ФІЗІОЛОГІЯ РОСЛИН І ГЕНЕТИКА. 2023. Т. 55. № 6

https://doi.org/10.15407/frg2023.06.493
UDC 633.15:577.34:58.087

METHODS OF INFRARED AND RAMAN SPECTROSCOPY USED TO STUDY PHOTOSYNTHETIC PIGMENTS AND OTHER ORGANIC MOLECULES IN MAIZE HYBRIDS INTENDED FOR FOOD AND FEED

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The present paper introduces results of studying the leaves and kernels of three maize hybrids rich in nutrients (ZP 341, ZP 434, and ZP 505). Methods of absorption, infrared and Raman spectroscopy were applied to study the role and functions of photosynthetic pigments and organic molecules. Absorption spectroscopy was used to determine the concentration of all types of chlorophyll (chlorophyll *a*, chlorophyll *b*) and carotenoids. Infrared spectroscopy showed the presence of different organic molecules in terms of origin and kinetic of the formation of the kernel spectrum, as a whole, and all its spectral bands with different amplitude intensities. Raman spectroscopy was used to reveal the content of carotenoids, organic molecules and some nutrients contained in kernels of maize hybrids. Organic molecules causing the formation of certain spectral bands in the Raman spectrum (carotenoids, glycogen, phosphates, amid III and others) were determined. Conformational and functional changes of photosynthetic pigments that occurred due to changes in the ratio (quotient), which had been determined by the intensity the spectral bands amplitudes, were analysed in particular. The obtained ratios (quotients) indicate different contributions of valence vibrations of their chemical bonds, which inevitably altered the conformation of molecules. The presented results of comprehensive studies point out to minor biogenic differences among the studied maize hybrids.

Key words: *Zea mays* L., hybrid, kernel, leaf, chlorophyll, carotenoids, infrared and Raman spectrum, spectral band, molecular conformational properties, vibrations of valence bonds.

The photosynthetic apparatus of maize hybrids leaves contains several structures of photosynthetic pigments: chlorophylls and carotenoids. The main role of chlorophyll *a* and chlorophyll *b* is to absorb the energy of sunlight and convert it into the energy of chemical processes and bonds [1].

Citation: Radenović Č.N., Ivanović M.R., Popović A.S., Nikolić V.V., Simić M.S., Sečanski M.D. Methods of infrared and Raman spectroscopy used to study photosynthetic pigments and other organic molecules in maize hybrids intended for food and feed. *Fisiol. rast. genet.*, 2023, **55**, No. 6, pp. 493—505. https://doi.org/10.15407/frg2023.06.493

An almost identical example of the role of chlorophyll as a transporter of solar energy in the photosynthetic apparatus is described in the monograph [2].

As it is known, carotenoids in higher plants are divided into two major classes: the class of carotenoids and the class of xanthophylls [3]. Carotenoids are molecules of tetraterpenes (linear or cyclic form) consisting of the carbon and hydrogen, while xanthophylls contain an oxygen atom [4]. Xanthophylls have three important roles in the structure of the photosynthetic apparatus of the maize hybrid leaf. The first function refers to the absorption of sunlight energy and its transport via chlorophyll in the photosynthetic reaction centre. The second function is the protection of the system by deactivation of the triplet state of chlorophyll, utilization of active forms of oxygen, peroxidation and other processes. The third function refers to the regulation based on the ability of β-carotene to regulate membrane microviscosity by epoxidation and de-epoxidation of xanthophylls in the thylakoid membrane [5, 6]. Carotenoids of the photosynthetic apparatus are predominantly in the *all-trans* configuration, except for carotenoids of the reaction centre, where the C15-*cis* configuration is activated [7].

There are several methods used to analyse the functions, roles and amounts of carotenoids. However, a prominent place belongs to the method of Raman spectroscopy, with a special study of oscillation occurrence of chemical bonds of atoms and molecules in functional groups of organic molecules. Raman spectroscopy also provides the possibility of studying the impacts of high temperatures, drought and other stressors on vital functions of the maize hybrid leaf [8—10], or on the application of the origin of a spectral band with forward or backward movements [11, 12]. Such processes occur due to the effects of laser radiation in the Raman spectrometer. Under such conditions, different shapes of spectral bands occur in the Raman spectra reflecting changes in the state of biomolecules in the kinetic form or shift the wavenumber (cm^{-1}) to one side or the other [12, 13].

