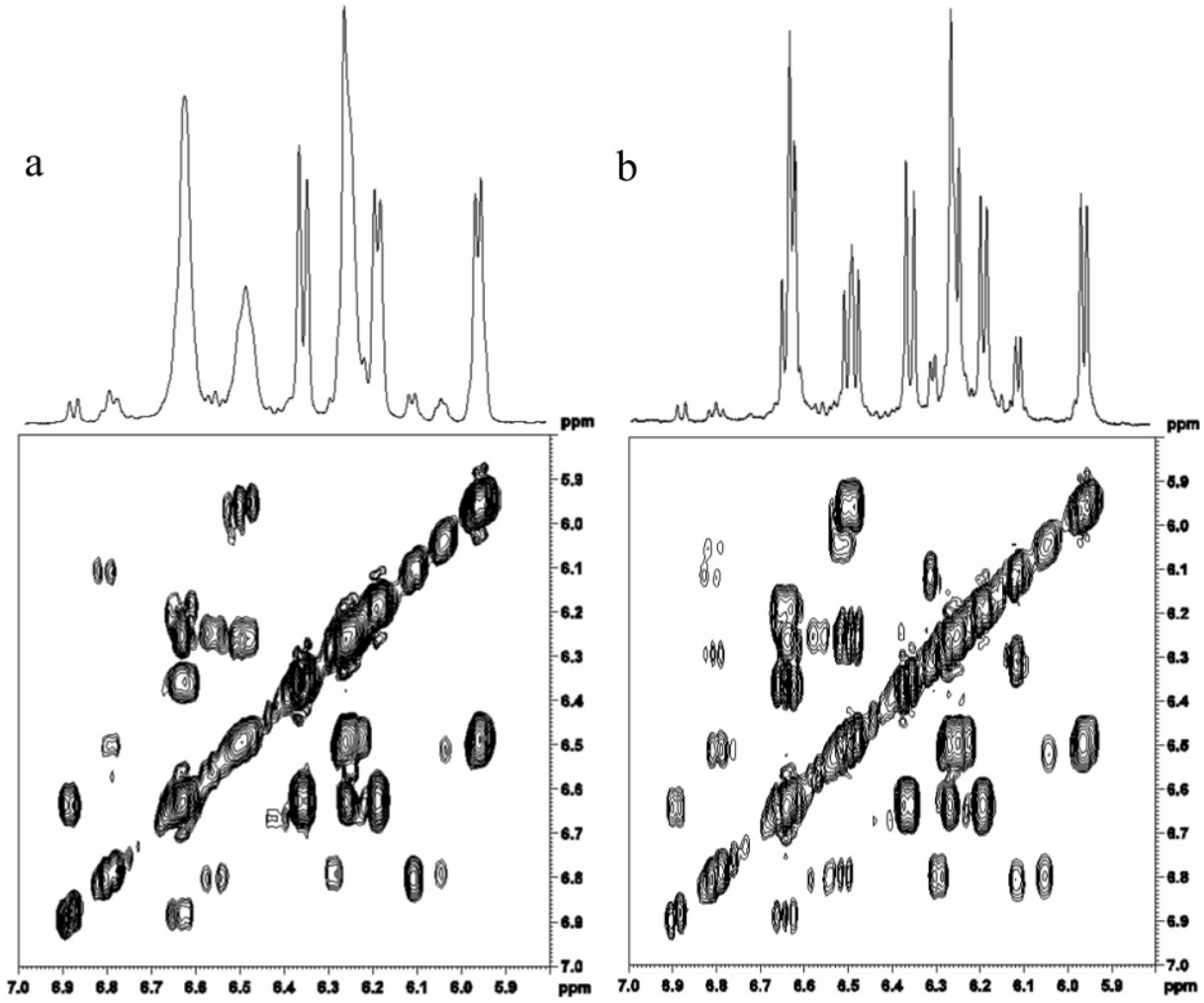

Figure 3. 2D spectrum (^{1}H–^{1}H COSY-45) of commercial (*all-E*)-lycopene standard (**a**) and carotenoids from a typical lipophilic mixture extracted from typical tomato juice (**b**) reported in the area related to the conjugated double bonds (5.8–7 ppm).

