ISSN 1061-9348, Journal of Analytical Chemistry, 2021, Vol. 76, No. 2, pp. 196–203. © Pleiades Publishing, Ltd., 2021.
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ARTICLES

Determination of Carotenoids of Tomato Fruits of Different Colors

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Received May 19, 2020; revised June 1, 2020; accepted July 2, 2020

Abstract—A method for the determination of the composition of carotenoids of tomato fruits of different colors is proposed using a combination of spectrophotometry and chromatography. An explanation of the elution order of mono-*cis*-isomers of lycopene under the conditions of reversed-phase chromatography on traditional "monomeric" reversed C18-phases is proposed for the first time. It was found that, for prolycopene, (7Z, 9Z, 7'Z, 9'Z)-lycopene, which is responsible for the orange color of fruits, there is an almost imperceptible transition in normal examination in the vibronic structure of the electronic absorption spectrum with the lowest energy, $\lambda_{max}(1) = 486.2$ nm. The main carotenoids of tomato fruits of different colors were determined: *trans*-lycopene and its *cis*-isomers for fruits of red and pink colors, protolycopene and other carotenes preceding its biosynthesis for fruits of orange color, and the carotenoid composition of yellow tomatoes significantly differing from that of tomatoes of first two colors by a considerable accumulation of lutein. For the quantitative assessment of the concentration of carotenoids with different chromophores, a calculation system ensuring the determination of the contribution of each of the components of complex mixtures was proposed.

Keywords: tomato carotenoids, prolycopene, electronic absorption spectra, reversed-phase HPLC, spectrophotometry, internal normalization

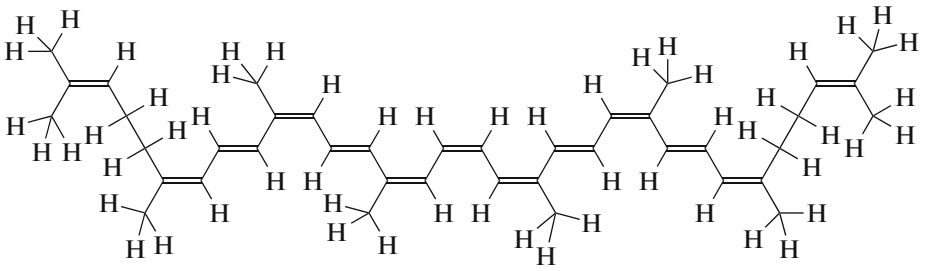
DOI: 10.1134/S1061934820120060

Recently, there has been noticeable interest in natural carotenoids that do not possess provitamin A activity. It has been found that xanthophylls lutein and zeaxanthin are responsible for the prevention of age-related macular degeneration [1, 2]. Astaxanthin has proven to be an effective agent in dermatology [3, 4]. Lycopene in a number of studies has shown activity in the fight against cancer and in the prevention of cardiovascular diseases [5, 6].

Like all carotenoids not synthesized in the human body, lycopene was found in edible plants used in food. The most important plant sources of all-*trans*-lycopene for a typical diet are tomato fruits of the usual red color and products of their processing (tomato paste, tomato juice, etc.) [7]. However, lycopene, like a number of other carotenoids, belongs to compounds with low bioavailability. Moreover, in some studies [8, 9], the concentration of *cis*-isomers in blood serum was found to be higher than that of all-*trans*-lycopene. Lycopene is better absorbed after heat treatment, which results in the appearance of *cis*-isomers [8]. In this respect, the tetra-*cis* isomer of lycopene – (7Z, 9Z, 7'Z, 9'Z)-lycopene or prolycopene – isomer with high bioavailability is of particular interest. In this regard, recommendations [9] for the consumption of preferably tomato varieties enriched with *cis*-lycopene isomers are not surprising.