Absorption spectroscopy methods were used to study the quantitative average content of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, carotenes and xanthophylls) in the leaves of maize hybrids. These methods provide the measurement of the light absorption coefficient in characteristic peaks (absorption maxima) of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and all carotenoids). This coefficient, proportional to the quantitative content of pigments dissolved in 100 % acetone, is presented in the manuscripts [14—17].

The aim of this study was to present a more detailed application of methods of absorption, infrared and Raman spectroscopy, including all procedural details in terms of the technique and operating conditions that were necessary for the determination of the concentrations and functions of photosynthetic pigments, organic compounds, in particular nutrients, as well as for the reveal of the conformational characteristics of organic molecules and nutritional compounds contained in the leaves and kernels of investigated maize hybrids.

Material and Methods

The kernels and leaves of the following three early and medium early maturity maize hybrids ZP 341 (FAO 115 days), ZP 434 (FAO 120 days) and ZP 505 (FAO 128 days) were studied. These hybrids widespread, yielding and enriched with nutrients, have been developed at the Maize Research Institute, Zemun Polje, Belgrade, Serbia. These maize hybrids are grown in Serbia and surrounding countries.

After sowing of seeds of the three maize hybrids (10 seeds each), the germinated seeds were grown under conditions of 16-hour daylight at air relative humidity of 75 % until the appearance of the third leaf, suitable for leaf experiments, i.e. until the appearance of the leaf that grew in 14, 20 and 25 days. It was possible to use leaves of different ages, but in this study, leaves with average values were used for the experimental measurements. A fraction of the third leaf was used to record the Raman spectrum of photosynthetic pigments: chlorophyll *a*, chlorophyll *b* and carotenoids in maize hybrid leaves. It was also used to determine the average concentration of chlorophyll and carotenoids in leaves of maize hybrids by absorption spectroscopy.

The methods of IR and Raman spectroscopy were used to study conformational changes in organic molecules in the kernels and leaves of maize hybrids [18]. In studies of the structure of certain molecules, such as carotenoids, in leaves and kernels of maize hybrids, Raman spectroscopy was more advantageous than IR spectroscopy. A fragment of the maize hybrid leaf (leaf blade) was placed on the spectrometer sample stand, and exposed to laser radiation of 5 mW for 10 seconds, after which the Raman spectrum with all spectral bands was recorded. Raman spectroscopy was used to study conformational changes in molecules of photosynthetic pigments and other compounds. Carotenoids in maize hybrid leaves were studied by the use of the Raman microspectroscopy method that included the application of the Renishow spectrometer microscope with excitation of $\lambda = 532$ nm.

The method of IR spectroscopy was applied to kernels of observed maize hybrids. Such IR studies have been regularly performed on kernels of maize hybrids [19—22]. In recent times, studies have been carried out on kernels and their structural parts: endosperm, pericarp and germ of maize hybrids intended for food and feed [12, 23—26].

The WiRE version 3.3 software was used for the primary processing of IR and Raman spectra, while the program package Origin version 8.1 was used for the detailed processing of these spectra. Absorption spectrometry methods were used to observe photosynthetic pigments in leaves of maize hybrids. The experimental material consisted of samples that were tested by the use of the Shimadzu UV-mini 1240 spectrophotometer (single beam photometric system). The spectral band width was 5 nm, wavelength accuracy was ±0.1 nm for the experiment duration. Photometric accuracy of this spectrophotometer ranged from ±0.003 to 0.005 Abs.

