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EXTRACTION AND PHYSICOCHEMICAL CHARACTERIZATION OF PECTIN POLYSACCHARIDES FROM AMARANTH LEAVES

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Abstract

Polysaccharides are one of the most important classes of natural compounds that have practical application in various fields of science and technology. Pectin remains one of the most essential polysaccharides, being a primary constituent of the structural elements of the cell wall in higher plants, performs the functions of binding and strengthening components of the cell wall, and also regulates water metabolism. Pectic substances are widely used in medicine as detoxicants of heavy metals and regulators of metabolic processes in the human body. In addition, they are also a universal food additive (E440). Despite significant amounts of traditional raw material resources (apple and citrus pomace, beet pulp), new alternative sources of raw materials are being searched for, including vegetable plants introduced in Russia for the production of pectins and their use in the production of functional foods. Among non-traditional plant resources, amaranth (*Amaranthaceae*) holds a significant rank. Due to its high yield and high content of biologically active substances and antioxidants, this crop acts as a potential source of obtaining valuable plant-derived substances for medicine, agriculture and the food industry. The *Amaranthus tricolor* L. cv. Valentina plants were used to isolate pectic substances by the classical method and ultrasonic treatment at a frequency of 22 kHz. Sugars were quantified using a Shimadzu 20-AD Prominence liquid chromatograph (Shimadzu Corporation, Japan) with a Shimadzu RID-10A refractometric detector. Infrared spectra were recorded on an IRS-113 instrument (Bruker, Germany) with a resolution of 1 cm^{-1} in the range 400-4000 cm^{-1}. The elemental composition was determined (a CHNSO-high-temperature analyzer Euro EA 3028-HT-OM, EuroVector Instruments & Software, Italy). All measurements by atomic force microscopy (AFM) were carried out on a Multi Mode V scanning probe microscope (Veeco Instruments, Inc., USA) in an intermittent contact mode. Structural study of the isolated polysaccharides by the IR spectroscopy method showed their possible affiliation to pectin substances. To study the monosaccharide composition, the samples of pectins were hydrolyzed with sulfuric acid (2 N) and partially hydrolyzed with trifluoroacetic acid (TFA). The high-performance liquid chromatography (HPLC) identified glucose, galactose, rhamnose, arabinose and galacturonic acid in the pectin fractions. Low galacturonic acid contents of 0.63 % and 1.68 % were determined in H_2SO_4 and TFA hydrolyzates, respectively. The conditions for hydrolysis-extraction which ensure the maximum yield of pectin substances were 0.5 % oxalic acid with complexone (0.5 % HDTA), 50-55 °C, 4 h, feed to extractant volume (hydromodule) ratio of 1:15. The physicochemical properties of pectin obtained under these conditions were studied using atomic force microscopy (AFM) and thermogravimetric/differential scanning calorimetry (TG/DSC) methods. After ethanol re-precipitation, this pectin sample showed an intense absorption band of stretching vibrations of carbonyls of carboxyl groups and ester groups at 1742 cm^{-1}. The TG/DSC indicated a two-step weight loss. The Fourier-transform infrared (FTIR) spectrum of the gaseous products derived from thermal decomposition of pectin sample showed that water was the main component of the gas phase at the first stage

of weight loss and at the second stage, pectin was decarboxylated. According to atomic force microscopy, the size of the aggregates was 2.4-2.5 μm maximum and ~ 330 nm minimum.

Keywords: *Amaranthus tricolor* L., amaranth, cv. Valentina, hydrolysis-extraction, ultrasonic disintegrator, pectin, IR spectroscopy, pectin thermostability, TG/DSC, AFM

Polysaccharides are one of the most important classes of natural compounds that have practical applications in various fields of science and technology. Pectin, a special polysaccharide of *Amaranthus* L. is a structural element of the cell tissue of higher plants, acts as a binding and strengthening component of the cell wall, and regulates water metabolism [1]. Pectins are widely used in medicine as heavy metal detoxifiers and metabolic regulators in humans, and also serve as a universal food additive (E 440) [2]. Despite the significant volumes of traditional raw materials (apple, citrus, and beet pulp), there is a search for new non-traditional raw materials for the pectins to be used in the functional food production [3-6]. Amaranth plants due to high yield and high content of biologically active substances and antioxidants is a potential source of valuable substances for medicine, agriculture and food industry [7-9]. The bioactive peptides derived from amaranth proteins exhibit antimicrobial, antioxidant, and antihypertensive activity [10-12], hypocholesterolemic effect [13] and antitumor activity [14].