The cross-peaks of COSY experiment were also compared with TOCSY, which, unlike the ^{1}H−^{1}H COSY, correlates all protons of a spin system. The comparison of TOCSY and COSY techniques (^{1}H−^{1}H COSY, **Figure 3**) showed that most of the cross-peaks are in common between commercial standard lycopene and carotenoids dissolved in the hydrophobic mixture (**Figure 3b**). The main difference was attributed to the presence of (15*Z*)-phytoene (*1*) (**Table 3**), one of the precursors of the lycopene biosynthesis (**Table 2**). The (*Z*)-configuration of this biomolecule was confirmed by ^{1}H chemical shifts and coupling constants (**Table 3**).

To further verify the presence of (15*Z*)-phytoene and other carotenoids, ^{1}H−^{13}C HSQC and ^{1}H−^{13}C HMBC techniques were used to correlate heteronuclear spins across a single bond (**Figure 4**) and over two or three bonds, respectively (*30*). The wider chemical shift spread of the ^{13}C NMR resonances and the different carbon chemical environments were exploited in these heteronuclear techniques to confirm homonuclear assignments and obtain additional information regarding other carotenoids. HSQC and HMBC techniques confirmed the assignments of (15*Z*)-phytoene. The proton and carbon chemical shift values of H−C(14 and 14′) and H−C(15 and 15′) (**Table 3**) were in good agreement with published results (*31*, *32*).

By application of 1D and 2D homonuclear spectroscopy, (*all-E*)-β-carotene signals were overlapped by (*all-E*)-lycopene peaks. (*all-E*)-β-Carotene was found by application of ^{1}H−^{13}C HSQC (**Figure 4**) and ^{1}H−^{13}C HMBC experiments as reported in **Table 3**. The ^{1}H−^{13}C connectivities related to the double bonds H−C(7) and H−C(8) and H−C(7′) and H−C(8′), close to the β-ionone ring structure, confirmed the presence of this biomolecule. The chemical shifts observed for this isomer are in excellent agreement with published values (*9*).

On the basis of the literature assignments (*9*, *10*, *29*), the presence of (*Z*)-lycopene isomers was found by combination of the mentioned 2D techniques. The loss of symmetry of (*all-E*)-lycopene in (*Z*)-isomers causes partial delocalization of π electrons (*9*) and, as a consequence, additional homonuclear and heteronuclear peaks can be observed (**Figures 3b** and **4**). These additional peaks were assigned to (9*Z*)- and (13*Z*)-lycopene isomers (**Table 3**). The use of the cryoprobe was critical in this instance when greater sensitivity was required for positive identification.

After the assignment of most carotenoids was established, dipolar coupling techniques were used to confirm the structure and the presence of additional lycopene stereoisomers. ^{1}H−^{1}H NOESY or ^{1}H−^{1}H ROESY techniques have been shown to be useful for the elucidation of the structure of stereoisomers, yet choosing an appropriate technique may prove to be difficult (*19*). The ROESY technique is often suggested for intermediate-sized molecules (700−1500 MW), whereas NOESY is suggested for smaller (MW < 600) and larger molecules (MW > 1500) molecules (*33*). Because the molecular weights of the

Table 2. ^{1}H and ^{13}C NMR Assignments of (*all-E*)-Lycopene, the Predominant Carotenoid Extracted from Tomato Juice[a]

	^{1}H	^{13}C
C(1) C(1′)		131.64
H–C(2) H–C(2′)	5.11 (m)	124.12
2H–C(3) 2H–C(3′)	2.11 (m)	26.83
2H–C(4) 2H–C(4′)	2.11 (m)	40.30
C(5) C(5′)		139.30
H–C(6) H–C(6′)	5.96 (d, $J = 11.4$)	125.62
H–C(7) H–C(7′)	6.50 (dd, $J = 11.4, 15$)	124.69
H–C(8) H–C(8′)	6.25 (d, $J = 15$)	135.32
C(9) C(9′)		136.15
H–C(10) H–C(10′)	6.19 (d, $J = 11.5$)	131.42
H–C(11) H–C(11′)	6.64 (dd, $J = 11.5, 14$)	125.00
H–C(12) H–C(12′)	6.36 (d, $J = 14.8$)	137.36
C(13) C(13′)		136.54
H–C(14) H–C(14′)	6.25 (m)	135.79
H–C(15) H–C(15′)	6.62 (m)	130.04
3H–C(16) 3H–C(16′)	1.688 (s)	25.66
3H–C(17) 3H–C(17′)	1.612 (s)	17.70
3H–C(18) 3H–C(18′)	1.818 (s)	16.97
3H–C(19) 3H–C(19′)	1.968 (s)	12.90
3H–C(20) 3H–C(20′)	1.968 (s)	12.81

