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INOSITOLS: BIOLOGICAL ROLE AND APPLICATION, METHODS OF EXTRACTION FROM PLANT RAW MATERIALS AND DETERMINATION, BIOTECHNOLOGICAL SYNTHESIS

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The *aim* of the work was to review modern extraction, detection and quantification analytical methods of inositols and their derivatives.

Methods. Inositols are extracted from vegetable raw materials by methods of liquid extraction, under pressure, microwave extraction and supercritical fluid extraction. Quantitatively analyzed by methods of gas and liquid chromatography with preliminary derivatization. The structure of inositols can be determined by the NMR spectroscopy.

Results. Inositols and their derivatives are biologically active compounds, wich are involved in the regulation of the intracellular calcium level, the transmission of hormonal signals, the breakdown of fats and the reduction of cholesterol in the blood, the modulation of the neurotransmitters activity, etc. Inositols are used in the production of vitamin preparations. The main source for inositols extraction is vegetable raw material, namely alfalfa, as well as wheat sprouts, grapefruit, hazelnuts and others. In the paper, the methods of inositols extraction with organic and inorganic solvents, including the use of a Soxhlet apparatus, liquid extraction under pressure, microwave extraction and supercritical fluid extraction are considered. The procedure of preliminary sample preparation and polyols derivatization for their further separation and quantitative determination is described. Modern chromatographic methods of polyols identification and quantitative determination are analyzed. The possibility of using ^{1}H, ^{13}C and ^{31}P NMR spectroscopy to identify the structure of inositols and their derivatives is described.

Conclusions. Inositols are biologically active compounds of a wide spectrum of action, therefore there is an urgent need to develop biotechnological processes for their production and extraction from plant raw materials and microorganisms.

Key words: polyols, inositols, extraction, chromatography, NMR spectroscopy, biotechnological synthesis.

Inositols are widely used in the food, pharmaceutical, medical and chemical industries [1]. They may be obtained through biotechnology. Plant biotechnology covers a wide range of tasks. Two main directions are plant medicinal raw materials obtaining and isolation the biologically active substances from them for medical purposes. The use of plant origin substances in modern medicine is constantly increasing. One of the urgent tasks of biotechnology is inositols obtaining, which play a biologically important role in metabolic pathways, participating in the metabolic transformations and intercellular

communications. Inositols and their derivatives are used in the production of medicines for immune system stimulation, as well as for antitumor preparations, and sedative drugs [2–4]. Inositol (cyclohexane-1,2,3,4,5,6-hexol) belongs to the group of vitamin-like substances, sometimes it is called vitamin B_8.

Inositols are one of the most common classes of organic compounds of natural origin, synthesized by living organisms. Inositols (sugar alcohols) are colorless, sweet substances that dissolve easily in water. Inositols in the free state are practically not found in living organisms, they contain cis-1,2,3,5-trans-4,6-cyclohexanehexaol, the trivial name is myo-inositol. Phospholipid derivatives of inositol are the part of cell membranes and blood plasma lipoproteins. Phytic acid (myo-inositol-1,2,3,4,5,6-hexaphosphate) is the main form of phosphorus storage in plants, it is found in bran and seeds. In plants, the structure of phytate consists of a myo-inositol ring with six phosphate groups, which can occur in acid form (inositol hexaphosphoric acid) or salt form (inositol hexaphosphate, InsPs). In cereal grains, inositol phosphates (InsPs) are contained mainly in the form of InsPs, although there are also small amounts of InsPs with a lower degree of phosphorylation [5].

Despite the gross formula $C_x(H_2O)_y$ similar to saccharides, inositol is not a carbohydrate by chemical nature, its taste is estimated to be half the sweetness of table sugar [6]. Food products contain inositol, inositol phosphates and phospholipid derivatives of inositol [7]. During food processing, phytate can impair the formation of InsPs with different degrees of phosphorylation. Phytate can be extensively degraded by microbial phytases such as lactobacilli and yeast, but phytases from grain sources other than oats can also hydrolyze oat phytate in foods with several grain ingredients [5]. Hydrolysis of phytate by phytase occurs as a stepwise reaction of InsPs formation with different degrees of phosphorylation at different stages of the reaction. The reaction rate slows down with a decrease in the degree of phosphorylation [8].

Inositol is the six-atom alcohol of cyclohexane. It can exist in nine stereoisomers [9], the formulas of which are presented in Fig. 1.

Table 1 lists food products with a relatively high content of myo-inositol [10].