Initially, a structure containing seven *cis*-double bonds (5Z, 9Z, 13Z, 15Z, 13'Z, 9'Z, 5'Z) was proposed for prolycopene (Scheme 1) [10]. When constructing it, the sterically unfavorable configuration was excluded because of the large overlap of spheres with van der Waals radii of hydrogen atoms of the methine and methyl groups (Scheme 1, variant (b)), i.e. actually *cis*-configuration at bonds 7, 11, 11', and 7'. However, studies by NMR spectrometry performed later [11] made it possible to clarify the structure of prolycopene, in which two parts of the molecule still have stains shown in Scheme 1 (variant (b)).

Previously, thin layer chromatography was widely used to separate and determine prolycopene and other lycopene isomers [10, 12]. Currently, reversed-phase HPLC is more often used for the separation of carotenoids [13], and special "polymer" reversed phases have been developed for the more efficient separation of *cis*- and *trans*-isomers. On the traditional C18 stationary phase [14], prolycopene is eluted immediately after the all-*trans*- and conventional *cis*-isomers of lycopene. When carotenoids are separated on the C30 "polymer" reversed phase with elution in a gradient mode from methanol to *tert*-butyl methyl ether [15], the retention of the identified substances increases in the following order (retention time is given in parentheses, min): lutein (14.3) < β-carotene (20.3) < prolycopene (20.6) < δ-carotene (22.3) < γ-carotene (23.5) < *cis*-lycopene (25.1) < *trans*-lycopene (26.4).



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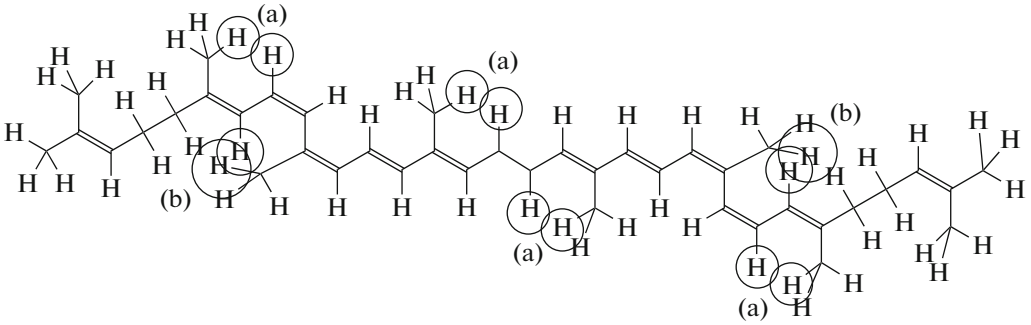

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Scheme 1. The structure of prolycopene according to the data of [10] (I) and [11] (II).

For the qualitative and quantitative determination of prolycopene (as for many carotenoids), the parameters of electronic absorption spectra were used. Thus, in [9], it was noted that the absorption maximum of prolycopene (438 nm) was significantly hypsochromically shifted relative to the absorption maximum of all-*trans*-lycopene (470 nm); at that the molar absorption coefficients were also different: 102900 and 184000 m^2/mol, respectively. However, in [16], to which the authors of [9] referred, somewhat different spectral characteristics were given for prolycopene: 461 (shoulder, 70000), 437 (105000), and 417 (shoulder, 90000), where the molar absorption coefficient was indicated in parentheses with the dimensionality L/(mol cm); the shoulder is the bend of the spectral line. However, the molar absorption coefficient of 102900 was also used in [17]. Finally, in a number of studies, in determining the carotenoid composition of complex mixtures containing lycopene and *cis*-isomers, including prolycopene, peak areas in the chromatogram are used without corrections for molar absorption coefficients [8, 18].

The aim of this work was to develop a method for the determination of prolycopene in orange tomatoes using reversed-phase HPLC on traditional C18 phases.

EXPERIMENTAL

Reagents and equipment. Acetone for UV-IR-HPLC-GPC, acetonitrile for UV-IR-HPLC-GPC (Panreac, Germany), and *n*-hexane for HPLC (Komponent-Reaktiv, Russia) were used.