Results and Discussion

Qualitative and quantitative content of photosynthetic pigments in the leaf of maize hybrids. Table 1 presents the average content of photosynthetic pig-

TABLE 1. Concentrations (mg/g fresh leaf tissue) of photosynthetic pigments in leaves of studied maize hybrids

Photosynthetic pigments	Maize hybrids		
	ZP 341	ZP 434	ZP 505
Chlorophyll *a*	2.275±0.114	1.473±0.074	2.000±0.100
Chlorophyll *b*	1.049±0.052	0.701±0.035	1.001±0.050
Chlorophyll (*a*+*b*)	3.323±0.166	2.174±0.109	3.000±0.150
Carotenoids	0.517±0.026	0.298±0.015	0.394±0.020

ments in leaves of observed maize hybrids. The highest concentration of all photosynthetic pigments was recorded in the hybrid ZP 341. A somewhat lower concentration of these pigments was detected in the maize hybrid ZP 505, while the lowest concentration was established in the maize hybrid ZP 434. These results indicate that higher amounts of chlorophyll in maize hybrid leaves enhance the energy supply capacity of leaf structures involved in photosynthesis. This in turn results in more efficient photosynthesis and a potential increase in maize hybrid yields.

It is important to note that concentrations of chlorophyll *a*+*b* and carotenoinds also vary over different maize hybirds, which can additionaly affect photosynethic efficiency and the yield, for several reasons: protection against oxidative stress, light collection efficiency, etc. With regard to photosynethic activity, concentrations of chlorphyll *a*+*b* and carotenoinds directly affect the ability of photosynthetic apparatus to absorb light energy and to convert it into chemical energy in the process of photosynthesis. Maize hybrids with higher concentrations of these pigments are usually more capable to absorb light energy, which contributes to photosynthesis that is more efficient. On the other hand, carotenoids, as well as other antioxidants, can protect photosynthetic apparatus against oxidative damages caused by excessive light radiation and stress. Maize hybrids with higher amounts of carotenoids can resist stressful conditions and preserve photosynthetic activity. Carotenoids also contribute to the absorption of a light spectrum that is not efficiently absorbed by chlorophyll, which increases the efficiency of light energy stored for photosynthesis.

Efficient photosynthesis and increased concentrations of photosynthetic pigments usually result in higher yields of plants, because more energy is converted into organic compounds, such as sugar, starch, etc. All these factors should be taken into account during the analysis and interpretation of the results, because different concentrations of pigments among various maize hybrids might indicate the differences in the photosynthetic capacity and ability of plants to adapt to diverse environments.

The determination of the ratio (quotient) of photosynthetic pigments in the leaf of studied maize hybrids. In Table 2 there are shown the ratios (quotients) of photosynthetic pigments of investigated maize hybrids: chlorophyll *a*/chlorophyll *b*, chlorophyll *a*+*b*/carotenoids. Ratios of chlorophyll *a* to chlorophyll *b* and chlorophyll *a*+*b* to carotenoids were higher in the maize hybrid ZP 341 than in maize hybrids ZP 434 and ZP 505, which points out to its potentially greater capability to absorb light energy and to its more efficient photosynthesis than in the remaining two maize hybrids.

TABLE 2. The ratio (quotient) of photosynthetic pigments concentrations in the leaves of studied maize hybrids

Ratio of photosynthetic pigments concentrations	Maize hybrids		
	ZP 341	ZP 434	ZP 505
Chlorophyll *a*/chlorophyll *b*	2.162±0.108	2.061±0.144	2.052±0.103
Chlorophyll (*a*+*b*)/carotenoids	8.131±0.569	7.676±0.384	6.862±0.643

Although a similar trend in the ratios of chlorophyll *a*+*b* to carotenoids were observed in all three maize hybrids, this ratio is more uniform in the maize hybrid ZP 434. Based on this uniformity, it can be concluded that this maize hybrid has more stable photosynthetic adaptations than the remaining two maize hybrids. More precisely, the maize hybrid ZP 434 can show minor variability in the response of photosynthetic system to various conditions or factors, which may be important for its photosynthetic efficiency and the yield as an ultimate gain.

Dissimilarities and similarities of the experimentally recorded Raman spectra of the studied maize hybrids. Fig. 1 shows the experimentally recorded resonance Raman spectra of leaves of the studied ZP maize hybrids (ZP 341, ZP 434, and ZP 505) with spectral bands. It is clearly seen that three spectral bands were the most pronounced in all spectra of maize hybrids at the wavenumbers of 1002 cm^{-1}, 1004 cm^{-1}, 1153 cm^{-1}, and 1520 cm^{-1} including corresponding amplitude intensities. Slightly greater dissimilarities can be observed in the spectrum Fig. 1, *c* — ZP 505. However, spectral bands of low and very low intensity were generally only indicated/hinted at or their occurrence was about to happen.