[a] Recorded in $CDCl_3$: chemical shift values are expressed as δ values (ppm) from TMS. Signal multiplicity and *J* coupling values (Hz) are shown in parentheses.

carotenoids under analysis are ≈500, they reside on the limit suggested in the literature for each technique; therefore, NOESY and ROESY experiments were performed and results compared. ROESY (**Figure 5a**) proved to be more useful as was found elsewhere for other carotenoids with similar molecular weights (*8*). In addition, the variant technique, T-ROESY, permitted the minimization of spurious TOCSY artifacts caused by coherent magnetization transfer between spin-coupled protons (*27*, *34*). The comparison between T-ROESY and TOCSY spectra (**Figure 5**) resulted in the elucidation of (5*Z*)-lycopene by ROE correlation between 2H−C(4) and H−C(7) (**Table 3**) and confirmed the peak at 2.23 ppm assigned to the proton located on 2H−C(4) of this (*Z*)-carotenoid.

Due to the incomplete carbon assignment in the literature (*29*, *31*, *34*), phytofluene, another precursor of the biosynthesis of lycopene (*3*), can be tentatively assigned only on the basis of proton signals. The complete identification of this compound

Table 3. ^{1}H and ^{13}C NMR Assignments of Minor Carotenoids Extracted from Typical Tomato Juice[a]

		^{1}H	^{13}C
(5*Z*)-lycopene	2H–C(4)	2.23 (m)	32.84
	H–C(8)	6.22 (d)	135.19
	3H–C(17)	1.622 (s)	17.65
	3H–C(18)	1.818 (s)	24.18
(9*Z*)-lycopene	H–C(8)	6.70 (d, $J = 11.5$)	127.28
	H–C(10)	6.04 (d)	130
	H–C(11)	6.80 (d, d)	123
	H–C(12)	6.28 (d)	135.4
	3H–C(19)	1.967 (s)	20.8
(13*Z*)-lycopene	H–C(8)	6.22	125.14
	H–C(8)	6.22	124.74
	H–C(10)	6.23	136.37
	H–C(12)	6.88	129.23
	H–C(14)	6.11 (m)	130.93
	H–C(15)	6.80 (m)	128.77
	H–C(15′)	6.56 (m)	129.35
	3H–C(20)	1.974 (s)	20.72
	3H–C(20′)	1.989 (s)	12.77
(*all-E*)-β-carotene	H–C(2) H–C(2′)	1.47	39.8
	2H–C(3) 2H–C(3′)	1.62	19.4
	2H–C(4) 2H–C(4′)	2.03	33.2
	H–C(7) H–C(7′)	6.16	126.7
	H–C(8) H–C(8′)	6.15	137.8
	3H–C(18) 3H–C(18′)	1.719	21.7
(15*Z*)-phytoene	H–C(14) H–C(14′)	6.31 (d, $J = 11.7$)	120.29
	H–C(15) H–C(15′)	6.11 (d, $J = 11.3$)	123.39

[a] Recorded in $CDCl_3$: chemical shift values are expressed as δ values (ppm) from TMS. Signal multiplicity and *J* coupling (Hz) values are shown in parentheses.

is pending the purification of phytofluene from tomato juice to serve as a standard.

Additional resonances from other compounds such as amino acids and other lipids were found, but their identification is beyond the scope of this paper.

After the identification of several carotenoids by 2D NMR techniques, the 1D NMR spectrum was used as a fast and noninvasive method to determine the average content of lycopene isomers. The integral of the 5.8−7 ppm region due to the resonance of conjugated double bonds was set at 100, and by integration of corresponding resonances in the 1D spectrum, the percentage of lycopene isomers was determined. The same procedure was applied to the aliphatic area (1.47−2.23 ppm) relative to the assigned carotenoid peaks (**Tables 2** and **3**). These data were compared with the relative concentration of these metabolites obtained by HPLC technique (**Table 1**). The percentage of (*all-E*)-lycopene using 1D NMR was found to be higher than that obtained by HPLC (**Table 1**). These results may suggest that the benefits of NMR spectroscopy as noninvasive technique combined with cryoprobe technology reduced the experimental time of the 1D spectrum to a few minutes (as compared to 60 min for the HPLC experiment), and therefore it may have limited the isomerization of (*all-E*)-lycopene to other (*Z*)-isomers.

