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USE OF NANOSTRUCTURED SILVER SUBSTRATES (COATINGS) TO STUDY THE CONTENT AND CONFORMATION OF β-CAROTENE

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Abstract

Interest in the industrial production of plant and microalgal biomass for biofuels and bioproducts has stimulated studies on microalgal physiology and mechanisms of valuable biomolecules synthesis and accumulation in algal cells. One of the most investigated biomolecules for commercial application are neutral lipids and carotenoids. However, until now the mechanism of the influence of different ambient factors, including application of inductors, on biosynthesis and accumulation of these molecules is not well understood. Partially, the lack of such knowledge is due to restricted technique of investigation. Raman Spectroscopy is one of the advanced methods of cell physiology investigation, which can fill some gaps in our understanding of such processes. The current techniques used in the papers presented show the advantages and other essential specifics of the method applied to plants (most importantly, microalgae) and other species/objects. It was found that the use of a substrate allows to enhance the Raman signal when diluting the sample by 10^5–10^6 times, at a substance concentration of 10^{-9} mol/l. In SERS conditions (i.e., mixing silver colloids to blood sample) hem markers show up, while in normal Raman conditions resonance enhancement allows direct observation

Keywords

Raman spectroscopy, surface-enhanced Raman spectroscopy, blood, carotenoids

of β-carotene features. This highlights the versatility of Raman technique and the prospects for biology and clinical chemistry

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Introduction. Over the recent decades we could see the utilization of Raman spectroscopy in many investigations of animal cells [1–3]. The method is very popular among biophysicists and life sciences researchers. Raman spectroscopy allows investigation of living cells without any damage [4, 5]. Nowadays we can see the increase of utilization of Raman spectroscopy in plants, and especially in algae investigations. Raman spectroscopy is a well-known approach that is being used in lots of biomedical studies. Since we are talking about biomolecules in low concentrations — the main obstacle that is connected to the utilization of such method in life sciences research is the low signal of Raman scattering. There are a lot of modifications of the Raman spectroscopy which actually allows to enhance the Raman scattering. Algae is the object, which is widely used in biotechnology [6–8]. It is the feedstock for biofuel [7], animal feed, pharmaceutical and cosmetic industries because they can synthesize a broad variety of nutraceuticals and biologically active compounds production. In addition to all the biotechnology methods existing for analysis of the quality and quantity of commercially used molecules in algae, Raman spectroscopy can be utilized for deeper and extensive study of the molecules of interest in blood and algae.

Due to the advantages (non-invasive, fast and highly sensitive) of the Raman spectroscopy and its modifications there is a high demand in its use in the biomedical research: investigation of the structure and conformation of the molecules of interest, investigation of the mechanisms of the drugs action and so on [9, 10]. Raman Spectroscopy and its modifications are widely used in cell research. With the use of SERS the variety of bacterial [11] and animal [1, 2] cells are being investigated. It has been shown that the information about membrane lipids can be obtained, first of all — the conformation of membrane lipids and the molecular surroundings [1].

It has been investigated that bioprospecting and mutagenesis are two important strategies in algae-based biofuel development [6]. Considering these two strategies there is a need for optimization of bio fuel production. Essential to any algal lipid bioproduction endeavor is the ability to rapidly characterize

accumulating algal lipids. Confocal Raman and RS microscopy can fulfill this role and also it is possible to locate lipid-rich regions within microalgae cells with a high spatial resolution [6]. It has been investigated that the chemical composition of lipid droplets can be obtained by the utilization of Raman spectroscopy [6]. It is stated that the combination of CARS (Coherent) and Raman microspectroscopy would allow precise determination of the harvesting times for algae [4]. This is supposed to be one of the valuable interests in modern biotechnology. It has been revealed that another modification of Raman spectroscopy — Single-cell Raman spectroscopy (SCRS) is applicable for gathering information of the cell lipid content and the lipid unsaturation degree [12].

