

Screening of Carotenoids in Tomato Fruits by Using Liquid Chromatography with Diode Array–Linear Ion Trap Mass Spectrometry Detection

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S Supporting Information

ABSTRACT: This paper presents an analytical strategy for a large-scale screening of carotenoids in tomato fruits by exploiting the potentialities of the triple quadrupole–linear ion trap hybrid mass spectrometer (QqQ_{LIT}). The method involves separation on C₃₀ reversed-phase column and identification by means of diode array detection (DAD) and atmospheric pressure chemical ionization–mass spectrometry (APCI-MS). The authentic standards of six model compounds were used to optimize the separative conditions and to predict the chromatographic behavior of untargeted carotenoids. An information dependent acquisition (IDA) was performed with (i) enhanced-mass scan (EMS) as the survey scan, (ii) enhanced-resolution (ER) scan to obtain the exact mass of the precursor ions (16–35 ppm), and (iii) enhanced product ion (EPI) scan as dependent scan to obtain structural information. LC-DAD-multiple reaction monitoring (MRM) chromatograms were also acquired for the identification of targeted carotenoids occurring at low concentrations; for the first time, the relative abundance between the MRM transitions (ion ratio) was used as an extra tool for the MS distinction of structural isomers and the related families of geometrical isomers. The whole analytical strategy was high-throughput, because a great number of experimental data could be acquired with few analytical steps, and cost-effective, because only few standards were used; when applied to characterize some tomato varieties (‘Tangerine’, ‘Pachino’, ‘Datterino’, and ‘Camone’) and passata of ‘San Marzano’ tomatoes, our method succeeded in identifying up to 44 carotenoids in the ‘Tangerine’ variety.

KEYWORDS: carotenoids, tomato, LC-DAD-MS, linear ion trap, information-dependent data acquisition, matrix solid phase dispersion

INTRODUCTION

Introduced in Europe from South America at the beginning of the 18th century, tomato along with its processing product, the passata, is one of the key foods of the Italian Mediterranean diet.¹ Juice, ketchup, paste, sauce, and soup are other processed tomato products, usually consumed in North America.² Recognized to be effective in preventing cardiovascular and neoplastic diseases in humans, the protective effects of tomato have mainly been attributed to carotenoids, which are one of the major classes of phytochemicals in this fruit.^{2,3}

Carotenoids are a group of fat-soluble pigments with color from yellow to red, which are synthesized by plants and many microorganisms.⁴ These tetraterpenes include more than 750 compounds distinguishable in carotenes, xanthophylls, and carotenic acids; they also comprise structures with fewer than 40 carbon atoms known as apocarotenoids and norcarotenoids.⁵

The beneficial effects of carotenoids on human health are attributed to their antioxidant properties, specifically to their ability to deactivate singlet oxygen $^1\text{O}_2$ and to interact with free radicals.⁶ The efficacy for physical quenching is especially high for carotenoids provided of more than nine conjugated double-bonds and an acyclic structure;² thus, lycopene is more active than β -carotene even if both compounds possess 11 conjugated double bonds.⁷ In addition to their antioxidant properties, some carotenoids exhibit provitamin-A activity on the condition that

they have a polyene chain with 11 carbon atoms and, at least, one unsubstituted β -ionone ring. β -Carotene (β,β -carotene), β -cryptoxanthin (β,β -caroten-3-ol), and γ -carotene (β,γ -carotene) own these requirements.⁸

To date, information on the composition of the most common varieties of tomato is still limited to few antioxidants, which are usually the most abundant ones. However, the quality and the properties of this functional food also depend on minor carotenoids and *cis* isomers which, compared to the *all-trans* forms, have superior bioavailability.⁹ For example, lycopene has been found in human plasma and tissues as a mixture of geometric isomers containing percentages of *cis* forms exceeding 50%.^{10,11} Therefore, knowing the detailed distribution of these fat-soluble antioxidants is essential to establish a closer correlation with the tomato’s nutritional properties. The methods available in the literature have mainly been based on liquid chromatography (LC) with UV-vis detection^{12–17} and have been able to identify up to a maximum of 28 compounds between geometric isomers and epoxides of the three most abundant carotenoids, i.e., lycopene, β -carotene, and lutein.¹³ In

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addition to these, the other carotenoids that have been identified so far are phytoene, phytofluene, ζ -carotene, γ -carotene, and neurosporene.^{12,18,19}

Even if the chromatographic behavior and the UV-vis absorption spectrum offer valuable clues for the identification of carotenoids, the MS detection has recently been combined with DAD to enhance identification power and sensitivity of their LC analysis in complex matrices.^{20–27} Besides molecular weight, MS can provide molecular structure information, based on fragmentation patterns, to support the UV-vis identification of structural and geometrical isomers^{11,28–30} as well as that of epoxidized forms.³¹

This work has aimed at developing an effective analytical approach, based on the HPLC-DAD-QqQ_{LIT} hyphenation, to profile carotenoids occurring in five of the most known Italian tomato varieties: 'Tangerine', 'Pachino', 'Datterino', 'Camone', and 'San Marzano'. Six carotenoids, whose authentic standards are commercially available and moderately expensive, were used as model compounds to predict the behavior of carotenes and xanthophylls toward the chromatographic retention on a C₃₀ stationary phase and the chemical ionization at atmospheric pressure. The product ion scan spectra (PIS) of these six compounds and the enhanced product ion (EPI) spectra of untargeted carotenoids, obtained by planning an adequate IDA experiment, allowed us to select MRM transitions useful (i) to support the LC-DAD identification of major carotenoids, (ii) to screen carotenoids occurring at low concentrations with high sensitivity, and (iii) to discriminate among structural isomers by using, for the first time, the ion ratio. Compared to the previous LC-DAD-MS methods,^{11,28–31} the developed approach has made possible the acquisition of a large number of experimental data to realize a large-scale screening by using a minimum number of analytical standards; since the latter ones are very expensive and/or difficult to find, our approach also offers a considerable economical and practical advantage.

All data on the composition of the different varieties of tomatoes will be presented and discussed in this paper along with the results related to the method development.

EXPERIMENTAL SECTION

Chemicals and Materials. *all-trans*- β -Carotene, *all-trans*-lutein, *all-trans*-zeaxanthin, *all-trans*- β -cryptoxanthin, and *all-trans*-lycopene were provided by Aldrich-Fluka-Sigma Chemical (Milan, Italy). *all-trans*- γ -Carotene was acquired from CaroteNature GmbH (Ostermundigen, Switzerland). All standards had a purity grade $\geq 90\%$ and were used without further purification.

Butylated hydroxytoluene (BHT), provided by Aldrich-Fluka-Sigma Chemical, was used as antioxidant both in standard solutions and during the several steps of extraction procedure. Acetonitrile and methanol were RS-Plus grade (special grade reagents); 2-propanol, hexane, and chloroform were RS grade (elevated purity grade); absolute ethanol was RPE grade (analytical grade). All of these solvents, potassium hydroxide (KOH), magnesium carbonate, and sodium sulfate were purchased from Carlo Erba (Milan, Italy). Distilled water, used in the extraction procedure based on the cold saponification, was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA).

Diatomaceous earth SPE-ED MATRIX 38 was purchased from Applied Separations (Allentown, PA, USA). Syringe-like polypropylene tubes (i.d. 26 mm, 75 mL capacity) and polyethylene frits were from Alltech (Deerfield, IL, USA).

Standard Solutions. After a stability study was performed over a period of 30 days, the individual stock solutions of the six carotenoids were prepared fortnightly by dissolving the weighed amounts (OHAUS DV215CD Discovery Semi-Micro and Analytical Balance 81 g/210 g

capacity, 0.01 mg/0.1 mg readability) in chloroform containing 0.1% (w/v) BHT at the following concentrations:

- 1 $\mu\text{g}/\mu\text{L}$, for *all-trans*- β -carotene and *all-trans*-lycopene
- 0.5 $\mu\text{g}/\mu\text{L}$, for *all-trans*-lutein (sonicating for 6 min at 30 °C), *all-trans*-zeaxanthin, *all-trans*- β -cryptoxanthin
- 0.2 $\mu\text{g}/\mu\text{L}$ for *all-trans*- γ -carotene

Working multistandard solutions were prepared from the individual stock solutions by diluting in methanol with 0.1% (w/v) BHT to obtain concentrations suitable for the several studies and experiments. When unused, all preparations were degassed with nitrogen and stored in amber glass flasks at -18 °C in the dark. Authentic standards were stored at -80 °C.