Myo-inositol (i-inositol; meso-inositol; 1,2,3,5-cis-4,6-cyclohexanehexaol; dambose [11]) and its derivatives, for example, pinitol (Fig. 2) [12] act as secondary messengers during signal transmission in intracellular signaling cascades in the form of inositol phosphates and phosphatidylinositols, taking part in the regulation of the intracellular calcium level [13], the transmission of hormonal signals, primarily from the insulin receptor [14], the breakdown of fats and the reduction of cholesterol levels in blood [15], modulation of neurotransmitter activity [16–17].

Table 1

Food products with a high content of myo-inositol

Product	Inositol content (slightly dried product), %	Inositol content (highly dried product), %
Flax seeds	2.15	2.78
Sesame flour	5.36	5.36
Almond	1.35	3.22
Brazil nuts	1.97	6.34
Coconut	0.36	0.36
Hazelnut	0.65	0.65
Peanut	0.95	1.76
Walnut	0.98	0.98
Corn	0.75	2.22
Oat	0.42	1.16
Bean	2.38	2.38

Lucerne (*Medicagosativa, alfalfa*) is one of the most cultivated fodder crops in the world due to its high nutritional value. Alfalfa is one of the richest natural inositol sources of plant origin [18]. Myo-inositol plays an important structural and signaling role in its cells. *Sinorhizobium meliloti*, a nitrogen-fixing bacterium of alfalfa, can use myo-inositol as the only carbon source [19].

Production of Inositols

Synthesis of myo-inositol. Inositols could be chemically synthesed [67,69]. Currently, myo-inositol is mainly produced in industry as a result of phytate acid hydrolysis (Scheme 1), which is isolated from rice bran and corn [20].

$$C_6H_{18}O_{24}P_6 + 3\,H_2O \overset{H^+}{\rightleftharpoons} C_6H_{12}O_6 + 6H_3PO_4$$

cis-inositol – cis-1,2,3,4,5,6-cyclohexane-hexanol	epi-inositol – cis-1,2,3,5,6-trans-4-cyclohexanehexanol	allo-inositol – cis-1,2,3,4-trans-5,6-cyclohexane-hexaol
neo-inositol – cis-1,2,3-trans-4,5,6-cyclohexanehexaol	myo-inositol - cis-1,2,3,5-trans-4,6-cyclohexane-hexaol	muco-inositol - cis-2,3,5,6-trans-1,4-cyclohexanehexaol
scyllo-inositol - cis-1,3,5-trans-2,4,6-cyclohexanehexaol	L-chiro-inositol – cis-2,3,5-trans-1,4,6-cyclohexanehexaol	D-chiro-inositol – cis-1,2,4-trans-3,5,6-cyclohexanehexaol

Fig. 1. **Inositol stereoisomers**

The direct reaction is inhibited by low pH or high pressure.

As an alternative to phytate acid hydrolysis in the production of myo-inositol, metabolic engineering deserves more attention due to its environmental safety and low cost. To obtain myo-inositol by the method of metabolic engineering, two microorganisms are mainly used: *Saccharomyces cerevisiae* [21] and *Escherichia coli* [22–24].

S. cerevisiae has its own myo-inositol biosynthetic pathway. Biosynthesis of myo-inositol by *S. cerevisiae* cell culture is characterized by a long fermentation cycle and low yield (0.359 g/l).

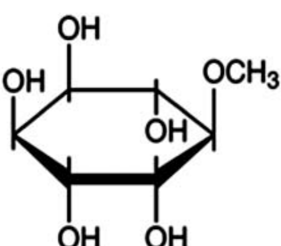

Fig. 2. **D-pinitol structure**

E. coli has been successfully used for the biosynthesis of many commercially valuable chemicals due to its distinct genetic background, short growth cycle, abundant substrate source, and simple cultivation [25–28]. Although myo-inositol is widely distributed in microorganisms, *E. coli* cannot synthesize it due to the lack of myo-inositol-1-phosphate synthase (MIP), which catalyzes the conversion of glucose-6-phosphate to myo-inositol-1-phosphate. In a study [29], the metabolic pathway from glucose to myo-inositol was constructed in *E. coli* by introducing the coding IPS synthase gene (INO1) from *S. cerevisiae* SC288. The INO1 gene was cloned and ligated into the plasmid pTrc99a to form the recombinant plasmid pTrcINO1, which was then transformed into *E. coli* K-12 (W3110). The transformant was tested and identified. The recombinant strain produced 797 mg/L myo-inositol from 10 g/L glucose in a 1L flask. This study confirms the biosynthesis of myo-inositol in recombinant *E. coli* and provides a basis for further myo-inositol industrial production.