It has been shown that Raman spectroscopy is a promising tool in biology and biochemical investigations [1, 2, 13]. The presence of molecules near the surface of metal nanoparticles, where strong electromagnetic fields are present under laser excitation, allows this strong enhancement of the Raman signal. The use of SERS can significantly extend the range of applicability of Raman techniques towards analytes that occur in biological samples at low concentrations. Carotenoids play an obvious role as pigments that give many natural objects their pleasing colors. b-Carotene is one of the widespread carotenoids which exhibits a manifold of biological functions. The key structural element of carotenoids is the β-conjugated polyene backbone. It is the delocalization of β-electrons that gives carotenoids their characteristic electronic spectra and is largely responsible for the photophysical and photochemical properties of these molecules, including cis–trans photoisomerization (a crucial step in the molecular mechanism of vision [14, 15]). The use of Raman spectroscopy to study the structure and conformational changes of carotene makes it possible to directly characterize the vibrations of individual bonds of a molecule and to reveal changes in the conformation of the molecule in pathology. However, the sensitivity of the Raman method is often insufficient for detecting changes in the structure of carotene. At the same time, giant Raman spectroscopy on nanostructured substrates (coatings) makes it possible to study the conformation of biomolecules with high sensitivity even in the case of transmembrane diagnostics of living cells [16]. In this study, for the first time, plasmonic nanostructures based on silver of ring morphology [1, 2] were used to enhance the Raman signal from carotene molecules.

The *aim of this work* is to study changes in the conformation of carotenoid molecules using nanostructured silver substrates (coatings), as well as to study the dependence of the intensity of Raman spectra on the concentration of carotenoids in the samples under study.

Materials and methods for solving problems. Synthetic samples of β-carotene (drug *Vetoron*) were used as the object of the study.

Raman scattering from β-carotene was recorded on an *in via* Basis spectrometer (*Renishaw*, Great Britain). A laser with a radiation wavelength of 532 nm, a power of 100 mW, a 50× objective was used for excitation. Raman scattering was recorded using a CCD detector (1024 × 256 pixels with Peltier cooling to –70 °C) with a grating of 1800 lines/mm. A *Leica* class microscope was used to visually select the location of the Raman scattering of nanostructured substrates (coatings). For the analysis of carotene, characteristic bands of the Raman spectrum were used in the range from 600 to 2000 cm^{-1}.

Preparation of nanostructured substrates for enhance the Raman scattering, we used substrates with a nanostructured coating based on silver, on which carotene samples were applied (2 — 0.02 μ/ml or 37 nMol/l — 3.7 nM/l). Nanostructured substrates were obtained using the technique described in [3]. Aqueous sodium hydroxide (0.1 M NaOH, high purity water, Milli-Q, Millipore) was added dropwise to freshly prepared 0.01 M aqueous silver nitrate solution until complete precipitation of a black-brown silver (I) oxide. This prepared oxide was thoroughly washed with deionized water and dissolved in a two-fold molar excess of a 10 % aqueous ammonia solution to have 0.01 M solution of a silver (I) complex. The obtained transparent silver complex solution was filtered through *Millex-LCR* syringe driven filter units (Millipore, 0.45 microns pores). Then this initial ammonia solution of silver (I) oxide was nebulized into mist and 1–5 micron droplets were streamed onto "warm" (270 °C) glass, alumina or microporous silica substrates during 40–60 min (possible 250 °C, deposition thickness 45 nm).

Results. *Study of the dependence (intensity) of Raman spectra on the concentration of carotene.* We base the vibrational assignments of the Raman lines of carotenoids is taken from [17]. The plot in Fig. 1 reveals strong lines at 1522, 1160 and 1004 cm^{-1} representing the marker bands of the β-carotene molecules [8, 18]. In particular, the strong line at 1522 cm^{-1} can be assigned to a collective C=C stretching mode whose frequency is a function of the effective conjugation length along the backbone of the β-carotene molecule (for details see [19] and references therein). Resonance effects selectively enhance the Raman signals of β-carotene even if they are a minor component of blood, since the wavelength of the 514 nm laser line is very close to the absorption maximum in the UV–vis electronic spectrum of the β-carotene [3].