Tomato and Passata Samples. The five tomato varieties ('Tangerine', 'Pachino', 'Datterino', 'Camone', 'San Marzano') selected for this study were purchased from some supermarkets and green-groceries of Rome. The passata of 'San Marzano' tomatoes was homemade or bought from local grocery stores.

Tangerine tomato owes its orange color to the prevalence of *cis*-lycopenes that, in its GMO variants, accumulate prevalently as prolycopene; in this work, we have studied the wild type.³² 'Pachino' is a protected geographical indication (GPI) variety, produced in a restricted area of Sicily; we have considered the cherry type, characterized by small, sweet, and bright red fruits. Also the 'Datterino' variety has small fruits, but they have an elongated form and a superior sweetness. The 'Camone' variety was introduced in the mid-1980s in the south of Sardinia; its fruits are red-green, crispy, and suitable for salads. Finally, the 'San Marzano' tomato is an Italian variety with protected designation of origin (PDO), and it is famous worldwide because its red pulpy fruits are particularly suitable for making passata. The passata preparation provides few fundamental steps: blanching of tomatoes in boiling water for 20 to 30 s, squeezing of the peeled fruits, sieving to separate the juice from the skins and flesh, bottling of the juice, and finally, sterilization at 95–100 °C.

Sample Treatment. A whole fruit was homogenized in an Ultra-Turrax homogenizer (Janke & Kunkle, Staufenim Breisgau, Germany), adding magnesium carbonate (in a ratio of 10:1, w/w) to neutralize the released organic acids and BHT (in a ratio of 10:0.1, w/w), in order to prevent isomerization and degradation of carotenoids. Tomato passata, ready to be used, was only spiked with BHT at the specified ratio.

Two different extraction procedures were used to exclude the potential formation of artifacts: overnight cold saponification and matrix solid phase dispersion (MSPD). In both procedures, the analytes were shielded from UV light by using low actinic glass tubes and plastic tubes wrapped with aluminum foil; for the same reason, all operations were performed in subdued light. Protection from oxygen was ensured by the addition of BHT to the solvents (0.1% (w/v)).

Overnight Cold Saponification. A 4 g aliquot of homogenized tomato was transferred into a 50 mL polypropylene centrifuge tube with a screw-cap (falcon). Six milliliters of absolute ethanol containing BHT and 1 mL of 50% (w/v) aqueous KOH were poured into the centrifuge tube. After flushing with oxygen-free nitrogen, the falcon was promptly closed and placed in a water bath kept at 25 °C, under magnetic stirring overnight (15 h).

At the end of the incubation period, the digest was diluted with 3 mL of Milli-Q water and the analytes were extracted with 4 mL of hexane with 0.1% (w/v) BHT. The extraction was repeated six times. After the addition of each aliquot, the mixture was stirred for 5 min, vortexed for 5 min, and then, centrifuged at 6000 rpm for 10 min to facilitate the separation of the insoluble residue, aqueous phase, and organic phase. Afterward, the combined hexane layers were washed with 8 mL portions of Milli-Q water; two washings were enough to remove alkalis completely (no color was observed on phenolphthalein addition). At this point, the extract was collected into a glass tube with conical bottom (i.d. 2 cm), evaporated up to 100 μL in a water bath kept at 30 °C under a slow nitrogen flow, and diluted to a final volume of 200 μL with 2-propanol:hexane (75:25, v/v) solution containing 0.1% (w/v) BHT. Eventually, after sonication for 10 min at 25 °C, 40 μL was injected into the HPLC-DAD-QqQ_{LIT} system.

Matrix Solid Phase Dispersion. Owing to the high water content of tomato fruit and passata, MSPD was performed by using diatomaceous earth, as a dispersing medium, in combination with sodium sulfate, a highly hygroscopic salt.

For each experiment, 4 g of sample, 6 g of diatomaceous earth, and 2 g of Na₂SO₄ were placed into a ceramic mortar and pounded with a pestle until the achievement of an evenly colored powder. At this point, the extraction cartridge was prepared by filling large-diameter syringe-like polypropylene tubes with a layer of C₁₈ sorbent (0.4 g) and a layer of the solid mixture, in this order. Polyethylene frits were placed below and above the chromatographic bed.

The analytes were eluted with 15 mL of methanol, 5 mL of 2-propanol, and 20 mL of hexane, with the aid of vacuum. The eluates were collected into a 50 mL falcon and centrifuged at 6000 rpm for 10 min. The supernatant was poured out into a glass tube with conical bottom (i.d. 2 cm) and evaporated up to 200 μL in a water bath kept at 25 °C under a gentle flow of nitrogen. The extract was then sonicated for 2 min, transferred into an Eppendorf tube, and centrifuged (Centrifuge 150, Tehnica, Slovenia) at 4000 rpm for 5 min. Forty microliters was injected into the chromatographic column for the HPLC-DAD-QqQ_{LIT} analysis.

Instrumentation. Liquid Chromatography. Liquid chromatography was performed by means of a micro HPLC series 200 (PerkinElmer, Norwalk, CT, USA) equipped with an autosampler, a vacuum degasser, and a column oven.

The analytes were separated on a ProntoSIL C30 column (4.6 mm i.d. × 250 mm, 3 μm particle size) from Bischoff Chromatography (Leonberg Germany) protected by a guard column of the same type (4.0 × 10 mm, 5 μm) under nonaqueous reversed-phase (NARP) conditions and with the column kept at 19 °C. In separating carotenoids, phase A was methanol and phase B a mixture 2-propanol/hexane 50:50 v/v. The elution profile was as follows: after the first minute in isocratic mode at 100% of eluent A, phase B was linearly increased from 0% to 50% in 20 min and then from 50% to 99.5% in 0.1 min; finally, phase B was maintained at 99.5% for 10 min. Flow rate of the mobile phase was 1 mL/min, and it was entirely introduced into the DAD-MS detection system. Phase B was also used to wash the autosampler injection device.

Diode Array Detection. Carotenoids were identified by a series 200 model (PerkinElmer) diode array detector, equipped with a flow Z cell (12 μL volume; optical path of 10 mm), coupled online between the chromatographic column and the mass spectrometer. The LC-DAD chromatograms were acquired by selecting the 450 nm wavelength (λ_{max} of *all-trans*-β-carotene, *all-trans*-zeaxanthin, and *all-trans*-β-cryptoxanthin) and a bandwidth of 10 nm. The UV-vis spectra were recorded in the range of 200–700 nm. The software packages used for running the instrument and acquiring spectral data were Totalchrom Navigator 6.3.1 and Iris (PerkinElmer) respectively.

Mass Spectrometry. The analytes were also identified by means of a 4000 Qtrap (AB SCIEX, Foster City, CA, USA) mass spectrometer. Detection was performed in positive ionization, placing the APCI probe in the Turbo V source and setting a needle current (NC) of 3 μA and a probe temperature of 450 °C. High-purity nitrogen was used as curtain (40 psi) and collision gas (4 mTorr), whereas air was used as nebulizer (55 psi) and makeup gas (30 psi).

A preliminary mass axis calibration of each quadrupole mass-analyzer, Q₁ and Q₃, was carried out by infusion of a polypropylene glycol solution at 10 μL/min. Unit mass resolution was established and kept in the each mass-resolving quadrupole by maintaining a full width at half-maximum (fwhm) of approximately 0.7 ± 0.1 u.

The APCI-Q1-full scan spectrum and the PIS spectra of *all-trans*-lutein, *all-trans*-zeaxanthin, *all-trans*-β-cryptoxanthin, *all-trans*-β-carotene, *all-trans*-γ-carotene, and *all-trans*-lycopeno were acquired by working in flow injection analysis (1–10 ng injected; 1 mL/min of flow rate).