The microbiological method for production of inositols has a fundamental drawback, namely the need to use relatively expensive substrates, such as glucose, fructose and maltose [30]. The source of carbon atoms and energy in the biotechnological process of polyols obtaining can be cheaper substrate, crude glycerol, which is a triatomic alcohol, that is, one of the simplest polyols [31–35].

Producers of polyols (erythritol, mannitol, arabidol) can be ascomycete fungi of the *Saccharomycetes* class, the genus *Yarrowia* (*Candida*) [30, 36].

Production technology of scyllo-inositol. Scyllo-inositol, a stereoisomer of inositol, is considered a promising therapeutic agent in the treatment of Alzheimer's disease because it penetrates into the brain and covers the surface of amyloid β–proteins, inhibiting the formation of toxic amyloid fibrils from them. However, it is relatively rare in nature. It is known that scyllo-inositol is produced by an expensive two-stage enzymatic transformation [37] from myo-inositol, as well as its derivatives, for example, phytic acid. The source of the latter are fruits, beans, grains and nuts [38].

The use of *Bacillus subtilis* cell culture is proposed for the effective production of scyllo-inositol from myo-inositol [30]. *Bacillus subtilis* can metabolize both myo-inositol and scyllo-inositol [39]. The iolABCDEFGHIJ operon encodes enzymes involved in many steps of inositol metabolism. The transcription of the operon is regulated by the transcription of the IolW repressor [40]. The iolABCDEFGHIJ operon encodes enzymes involved in multiple steps of the inositol metabolism, and the transcription of the operon is regulated by the IolR transcriptional repressor. At the first stage, myo-inositol is converted to scyllo-inosose by the enzyme iolG. *B. subtilis* has two additional inositol dehydrogenases, iolG and IolW, which specifically act on scyllo-inositol, converting it to scyllo- inosose [41]. IolX plays a major role in scyllo-inositol catabolism.

Scyllo-inositol is metabolized sequentially in several steps involving the enzymes IolE, IolD, IolB, IolC, IolJ, and IolA to produce the common intermediates, dihydroxyacetone phosphate and acetyl CoA [42].

All "useless" genes, including iolABCDEFHIJ, iolG, and iolW and overexpressed iolG and iolW, were deleted from the *B. subtilis* chromosome under the control of a strong and constitutively active promoter to create a cell factory with complete bioconversion of myo-inositol at a concentration of 10 g/L in that the same amount of scyllo-inositol released into the nutrient medium within 48 hours [43].

Phytases are a class of phosphatases that catalyze the hydrolysis of phytic acid with the release of myo-inositol and phosphate [44–46] *B. subtilis* has a high ability to secrete enzymes [47, 48]. Scyllo-inositol is obtained directly from rice bran, which is rich in phytic acid. Myo-inositol is synthesized from glucose-6-phosphate in two steps by many organisms [49]. In *B. subtilis*, glucose-6-phosphate is the starting substance of glycolysis, which appears when glucose is incorporated into the cell through the phosphotransferase system [50]. Glucose-6-phosphate is converted by inositol-3-phosphate synthase into myo-inositol 1-phosphate, which is then dephosphorylated by inositol monophosphatase to obtain myo-inositol.

Inositol Extraction Methods

Solvent extraction. Extraction of sugar alcohols from vegetable raw materials is usually carried out with hot water-ethanol solutions with different percentages of ethanol: 70%, 80%, 96% by volume [51]. In the literature, the following basic methods of extraction are given:

1) extraction with 80% ethanol at boiling for a day followed by extraction for 5 minutes with 20% ethanol also at boiling;

2) three times extraction for 5 min with 80% ethanol at boiling;

3) extraction with 80% methanol at a temperature of 60 °C;

4) extraction with 80% ethanol at a temperature of 13 °C for 14 hours [52].

After centrifugation, the supernatant is kept at 60 °C for 20 minutes, and then dried. Cold 80% ethanol is added to the dry residue and dried again. The procedure is repeated three times.

For better extraction of polyatomic alcohols mannitol, sorbitol and inositol, the fruit puree is thoroughly homogenized. Extraction is provided with 80% methanol solution. Ethanol can be used for the extraction, with 50% ethanol giving a higher yield of polyols than 89% ethanol. After 15 minutes of refluxing, the supernatant can be filtered off [7].