This work showed that the extraction of carotenoids using $CDCl_3$ can be an alternative rapid procedure prior to NMR analysis that is able to minimize isomerization/degradation and

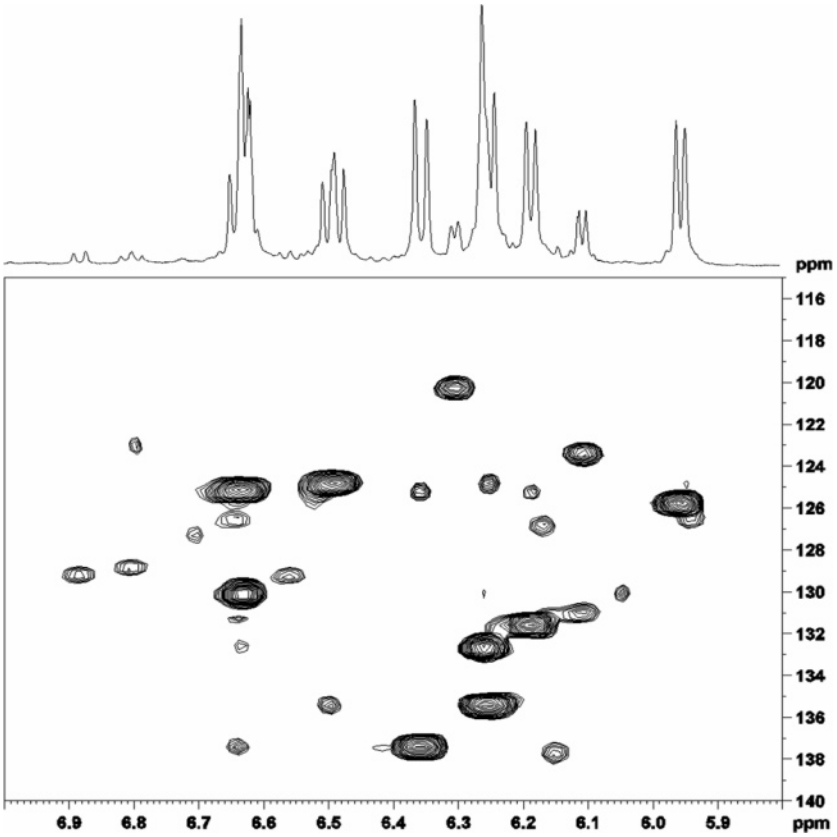

Figure 4. ^{1}H–^{13}C HSQC spectrum of carotenoids from a typical lipophilic mixture extracted from typical tomato juice reported in the olefinic region related to conjugated doubles bonds.

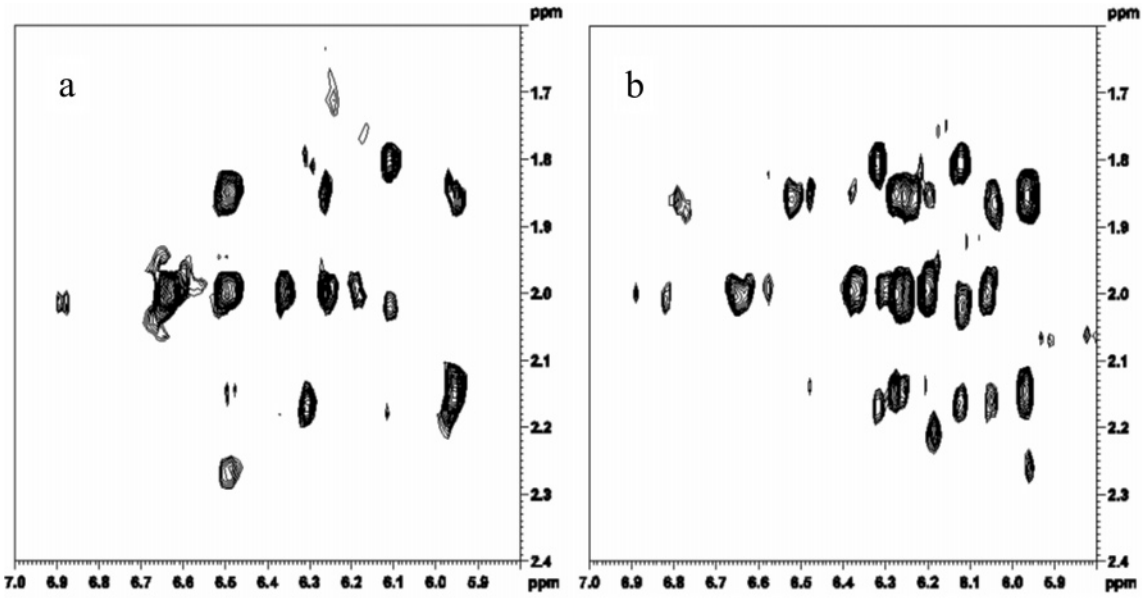

Figure 5. Comparison between ^{1}H–^{1}H T-ROESY (**a**) and ^{1}H–^{1}H TOCSY (**b**) spectra to assess correlation through space and bonds.