Structural information on untargeted carotenoids in the real samples was obtained by acquiring an IDA scan cycle, including an enhanced-mass scan (EMS) as the survey scan, an enhanced-resolution (ER) scan to obtain high resolution *m/z* values for the precursor ions of interest, and dependent EPI scan. With the threshold of the ion intensity at 10000 counts per second (cps), the IDA criteria were set to allow the

most abundant ions in the EMS to trigger EPI scans. The EMS scan was carried out from *m/z* 500 to *m/z* 610 at 100 ms of dwell time. The EPI scan was in the range *m/z* 100–610, scanned at 4000 Da/s, under a collision energy (CE) of 60 eV with a CE spread of 20 eV (60 ± 20 eV).

A sensitive LC-DAD-MRM screening of carotenoids in real samples was performed selecting two MRM transitions per carotenoid on the basis of the fragmentation spectra previously acquired; when such fragmentation spectra were not available, two theoretical MRM transitions were “guessed” by using ChemBioDraw Ultra 12.0.

The software used for running the instrument and acquiring LC-MS data was Analyst 1.5.1 (AB Sciex).

RESULTS AND DISCUSSION

Selection of Nonaqueous Reversed-Phase Chromatographic Conditions as a Compromise between Requirements of Separation and MS Detection. The NARP chromatography has often been used for the separation of carotenoids and fat-soluble vitamins.^{30,33} This technique makes use of C₁₈ or C₃₀ columns and of poorly polar mobile phases. The triacontyl stationary phase is superior to the octadecyl one for shape selectivity, which significantly improves at subambient temperature. A typical nonaqueous mobile phase consists of a polar solvent (for instance, acetonitrile or methanol) and a solvent of lower polarity (for example, dichloromethane) to solubilize the analytes and to adjust the eluting strength of the mobile phase. It is well-known that the APCI ionization process uses the mobile-phase vapor as reactant gas in addition to the nebulizer gas and makeup gas; nevertheless, the composition of a nonaqueous mobile phase is, in general, not completely suitable to support APCI ionization. For this reason, our chromatographic conditions were selected as a compromise between requirements of separation and MS detection. The six model compounds were used to refine the separative conditions which included the use of

- a C₃₀ analytical column, kept at 19 °C to enhance shape selectivity and efficiently solve structural and geometrical isomers
- methanol and a mixture 2-propanol:hexane (50:50, v/v) as mobile phase. Hexane is not involved in the acid–base reactions during APCI ionization, but it was indispensable to reduce the retention time of carotenes (especially lycopene) and to avoid peak broadening phenomena due to longitudinal diffusion. Both alcohols participate in the acid–base reactions; in particular, in order to assist the ionization of the analytes with high capacity factors, a percentage of 0.5% methanol was maintained in the final part of the chromatographic run.

The chromatographic selectivity was adjusted by modulating the polarity difference between stationary phase and mobile phase: the smaller was the difference, the higher was the selectivity. Selecting this mobile phase we have achieved good results in terms of resolution.

Identification Criteria of Carotenoids by HPLC-DAD-QqQ_{LIT}. The model compounds were also used to study their APCI ionization and fragmentation, and to forecast the chromatographic retention of untargeted carotenoids on the triacontyl-bonded stationary phase. Table 1 shows the parameters applied for their identification in tomato and passata samples (retention time, two MRM transitions for each analyte, ion ratio).

Expected Retention Time. The expected retention time is an important parameter to support the UV-vis/MS identification of a specific carotenoid. In this work, it has been deduced both on

Table 1. LC-MRM Parameters Used for the Identification of the Six Model Carotenoids

carotenoid	retention time: mean \pm SD ^a (min)	MRM transitions	ion ratio: ^b mean \pm SD ^a	DP ^c (V)	CE ^d (eV)
<i>all-trans</i> -lutein	10.6 \pm 0.4	551.5/175	0.6 \pm 0.1	55	40
		551.5/135			30
<i>all-trans</i> -zeaxanthin	11.2 \pm 0.4	569.6/175	0.5 \pm 0.1	30	60
		569.6/119			40
<i>all-trans</i> - β -cryptoxanthin	13.8 \pm 0.4	553.5/175	1.3 \pm 0.1	30	70
		553.5/119			60
<i>all-trans</i> - β -carotene	17.9 \pm 0.5	537/177	1.5 \pm 0.1	70	30
		537/119			60
<i>all-trans</i> - γ -carotene	20.4 \pm 0.5	537/177	0.8 \pm 0.1	70	30
		537/119			60
<i>all-trans</i> -lycopene	26.8 \pm 0.5	537/177	0.4 \pm 0.1	70	30
		537/119			60

^aStandard deviation. ^bThe ion ratio was calculated as follows: $I_r = (\text{intensity}_{(\text{MRM on the first line})}) / (\text{intensity}_{(\text{MRM on the second line})})$. ^cDeclustering potential. ^dCollision energy.

the basis of the chromatographic behavior of the six model compounds under the NARP conditions, described in the **Liquid Chromatography** section, and on the basis on data from the literature.³³ The following elution patterns have been considered for the prediction of retention times:

- (i) The increase of molecular weight or the conjugation extension corresponds to longer retention time.
- (ii) The retention time decreases as the number of hydroxyl and/or epoxy groups increases, because the increase of polarity prevails over the corresponding increase of the chromophore length; therefore, among β,β -type carotenoids, zeaxanthin elutes before β -cryptoxanthin, which, in turn, elutes before β -carotene. This behavior has allowed us to simplify the carotenoid identification, subdividing each LC-DAD-MS chromatogram into three regions: the first one including xanthophylls with two and more than two polar groups (t_r up to 12 min); the second one comprising xanthophylls with only one polar group (t_r between \sim 12 and 15 min); finally, the third one encompassing carotenes (t_r between \sim 15 and 26 min).

Within each of these ranges, further considerations can be made to postulate the retention times. For example:

- Comparing the chromatographic behavior of the pairs lutein/zeaxanthin and α -carotene/ β -carotene (mixed carotenes from carrots; purchased Aldrich-Fluka-Sigma Chemical, Milan, Italy), it can be deduced that β,ϵ -carotenoids have a lower retention time than β,β -carotenoids.
- A bicyclic carotenoid elutes before a monocyclic carotenoid that, in turn, elutes before an acyclic carotenoid; for example, β -carotene (bicyclic β,β) elutes before the γ -carotene (β,γ -carotene), which in turn elutes before lycopene (γ,γ -carotene).
- (iii) The molecule shape can affect the retention of carotenoids, sometimes representing an exception to the rule in the point (i). For example:
 - 5,8-Epoxy carotenoids (mutatoxanthin) elute later than the 5,6-epoxy carotenoids (antheraxanthin, taraxanthin), although 5,8-epoxy carotenoids have a shorter chromophore. This happens because the 5,8-epoxide group affects the molecule shape and exerts a deep interaction with the stationary phase despite of the less extensive conjugation length.³³
 - A *cis* double bond affects the elution order of equal length chromophores, depending on its location in the system of conjugated double bonds.³³ The 15-*cis*-isomers are the least retained because their molecules are more twisted than the 13-*cis*- and 13,9-di-*cis* isomers. On the other hand, carotenoids with peripheral *cis* double bonds (9-*cis*- and 9,9'-di-*cis*-isomers for bicyclic carotenoids; 5-*cis*-isomers for monocyclic and acyclic carotenoids) are more retained than the *all-trans* isomers which, thus, elute between the next-to-central and the peripheral geometrical isomers.