Screening of the chemical composition of amaranth plants of various species showed that its biomass (leaves, inflorescences, stems) contains a number of chemical compounds of practical value. These are waxes, carotenoids, flavonoids, proteins, polysaccharides and other substances. Determination of their chemical composition and development of isolation methods open up prospects for obtaining functional products [6, 16, 17].

In this work, for the first time, the influence of the hydrolyzing agent and ultrasonic treatment (22 kHz) on the efficiency of hydrolysis, extraction, and the yield of pectin during isolation from amaranth variety Valentina plants is shown compared to the classical method.

Our goal was to develop methods for extracting pectin polysaccharides from *Amaranthus tricolor* L. cv. Valentina plants and to characterize the physicochemical properties and structural features of the extracted compounds.

Materials and methods. Vegetable amaranth cv. Valentina (bred at the All-Russian Research Institute of Vegetable Breeding and Seed Production VNIISSOK; included in the State Register and approved for industrial use) [18, 19] were grown without the use of pesticides and herbicides (the VNIISSOK experimental fields, Moscow Province).

In experiment 1, a flat-bottomed flask with dry ground *A. tricolor* leaves (100 g) were filled with distilled water and allowed at room temperature to isolate the amaranthine pigments. The dried depigmented raw material (85 g) was poured with 0.5% oxalic acid and added with a complexone (0.5% hexamethylenediaminotetraacetic acid, HDTC) to bind divalent metal ions. After 4-hour extraction (a hydromodulus of 1:15, pH 3.8, 50-55 °C) the extract was separated by filtration, evaporated to 200 ml at 60 °C under vacuum and precipitated with acetone (1:1 v/v). The precipitates were dried (a SNOL 58/350 laboratory oven, JSC Umega, Lithuania) at 40-50 °C to form films. The mass of dried pectin polysaccharide (sample 1) was 2.980 g.

In experiment 2, a flat-bottomed flask with dry ground raw materials (165 g) was filled with distilled water. Portions of dried depigmented raw material (108 g) was added with 0.5% citric acid and extracted without a complexone for 5 hours (a hydromodule of 1:14, pH 3.8, 50-55 °C). The extract was separated using a fabric filter, evaporated to 200 ml at 60 °C under vacuum and precipitated with acetone (1:1.5 v/v). The precipitate was dried at 40-50 °C; the weight of the

dried precipitate (sample 2) was 0.570 g.

To intensify hydrolysis and extraction, in two other experiments, ultrasonic treatment was applied (a disintegrator UZDN-1, Russia). Amaranthine was preliminarily removed with water from ground amaranth leaves (100 g). The resulting depigmented raw material was divided into two parts for extractions with 1.0% oxalic or 1.0% citric acid.

In experiment 3, pectin substances were extracted from raw materials (32 g) with 1.0% oxalic acid during ultrasonic treatment (UZDN-1, 22 kHz) for 5 min (a hydromodule of 1:10) with heating (11-37 °C). The extract was filtered and centrifuged (a centrifuge Sigma 4-15, Sigma-Aldrich, Germany). The product was precipitated with an equal volume of acetone and dried at 60 °C. The weight of the dried precipitate (sample 3) is 0.420 g.

In experiment 4, ground raw materials (32 g) were extracted with 1.0% citric acid under sonication (UZDN-1, 22 kHz) for 5 min with heating (23-45 °C, a hydromodule of 1:10). The extract separated from the pulp was added with an equal volume of acetone to precipitate pectin polysaccharides. The weight of the dried product (sample 4) is 0.560 g.

The isolated pectin substances were purified by re-precipitation with pretreatment with an ion-exchange resin (KU-2 cation exchanger) for demineralization. The concentration of pectin in the extracts was measured by the calcium pectate method.

Partial hydrolysis was performed to identify sugars in the side links of pectin polymers. A portion of the sample was hydrolyzed with trifluoroacetic acid (TFA) at 120 °C for 1 hour. TFA was distilled off under vacuum. To determine the monosaccharide composition, a sample of pectin was hydrolyzed with 2 N sulfuric acid at 110 °C for 5 hours. The hydrolyzate was neutralized with barium hydroxide, the precipitate was separated by filtration. The filtrates were analyzed by paper chromatography (ZMM chromatographic paper, Czech Republic; n-butanol:acetic acid:water solvent system, 5:1:4; aniline hydrogen phthalate as a reagent for developing chromatograms). Silufol UV-254 plates (Kavalier, Czech Republic) were used for thin-layer chromatography (n-butanol:acetic acid:water solvent system, 5:4:1; aniline hydrogen phthalate as a developer); monosaccharides (Sigma-Aldrich, Germany) were used as markers.