Pinitol, mannitol, sorbitol, and inositol were extracted from plants with 5% perchloric acid at 4 °C, after which pH was adjusted to 3–3.5 using 1.0 mol/l potassium carbonate solution. In the case of extracting only sucrose, to prevent its acid hydrolysis, the samples were dissolved in a 50% (v/v) ethanol solution together with 0.1 mol/l imidazole buffer, pH 7.0. The obtained extracts were purified using Sep-Pak C18 cartridges, which were previously kept in methanol and activated by washing with Milli-Q deionized water, and then with a 30% (v/v) solution of acetonitrile in 1.0 mmol/L of hydrochloric acid.

In work [13], extraction of polyols was carried out according to the following scheme. First, the plant sample was ground in a mill for 1 min. Then the homogenate was centrifuged at a speed of 20,000 g at 4 °C for 10 min, the supernatant was passed through a cartridge (1–2 cm filled with styrene-divinylbenzene strongly acidic cationite in H^+ form, grain size 200–400 mesh, then through a cartridge (1–2 cm) with anionite (with the same matrix as the previous one) in the Cl^- form, 200–400 mesh. The eluate was heated to obtain a dry residue, which was dissolved in a solvent suitable for chromatography.

Extraction of inositol phosphates [7] from plant cells was carried out with ice-cooled perchloric acid with a concentration of 0.5 mol/l. This extract must be free of sulfate and phosphate ions, ethylene glycol-bis-(2-aminoethyl ether)-N,N,N′N′-tetra acetic acid and other multi-charged anions. Tissue samples were frozen and ground into powder, in the form of powder, they were placed in a liquid-N_2-cooling steel ball press and cooled in an ice bath, homogenized in 2 ml of the same cooled 0.5 mol/l perchloric acid solution for 20 seconds. The sediment was separated by centrifugation for 20 min under cooling at a speed of 5,000 g, the pH of the supernatant was adjusted to 5.0 with potassium hydroxide. The precipitate of potassium perchlorate formed after keeping the mixture for 20 min on ice was removed by centrifugation.

If the samples had a high content of inositol phosphate [53], they were diluted with water and passed through a cartridge filled with 0.5 ml of Q-sepharose in the Cl-form. After washing the cartridge twice with 4 ml of 2.5 mmol/l perchloric acid solution, inositol phosphate was desorbed twice with 2.5 ml with 0.6 mol/l perchloric acid solution [15]. Then the desorbate was dried by freezing. The dried sample was dissolved in 2.2 ml of 5 mmol/l sodium acetate solution, pH 5.0. For further ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR, dried extracts containing polyatomic alcohols were dissolved in 0.7 ml of a deuterium oxide solution (99.996% deuteration), creating a pH of the resulting solution of 6.0 or 9.0, by adding deuterated formic acid or deuterated ammonia, respectively.

The authors of work [8] extracted inositol phosphates from oat products. Extraction was carried out by 0.3–0.5 g of each sample weighing and 5 ml of 0.66 M hydrochloric acid adding to each sample. The suspensions were shaken overnight to extract inositol phosphates. The extracts were filtered by centrifugation of the solution using filter materials (Regenerated cellulose, MerckMillipore, Ireland). The total content of inositol phosphates, determined by anion exchange chromatography, ranged from 1.34 mg/g to 13.6 mg/g.

Extraction of inositol was also carried out from alfalfa. The collected alfalfa was dried in a place without access to direct sunlight. 1.20 g of alfalfa ground in an electric coffee grinder was poured into a flat-bottomed conical flask, 200 ml of distilled water was added. The mixture was infused for 3 hours, shaken for 1 hour and cooled in the refrigerator for a day. The extract was filtered from the millcake using a Schott filter and a Bunsen flask using a vacuum water jet pump. The filtrate was discarded, the millcake was washed on the filter with water in a volume of 40 ml, then 200 ml of water was added and infused again for 3 hours, filtered. The resulting filtrate was evaporated to oil on a rotary evaporator at a temperature of 50 °C in a round-bottom flask with a volume of 2 liters. The washing water and filtrate were combined and evaporated

on a rotary evaporator in order to reduce the volume of the solution for its passage through cationite KU-2 and anionite AB-17-8.

KU-2 characteristics: polymer matrix of styrene and divinylbenzene gel copolymer with active functional sulfate groups, grain size in the swollen state 0.315–1.25 mm, total static exchange capacity 2.0 mmol/cm^3. 0.1 M HCl was passed through the cationite in order to convert it into a protonated form, the acid was washed with water. Then the solution itself was passed.