In the course of the study, Raman spectra were obtained at various carotene concentrations (see Fig. 1). It was found that the Raman spectroscopy intensity with an increase in carotene concentration (in the range from 0.02

to 20 mg/ml or 37 μmol/l — 37 mmol/l) increases, and the maximum changes were found in the linear region (from 0.25 to 5 mg/ml (466 μM/l — 931 μM/l)) (Fig. 2). Thus, the sensitivity of Raman spectroscopy for recording the concentration of carotene in solution is 0.02 mg/ml (37 μmol/l).

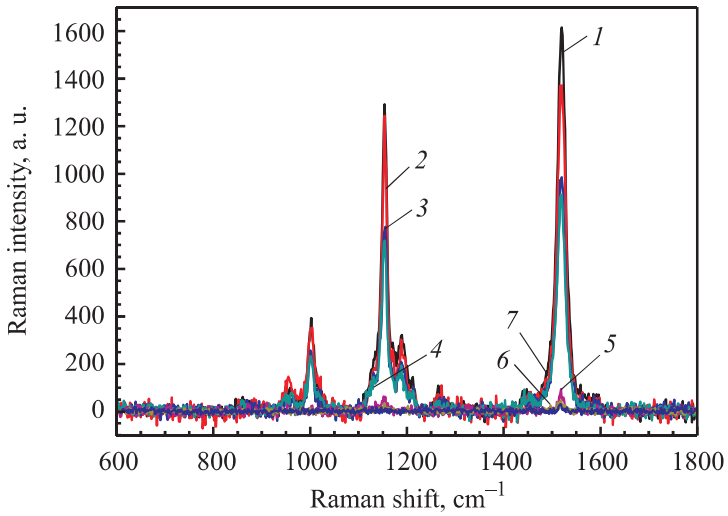

Fig. 1. Raman spectra obtained at various concentrations of β-carotene in the sample:
1 — concentration of β-carotene in the sample 20 mg/ml (37.2 mMol/l);
2 — 4 mg/ml (7.4 mmol/L); *3* — 2 mg/ml (3.72 mmol/l); *4* — 0.4 mg/ml (740 μmol/l);
5 — 0.2 mg/ml (372 μmol/l); *6* — 0.04 mg/ml (74 μmol/l); *7* — 0.02 mg/ml (37.2 μmol/l)

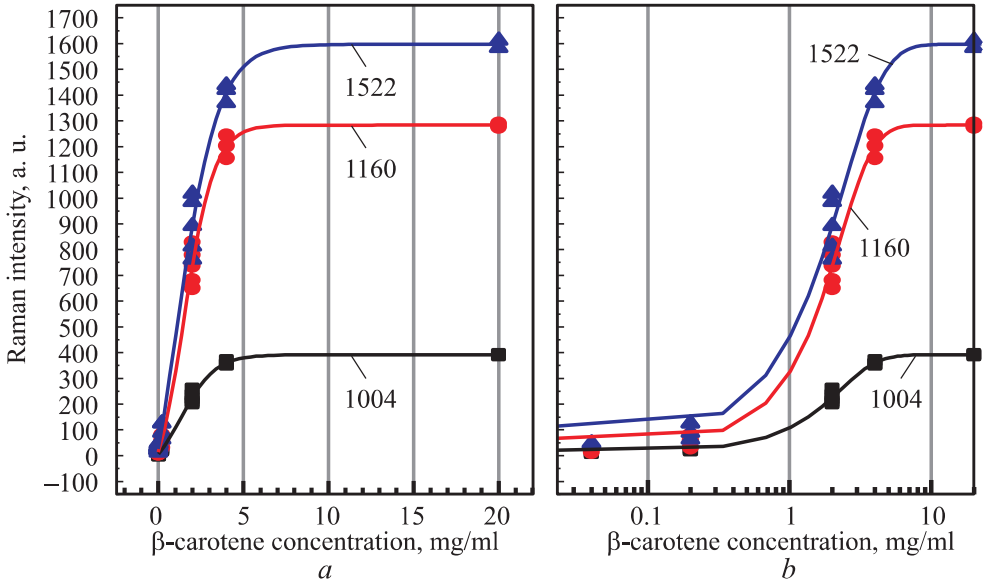

Fig. 2. Calibration graph for determining the concentration of β-carotene:
a) direct relationship; *b*) decimal logarithm