Electronic absorption spectra were recorded on a Shimadzu UV 2550 spectrophotometer in quartz cells (l = 1 cm). The total carotenoid concentration in terms of the main component of the mixture was determined by the equation:

$$c(i) = \frac{A(i)V(i)k(i)M(i)\times 100}{\varepsilon_\lambda(i)m(i)}, \text{ mg/g}, \qquad (1)$$

where $A(i)$ is the absorbance of the extract at a given wavelength, λ, nm; $V(i)$ is the volume of the extract; $k(i)$ is the ratio of the dilution of the initial extract before measurements, $M(i)$ is the molar mass of the main component, g/mol; $\varepsilon_\lambda(i)$ is molar absorption coefficient of the component at a given wavelength; $m(i)$ is the weight of the freeze-dried sample, g; and 1000 is conversion factor of the results in mg/g. The wavelengths, molar absorption coefficient, and molar masses are given in Table 1.

An Agilent 1260 Infinity chromatographic system with a diode array detector was used to control the species composition and determine the areas of carotenoid peaks. Chromatographic column: 150 × 4.6 mm, Reprosil-Pur C18-AQ, 3.5 μm with a 10 × 4.6 mm Kromasil 100-5C18 guard column was used at

Table 1. Individual carotenoids of tomato fruits of different colors

No.*	Name	λ, nm [17]	ε, L/(mol cm) [17]	t_R, min	Accumulation level (μg/g) for tomatoes of different colors			
					red	pink	orange	yellow
1	Lutein	445	145100	1.83	0.5	2.2	0.8	1.7
2	*trans*-Lycopene (with *cis*-isomers)	472	185200	5.19	50.2	43.3	4.0	0.6
3	Prolycopene	434	102900	6.02	<0.1	<0.1	38.3	<0.1
4	β-Carotene (with *cis*-isomers)	448	139200	8.83	3.6	6.4	2.8	2.5
5	Neurosporene	435	134500	6.33	<0.1	<0.1	5.0	<0.1
6	Proneurosporene	430	83900	6.80	<0.1	<0.1	15.1	<0.1
7	ξ-Carotene (with *cis*-isomers)	399	135200	7.80	<0.1	<0.1	19.0	<0.1

* Numbering as in Fig. 3.

a column thermostat temperature of 30°C. The mobile phase of the composition of 30 vol % of acetonitrile in acetone was passed in at a rate of 0.8 mL/min in an isocratic mode. Chromatograms were recorded at a detector wavelength adjusted to the determination of specific major carotenoids, see Table 1. Chromatograms were recorded, stored, and processed using the Agilent ChemStation software.

Sample preparation. Thin peels were removed from tomatoes of different colors purchased on the market, and the mesocarps were homogenized, frozen in a freezer (−20°C), followed by lyophilization in a FreeZone 6L Labconco freeze dryer, and the product was ground into powder in a porcelain tube. The powder was stored in a refrigerator.

The extract was prepared by grinding a weighed portion (m) of a powder moistened with a small amount of water under a layer of *n*-hexane. Portions of extracts were taken from the mortar and transferred to a funnel with a filter paper. A new portion of the extractant was added to the solid residue and extraction was repeated. Extraction was repeated until an almost colorless portion of the extract was obtained. All portions of the extract were combined and the solvent was removed on a vacuum rotary evaporator. The residue was dissolved in a known volume (V) of the mobile phase for the HPLC determination of carotenoids. The resulting solution was filtered through a packed filter into vials, transferring them to the cells of the autosampler of the chromatograph.

Calculation methods. To process peak areas by the method of internal normalization, two methods were used.

Method 1 was used only to assess the contribution of the main component to the total absorbance at the selected detection wavelength:

$$\alpha^*(i) = \frac{S(i)}{\sum_j S(j)}, \quad (2)$$

where $\alpha^*(i)$ was the proportion of component i (lycopene or prolycopene) in the sum of carotenoids, to which the total concentration found from spectrophotometric analysis data was recalculated (Eq. (1)); $S(i)$ was the peak area of the main component; $\Sigma S(j)$ was the sum of all peak areas peaks in the chromatogram. At that, the concentration of component i ($c^*(i)$) corrected for an individual component was determined by the equation:

$$c^*(i) = \alpha^*(i)c(i). \quad (3)$$

Method 2 was used to determine the fractions of components in the sum of carotenoids of the analyzed mixture. For this, the electronic absorption spectrum of each substance (recorded in the detector cell) was exported to Excel. Then the spectra were used to find $A(\lambda_{max}(i))$, the absorbance of the peak in the i-th absorption maximum characteristic for the substance, and $A(\lambda_{det}(i))$, the absorbance of the same carotenoid at the wavelength of chromatogram recording and using the values of molar absorption coefficients $\varepsilon(\lambda_{max}(i))$ given in the literature:

$$\alpha(i) = \frac{S(i)/\varepsilon(\lambda_{max}(i))\, A(\lambda_{max}(i))/A(\lambda_{det}(i))}{\sum_j S(j)/\varepsilon(\lambda_{max}(j))\, A(\lambda_{max}(j))/A(\lambda_{det}(j))}, \quad (4)$$

where $\alpha(i)$ were corrected fractions of carotenoid species in the mixture; $S(i)$ were peak areas of the corresponding components in the chromatogram; and Σ

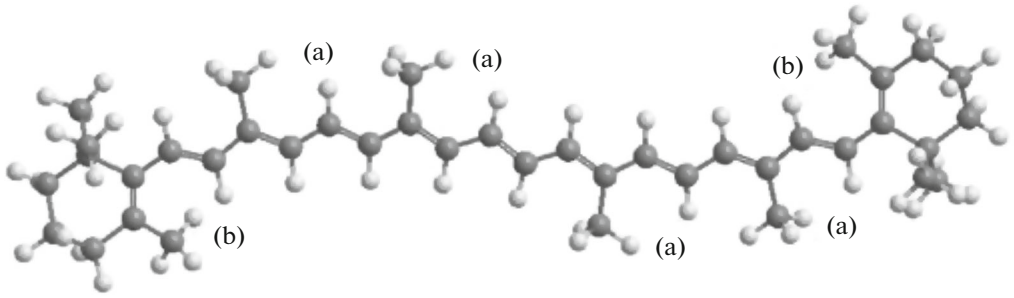

Fig. 1. Structure of β-carotene determined by steric stains of types (a) and (b).

was the sum of such values for all components, were calculated at the characteristic wavelength for each component. At that, the concentrations of all other carotenoids (except for the concentration of the main component determined earlier by Eq. (3)) ($c^*(j)$) were calculated by the equation:

$$c^*(j) = c^*(i)\frac{\alpha(j)}{a(i)}. \quad (5)$$

The geometry of β-carotene was optimized by the MM2 method in the Chem3D program of the ChemOffice2016 software package (PerkinElmer, GB).

RESULTS AND DISCUSSION

Study of electronic absorption spectra. Type (a) strains in Scheme 1 are sufficient for the distortion of the linear shape of the polyene chain of isoprene fragments to the S-shape, which can be demonstrated by the structure, the energy of which is minimized in the MM2 program (Fig. 1). The planar central part of the molecule (between the ionone rings) is distorted in the plane to an S-shape because of steric strains of type (a). Such curved structure in this part is retained for all carotenoids. However, two steric stains of type (b) remove the double bonds of the rings from complete conjugation with the polyene system of the middle part. This results in the fact that, for β-carotene, in comparison with lycopene, at the equal conjugation chain length (11 C=C bonds), the maxima of absorption bands are hypsochromically shifted by 27 nm: the wavelengths for the first (in energy) electronic transitions are 478 and 505 nm for β-carotene and lycopene, respectively [19], i.e. differ by 27 nm. For 7,7'-di-*cis*-lycopene, the hypsochromic shift turns out to be relatively small, only about 9 nm, while for 9,9'-di-*cis*-lycopene it is somewhat larger, 12 nm [16]. In this case, the decrease in the wavelength by 41 nm for the same (first) transition for prolycopene (7,9,7', 9'-tetra-*cis*-lycopene) is difficult to explain.