MS Identification Criteria. On the basis of what was observed in this work and described in previous papers^{20,34} and reviews,^{30,35,36} it can be stated that both carotenes and xanthophylls show $[M + H]^+$ as base peak on APCI(+) full scan mass spectra. For *all-trans*- β -carotene, *all-trans*- γ -carotene, and *all-trans*-lycopene, such an ion is observed at m/z 537; when

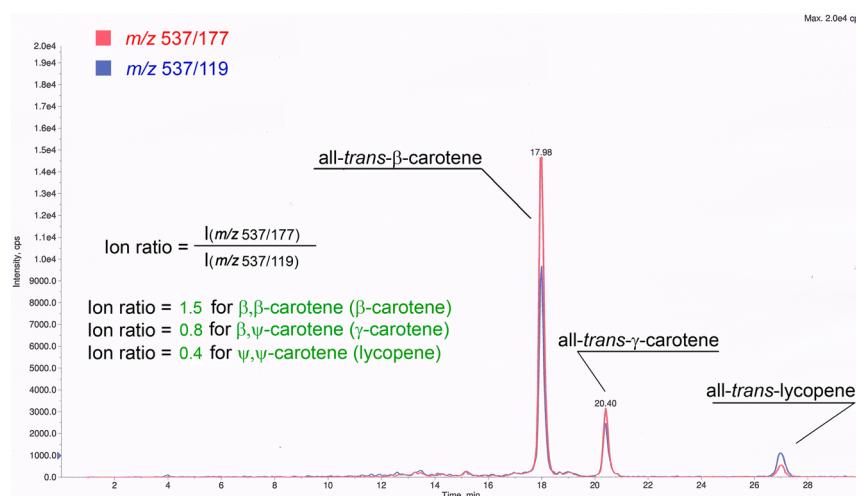


Figure 1. Relative abundance of two MRM transitions, shared by isomeric carotenoids, is an effective parameter to distinguish and identify *all-trans*- β -carotene, *all-trans*- γ -carotene, *all-trans*-lycopene, and the related families of geometrical isomers in real samples (see also Figure 4).

selected as precursor ion in MS/MS experiments, it exhibits a similar fragmentation pattern with two series of fragment ions: the first series is originated from breaking the double bonds (C7–C8, C9–C10, C11–C12, C13–C14, and C15–C15') and generates intense product ions at m/z 137 and m/z 177; the second series is due to the cleavage of single bonds (C6–C7, C10–C11, C12–C13, and C14–C15) and produces an abundant ion at m/z 119. The main distinctions between the PIS spectra of these three structural isomers consist of the different intensities of the common fragment ions.

Besides $[M + H]^+$, xanthophylls also show the dehydrated ion $[MH - H_2O]^+$. Zeaxanthin and β -cryptoxanthin are β,β -carotenols and exhibit $[M + H]^+$ as base peak, while lutein is a β,ϵ -carotene and has $[MH - H_2O]^+$ as base peak. In general, all carotenols with a hydroxyl group in an allylic position (for example lutein and α -cryptoxanthin) lose a water molecule, producing an intense fragment ion which is stabilized by mesomeric effects.³⁵ We have exploited this behavior to distinguish isomeric xanthophylls from each other such as lutein from zeaxanthin or lutein epoxide from antheraxanthin. Product ions common to all xanthophylls are the fragments at m/z 135 and m/z 175, corresponding to a dehydrated terminal ring with cleavage at the C7–C8 bond and the C9–C10 bond, respectively: these ions can be considered diagnostic for this carotenoid class. Like β -carotene, γ -carotene, and lycopene, also lutein and zeaxanthin are structural isomers which share similar fragmentation pattern; nevertheless, unlike carotenes, lutein exhibits two exclusive fragment ions at m/z 495 and m/z 430.³⁷ However, their intensity is too low to detect lutein (and to distinguish it from zeaxanthin) at the concentrations naturally occurring in tomato samples. To this end, the abundant ion at m/z 119 is more useful despite its lower specificity.

Relying on the PIS spectra, we have selected the two most intense MRM transitions to identify each model compound as well as their isomeric unknowns (sharing the same ion currents). Although there is no unique fragment to differentiate carotenes that are structural isomers, we have found a simple but efficacious solution to this problem: the relative abundance between the two MRM transitions, shared *all-trans*- β -carotene, *all-trans*- γ -carotene, and *all-trans*-lycopene, provides a characteristic ion ratio (see Table 1 and Figure 1) which we have used successfully to distinguish the three carotenes and their families of geometrical isomers in the real samples (see the paragraph entitled Characterization of the Carotenoid Fraction of Some Italian Varieties of Tomatoes).

For carotenoids that are not isomers of the six model compounds, the MRM transitions have been deduced from the EPI spectra acquired by the IDA experiments. When the sensitivity of the IDA analysis was poor, theoretical MRM transitions were inferred by using the Fragmentation Tools, provided by the software ChemBioDraw 12.0.

In general, the MRM scan mode is particularly effective in the identification of low-abundance compounds. Moreover, the partial specificity of MRM ion currents of isomeric carotenoids is advantageous because it allows their screening without having the availability of all the authentic standards, but on the condition that their chromatographic separation is guaranteed. To this aim, we used the model compounds also to verify the complete chromatographic separation of the isomeric groups lycopene/ γ -carotene/ β -carotene and lutein/zeaxanthin. A good resolution was achieved as a result of the chromatographic selectivity related to the small polarity difference between nonaqueous reversed mobile phase and C₃₀ stationary phase.

In this work, the UV-vis data have been correlated with the MS data, considering that the retention times on the LC-MS chromatogram are about 0.8 min later compared to those on the corresponding LC-DAD chromatogram.

UV-Vis Identification Criteria. Carotenoids are characterized by a strong absorption in the UV-vis region of electromagnetic spectrum due to the extensive conjugated double-bond system. The majority of these compounds exhibit a three-peak spectrum^{5,7} whose fine structure and absorption maxima allow the chromophore identification. In this work, the experimental UV-vis data have been compared to both the literature data and theoretical data. The latter has been obtained by applying the Fieser rules,³⁸ which allows foreseeing the wavelength of the central peak (λ_{II} or λ_{max}) on the basis of the hypothesized structure:

$$\lambda_{max} = 114 + 5M + n(48 - 1.7n) - 16.5R_{endo} - 10R_{exo} \quad (1)$$

for carotenes

$$\lambda_{max} = 114 + 5M + n(55.5 - 2.1n) - 16.5R_{endo} \quad (2)$$

for carotenals and carotenic acids

$$\lambda_{max} = 118 + 5M + n(48 - 1.7n) - 23R_{endo} \quad (3)$$

for epoxycarotenoids

where n = number of conjugated double bonds; M = number of alkyl or alkyl-like substituents on the conjugated system; R_{endo} = number of rings with endocyclic double bonds in the conjugated system; R_{exo} = number of rings with exocyclic double bonds.

Such rules are valid only if the polyene moiety contain more than 4 conjugated double bonds.

In all cases, the degree of fine structure has been expressed as the percentage ratio between the heights of the peaks II and III, measured from baseline (i.e., % III/II).

The identification of a *cis* isomer⁵ has relied on the comparison between its absorption spectrum and that of the corresponding *all-trans* isomer. The following differences have been evaluated: (i) a decrease of % III/II; (ii) a hypochromic effect; (iii) a hypsochromic shift of about 2–6 nm for mono-*cis* isomers, 10 nm for di-*cis* isomers, and 50 nm for poli-*cis* isomers; (iv) the appearance of a *cis*-peak (peak B) in the UV region. Since the height of the *cis*-peak increases as the *cis*-double bond becomes closer and closer at the center of the conjugated system, the Q-ratio has been used as parameter to establish the position of such a bond.⁷ This parameter has been calculated as the ratio between the heights of the peaks II and B, measured from the baseline of spectrum; in the literature, it is sometimes calculated also as (B/II).

Extraction of Carotenoids from Tomato Fruits and Passata. In this work, the extraction of carotenoids from the real samples has been achieved by applying two different extraction methodologies: (a) cold saponification and (b) MSPD. The definitive conditions were established after optimizing several parameters including sample amount, type of extractants, and volume of extractants.