In the next series of experiments, we investigated the ability of the obtained nanostructured substrates to enhance the Raman signal of carotene (Fig. 3). It was found that the use of a substrate allows to enhance the Raman signal when diluting the sample by 10^5–10^6 times, at a substance concentration of 10^{-9} mol/l (see Fig. 3, Table). Thus, the use of nanostructured silver substrates makes it possible to enhance the Raman scattering of individual carotene molecules, which is important for biomedical research.

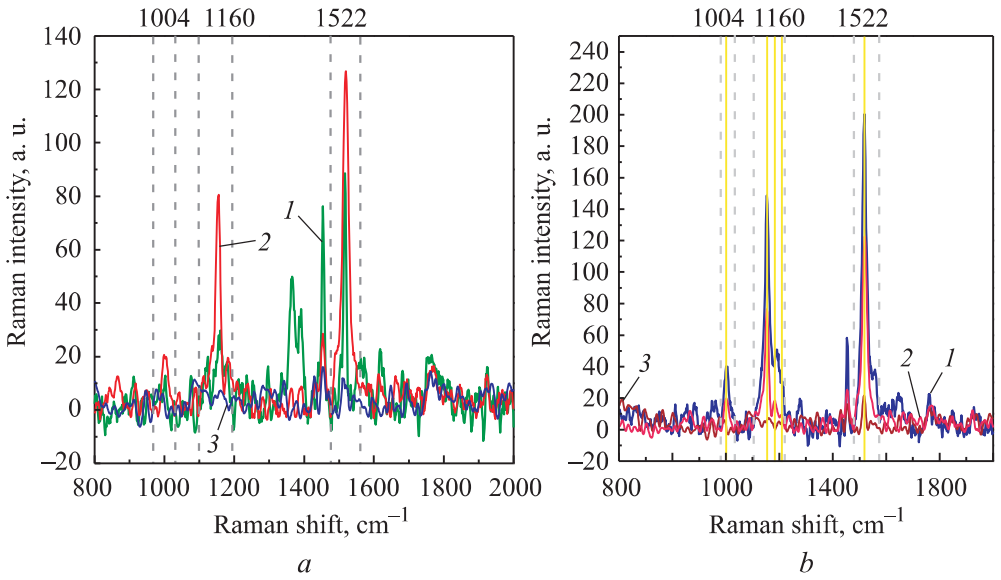

Fig. 3. Raman spectra obtained by diluting the sample 10^6 (*a*) and 10^5 (*b*) times:
1 — on a silver substrate; *2* — when the sample is diluted 100 times;
3 — without using a substrate (coating)

Gain factor

1004	1160	1522
Dilution 100,000 times, final concentration of β-carotene 0.0002 mg/ml		
7 702	6 444	10 617
6 935	4 678	8 655
12 849	12 819	14 527
9 954	9 550	13 454
11 869	10 912	15 822
15 103	11 496	15 877
10 735	9 316	13 159
Dilution 1,000,000 times, final concentration of β-carotene 0.00002 mg/ml		
–	38 716	47 056
–	46 879	47 801

End of the Table

1004	1160	1522
Dilution 1,000,000 times, final concentration of β-carotene 0.00002 mg/ml		
59 491	44 380	59 324
–	21 624	–
–	33 210	71 116
–	29 430	–
–	53 297	–
–	54 041	85 642
–	30 006	71 878
–	30 437	54 382
59 491	38 202	62 457
$EF = \left(I_{\text{SERS}} / I_{cr}\right)\left(C_{cr} / C_{\text{SERS}}\right)$.		