Characteristics of anionite AB-17-8: polymer matrix of styrene and divinylbenzene gel copolymer with active functional benzyl trimethyl ammonium groups, grain size in the swollen state 0.315–1.25 mm, total static exchange capacity 1.15 mmol/cm^3. 5% NaOH solution was passed through the anionite in order to convert it into hydroxide form, the alkali was washed with water. Then the solution itself was passed. The resulting solution was evaporated on a rotary evaporator, transferred to jars with a volume of 8 ml and dried in centrifuges at 40 °C to oil. The mass of the sample containing myo-inositol was 0.168 g. The sample was qualitatively analyzed by HPLC-MS. Molecular ions 73, 147, 217 and 318 are repeated on the mass spectra of derivatized myo-inositol from the database and on the chromatograms of the extract, which is confirmation of the myo-inositol presence in the plant extract. The HPLC-MS conditions under which the spectra of myo-inositol derivatized with phthalic anhydride were recorded are as follows: liquid chromatograph AgilentTechnologies 1260 InfinityLC/MSD system with DAD/ELSDAlltech 3300 and AgilentLC/MSDG6120B mass spectrometer, column Poroshell 120 SB-C18 4.6–30 mm with a grain size of 2.7 μm, column temperature of 60 °C, mobile phase eluent A — ACN:H_2O (99:1), 0.1% HCOOH, eluent B — H_2O (0.1% HCOOH), flow rate 3 ml /min, gradient 0.01 min — 99% V; 1.5 min — 0% V; 1.73 min — 0% B; 1.74 min — 99% B, injection volume 0.5 μl, ESI, scan range m/z 83–600.

Extraction in a Soxhlet apparatus. Soxhlet extraction is another method of solvent extraction of polyols, including inositols, from plants [54, 55], which involves heating the solvent to boiling point and then returning the condensed vapor to the flask in which concentrating takes place. This method has such disadvantages as high solvent consumption (most often — ethanol) and considerable duration of execution (from 24 to 72 hours). But at the same time, the efficiency of extraction is comparatively better than with the use of traditional liquid-liquid extraction.

Pressurized liquid extraction (PLE). Unlike extraction in a Soxhlet apparatus, pressurized liquid extraction is an environmentally safe method of biologically active compounds extraction using solvents at such pressure and temperature values, when the critical point has not yet been reached. Different solvents (methanol, ethanol) or a mixture of solvents can be used in this method. If water is used as an extraction solvent, the method is called subcritical water extraction (SWE). In SWE, water is heated to 200 °C. An increase the water temperature to 190 °C reduces its permittivity to 36.5 (under normal conditions, the permittivity is 81). Under such conditions, water behaves like an organic solvent such as methanol. The last one has a dielectric constant of 32.6 at 25 °C [55, 20].

In recent years, the PLE method has been successfully used to extract polyols from various plant materials, such as pine nuts [56], oak wood [52], mulberry leaves [57], etc. In work [58], liquid extraction under pressure and solid-liquid extraction were used to extract inositols from pine nuts. The obtained inositol concentration using the PLE method reached 5.7 mg/g (conditions: 50°C, 18 min, 3 cycles by 1.5 ml of water each, 10 MPa) and it was higher than the determined inositol concentration for the SVE method — 3.7 mg/g (conditions: room temperature, 2 hours, 2 cycles by 5 ml of water each). Moreover, with the help of PLE, 2 times less solvent was used.

The work [57] compared the effectiveness of the PLE method and traditional extraction using different polar solvents. It was shown that when using methanol, ethanol, water at a temperature of 60 °C and water with ultrasonic treatment for the inositols extraction, their total content was: 3.41; 0.92; 4.7; 5.13 and 3.6 mg/g of dry product, respectively. The PLE method can extract 5.0 mg inositols per g of dry pine nut product, which is close to the content obtained using water at a temperature of 60 °C.

The authors of the work [59] determined the content of myo-inositol extracted with 80% ethanol as 3.8 mg/g of dry soybeans.

Microwave extraction. Microwave extraction (ME) is used to extract polyols from plant material. In work [60], the production of polyols from such plants as pithecellobium sweet or Manila tamarind (*Pithecellobiumdulce*) and artichoke (*Cynarascolymus L.*) was considered.

ME can be an alternative to traditional extraction methods. The advantages are, first of all, significant savings in raw material sample processing time and the amount of solvent used, energy consumption, as well as an increase in extraction efficiency [61]. Microwaves are non-ionizing electromagnetic waves with frequencies ranging from 0.3 to 300 GHz. According to [61], the use of microwaves can disrupt the cellular structure due to the absorption of microwaves by polar molecules, primarily water molecules. The release of heat increases, which increases the diffusion of the solvent into the sample and the extraction of the sample solutions into the solvent.