cross-contamination of these biomolecules during extraction. The availability of a cryoprobe decreased the experimental time of high-field NMR spectra and thereby minimized further isomerization of lycopene metabolites. These results suggest that high-resolution multidimensional NMR experiments can be used as an excellent means to rapidly identify (*all-E*)-, (5*Z*)-, (9*Z*)-, and (13*Z*)-lycopene isomers and other carotenoids such as (*all-E*)-β-carotene and (15*Z*)-phytoene with minimal purification procedures.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; 2D, two-dimensional; 1D, one-dimensional; $CDCl_3$, chloroform deuterated; MTBE, methyl *tert*-butyl ether; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; J-RES, *J*-resolved; HSQC, heteronuclear single quantum correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; PDA, photodiode array; T-ROESY, transverse rotating frame nuclear Overhauser enhancement spectroscopy;

NOESY, nuclear Overhauser effect spectroscopy; SD, standard deviation; TPPI, time proportional phase increment; MW, molecular weight.

LITERATURE CITED

(1) Clinton, S. K. Lycopene: chemistry, biology, and implications for human health and disease. *Nutr. Rev.* **1998**, *56* (2), 35−51.

(2) Britton, G. Structure and properties of carotenoids in relation to function. *FASEB J.* **1995**, *9* (15), 1551−1558.

(3) Ladygin, V. G. Biosynthesis of carotenoids in the chloroplasts of algae and higher plants. *Russ. J. Plant Physiol.* **2000**, *47* (6), 796−814.

(4) Nguyen, M.; Francis, D.; Schwartz, S. J. Thermal isomerisation susceptibility of carotenoids in different tomato varieties. *J. Sci. Food Agric.* **2001**, *81* (9), 910−917.

(5) Hadley, C. W.; Miller, E. C.; Schwartz, S. J.; Clinton, S. K. Tomatoes, lycopene, and prostate cancer: progress and promise. *Exp. Biol. Med.* **2002**, *227* (10), 869−880.

(6) Unlu, Z. N.; Clinton, S. K.; Schwartz, S. J. Absorption of lycopene isomers following single meals containing tomato sauces with varying isomer patterns. *FASEB J.* **2003**, *17* (5), A756−A757.

(7) Giovannucci, E. A review of epidemiologic studies of tomatoes, lycopene, and prostate cancer. *Exp. Biol. Med.* **2002**, *227* (10), 852−859.

(8) Englert, G. NMR of carotenoids—novel experimental-techniques. *Pure Appl. Chem.* **1991**, *63* (1), 59−70.

(9) Englert, G. NMR spectroscopy. In *Carotenoids. Vol. 1B: Spectroscopy*; Birkhäuser Verlag: Basel, Switzerland, 1995; pp 147−260.

(10) Englert, G. Homonuclear Overhauser proton NMR. Experiments on the carotenoid pigments lycopene and prolycopene. *Helv. Chim. Acta* **1979**, *62*, 1497−1500.

(11) Englert, G.; Noack, K.; Broger, E. A.; Glinz, E.; Vecchi, M.; Zell, R. Synthesis, isolation, and full spectroscopic characterization of 11 (Z)-isomers of (3*R*,3′*R*)-zeaxanthin. *Helv. Chim. Acta* **1991**, *74* (5), 969−982.

(12) Craft, N. E.; Soares, J. H. Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents. *J. Agric. Food Chem.* **1992**, *40*, 431−434.

(13) Scott, K. J. Observations on some of the problems associated with the analysis of carotenoids in foods by HPLC. *Food Chem.* **1992**, *45* (5), 357−364.

(14) Su, Q.; Rowley, K. G.; Balazs, N. D. H. Carotenoids: separation methods applicable to biological samples. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2002**, *781* (1−2), 393−418.