The sugars were quantified using a Shimadzu 20-AD Prominence liquid chromatograph (Shimadzu Corporation, Japan) with a Shimadzu RID-10A refractometric detector and ReproSil-Pur NH_2 (250 × 4 mm) (Dr. Masch GmbH, Germany) and YMC-Pack Polyamine II (250 × 4.6 mm) (YMC Europe GmbH, Germany) chromatographic columns. The acetonitrile:water 75:25 (v/v) eluent was the mobile phase (the eluent flow rate 1 ml/min). Calibration was carried out using standard solutions of monosaccharides (Sigma, Germany).

Infrared spectra (an infrared spectrographn IRS-113, Bruker, Germany) were recorded with a 1 cm^{-1} resolution in the range of 400-4000 cm^{-1}in KBr pellets. The elemental composition was determined (an elemental CHNSO-high-temperature analyzer Euro EA 3028-HT-OM, EuroVector Instruments & Software, Italy) with streptocide as an analytical standard (C — 41.85%, H — 4.65%, N — 16.26%, and S — 18.62%).

All measurements by atomic force microscopy (AFM) were performed on a Multi Mode V scanning probe microscope (Veeco Instruments, Inc., USA) in the intermittent-contact mode. Scanning was performed using RTESP rectangular cantilevers (Veeco Instruments, Inc., USA) with silicone probes. The resonant frequency of the cantilevers ranged was 250-350 kHz, the probe curvature radius was 10-13 nm. Microscopic image resolution was 512×512 points per

frame at a scanning speed of 1 Hz. To eliminate the distortions associated with the external noise-caused "trembling" of the microscope, an anti-vibration system capable of smoothing oscillations up to 0.5 Hz (lower limit) (TMC Vibration control, USA) was used. IR samples were prepared by applying 7 μl of a pectin solution onto a mica substrate, followed by deposition of pectin particles on it.

Ultrafiltration was carried out using an automatic ultrafiltration unit AUF-0.6 (TOO Vzor, Russia) with hollow fibers. The filtration limit is 50 kDa, the operating pressure during filtration is 0.8-1.2 MPa, and the filtering surface area is 200 cm2.

To study the thermal decomposition of amaranth pectin, we used the method for synchronous thermal analysis — thermogravimetry/differential scanning calorimetry with IR-Fourier spectroscopy (TG/DSC) was used which records the change in the mass of the sample under thermal effects vs. temperature. A coupled system consisting of a TG/DSC STA449-F3 simultaneous thermal analysis instrument (Netzsch, Germany) and a Tensor 27 IR-Fourier spectrometer (Bruker, Germany) was used.

Results. In our experiments on the isolation of amaranthine from the leaves of amaranth variety Valentina by water extraction followed by extraction of pectin substances, oxalic and citric acids were hydrolyzing agents at the hydrolysis-and-extraction stage. The optimized technological parameters were temperature, pH, hydromodules, and time. We believed that ultrasonic treatment intensifies the procedures. Table 1 shows the extraction parameters and the yield of pectins.

The results showed that the UZDN-1 treatment significantly reduces the time of the extraction. In addition, when using citric acid, the weight of isolated pectin polysaccharides turned out to be higher than when extracting with oxalic acid (see Table 1). Perhaps the difference is due to the extraction conditions, namely, heating the sample with citric acid to 20 °C prior to ultrasonication.

1. Isolation of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants by acid hydrolysis

Hydrolyzing agent	Extraction mode				Pectin, g	Yield (per absolute dry weight), %
	time	T, °C	hydromodule	kHz		
0.5 % oxalic acid + 0.5 % HDTA	4 h	50-55	1:15	–	2.98	3.51
0.5 % citric acid	5 h	50-55	1:14	–	0.57	0.53
1.0 % oxalic acid	5 min	11-37	1:10	22	0.42	0.63
1.0 % citric acid	5 min	23-45	1:10	22	0.56	1.75

N o t e. Ultrasonication was performed using a UZDN-1 device (Russia). Dashes mean that processing was not carried out. HDTA — hexamethylenediamine tetraacetic acid.