In work [62], microwave extraction and liquid extraction under pressure were used to extract inositols from artichoke using water as a solvent. ME was carried out under the following conditions: the mass of the artichoke sample m = 0.1–0.3 g, the volume of Milli-Q water 10 ml, the temperature in the range of 50–120 °C, the extraction time 3–30 min. The obtained extracts were immediately cooled, centrifuged at 4,400 g at 4 °C for 10 min and stored in a refrigerator at −18°C until the start of the analysis. The total concentration of inositols (chiro-, scyllo-, and myo-inositol) varied between 6.7 and 11.6 mg/g of dry sample. For the PLE, a dry artichoke weighing 0.3 g was placed in an 11 mL steel extraction set. The extraction was carried out under a pressure of 100 Bar. It was shown that a greater amount of inositol (11.6 mg/g of dry raw material) was extracted by the ME method than by PLE (7.6 mg/g).

Supercritical fluid extraction. Supercritical fluid extraction (SFE) is another environmentally safe method used to extract and concentrate many biologically active components and is a promising alternative to traditional extraction methods. The supercritical state is reached when the temperature and pressure of the fluid rise above their critical point. Fluid carbon dioxide is considered the ideal extractant in SFE, which makes this method effective for the extraction of non-polar compounds. Polar co-solvents such as methanol, ethanol, acetonitrile, acetone, water, ethyl ether or dichloromethane can be added to increase solubility [63]. In work A. Cháfer et al. [64] this method was successfully applied to extract D-pinitol from carob tree pods.

The conditions of sample preparation and subsequent polyols determination in plant materials are summarized in Table 2.

Methods of Polyols Separation and Determination

High performance liquid chromatography. Modern high-performance liquid chromatography (HPLC) is one of the most effective methods of separation and analysis of complex mixtures of non-volatile components, including polyols [20]. It is widely used in biochemistry, molecular biology, and pharmaceutics [54].

In work [68] in 1984, for the first time, the method of determining nanogram quantities of inositol derivatives with p-nitrobenzoate by HPLC on a Porasil column was described. Inositols determination by the HPLC method using refractometric and photometric detectors, as well as by the method of pulsing amperometry without prior derivatization was developed and described [11–12]. The HPLC method with pre-column derivatization using benzoyl chloride was used to determine inositol from aqueous solutions. A linear range was observed in the range of 1.4–89 nmol of myo-inositol [13–14].

In work [69], the separation of polyols was carried out by the method of hydrophilic interaction chromatography (HIC) on a Lichrosphere100 DIOL column, 150 — 4.1 mm, 5 µm using the ACN: H_2O mobile phase (90:10).

In works [70, 71], various polyols were separated on a C_{18} reversed-phase column 250×4.6 mm, 5 µm using the mobile phase ACN: NaH_2PO_4 (20 mM aqueous solution, pH 3.5) in ratio of 70:30. In work [9], columns Lichrospher Si60 and Lichrospher 100 RP18 (250×8 mm, 10 µm) were used. Recently, columns with aminopropyl groups $LunaNH_2$ (250×4.60 mm, 5 µm) have also become popular for the analysis of polyols [72].

In liquid chromatography, for the selective separation of substances in addition to the correctly selected stationary phase, the correct choice of the corresponding mobile phase is important. Universal mobile phases in reversed-phase HPLC are mixtures of organic solvents (acetonitrile, methanol) with water [73, 74]. To avoid dissociation of polyols during chromatography and to improve the shape of the peaks, 0.1% formic acid [75], trifluoroacetic acid [73] or 5–100 mmol of ammonium acetate [74] were added to the mobile phase.

To detect polyols, the following are used: a refractometric detector (RD) [54], a UV detector [71, 74], an evaporative light scattering detector (ELSD) [73, 66, 72], an electrochemical detector (ECD), a photodiode

Table 2. **Conditions of sample preparation and polyols determination in plants**