It can be supposed that there is one more slight inflection in the spectrum of prolycopene. To confirm the assumption about the existence of one more (namely the first) electronic transition, the total spectrum was decomposed into individual bands. Decomposition was performed using Gaussian functions in spectra with changed coordinates: instead of the wavelength scale (nm), the energy scale (eV) was used. At that, for all-*trans*-lycopene, the first five transition bands were found at the wavelengths 503.8, 472.9, 445.5, 421.1, and 399.2 nm. Equal energy differences of 0.161 eV were found between the bands, which is characteristic of transitions to different vibrational states of the excited electronic state (Fig. 2a). For prolycopene, the wavelengths of the first five transition bands, 485.2, 464.7, 441.5, 420.6, and 401.5 nm (at equal energy differences of 0.140 eV, Fig. 2) corresponded to a transition that was almost impossible to detect in the spectrum. The difference between the wavelengths of the first transitions for all-*trans*-lycopene and prolycopene was 18.6 nm, which no longer raises questions.

Separation and quantitative determination of carotenoids in multicolored tomatoes. Extracts of red, pink, orange, and yellow tomatoes were studied by reversed-phase HPLC on the traditional "monomeric" C18 phase (Fig. 3). Carotenoids were identified by their electronic absorption spectra taking into account changes in chromatographic mobility. At that, it was found that lycopene was retained significantly more strongly than lutein, which corresponded to a decrease in the lipophilicity of analytes with the addition of hydroxyl groups. Prolycopene was retained more strongly than lycopene. Special attention should be paid to this fact. Thus, according to the published data for *trans*-β-carotene and *trans*-lycopene and their mono-*cis*-isomers, the retention times on "monomeric" reversed C18 phases increased in the order [20]: t_R(*trans*-lycopene) < t_R(9-*cis* -lycopene) < t_R(13-*cis*-lycopene) < t_R(15-*cis*-lycopene). To explain this order, it should be taken into account that the retention of *trans*-lycopene according to our data decreased when the C18 phase was replaced by a phase with a shorter length of attached alkyl groups (C8 and C4), which indicated the introduction of molecules into the attached phase. According to the previously proposed structure of the attached phase [21], alkyl groups of

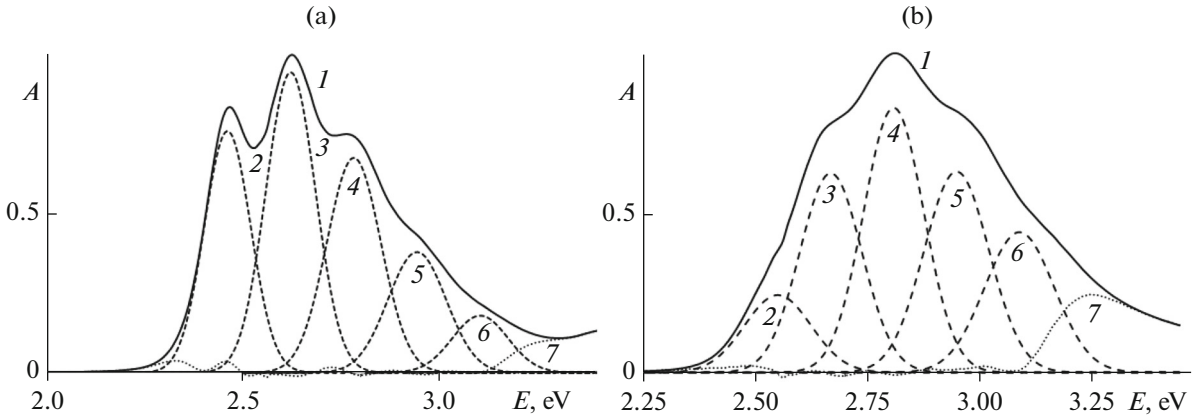

Fig. 2. Decomposition of vibronic absorption spectra of (a) lycopene and (b) prolycopene into components.