Data of the elemental analysis of pectin substances from amaranth cv. Valentina (C — 41.87%, H — 6.56%) showed the absence of protein when extracted with 0.5% oxalic acid + 0.5% HDTA and a high protein content (N — 4.43%) when extracted with 0.5% citric acid. After ultrasonication, extraction with both 0.5% oxalic acid and 0.5% citric acid resulted in 2.0% nitrogen content.

To determine the monosaccharide composition of the isolated pectin, we applied hydrolysis with 2 N sulfuric acid and partial TFA-assisted hydrolysis. Thin-layer chromatography showed a significant proportion of arabinose and galactose polymers (arabinans and galactans or arabinogalactans), and traces of rhamnose. HPLC revealed glucose, galactose, rhamnose, arabinose and galacturonic acid (8.3:7.7:4.1:6.6:71.0) in the pectin fractions, which is consistent with the report of Sarkar et al. [20]. A low content of galacturonic acid was characteristic of both hydrolysates (0.63 and 1.68% for H_2SO_4 and TFA, respectively). When extracted with 0.5% oxalic acid + 0.5% HDTA, rhamnose was not detected. Similar results were obtained by paper chromatography.

Infrared spectroscopy (IRS) is one of the most common methods for studying plant polysaccharides [21] used both to monitor extracts and to quantify acidic and neutral sugars in a polysaccharide. Structural study of the isolated polysaccharides by IR showed their correspondence to pectin substances (Table 2). In the IR spectra, a wide intense band at 3000-3600 cm^{-1} appeared in all samples, which is characteristic of the stretching vibrations of hydroxyl groups. The characteristic shift indicated that this is a band of stretching vibrations of −OH groups with a hydrogen bond. It was also shown that in the IR spectra of pectin substances there were characteristic absorption bands of different intensity corresponding to the stretching vibrations of carbonyls of carboxyl groups (at 1700-1750 cm^{-1}) and ester groups (at 1730-1750 cm^{-1}).

At 1632-1647 cm^{-1}, absorption bands corresponded to stretching vibrations of dissociated carboxyl groups. The absorption bands at 1020-1100 cm^{-1} corresponded to the stretching vibrations of the pyranose rings, and at 1325 cm^{-1} to in-plane vibrations of the −CH groups (for IR spectra of pectin extracted from amaranth cv. Valentina plant by acidic hydrolysis, see http://www.agrobiology.ru).

2. Peaks (cm^{-1}) **of the main characteristic absorption bands in the infrared spectra of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants extracted by acid hydrolysis** (IR-Fourier spectrometer IRS-113, Bruker, Germany)

Sample 1	Sample 2	Sample 3	Sample 4	Main types of vibrations
3444	3445	3427	3412	ν(OH), ν(H_2O)
2925	2923	2924	2921	ν(CH)
1739	1738	–	–	ν(C=O)
1634	1632	1647	1647	ν(COO^-)
1401	1406	1402	1403	ν, δ(C−OH)
1382, 1228	1238	1325, 1224	1375, 1325, 1294	δ(CH)
1022-1101	1022-1104	1029	1029-1155	ν(C−O−C), ν(C−C), ν(C−O)

N o t e. For extraction procedure, see the Materials and Methods section. Dashes indicate the absence of corresponding peaks.

Extraction with 0.5% oxalic acid + 0.5% HDTA without sonication resulted in the highest degree of pectin esterification. Salt carboxyls predominated in pectin isolated with UZDN-1 treatment (the sample with oxalic acid also contained a minor amount of free carboxyl groups). In both samples, there was a band at 1325 cm^{-1} (more pronounced for citric acid application), corresponding to in-plane deformation vibrations of −CH groups. In all treatments, apart from polysaccharides, proteins were extracted, and absorption bands of amide groups appeared in the IR spectra.