Name of the plant / Polyols	Conditions of sample preparation	Methods	Literature
Carob tree pods / pinitol, myo-inositol, D-chiro-inositol	– Extraction with water at a temperature of 50 °C (2 hours). Addition of 0.01% (v/v) dry yeast at a temperature of 30 °C (7 days). – Removal of residual sugars in a cartridge (120×1.5 cm) filled with Dowex 1-X8 anionite in OH^- form, 100–200 mesh. – Recrystallization of the obtained pinitol crystals using ethanol. – Separation of various polyols from the mother liquor using cationite Dowex 50W-X4 in Li^+ form, 50–100 mesh, and further elution with 87% ethanol. – Recrystallization of myo-inositol and D-chiro-inositol using ethanol. – Derivatization by silylation with trimethylchlorosilane and N-methyl-N(trimethylsilyl) heptafluorobutyramide.	TLC GC, GC– MS	[65]
Pine nut / Muco-inositol chiro-inositol scyllo-inositol myo-inositol	PLE – Extraction with hot water (under a pressure of 10 MPa, at a temperature of 50 °C, time 20 min). – Mixing with a 70% ethanol solution of phenyl-β-D-glucoside. – Conversion into trimethylsilyl oxime derivatives	GC — MS	[56]
Artichoke / Chiro-inositol, scyllo-inositol, myo-inositol	ME – Dried artichoke outer leaves (0.1–0.3 g) and 10 ml of ultra-pure water were transferred to a vessel with a capacity of 100 ml (temperature 50–120 °C, extraction time 3–30 min). PLE – Dried artichoke leaves (0.3 g) were placed in an 11 mL stainless steel extraction vessel between layers of sand. – Extraction solvent — Milli-Q water, one-time static extraction cycle (pressure — 10 mPa).	GC — FID	[62]
Soybean roots / Pinitol, myo-inositol, D-chiro-inositol	– Extraction of frozen roots with 80% ethanol at a temperature of 70 °C. – Derivatization with trimethylsilylimidazole. – Addition of sodium sulfate and dilution with toluene	GC — MS	[59]
Argyrolobium pink / D-pinitol	– Extraction with 95% ethanol (15 h). – Washing with hexane to remove non-polar accompanying substances. – Pass through a cartridge filled with 520 g of silica gel, 60–120 mesh, eluent EtOAc. – Elution with a mixture of EtOAc: MeOH (1: 1) solvents. – Recrystallization of fractions in hot ethanol.	^{1}H NMR	[66]
Mountain Slime / Myo-inositol	– Extraction with 80% ethanol at a temperature of 60–70 °C (2 days). – Further purification using the RPLobar-Acolumn cartridge, 45% MeOH eluent.	FAB — MS NMR	[67]

Notes. TLC — thin–layer chromatography, GC — gas chromatography, MS — mass spectrometry, FID — flame ionization detector, NMR — nuclear magnetic resonance, FAB — fast atom bombardment, EtOAc — ethyl acetate, MeOH — methanol, *n*-BuOH — *n*-butanol.

array detector (PDA) [76] and mass selective detector (MSD) [77].

Polyols do not absorb UV radiation in the range of 200–400 nm [73, 78–79] These compounds can be detected using a UV detector only at a wavelength of 195 nm when using the purest (gradient mode) mobile phases.

In work [7], polyphosphate inositol isomers were detected using a UV detector in the form of orthophosphates, using a dephosphorylation reaction in an enzyme-loaded post-column reactor.

Pre-column polyols derivatization with benzene-containing chromophoric reagents containing a benzene ring, such as sodium benzoate and 2,4-dinitrobenzoate, allows detection in the near UV range [80]. The use of *n*-nitrobenzoyl chloride for polyols derivatization made it possible to quantitatively determine their derivatives, which absorb intensively at 231 nm [81–82].

The evaporative light scattering detector (ELSD) is used to detect substances that do not absorb UV radiation. For example, ELSD was

used to determine inositols and methyl-inositol derivatives extracted from sea buckthorn berries after their separation on a $LunaNH_2$ column (250×4.60 mm, 5 μm) [72]. Higher sensitivity and selectivity for polyols were obtained by combining high-performance anion exchange chromatography with a photodiode array detector (PDA) [83, 84]. ELSD and PDA have the same order of detection limit when determining inositols: the detection limit of myo-inositol when using ELSD [69] is 9 mg/l, when using PDA [78] — 1.67 mg/l. When using an UV detector, the detection limit for D-pinitol is 99.69 mg/l [59]. The linear range for the UV detector is within 10–100 mg/l, for PDA — 60.4–302.0 mg/l, for ELSD — 2.79 mg/l for inositol [85].

The most selective and sensitive detector for the polyols determination is a mass detector. Thus, the HPLC use in the combination with a mass detector made it possible to simultaneously obtain information on the type, number and molecular mass of ions [77]. Electrospray ionization (ESI) of molecules is the most common in the polyols analysis [86]. In work [87], the HPLC method was combined with a triple tandem quadrupole spectrometer (MicroMassQuattro, Waters, Manchester, Great Britain) in negative ion mode, using the following parameters: 3.2 kV capillary, temperature of 150 °C source, desalination temperature of 350 °C, voltage cone of 20 V, cone gas flow rate of 90 l/h and desolvation gas flow rate of 900 l/h. The mass spectra of myo-inositol in the deprotonated form [M-H]- show such molecular ions as 178.8; 160.8, and 86.4.