(15) Scott, K. J.; Finglas, P. M.; Seale, R.; Hart, D. J.; deFroidmont-Gortz, I. Interlaboratory studies of HPLC procedures for the analysis of carotenoids in foods. *Food Chem.* **1996**, *57* (1), 85−90.

(16) Aust, O.; Sies, H.; Stahl, W.; Polidori, M. C. Analysis of lipophilic antioxidants in human serum and tissues: tocopherols and carotenoids. *J. Chromatogr. A* **2001**, *936* (1−2), 83−93.

(17) Feltl, L.; Pacakova, V.; Stulik, K.; Volka, K. Reliability of carotenoid analyses: a review. *Curr. Anal. Chem.* **2005**, *1* (1), 93−102.

(18) Emenhiser, C.; Englert, G.; Sander, L. C.; Ludwig, B.; Schwartz, S. J. Isolation and structural elucidation of the predominant geometrical isomers of α-carotene. *J. Chromatogr. A* **1996**, *719* (2), 333−343.

(19) Croasmun, W. R.; Carlson, R. M. K. *Two-Dimensional NMR Spectroscopy: Applications for Chemists and Biochemists*; VCH: New York, 1994.

(20) Lambert, J. B.; Mazzola, E. P. *Nuclear Magnetic Resonance Spectroscopy: An Introduction to Principles, Applications, and Experimental Methods*; Pearson Education: Upper Saddle River, NJ, 2004.

(21) Kovacs, H.; Moskau, D.; Spraul, M. Cryogenically cooled probes—a leap in NMR technology. *Prog. Nucl. Magn. Reson. Spectrosc.* **2005**, *46* (2−3), 131−155.

(22) Robertson, D. G. Metabonomics in toxicology: a review. *Toxicol. Sci.* **2005**, *85* (2), 809−822.

(23) Lindon, J. C.; Holmes, E.; Nicholson, J. K. Toxicological applications of magnetic resonance. *Prog. Nucl. Magn. Reson. Spectrosc.* **2004**, *45* (1−2), 109−143.

(24) Griffin, J. L. Metabonomics: NMR spectroscopy and pattern recognition analysis of body fluids and tissues for characterisation of xenobiotic toxicity and disease diagnosis. *Curr. Opin. Chem. Biol.* **2003**, *7* (5), 648−654.

(25) Viant, M. R. Improved methods for the acquisition and interpretation of NMR metabolomic data. *Biochem. Biophys. Res. Commun.* **2003**, *310* (3), 943−948.

(26) Fan, W. M. T. Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *28*, 161−219.

(27) Hwang, T. L.; Shaka, A. J. Cross relaxation without TOCSY—transverse rotating-frame Overhauser effect spectroscopy. *J. Am. Chem. Soc.* **1992**, *114*, 3157−3159.

(28) Mercadante, A. Z.; Britton, G.; Rodriguez-Amaya, D. B. Carotenoids from yellow passion fruit (*Passiflora edulis*). *J. Agric. Food Chem.* **1998**, *46*, 4102−4106.

(29) Mercadante, A. Z.; Steck, A.; Pfander, H. Carotenoids from guava (*Psidium guajava* L.): isolation and structure elucidation. *J. Agric. Food Chem.* **1999**, *47*, 145−151.

(30) Claridge, T. D. W. *High-Resolution NMR Techniques in Organic Chemistry*; Pergamon: New York, 1999.

(31) Clough, J. M.; Pattenden, G. Stereochemical assignment of prolycopene and other poly-Z-isomeric carotenoids in fruits of the tangerine tomato *Lycopersicon esculentum* Var. Tangella. *J. Chem. Soc., Perkin Trans. 1* **1983**, (12), 3011−3018.

(32) Granger, P.; Maudinas, B.; Herber, R.; Villoutreix, J. Proton and carbon-13 NMR spectra of *cis*- and *trans*-phytoene isomers. *J. Magn. Reson.* **1973**, *10* (1), 43−50.

(33) Neuhaus, D.; Williamson, M. P. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; Wiley-VCH: New York, 2000.

(34) Englert, G.; Aakermann, T.; Liaaenjensen, S. NMR studies on natural *all-trans*-(9′*Z*,11′*Z*)-(3*R*,3′*S*,5′*R*,6′*R*)-pyrrhoxanthin, an acetylenic-C-37-skeletal nor-carotenoid butenolide. *Magn. Reson. Chem.* **1993**, *31* (10), 910−915.

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