Resonance Raman spectrum of studied maize hybrids. A common resonance Raman spectrum (Fig. 2) was recorded on the leaf segments of the maize inbred lines and maize hybrids. Spectral bands appeared within ranges of 960 cm^{-1}, 1004 cm^{-1}, 1156 cm^{-1}, 1188 cm^{-1}, 1245 cm^{-1}, and 1520 cm^{-1}. Fig. 2 shows the existence of spectral bands with higher intensity amplitudes — 1520 cm^{-1}, 1156 cm^{-1}, 1004 cm^{-1}, and spectral bands with low intensity amplitudes — 960 cm^{-1}, 1188cm^{-1}, 1245cm^{-1}. It was established that the formation of the mentioned spectral bands had been caused by certain organic molecules, compounds and nutrients. Thus, a spectral band at 960 cm^{-1} (or 962 cm^{-1}) was caused by phosphates, while the spectral band at 1004 cm^{-1} (or 1026 cm^{-1}) was caused by glycogen. The spectral band at 1188 cm^{-1} (or 1118 cm^{-1}) was also caused by phosphates, while the spectral bands at 1245 cm^{-1} (or 1206 cm^{-1}) and 1520 cm^{-1} were caused by amide III and carotenoids, respectively [27].

Change in the ratio (quotient) of the amplitude intensities of spectral bands of the Raman spectrum of the leaves of studied maize hybrids characterizes the nature and changes in the structure of organic molecules. The ratios (quotients) of the amplitude intensities of the spectral bands of the Raman spectrum are presented in Table 3. The ratios (quotients) of the intensity of amplitudes of the spectral bands of the Raman spectrum are presented in the order of their recording in the wavenumber range from 400 to 1600 cm^{-1} [28]. The existence of the following quotient ratios was determined:

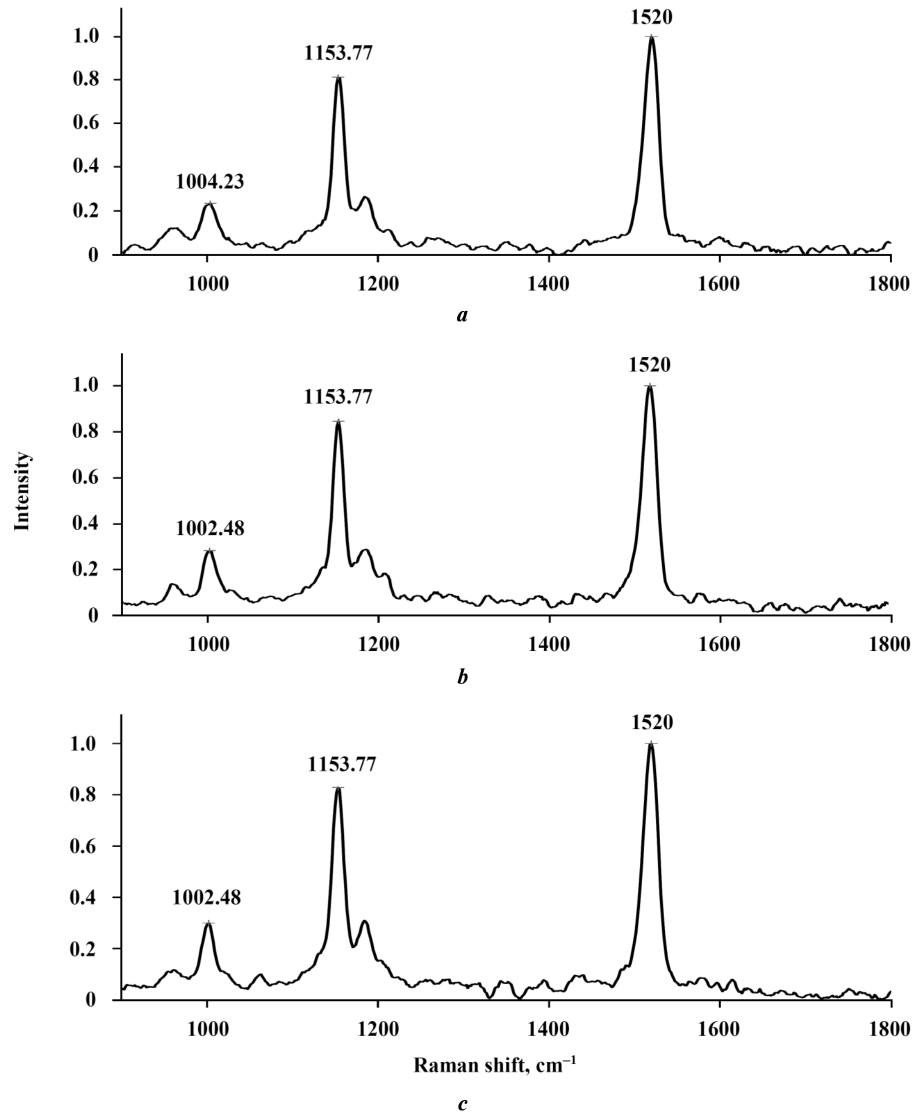

Fig. 1. Resonance Raman spectra with spectral bands of the leaf of studied maize hybrids: *a* — ZP 341; *b* — ZP 434; *c* — ZP 505

- the I_{960}/I_{1004} ratio characterizes the proportion of the formation of a new shape of the conformation of molecules or compounds that are not known yet;

- the I_{1004}/I_{1156} ratio characterizes the proportion of valence vibrations of the C–CH$_3$ in relation to the valence vibrations of =C–C= bonds;

- the I_{1156}/I_{1188} ratio characterizes the proportion of valence vibrations of carbon in the carotenoid molecule [4];

- the I_{1156}/I_{1245} and I_{1245}/I_{1520} ratios characterize the proportion of valence vibrations of unknown organic molecules and compounds.

Besides, the following ratios (quotients) are added:

- the I_{1520}/I_{1156} ratio characterizes the proportion of valence vibrations of double –C=C– bonds, in relation to single =C–C= bonds;

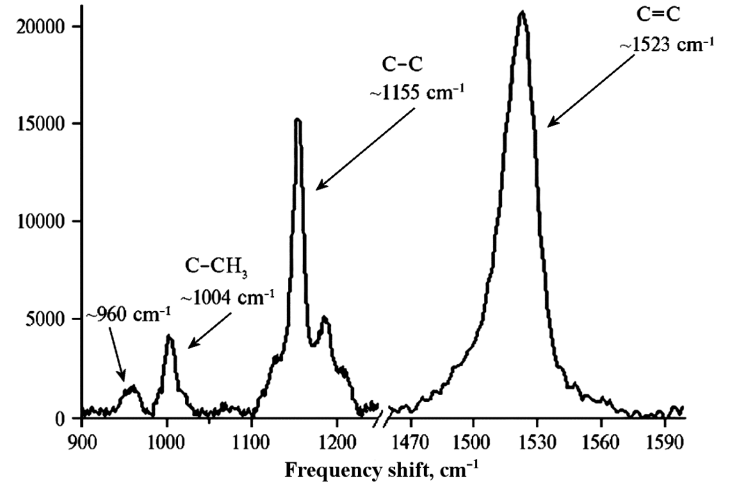

Fig. 2. Resonance Raman spectrum of studied maize hybrids

- the I_{1004}/I_{1520} ratio characterizes the proportion of valence vibrations of C—CH$_3$ in relation to —C=C— bonds.

The I_{960}/I_{1004} ratio might indicate changes in the conformation of molecules or the presence of new unknown substances. The I_{1004}/I_{1156} ratio may contribute to comprehension of the relationship between vibrations of C—CH$_3$ and vibrations of =C—C= bonds, which is significant in the characterization of organic compounds. The I_{1156}/I_{1188} ratio may indicate valence vibrations of carbon in carotenoid molecules, while I_{1156}/I_{1245} and I_{1245}/I_{1520} ratios might point out to the presence of valence vibrations of unknown organic molecules and compounds.

The presentation of all results in this section was done analogously to the analysis of transient oscillations in the processes of induced delayed chlorophyll fluorescence both in maize inbred lines and in maize hybrids [2, 29].