It is known that the morphology (size and localization of silver nanoparticles) of a nanostructured substrate changes the character of interactions and, accordingly, the optimal conditions for enhancing Raman scattering [1, 2]. In this regard, we carried out a study of the dependence of the Raman signal (the ratio of the characteristic bonds of the Raman spectrum: I_{1522} / I_{1160} (to assess the contribution of the stretching vibrations of the double bonds of the polyene chain to the Raman spectrum), I_{1160} / I_{1004}, I_{1522} / I_{1004}) on the concentration of carotene in solution and on the substrate (normalization of the signal according to the "intramolecular reference" (Fig. 4).

Use of nanostructured substrates to enhance the Raman signal from blood plasma carotenoids. As commented above, the resonant Raman spectrum mainly shows lines ascribed to β-carotene. With respect to previous investigation [1, 2], the SERS spectrum reported here shows more defined lines and some more detailed.

In the next series of experiments, the Raman signal from blood plasma carotene was recorded, diluted 10 to 100 times and fixed on a nanostructured substrate (Fig. 5). It was found that dilution of plasma by a factor of 100 makes it possible to identify and record the main parameters (band intensity, band position in the spectrum, ratio of the main bands) of the Raman spectrum of plasma carotene. So, with the help of Raman, it is possible to control changes in the concentration and conformation of the carotene molecule, registering both the magnitude of the Raman intensity and the ratio of the intensities of the bands in the Raman spectrum of carotene. The fixation of carotene molecules on a nanostructured substrate significantly increases the sensitivity of the method