In general, hot saponification is one of the most used procedures to extract carotenoids from foods because it allows the effective removal of lipids and chlorophylls. In alkaline media, carotenoids are quite stable, but isomerization and degradation can occur to an extent depending on the applied conditions, especially for xanthophylls.³⁹ To avoid these processes, we applied milder conditions by digesting the real samples overnight

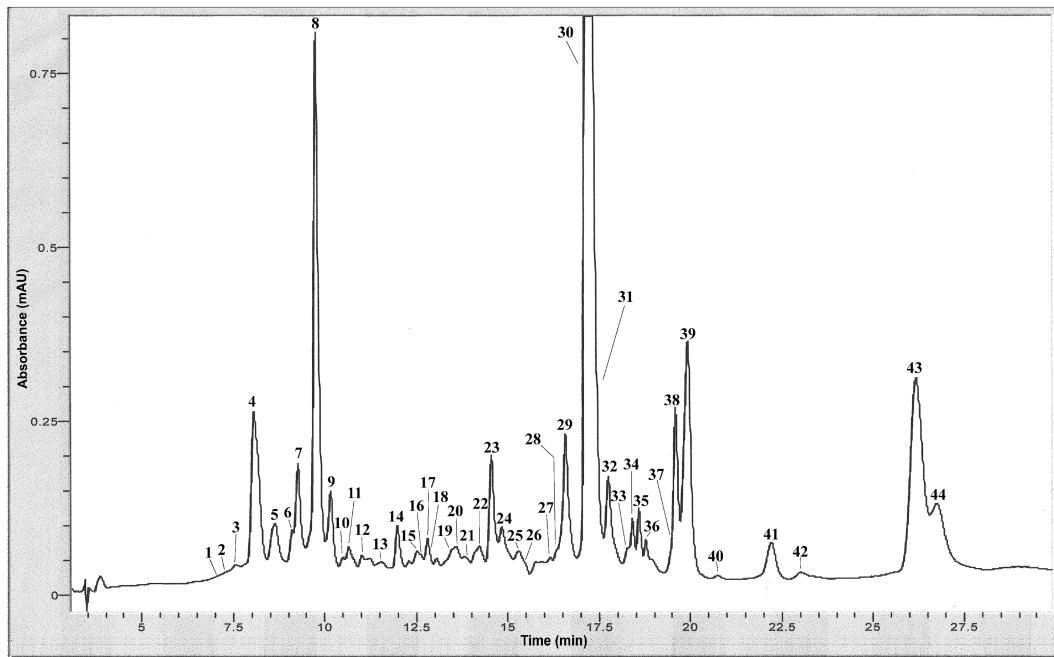


Figure 2. LC-DAD chromatogram, acquired at 450 nm, of a saponified extract from ‘Wild Tangerine’ tomato.

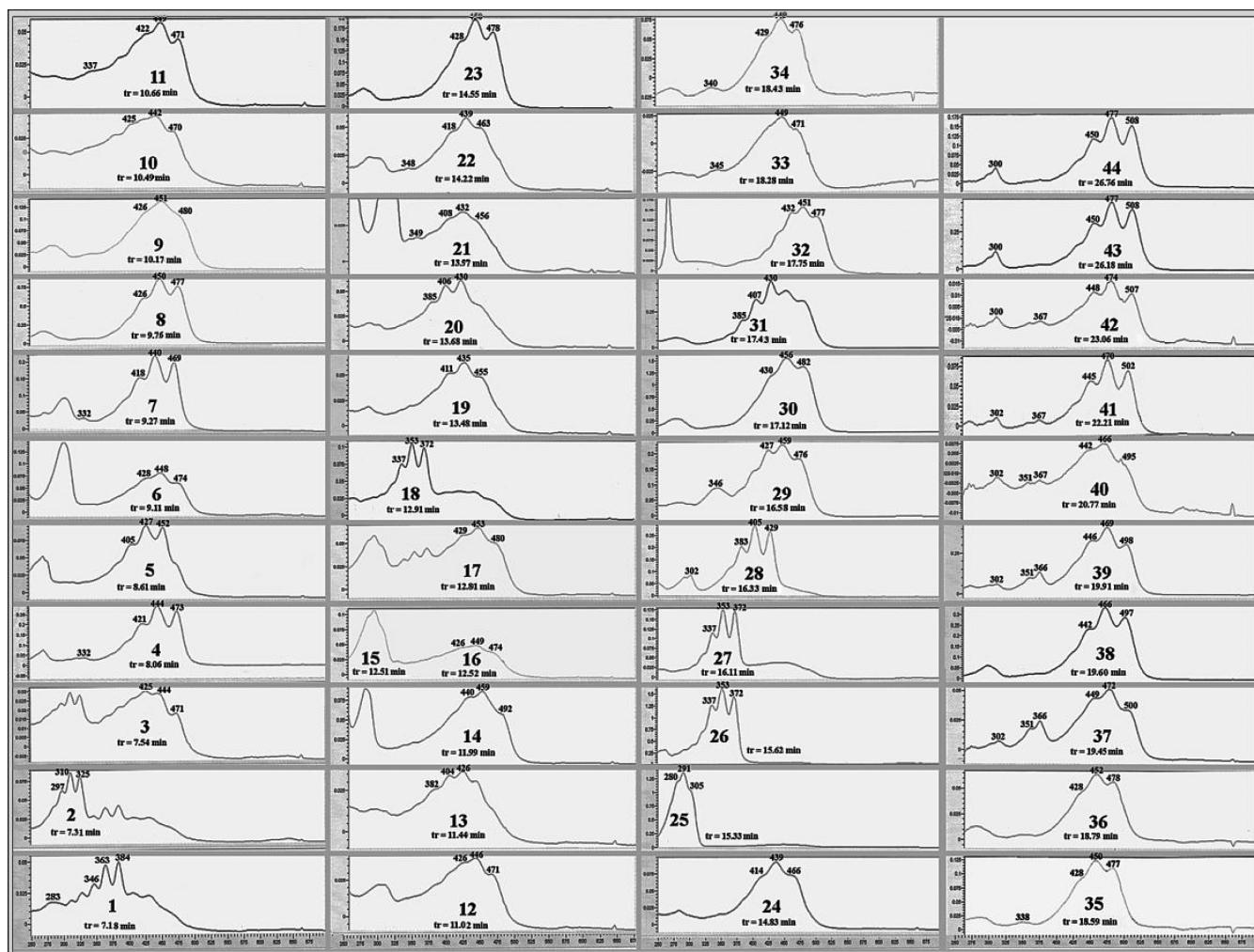


Figure 3. UV-vis absorption spectra (200–700 nm) extracted from the corresponding chromatographic peaks of Figure 2.

Table 2. LC-UV/Vis Data Used for the Identification of Carotenoids in 'Wild Tangerine' Tomato and Other Tomato Varieties