Dynamics of the formation of typical infrared spectra of studied maize hybrids with all spectral bands. Infrared spectra of organic and natural compounds have been included into the program of research on conformational and functional characteristics [30, 31]. This paper presents our studies on IC spectra with the analyses of spectral bands with high and very high

TABLE 3. The ratio (quotient) of the amplitude intensities of the spectral bands of the Raman spectrum of maize hybrid leaves which are characterised by the structure of organic molecules, compounds and nutrients

Ratio (quotient) of the amplitude intensities of spectral bands (relative ratio)	Maize hybrids		
	ZP 341	ZP 434	ZP 505
960/1004	0.395±0.020	0.470±0.023	0.347±0.017
1004/1153	0.236±0.012	0.156±0.008	0.120±0.006
1188/1153	0.285±0.014	0.339±0.017	0.359±0.018
1004/1520	0.190±0.010	0.280±0.014	0.287±0.014
1520/1153	1.243±0.062	1.184±0.059	1.212±0.061

intensity of amplitudes. In addition, spectral bands of low and very low intensities of amplitudes that were of the excited state and caused vibrations of valence bonds in the functional groups of organic molecules, were also analysed.

A typical IC spectrum of kernels of the maize hybrid ZP 341 (Fig. 3, *a*) was characterized by three strongly pronounced spectral bands within the range of the wavenumber from 1000 cm^{-1}, over 2900 cm^{-1} to 3400 cm^{-1}. Likewise, spectral bands at 1145 cm^{-1}, 1175 cm^{-1}, 1650 cm^{-1}, and 2850 cm^{-1} deserve to be studied separately. In the course of the further analysis, special attention should also be paid to spectral bands with low amplitude intensities: 600 cm^{-1}, 700 cm^{-1}, 775 cm^{-1}, 825 cm^{-1}, 925 cm^{-1}, 1100 cm^{-1}, 1145 cm^{-1}, 1550 cm^{-1}, 2300 cm^{-1}, and 3780 cm^{-1}. It should also be noted that several unstable spectral bands at 650 cm^{-1}, 1900 cm^{-1}, and 3000 cm^{-1} had occurred within the entire range from 400 to 4000 cm^{-1}.

With respect to the maize hybrid ZP 434 (Fig. 3, *b*) the IC spectrum of kernels had four spectral bands with the pronounced intensity of amplitudes: 1000 cm^{-1}, 1700 cm^{-1}, 2950 cm^{-1}, and 3400 cm^{-1}. In addition, there were spectral bands with sufficiently clearly pronounced intensities of amplitudes: 1185 cm^{-1}, 1775 cm^{-1}, and 2825 cm^{-1}. Moreover, there were also several spectral bands with the distinctively low intensity of amplitudes: 600 cm^{-1}, 700 cm^{-1}, 800 cm^{-1}, 900 cm^{-1}, 975 cm^{-1}, 1100 cm^{-1}, 1225 cm^{-1}, 1500 cm^{-1}, and 2375 cm^{-1}. The IC spectrum of this hybrid also had several unstable states in spectral bands at: 450 cm^{-1}, 1400 cm^{-1}, 1900 cm^{-1}, 2300 cm^{-1}, and 3900 cm^{-1}.

The maize hybrid ZP 505 (Fig. 3, *c*) differed from the other two maize hybrids as it had eight spectral bands with the highly pronounced intensity of amplitudes: 1000 cm^{-1}, 1150 cm^{-1}, 1450 cm^{-1}, 1700 cm^{-1}, 1750 cm^{-1}, 2850 cm^{-1}, 2900 cm^{-1}, and 3400 cm^{-1}. Then, there were 12 spectral bands with medium amplitude intensity: 500 cm^{-1}, 517 cm^{-1}, 700 cm^{-1}, 775 cm^{-1}, 900 cm^{-1}, 1000 cm^{-1}, 1100 cm^{-1}, 1300 cm^{-1}, 1550 cm^{-1}, 2350 cm^{-1}, 3025 cm^{-1}, and 3750 cm^{-1}. Finally, there were five spectral bands with the unstable state of their formation.