the analyte of normal structure can be freely located between the alkyl groups of the attached phase. However, molecules longer than the thickness of the attached phase should be sorbed either horizontally or at an angle to the substrate surface, and the length of the lycopene molecule is about twice the thickness of the attached C18 layer. In this case, the following is obvious. First, the longer the inserted (horizontally relative to the attached phase) molecule, the less likely is that the voids between the attached radicals are arranged linearly. The immersion of a molecule inside such a phase is associated with a change in the conformation of the attached radicals with the formation of such voids; in this case, the energy gain due to sorption decreases, and the retention time should also decrease. Second, the steric stresses arising in this process inside the attached layer increase with a decrease in the distance between the inserted molecule to the site of attachment of the radical to the silica substrate. For 15-*cis*-lycopene, the bending of the molecule will favor sorption due to the removal of the ends of the molecule from the silica substrate (Fig. 4), which explains the increase in the sorption of the analyte. In going from 15-*cis*-lycopene to other isomers, the

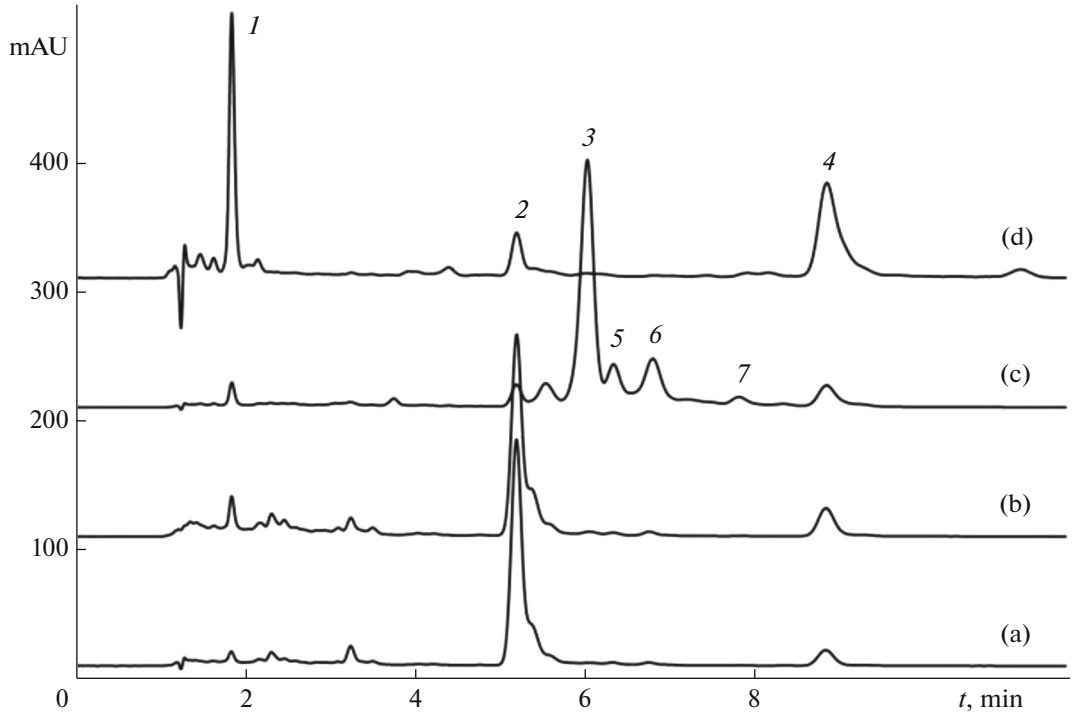

Fig. 3. Separation of carotenoids of tomato fruits of (a) red, (b) pink, (c) orange, and (d) yellow colors. Carotenoids: (*1*) lutein, (*2*) all-*trans*-lycopene, (*3*) prolycopene, (*4*) β-carotene, (*5*) neurosporene, (*6*) proneurosporene, and (*7*) ξ-carotene.