peak no.	LC-DAD retention times (min)	identified carotenoids	calcd λ_{\max}^a (nm)	λ (nm)		(III/II) %		Q-ratio (II/B)	
				obsd ^b	lit. ^c	obsd ^b	lit. ^c	obsd ^b	lit. ^c
1	7.18	9-cis-valenciananthin	368–364	283, 346, 363, 384	282, 348, 366, 388	102			
2	7.31	cis-phytofluene diepoxide	319	297, 310, 325		73			
3	7.54	deepoxyneoxanthin or trollein	442.5	425, 444, 471	420, 442, 469				
4	8.06	9-cis-antheraxanthin	443–439	332, 421, 444, 473	325, 417, 440, 468 ^d	75	63 ^d	3.3	7 ^d
5	8.61	9-cis-mutatoxanthin	430–426	310, 405, 427, 452	310, 405, 427, 452 ^d	81	78 ^d	4.1	
6	9.11	zeaxanthin epoxide	447	428, 448, 474					
7	9.27	9-cis-lutein epoxide or cis-taraxanthin	440–436	332, 418, 440, 469	420, 440, 470 ^e	78		6 ^e	
8	9.76	all-trans-lutein	448	426, 450, 477	422, 445, 474	57	60		
9	10.17	all-trans-zeaxanthin	453	426, 451, 480	428, 450, 478	25	25		
10	10.49	cis- β -cryptoxanthin 5,6-epoxide	443–439	425, 442, 470		20			
11	10.66	9-cis-lutein	446–442	337, 422, 444, 471	418, 441, 469 ^f	22	58 ^f		
12	11.02	9'-cis-lutein	446–442	426, 446, 471	335, 417, 441, 469 ^f	-22	60 ^f		
13	11.44	all-trans- ζ -carotene diepoxide	401	382, 404, 426					
14	11.99	apo-10'-lycopenal	454	440, 459, 492	460 ^g				
15	12.51	phytoene epoxide	^h	280, 290, 302	275, 285, 296				
16	12.52	cis- β -cryptoxanthin	447–451	326, 426, 449, 474					
17	12.81	all-trans- β -cryptoxanthin	453	429, 453, 480	425, 449, 476	25	25		
18	12.91	phytofluene epoxide	341	337, 353, 372		82			
19	13.60	prolycopene		411, 435, 460	414, 436, 463				
20	13.69	9,15,9'-tri-cis- ζ -carotene		289, 385, 406, 430	377, 399, 424 ⁱ	76 ⁱ	2.3	4.2 ⁱ	
21	14.00	di-cis- γ -carotene-epoxide		408, 432, 456					
22	14.22	di-cis- γ -carotene-1,2-epoxide		418, 439, 463					
23	14.55	all-trans- β -carotene-5,6-epoxide	445	428, 450, 478	422, 445, 472	38			
24	14.83	tri-cis- γ -carotene		414, 439, 466					
25	15.33	15-cis-phytoene	^h	280, 291, 302	276, 285, 297				
26	15.62	15-cis-phytofluene	350–346	262, 337, 353, 372		89		1.7	
27	16.11	all-trans-phytofluene	352	337, 353, 372	331, 347, 367 ⁱ	97	91 ⁱ		
28	16.33	15-cis- ζ -carotene	396–392	292, 302, 383, 405, 429		92		1.3	
29	16.58	13-cis- β -carotene	451–447	346, 427, 450, 476	339, 422, 445, 470 ⁱ	10	5 ⁱ	2.4	2.6 ⁱ
30	17.12	all-trans- β -carotene	453	430, 456, 482	425, 450, 477	25	25		
31	17.43	all-trans- ζ -carotene	398	385, 407, 430	378, 401, 426 ⁱ		102 ⁱ		
32	17.75	9-cis- β -carotene or neo- β -carotene	451–447	347, 451, 477	340, 424, 446, 474 ⁱ	22	29 ⁱ	4.1	10 ⁱ
33	18.28	15,15'-di-cis-lycopen		345, 449, 471		22		3.7	
34	18.43	9'-cis- γ -carotene	463–459	340, 428, 452, 478		33		5.5	
35	18.59	9-cis- γ -carotene	463–459	338, 428, 450, 477		30		5.8	
36	18.79	13,15-di-cis-lycopen		340, 428, 452, 478		33		5.5	
37	19.45	15-cis-lycopen	474–470	351, 366, 449, 472, 500	362, 446, 470, 500 ^j		1.7	1.6 ^j	
38	19.60	all-trans- γ -carotene	465	442, 466, 497	437, 462, 494	53	40		
39	19.91	13-cis-lycopen	474–470	351, 366, 446, 469, 498	362, 440, 470, 508 ^j	18		2.7	1.8 ^j
40	20.77	9,13-di-cis-lycopen	466	351, 367, 442, 466, 495	344, 434, 464, 494 ^j			2.0	
41	22.21	9-cis-lycopen	474–470	367, 445, 470, 502	362, 446, 470, 500 ^k	67		4.6	4.2 ^k
42	23.06	5-cis-lycopen-epoxide	478–474	367, 448, 474, 507		44		2.6	
43	26.18	all-trans-lycopen	476	300, 450, 477, 508	452, 476, 506 ^j	75	76 ^j		
44	26.76	5-cis-lycopen	476	300, 367, 450, 477, 508	344, 446, 476, 506 ^j	71	76 ^j		20 ^j

^a λ_{\max} was calculated by using the Fieser rules as explained in the UV-Vis Identification Criteria paragraph. ^bIn this work, the solvents used for the carotenoid separation and, therefore, for the acquisition of the UV-vis spectra were methanol, 2-propanol, and hexane. ^cThe majority of the UV-vis data have been taken from ref 5; references different from ref 5 are given explicitly next to the corresponding datum. ^dSee ref 44. ^eSee ref 45. ^fSee ref 46. ^gReference 47 reports λ_{\max} but does not show the UV-vis spectrum. ^hFor polyenes with less than 4 conjugated double-bonds, the Fieser rules cannot be applied. ⁱSee ref 42. ^jSee ref 13. ^kSee ref 48.

Table 3. LC-MS and LC-MS/MS Data Used for the Identification of Carotenoids in 'Wild Tangerine' Tomato and Other Tomato Varieties

peak no.	LC-MS retention time ^a (min)	identified carotenoids	monoisotopic mass ^b (u)	LC-IDA method		LC-MRM method MRM transitions (m/z)
				EMS data (m/z)	EPI data ^d (m/z)	
1		9-cis-valenciaxanthin	412, 2977	c		413/221 ^e 413/181
2		cis-phytofluene diepoxide	576, 4906	[M + H] ⁺ = 577.5		577.5/503 ^e 557.5/429
3		deepoxyneoxanthin or trollein	584, 4229			585.4/567.5 ^e 567.5/549.5
4	8.84	9-cis-antheraxanthin	584, 4229	[MH - H ₂ O] ⁺ = 567.4	[MH-H ₂ O] ⁺ = 549.4; [MH - 56] ⁺ = 529; 221; 181	567.4/221 567.4/181
5	9.39	9-cis-mutatoxanthin	584, 4229	[M + H] ⁺ = 585.4	[MH-H ₂ O] ⁺ = 567.4; [MH-2H ₂ O] ⁺ = 549.4; [MH - 92] ⁺ = 493; 221; 181	585.4/221 585.4/181
6		zeaxanthin epoxide	584, 4229	[M + H] ⁺ = 585.4		585.4/221 ^e 585.4/135
7	10.05	9-cis-lutein epoxide or cis-taraxanthin	584, 4229	[MH - H ₂ O] ⁺ = 567.4	[MH-H ₂ O] ⁺ = 567.4; [MH - 92] ⁺ = 493; 221	567.4/221 567.4/175
8	10.54	all-trans-lutein	568, 4280	[MH - H ₂ O] ⁺ = 551.4	[MH-2H ₂ O] ⁺ = 533; 175; 135; 119	551.4/135 551.4/175
9	10.95	all-trans-zeaxanthin	568, 4280	[M + H] ⁺ = 569.4	[MH-H ₂ O] ⁺ = 551.4; 175; 135; 119	569.4/119 569.4/175
10	11.27	cis-β-cryptoxanthin 5,6-epoxide	568, 4280			569.4/181 ^e 569.5/221
11	11.43	9-cis-lutein	568, 4280			551.4/135 551.4/175
12	11.80	9'-cis-lutein	568, 4280			551.4/135 551.4/175
13	12.22	all-trans-ζ-carotene diepoxide	572, 4593	[M + H] ⁺ = 573.5	317, 243, 173, 135, 119	573.5/119 ^e 573.5/135
14	12.77	apo-10'-lycopenal	376, 2766	c		377/308 ^e 377/69
15	13.24	phytoene epoxide	560, 4957	[M + H] ⁺ = 561.5		553.5/135 ^e 553.5/69
16	13.30	cis-β-cryptoxanthin	552, 4331	[M + H] ⁺ = 553.4		553.5/119 553.5/175
17	13.81	all-trans-β-cryptoxanthin	552, 4331	[M + H] ⁺ = 553.4		553.5/119 553.5/175
18	13.69	phytofluene epoxide	558.4800	[M + H] ⁺ = 559.5		559.5/109 ^e 559.5/69
19	14.37	polycopene	536, 4382	[M + H] ⁺ = 537.4	321; 177; 137; 119	537.5/119 537.5/177
20	14.47	9,15,9'-tri-cis-ζ-carotene	540, 4695			541.5/173 ^e 541.5/69
21	14.66	di-cis-γ-carotene-epoxide	552, 4331	[M + H] ⁺ = 553.4		553.5/119 ^e
22	15.00	di-cis-γ-carotene-1,2-epoxide	552, 4331	[M + H] ⁺ = 553.4	[MH-H ₂ O] ⁺ = 535.5; 119	553.5/119
23	15.32	all-trans-β-carotene-5,6-epoxide	552, 4331	[M + H] ⁺ = 553.4	[MH-H ₂ O] ⁺ = 535.5; 135; 119	553.5/135 553.5/119
24	15.61	tri-cis-γ-carotene	536, 4382	[M + H] ⁺ = 537.4	321; 177; 137; 119	537.5/119 537.5/177
25	16.11	15-cis-phytoene	544, 5008	[M + H] ⁺ = 545.5	203; 177; 109	545.5/177 545.5/109
26	16.40	15-cis-phytofluene	542, 4852	[M + H] ⁺ = 543.5	461; 173; 137; 109	543.5/173 543.5/109
27	16.89	all-trans-phytofluene	542, 4852	[M + H] ⁺ = 543.5	461; 173; 137; 109	543.5/173 543.5/109
28	17.11	15-cis-ζ-carotene	540, 4695	[M + H] ⁺ = 541.5	173; 123.5	541.5/173 541.5/123.5