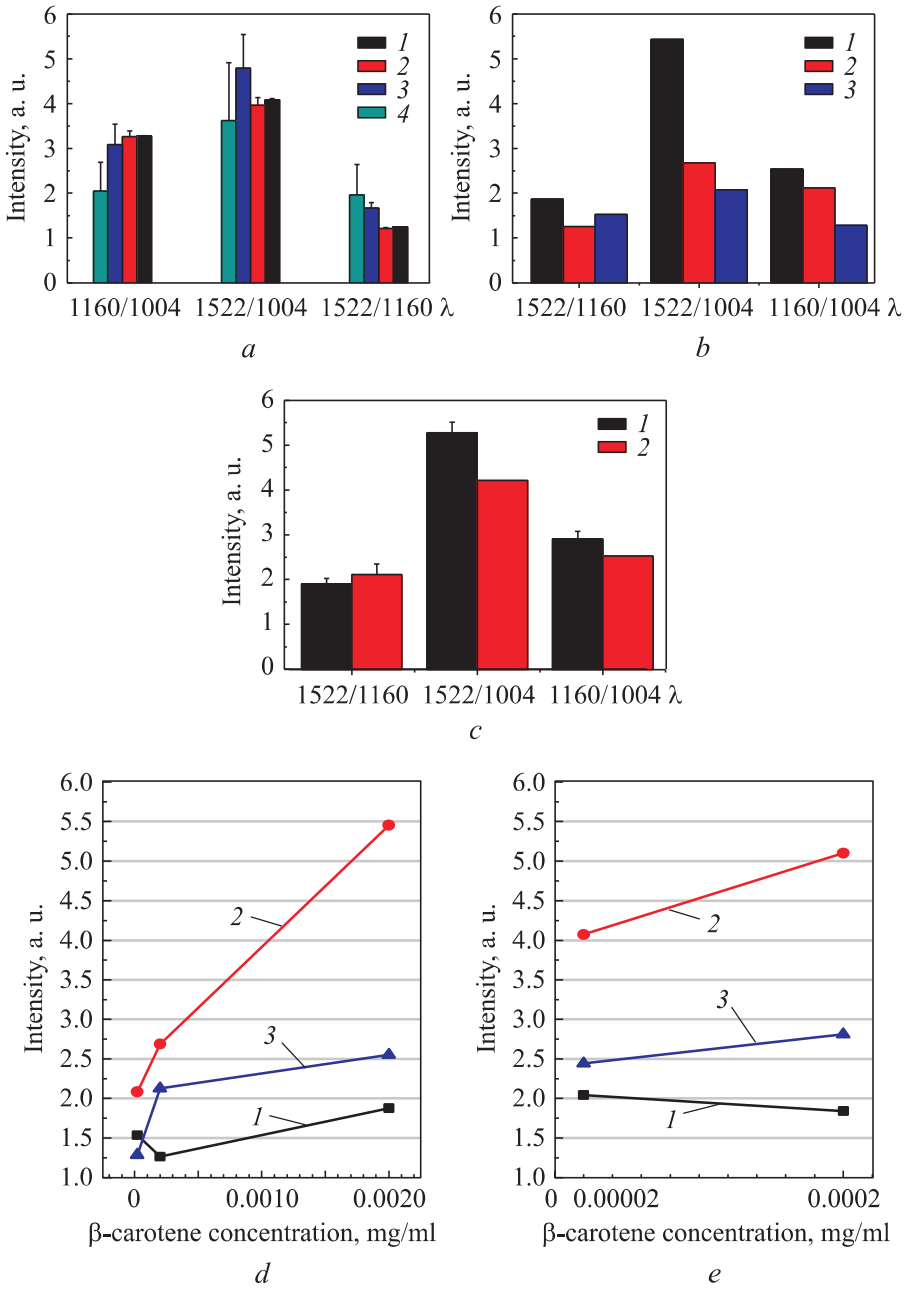

Fig. 4. Peak ratios for Raman spectra β-carotene:

a) in aqueous solution (*1* — 20 mg/ml; *2* — 2 mg/ml; *3* — 0.2 mg/ml; *4* — 0.02 mg/ml); *b*) dried sample on a silver support (*1* — 0.002 mg/ml; *2* — 0.0002 mg/ml; *3* — 0.00002 mg/ml); *c*) an aqueous solution on a silver support (*1* — 0.0002 mg/ml; *2* — 0.00002 mg/ml); *d*) = *b*) and *e*) = *c*) (*1* — 1522/1160; *2* — 1522/1004; *3* — 1160/1004)

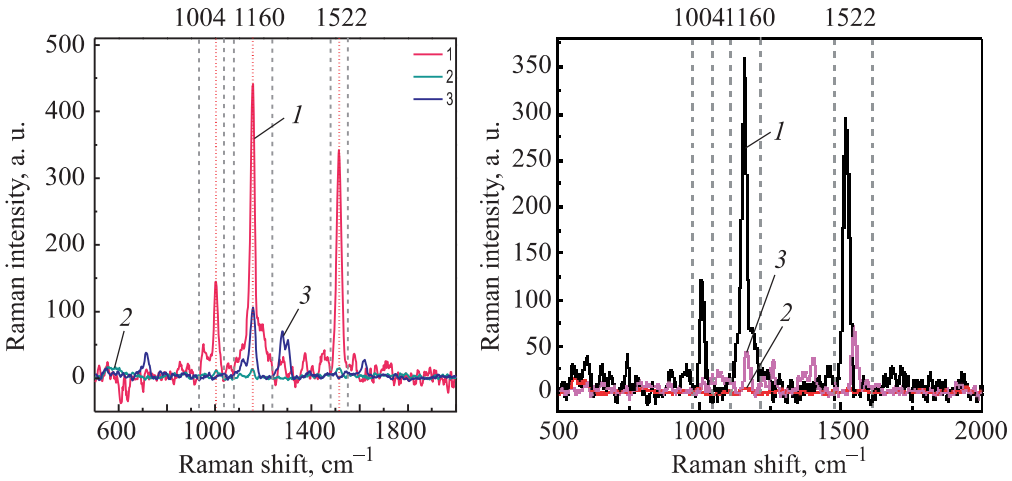

Fig. 5. Raman spectra from blood plasma when diluted:
a) 10 times — in solution; *b*) 100 times — dried (*1* — spectrum from blood plasma; *2* — from sample dilution; *3* — from a diluted sample on a silver substrate)

(the amplification factor reaches 10^4), and also allows recording the Raman spectra of carotene in blood plasma, which is important for clinical diagnostics. This study indicates that Raman spectroscopy with λ = 534 nm excitations can be used as a probe specific to β-carotene present in blood and involved in human functional changes [20–22]. The analysis of the Raman and SERS spectra recorded at 534 nm has shown that with the same excitation line and Raman equipment it is possible to selectively probe either β-carotene depending on blood sample preparation.