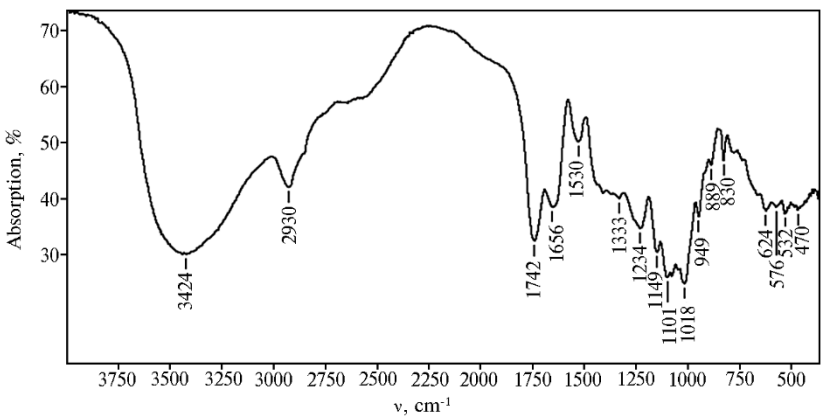

Fig. 1. Infrared spectrum of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants extracted by acid hydrolysis (0.5% oxalic acid solution + 0.5% hexamethylenediamine tetraacetic acid) and purified by re-precipitation with ethyl alcohol (IR-Fourier spectrometer Tensor 27, Bruker, Germany).

A combination of 0.5% oxalic acid + 0.5% HDTA as a hydrolyzing agent ensured the maximum pectin yield. Therefore, we additionally purified the pectin of *A. tricolor* cv. Valentina (sample 1) by re-precipitation with ethyl alcohol and characterized by IR spectroscopy, TG/DSC, and AFM. After re-precipitation, this sample had an intense absorption band of stretching vibrations of carbonyls of carboxyl groups and ester groups at 1742 cm^{-1} (Fig. 1).

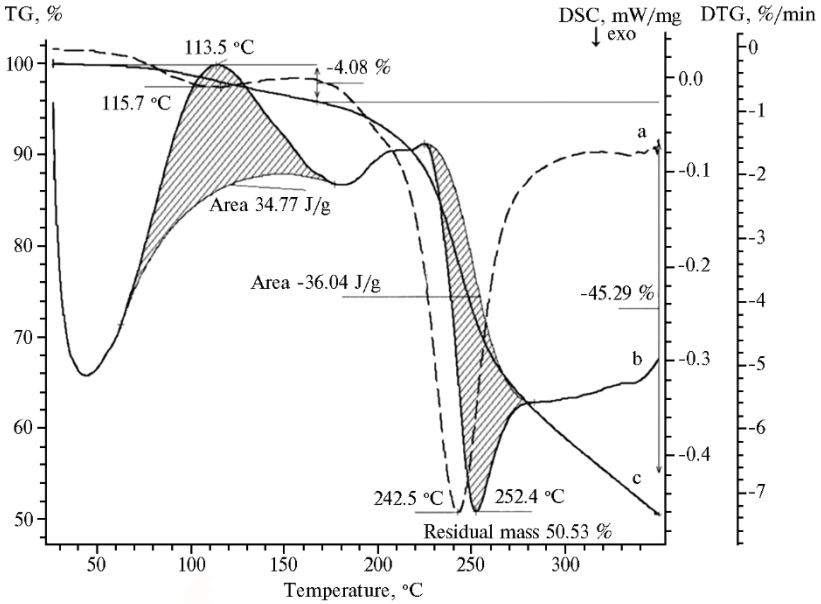

Fig. 2. Thermogravimetry/differential scanning calorimetry (TG/DSC) of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants extracted by acid hydrolysis (0.5% oxalic acid solution + 0.5% hexamethylenediamine tetraacetic acid) after re-precipitation with ethyl alcohol: a — Gram-Schmidt curve, b — DSC, c — TG. A STA449-F3 synchronous thermal analysis instrument (Netzsch, Germany) combined with a Tensor 27 IR-Fourier spectrometer (Bruker, Germany).

The thermal stability of pectin is one of the most important characteristics that determines the conditions for their storage and use in the confectionery industry. The TG/DSC data of pectin, which we isolated from amaranth cultivar Valentina (sample 1) and further purified by re-precipitation with ethyl alcohol, indicated a two-stage weight loss. The first stage had a peak at 117.3 °C with a weight loss on the TG curve of ~ 4.1% and was accompanied by an endothermic peak at 113.5 °C. Then, at 243.1 °C, the second stage of weight loss (~ 45.3%) occurred together with an exothermic peak with a ~ 36.04 J/g enthalpy (Fig. 2). The IR-Fourier spectrum of the gaseous products of the thermal decomposition of amaranth pectin showed that water was the main component of the gas phase at the first stage of weight loss; at the second stage, the decarboxylation of pectin occurred.