Table 3. continued

peak no.	LC-MS retention time ^a (min)	identified carotenoids	monoisotopic mass ^b (u)	LC-IDA method		LC-MRM method MRM transitions (m/z)
				EMS data (m/z)	EPI data ^d (m/z)	
29	17.35	13-cis-β-carotene	536, 4382	[M + H] ⁺ = 537.4	203; 177; 137; 121; 119	537.5/119 537.5/177
30	17.97	all-trans-β-carotene	536, 4382	[M + H] ⁺ = 537.4	321; 177; 137; 119	537.5/119 537.5/177
31	18.21	all-trans-ζ-carotene	540, 4695	[M + H] ⁺ = 541.5	173; 123.5	541.5/173 541.5/123.5
32	18.51	9-cis-β-carotene or neo-β-carotene	536, 4382	[M + H] ⁺ = 537.4	321; 177; 137; 119	537.5/119 537.5/177
33	19.1	15,15'-di-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4	321; 177; 137; 119	537.5/119 537.5/177
34	19.21	9'-cis-γ-carotene	536, 4382	[M + H] ⁺ = 537.4	209; 177; 157; 137; 119; 109	537.5/119 537.5/177
35	19.44	9-cis-γ-carotene	536, 4382	[M + H] ⁺ = 537.4	209; 177; 157; 137; 119; 109	537.5/119 537.5/177
36	19.57	13,15-di-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4		537.5/119 537.5/177
37	20.23	15-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4	468; 209; 171; 157; 119	537.5/119 537.5/177
38	20.36	all-trans-γ-carotene	536, 4382	[M + H] ⁺ = 537.4	209; 177; 157; 137; 119; 109	537.5/119 537.5/177
39	20.68	13-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4	468; 209; 171; 157; 119	537.5/119 537.5/177
40	21.53	9,13-di-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4		537.5/119 537.5/177
41	22.97	9-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4	468; 209; 171; 157; 119	537.5/119 537.5/177
42	23.80	5-cis-lycopene-epoxide	552, 4331	[M + H] ⁺ = 553.4		553.5/135 ^e 553.5/119
43	27.01	all-trans-lycopene	536, 4382	[M + H] ⁺ = 537.4	468; 209; 171; 157; 119	537.5/119 537.5/177
44	27.55	5-cis-lycopene	536, 4382	[M + H] ⁺ = 537.4	169; 145; 107	537.5/119 537.5/177

^aThe retention times on the LC-MS chromatogram appear with a delay of about 0.78 min compared to the corresponding LC-DAD chromatogram.

^bMonoisotopic masses have been calculated by Chem & Bio Draw 12.0. ^cThe mass range of the EMS experiment includes m/z values from 500 to 610. ^dOnly the most intense or characteristic fragment ions have been listed. ^eFor these compounds the MRM transitions are theoretical.

at room temperature with the minimal amount of KOH (established comparing the results obtained with 0.5, 1.0, and 2 mL of KOH). Compared to MSPD, the advantages of this procedure (see the detailed conditions in the corresponding paragraph) are several: (i) a very clean extract; (ii) slightly superior yields; (iii) the achievement of a greater enrichment factor; (iv) simplification of the carotenoid analysis by hydrolyzing esters of carotenols with fatty acids.

MSPD is a valuable technique for a mild and rapid isolation of carotenoids from foods (especially for solid samples). Its extraction efficiency without the formation of artifacts during the sample preparation process has been already verified previously.^{20,40}

The comparison of the LC-DAD-MS chromatograms, acquired after applying the two different extraction protocols, is an easy and quick way to deduce the occurrence of esterified carotenols in real matrices. When this comparison was performed for the tomato samples, the matching between the LC-DAD-MS chromatograms has proved the absence of bound carotenols as well as of artifacts during the saponification.

Characterization of the Carotenoid Fraction of Some Italian Varieties of Tomatoes. The developed method was applied to the analysis of the previously mentioned tomato varieties (see Tomato and Passata Samples paragraph). Like a representative example, Figure 2 depicts the LC-DAD chromatogram of a sample of 'Wild Tangerine' tomato, after cold saponification. Figure 3 shows the UV-vis spectra extracted from each chromatographic peak of Figure 2, while Tables 2 and 3 summarize the LC-DAD and LC-MS data used for the identification of carotenoids in the 'Tangerine' tomato as well as in the other tomato varieties.

Most of the assignments comply with the biosynthetic sequence of carotenoids in plants and confirm the supposition that the biosynthetic pathway involves *cis* configurations of the precursor (15-cis-phytoene) and the intermediates (*cis* isomers of ζ-carotene and lycopene).^{41,42} For almost all the analyzed varieties, we have observed the presence of phytoene only in the 15-cis configuration and of phytofluene in the 15-cis and *all-trans* forms.

9,15,9'-Tri-cis-ζ-carotene is the intermediate that follows 15-cis-phytoene in the biosynthetic pathway.⁴² In this work, we have

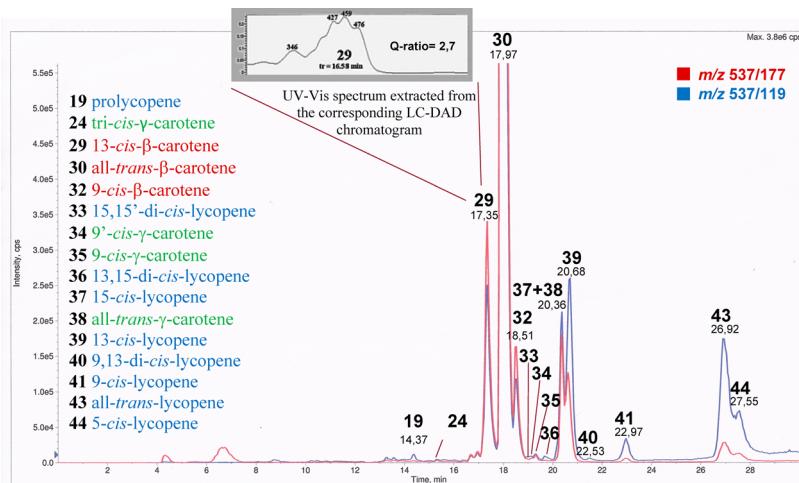


Figure 4. LC-MRM profiles of a saponified extract from 'Wild Tangerine' tomato: the ion currents m/z 537/177 and m/z 537/119 are shared by *all-trans*-lycopene, *all-trans*- β -carotene, *all-trans*- γ -carotene, and their corresponding *cis* isomers; each family of geometrical isomers can easily be distinguished on the basis of the characteristic ion ratio (see Table 1 and Figure 1).

identified 9,15,9'-tri-*cis*- ζ -carotene along with 15-*cis*- ζ -carotene and *all-trans*- ζ carotene in all tomato varieties. The *all-trans* isomer was prevalent only in the passata samples, probably because of the isomerization of 15-*cis*- ζ -carotene to the more stable *all-trans* isomer during the tomato processing. Neurosporene, another intermediate in the synthesis of lycopene,⁴² was never detected in any of the analyzed varieties.