In SERS conditions (i.e., mixing silver colloids to blood sample) hem markers show up, while in normal Raman conditions resonance enhancement allows direct observation of β-carotene features. This highlights the versatility of Raman technique and the prospects for biology and clinical chemistry (Fig. 6).

Discussion of the obtained results and conclusions. Interest in the industrial production of plant and microalgal biomass for biofuels and bioproducts has stimulated studies on microalgal physiology and mechanisms of valuable biomolecules synthesis and accumulation in algal cells. One of the most investigated biomolecules for commercial application are neutral lipids and carotenoids. However, until now the mechanism of the influence of different ambient factors, including application of inductors, on biosynthesis and accumulation of these molecules is not well understood. Partially, the lack of such knowledge is due to restricted technique of investigation. Raman spectroscopy is one of the advanced methods of cell physiology investigation, which can fill some gaps

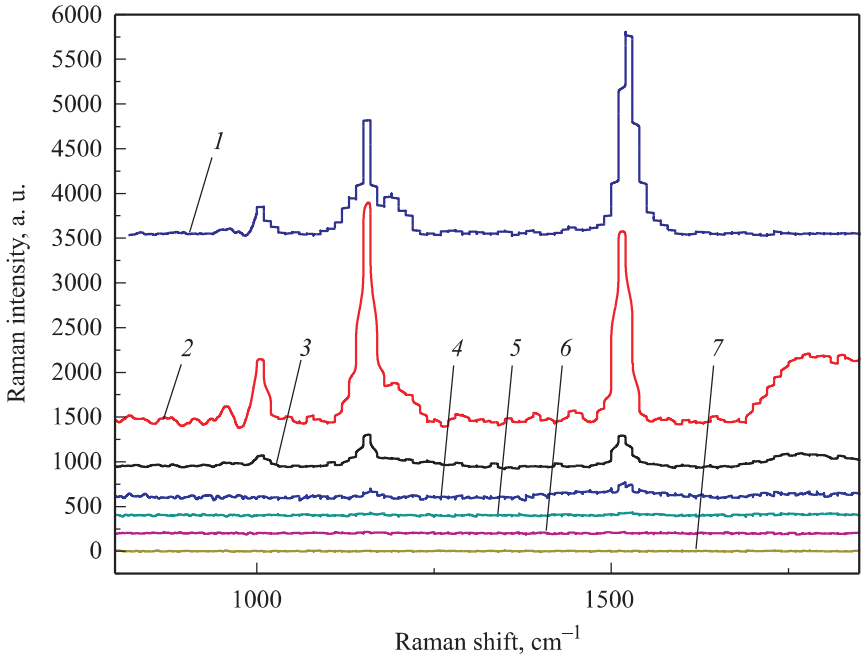

Fig. 6. Raman spectroscopy spectra of carotenoids, obtained from different objects: *1* — apple (λ = 473 nm); *2* — undiluted plasma dried on a glass slide; *3* — whole plasma; *4* — plasma diluted with Alena's buffer 2 times; *5* — plasma diluted with Alena's buffer 10 times; *6* — 100 times; *7* — 1000 times (λ = 532 nm) (the 1520–1526 cm^{-1} band is associated with stretching vibrations of the C=C bond in the polyene chain, 1160 cm^{-1} with stretching vibrations of C–C in the polyene chain, 1008 cm^{-1} with stretching vibrations of the C–CH_3 bond between the carbon of the polyene chain and a carbon of a pendant methyl group)

in our understanding of such processes. The current techniques used in the papers presented show the advantages and other essential specifics of the method applied to plants (most importantly, microalgae) and other species/objects. It was found that the use of a substrate allows to enhance the Raman signal when diluting the sample by 10^5–10^6 times, at a substance concentration of 10^{-9} mol/l. In SERS conditions (i.e., mixing silver colloids to blood sample) hem markers show up, while in normal Raman conditions resonance enhancement allows direct observation of β-carotene features. This highlights the versatility of Raman technique and the prospects for biology and clinical chemistry.

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