In general, the spectral bands of IC spectra of kernels of all three studied maize hybrids provided information about organic molecules (compounds, nutrients, etc.) that caused their formation. This conclusion especially applies to certain spectral bands with very high and high amplitude intensities, but also to spectral bands with low amplitude intensities. In the case of spectral bands with low amplitude intensities, the excited states of valence vibrations of chemical bonds in functional groups of organic molecules should be analysed [21, 32]. Based on presented results on IC spectra of kernels and on spectral bands it is possible to reach a conclusion about conformational changes of organic molecules, compounds and macro- and micronutrients contained in kernels and their structural parts: endosperm, pericarp and germ [12, 24—26].

Conclusion. Thus, we determined the average concentrations of photosynthetic pigments in leaves of the studied maize hybrids (ZP 341, ZP 434, and ZP 505) for chlorophyll *a*, chlorophyll *b*, chlorophyll (*a*+*b*) and carotenoids. Origin and the kinetic form of all spectral bands of the

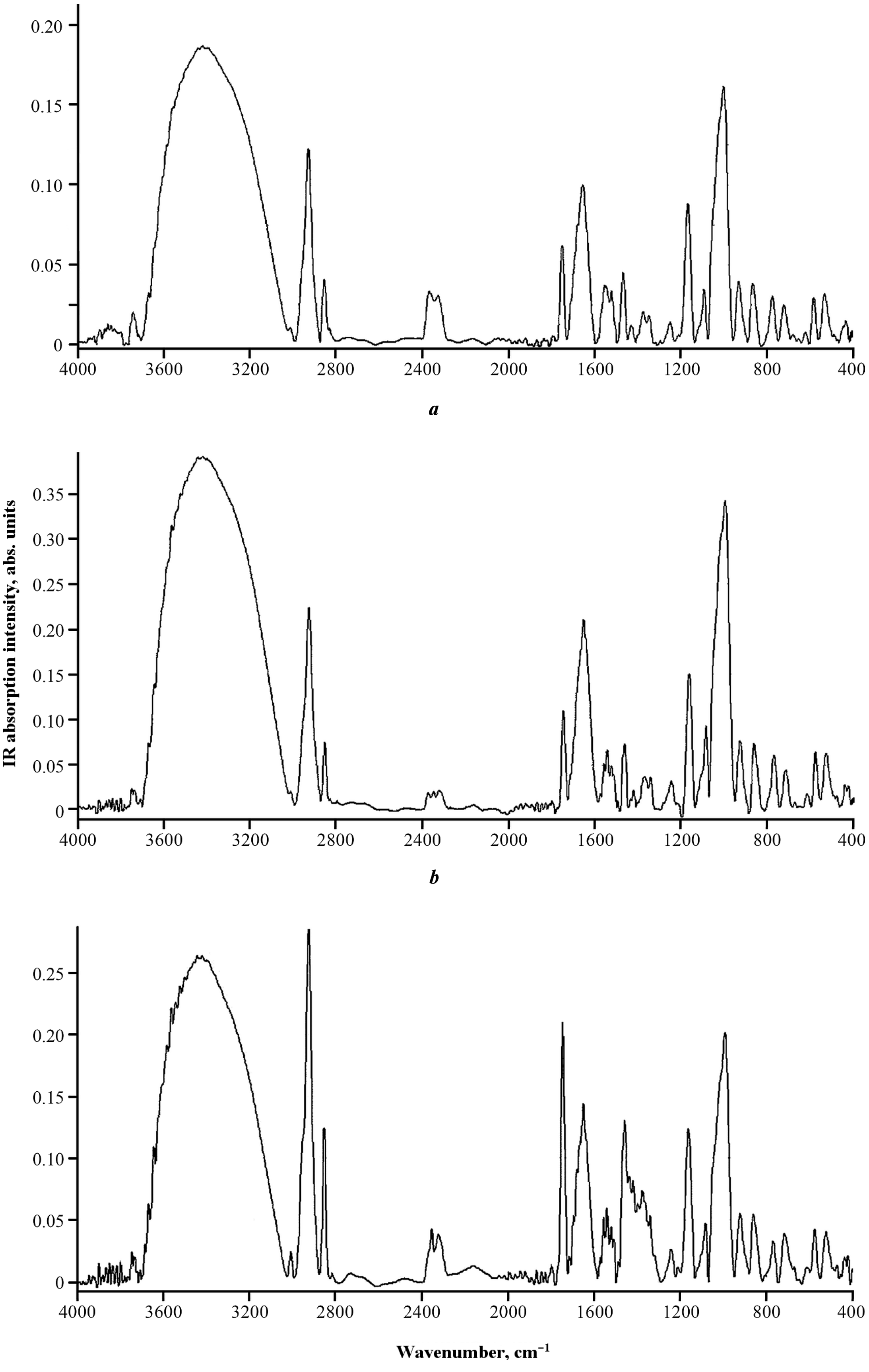

Fig. 3. Typical IC spectrum of kernels of studied maize hybrids intended for food and feed with all spectral bands: *a* — IC spectrum of ZP 341; *b* — IC spectrum of ZP 434; *c* — IC spectrum of ZP 505

infrared and Raman spectra were obtained. The value of changes in the ratio (quotient) of the intensity of spectral bands of the Raman spectrum of leaves for each of studied maize hybrids was established. All spectral bands of Raman spectrum produced by molecules and chemical compounds (phosphates, glycogen, amid III, carotenoids and other nutrient substances — macro and micro nutrients) were presented.

It was shown that amounts of photosynthetic pigments differed over observed maize hybrids. The highest concentration of photosynthetic pigments was detected in the maize hybrid ZP 341. Based on the concentration of these pigments in maize leaves, the selection of maize hybrids is performed. The greater amounts of photosynthetic pigments are — the greater energy supply capacity of photosynthetic apparatus is.