Polycopene is known as the final product of the desaturation reactions that a specific isomerase converts into *all-trans*-lycopene;⁴¹ subsequent cyclizations of *all-trans*-lycopene create a series of carotenes that have one or two rings of β -type and/or of ϵ -type. Unlike their precursors, lycopene, γ -carotene and β -carotene occur as *all-trans*-carotenes in wild-type tomato fruits; nevertheless, we have also observed their geometrical isomers and epoxidic forms in the 'Wild Tangerine' variety and passata (Tables 2 and 3). Figure 4 depicts the MRM profiles shared by lycopene, β -carotene, and γ -carotene, for a saponified sample of 'Wild Tangerine' tomato. These ion currents, together with the UV-vis data (Table 2; Figure 3), were used to identify 16 carotenes including three geometrical isomers of β -carotene, nine geometrical isomers of lycopene, and four geometrical isomers of γ -carotene. As an example, the peak 29 has been ascribed to 13-*cis*- β -carotene for the following reasons:

- the ion ratio value (ion ratio = $(I_{(m/z\ 537/177)} / I_{(m/z\ 537/119)}) = 1.5$) indicates that it is one of the geometrical isomers of β -carotene (Table 1; Figure 2)
- the retention time suggests that it is a next-to-central *cis*-isomer, because it elutes before the *all-trans* form
- the Q-ratio, calculated as $(II/B) = 2.4$, is in agreement with the data of the literature (Table 2); the hypsochromic shift of λ_{II} of 6 nm (from 456 nm for *all-trans*- β -carotene to 450 nm for the unknown) and the decrease in the intensity of peak III reinforce the attribution

Similarly, the peaks 37 and 39 have been attributed to 13-*cis* and 15-*cis* isomers of lycopene. The peaks 43 and 44 have been assigned to *all-trans*-lycopene and 5-*cis*-lycopene, respectively; the peak assignment has been based on the retention time of the authentic standard of *all-trans*-lycopene in solvent and on the occurrence of *cis*-peak at 367 nm detected only for the unknown 44. It is interesting to highlight that 5-*cis*-lycopene (peak 44), 13-*cis*- β -carotene (peak 29), and 9-*cis*- β -carotene (peak 32) were

detected only in the extracts of 'Wild Tangerine' tomato and passata of 'San Marzano' tomatoes (probably due to the isomerization of *all-trans* forms during tomato processing). In the other tomato varieties, *all-trans*-lycopene and *all-trans*- β -carotene did not accompany the above-mentioned geometrical isomers.

The presence of γ -carotene in tomato and tomato products has previously been reported by Tonucci et al.¹⁸ and Kimura et al.,¹⁹ based on the online acquisition of UV-visible spectra without peak verification using more specific techniques. In this work, the occurrence of *all-trans*- γ -carotene (peak 38 of Figure 4) has been confirmed by using its authentic standard (Table 1; Figure 1); the key element of the identification has been the matching of the retention time, the perfect overlapping of the UV-vis spectra (λ_I , λ_{II} , λ_{III} , and % III/II), and the MS data. Even if *all-trans*- γ -carotene coelutes with 15-*cis*-lycopene (peak 37 + 38), their UV-vis spectra are perfectly distinguishable (see spectra 37 and 38 of Figure 3); moreover, the ion ratio close to 1 (compare Figure 1 and Figure 4) indicates that *all-trans*- γ -carotene is more abundant than 15-*cis*-lycopene.

Apo-lycopenals are hypothesized to be lycopene metabolites in mammals; nevertheless, they have recently been found in raw tomato and processed tomato products,⁴³ suggesting that these compounds may also be absorbed from food. In this work, the occurrence of apo-10'-lycopenal (peak 14) has been supposed on the basis of the UV-vis spectrum (Figure 2), the correspondence with the λ_{max} calculated according to the Fieser rule modified for aldehydes (eq 2; Table 2), and the LC-MRM profile obtained by acquiring the m/z 377/308 ion current (Table 3). For this compound, the two theoretical MRM transitions, listed in Table 3, have been deduced by hypothesizing an allylic cleavage of the C4-C5 bond.

Finally, among the xanthophylls identified in the 'Wild Tangerine' tomato, relevant for abundance were *all-trans*-lutein and 9-*cis*-antheraxanthin, followed by *cis*-lutein epoxide and *all-trans*-zeaxanthin. A series of *cis* isomers of low intensity were observed in particular for lutein, together with epoxides of zeaxanthin and β -cryptoxanthin (Tables 2 and 3). Traces of valencianaxanthin, a norcarotenoid occurring in some orange varieties, were found in the 'Pachino' tomato along with antheraxanthin and mutatoxanthin, all of them in the 9-*cis*-configuration. Usually, epoxidic forms are difficult to identify

using only the UV-vis detection (**UV-Vis Identification Criteria** paragraph, [eq 3](#)); LC-MS supports their LC-DAD identification since fragments at *m/z* 352, 221, and 181 indicate the presence of an epoxy group in a ring with a hydroxyl,³⁰ while fragment ions at *m/z* 165 and *m/z* 205 are characteristic of an epoxy group as the only substituent in the β -ring.³⁰ In passata and raw tomatoes, we also have identified epoxides of some carotenoids ([Tables 2](#) and [3](#)).

In conclusion, the approach described in this paper has allowed us to realize a large-scale screening of carotenoids with the support of few authentic standards by exploiting the versatility of the LC-DAD-QqQ_{LIT} hyphenation. The identification power of this hyphenated technique is quite strong when all data (retention time; λ_{I} , λ_{II} , λ_{III} ; % III/II; Q-ratio; *m/z* of pseudomolecular ion; fragmentation spectrum; relative abundance between the MRM transitions) are available, otherwise the assignment has to be considered tentative. Our methodology has been proved to be time-saving, because it can collect a large number of data with few analytical steps, and money-saving, because it uses a small number of standards commercially available at a reasonable cost. The 'Wild Tangerine' tomato has shown a number of carotenoids significantly larger than those of other analyzed varieties (up to forty-four compounds). In passata from 'San Marzano' fruits, some *cis*-forms of β -carotene and lycopene have been found particularly abundant since they were probably formed during the manufacturing process (isomerization due to the heat treatment and/or the occurrence of the organic acids freed during the squeezing step). The presence of *cis*-isomers in food has recently been revalued due to the superior bioavailability of these carotenoid forms.^{9–11}

This study has not examined the effect of geographic area, the season, the ripening grade, and other variability parameters which can potentially affect the concentrations of these micronutrients. In the future, such an analytical approach could be extended to the comprehensive characterization of antioxidants in the genetically engineered varieties of tomato, enriched with anthocyanins to enhance the anticancer power of these fruits that, in the wild types, is only due to the carotenoids.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jafc.5b02910](https://doi.org/10.1021/acs.jafc.5b02910).

Characteristics of the tomatoes, list of identified carotenoids, LC-MS distinction of lutein and zeaxanthin, structures of selected carotenoids, and EPI spectra ([PDF](#))

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

QqQ_{LIT}, hybrid triple quadrupole-linear ion trap mass spectrometer; DAD, diode array detection; APCI-MS, atmospheric pressure chemical ionization-mass spectrometry; IDA, information dependent acquisition; EMS, enhanced-mass scan; ER, enhanced-resolution; EPI, enhanced product ion; MRM, multiple reaction monitoring; LC, liquid chromatography; λ_{max} wavelength of maximum absorption; PIS, product ion scan spectra; BHT, butylated hydroxytoluene; MSPD, matrix solid

phase dispersion; NARP, nonaqueous reversed-phase; CE, collision energy

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