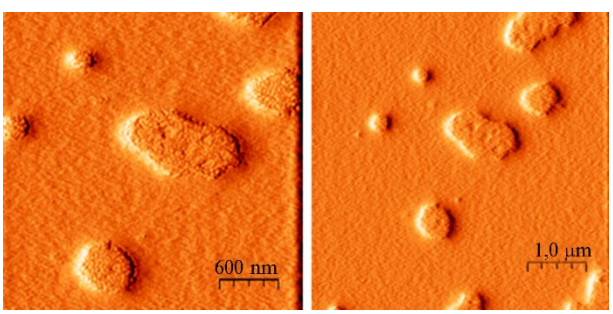

Fig. 3. Surface image of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants obtained by acid hydrolysis (0.5% oxalic acid + 0.5% hexamethylenediamine tetraacetic acid) and re-precipitation with ethyl alcohol (atomic force microscopy method, scanning probe microscope Multi Mode V, Veeco Instruments Inc., USA).

AFM analysis showed that the maximum aggregate size in the pectin

sample 1 (concentration of solutions 10^{-4}%) was 2.4-2.5 μm, the minimum size was ~ 330 nm (Fig. 3). The aggregates mostly sized from 660 to 880 nm, the size of individual particles that made up the aggregates was 30-45 nm.

The results we obtained on the yield of *A. tricolor* cv. Valentina pectin per absolute dry mass and the physicochemical properties of the product are of practical interest for large-scale production for the food industry and pharmacology. The deterioration of the ecological situation and the increase in the number of various diseases necessitate detoxifying drugs and effective long-acting drugs based on pectin polysaccharides. It is known that amaranth pectin has a wide range of physiological activity [22, 23].

The traditional technology of pectin production widely uses the method of hydrolysis of protopectin with strong mineral acids. Currently, hydrochloric acid (HCl) is the main extracting solvent for the release of pectin from plant tissues. However, the use of mineral acids creates environmental problems [5, 9]. Green Chemistry provides for the development of fundamentally innovative approaches to reduce the anthropogenic load on the environment and avoid a harmful effect on humans. For environmentally friendly production, Cho et al. [24] extracted pectin from apple pomace with organic acids (tartaric, malic, and citric) at 85 °C. The obtained pectin was highly esterified and had high molecular weight (citric acid led to the maximum molecular weight). The yield of pectin extracted with organic acids was comparable to that during extraction with 0.1 M hydrochloric acid widely used f in industry. The results of these authors confirm the prospects and efficiency of using organic acids as a hydrolyzing agent and are consistent with our choice of oxalic and citric acids for obtaining pectin from *A. tricolor* cv. Valentina.

It should be noted that the extraction of pectin from non-traditional raw materials requires a thorough refinement of existing technologies, including not only the parameters of the main stages, but also searching for additional stages of processing raw materials and the resulting products to improve their quality. There is a technology based on the use of electro-activated water obtained by electrodialysis as a hydrolyzing agent. Rotary-pulsation-based and ultrasonication-based environmentally friendly technologies for pectin production are also reported, which intensify hydrolysis and extraction of pectin-containing raw materials and replace the traditionally used strong mineral acids with “soft” food acids [25]. In addition, there is a technology based on the use of microwave radiation [26]. Maran et al. [27] proposed the optimal conditions for microwave extraction (microwave power 413 W, pH 2.7, 134 s, hydromodule 1:18) providing the maximum pectin yield (28.86%).

Thus, the efficiency of hydrolysis and extraction of pectin substances depends on the hydrolyzing agent, pH, a hydromodule, time and temperature. We have proposed the protocol that provides the maximum yield (3.51%) of pectin from the *Amaranthus tricolor* L. cv. Valentina biomass extracted by the classical method: 0.5% oxalic acid + 0.5% hexamethyleneamine tetraacetic acid, 50-55 °C, 4 hours and hydromodule 1:15. Ultrasonic treatment at 22 kHz reduces the extraction time from 4 hours to 5 min, providing a pectin yield of 1.75%. Structural study of the extracted polysaccharides by infrared spectrometry showed their correspondence to pectin substances. High-performance liquid chromatography showed that the pectin fractions contained glucose, galactose, rhamnose, arabinose, and galacturonic acid monosaccharides. The content of galacturonic acid was 0.63% in the H_2SO_4 hydrolysate and 1.68% in the trifluoroacetic acid hydrolyzate. High molecular weight and degree of esterification allow us to recommend pectin from the amaranth cv. Valentina for use in the food industry.

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