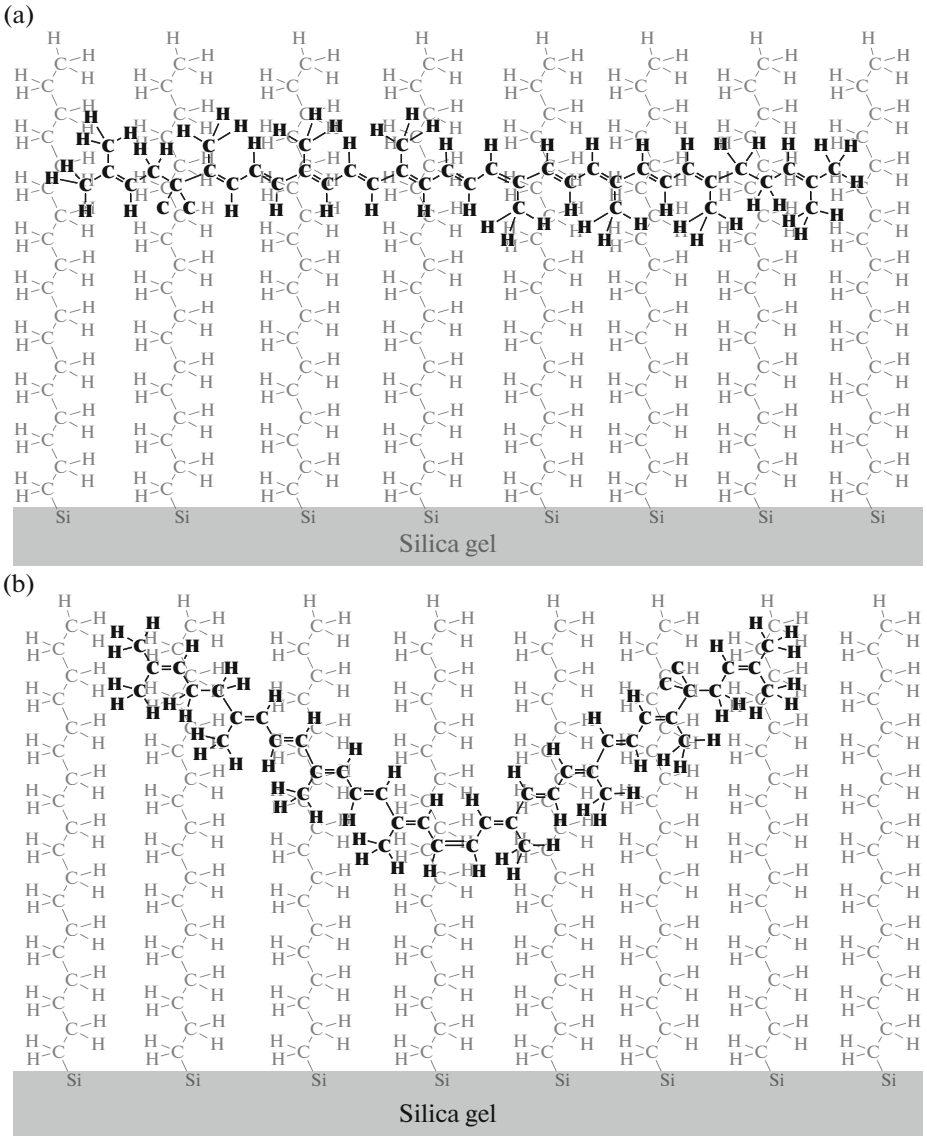

Fig. 4. Simplified scheme of sorption of (a) call-*trans*-lycopene and (b) 15-*cis*-lycopene on "monomeric" reversed C18 phase.

length of one of parts of the molecule (with all *trans*-bonds) increased, which corresponded to a relatively lower retention.

The increase in retention times in the series lycopene – neurosporene – ξ-carotene (Table 1), as well as the further transition to phytofluene, was a consequence of a sequential increase in the lipophilicity of molecules upon the replacement of the C=C bond by two methylene groups.

As follows from the presented chromatograms, red tomatoes accumulate all-*trans*-lycopene with *cis*-isomers (about 90%) and β-carotene (slightly more than 6%) as the main carotenes. In a pink tomato, the same carotenes were found in a slightly different ratio. In a yellow tomato, β-carotene accounted for about half of all carotenoids, and about one third of all carotenoids was xanthophyll—lutein.