Above-mentioned and other studies of the structures, roles and functions of photosynthetic pigments in the leaf and photosynthetic apparatus contributed to the increase of general knowledge about insufficiently studied processes within photosynthetic structures (chloroplasts, pigment-protein complexes, activity of antennas and reaction centers), by which efficient photosynthesis was achieved and higher grain yields of observed maize hybrids were obtained.

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Received 20.11.2023

МЕТОДИ ІНФРАЧЕРВОНОЇ ТА КОМБІНАЦІЙНОЇ СПЕКТРОСКОПІЇ ДЛЯ ДОСЛІДЖЕННЯ ФОТОСИНТЕТИЧНИХ ПІГМЕНТІВ ТА ІНШИХ ОРГАНІЧНИХ МОЛЕКУЛ У ГІБРИДАХ КУКУРУДЗИ, ПРИЗНАЧЕНИХ НА ХАРЧУВАННЯ ТА КОРМИ

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Наведено результати дослідження листків і зерен трьох багатих на поживні речовини гібридів кукурудзи (ZP 341, ZP 434 і ZP 505). Для вивчення ролі та функцій фотосинтетичних пігментів і органічних молекул застосовано методи абсорбційної, інфрачервоної та комбінаційної спектроскопії. За допомогою абсорбційної спектроскопії визначено вміст всіх типів хлорофілу (хлорофілу *a*, хлорофілу *b*) та каротиноїдів. Інфрачервона спектроскопія показала наявність різних органічних молекул за походженням і кінетичною формою спектра зернини в цілому та всіх його спектральних смуг з різною амплітудною інтенсивністю. За допомогою спектроскопії комбінаційного розсіювання досліджено вміст каротиноїдів, органічних молекул і деяких поживних речовин, що містяться в зерні гібридів кукурудзи. Визначено органічні молекули, сполуки та поживні речовини, які зумовлюють утворення певних спектральних смуг у спектрі комбінаційного розсіювання (каротиноїди, глікоген, фосфати, амід III та інші). Зокрема, проаналізовано конформаційні та функціональні зміни фотосинтетичних пігментів, що відбуваються внаслідок зміни співвідношення (частки), яке визначено за інтенсивністю амплітуд спектральних смуг. Отримані співвідношення (частки) вказують на різні внески валентних коливань їхніх хімічних зв'язків, які неминуче змінювали конформацію молекул. Представлені результати комплексних досліджень вказують на незначні біогенні відмінності досліджуваних гібридів кукурудзи.

Ключові слова: Zea mays L., гібрид, зерно, листок, хлорофіл, каротиноїди, інфрачервоний та раманівський спектри, спектральна смуга, молекулярні конформаційні властивості, коливання валентних зв'язків.

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