The most complex was the carotenoid composition of orange tomatoes: with small amounts of lutein, *trans*-lycopene, common simple *cis*-isomers and β-carotene, the main peak in the chromatogram was represented by prolycopene (45% of the total carotenoids), which was accompanied by a group of carotenes preceding prolycopene with biosynthetic chains [14]. The fact that the small peak of ξ-carotene in the chromatogram in Fig. 3c corresponds to a significant amount (22.4%) among all carotenoids was due to the peculiarity of detection at 440 nm – in the region with the low absorbance of substances with strongly hypsochromically shifted bands. In the 3D chromatogram (Fig. 5), the peak of phytofluene (*8*), which was difficult to separate from β-carotene, was noticeable and

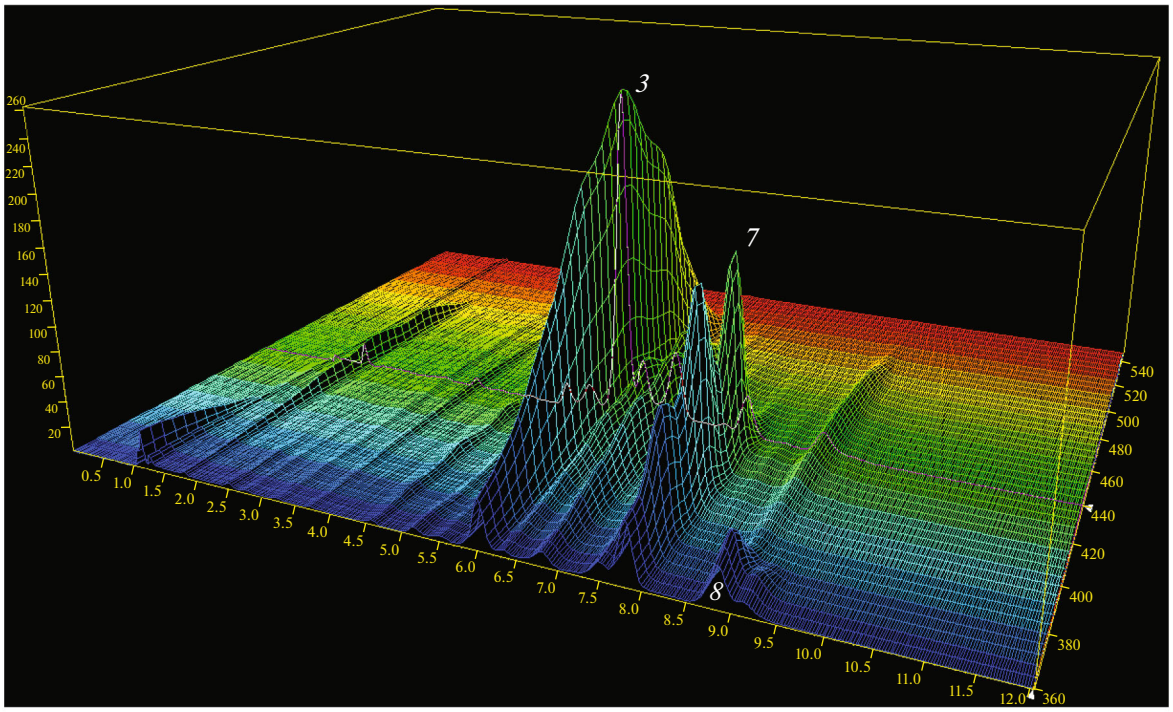

Fig. 5. 3D chromatogram of carotenoids of orange-colored tomato fruits.

could not be detected at the usual wavelengths in recording chromatograms of carotenoids. Thus, the use of peak areas without correction factors can result in very large errors in determining the fractions of carotenoids in a complex mixture; therefore, the proposed calculation method according to Eq. (3) for the control of the composition of carotenoids should be considered mandatory.

To determine the level of accumulation of carotenoids in complex mixtures, an approach can be proposed that combines spectrophotometric and chromatographic methods. According to the proposed method, an electronic spectrum of a mixture of carotenoids should be recorded and absorbance should be determined at the wavelength recommended for the determination of the main component – usually this is the second (in terms of energy) maximum in the spectra of individual carotenoids, e.g., 472 nm for lycopene. Then a chromatogram with detection at the same wavelength should be recorded and calculations as indicated in the Experimental section should be performed.

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Translated by V. Kudrinskaya