Also, in the analysis of polyols, HPLC is used in combination with electrochemical detection (potentiometry and amperometry) [83]. Several methods based on electrochemical oxidation using various metal electrodes are known. Copper, nickel, silver and chemically modified electrodes were developed for the electrocatalytic carbohydrates oxidation in an alkaline medium. In the last variant, high concentrations of sodium hydroxide (0.1–0.7 mol/l) were created in the mobile phase isocratically or in a gradient with water containing or not containing the same modifiers.

The combination of pulsating amperometry with a gold working electrode, the surface of which catalyzes the oxidation of –COH groups in solutions with a high pH value, provides selective and sensitive myo-inositol determination [84].

An electrode modified by anodic electrosedimentation of cobalt oxyhydroxide on a polished glassy carbon surface was used to detect inositol, xylitol, sorbitol, and mannitol in a mixture of compounds. It is known that in an alkaline medium, high degrees of cobalt oxidation in its hydroxide indicate electrochemical activity for the electrooxidation of polyatomic organic compounds and, thus, the catalytic oxidation of aldols occurs at potentials above 0.3 V using a saturated calomel electrode as a reference electrode.

Copper electrode wires were used for the simultaneous detection of sugars, polyols, and carboxylic acids by HPLC with amperometric or potentiometric detection, and aqueous solutions of acids were used as eluents for the separation of carbohydrates and carboxylic acids in ion-exclusion chromatography with potentiometric or amperometric detection. In this case, sodium hydroxide was added to the eluate [88].

Table 3 shows examples of the HPLC use for the polyols determination in various plants.

Planar chromatography. Along with liquid column chromatography and gas chromatography, the separation of mixtures of organic substances of natural origin is carried out both in a thin layer (TLC) of a sorbent and on a paper [91].

Thus, TLC was used to separate inositol phosphates [92]. 25 samples were applied to a silica gel plate with dimensions of 20×20 cm. A mixture of chloroform, methanol and 30% ammonia (3:10:5, vol) was used as an eluent. To detect myo-inositol 1-phosphate, the plate was treated with the reagent for 24 hours. The reagent was prepared by mixing sodium fluorescein (0.15 g in 15 ml of 95% methanol) and lead tetraacetate (1 g in 33 ml of glacial acetic acid). Fluorescence intensity was recorded from 0.1 to 50 nmol of myo-inositol 1-phosphate. Di- and triphosphates of myo-inositol do not interfere with the determination of myo-inositol 1-phosphate in this way.

TLC determination of D-pinitol was performed on a plate with a layer of silica gel. The plate was lowered into a chamber with a mobile phase of ethyl acetate : methanol (3:2). After chromatography, the plate was dried in an oven at 100 °C for 15 min. The dried plate was immersed in an iodine solution to develop separated spots. The R_f values (short for “retention factor”) of the spots were calculated. After treatment the TLC plate with iodine solution, two yellow spots were obtained with different R_f values of 0.75 and 0.59, respectively. According to the standard,

Table 3

Determination of plant polyol by HPLC

The name of the plant	Found polyols	Column (d, mm×L, mm; grain diameter, μm) / Detector	Solvents (vol:vol or gradient)/ Flow rate	t, °C	Literature
Sea buckthorn berries	Myo-inositol, chiro-inositol	LunaNH$_2$ (4.60×250; 5) /ELSD	CH_3CN:H_2O: CH_3OH (85:10: 5) / 1.2 ml/min	45	[72]
Pitaya	Myo-inositol	ZorbaxEclipseXDBC 18 (2.1×150; 3.5) / UV-visible detector	H_2O:CH_3OH (5:95), 0.1% of formic acid / 0.3 ml/min	40	[74]
Tinospora heartleaf	Myo-inositol	Zorbax-NH$_2$ (4.5×250; 5) / ELSD	CH_3CN:H_2O Gradient: CH_3CN 86% (0–10 min), CH_3CN linear to 88% (10-20 min), 78% (20–30 min), 74% (30–40 min), 86% (40–50 min) and getting out of balance (50–60 min) / 0.5 ml/min	25	[89]
Tatar buckwheat	D-chiro-inositol	Prevail Carbohydrates ES (4.6×250; 5) / ELSD	CH_3CN:H_2O Gradient: CH_3CN 80% (20 min), CH_3CN 65% (20–50 min) / 1 ml/ min	95	[73]
Tung beans	D-chiro-inositol	Prevail carbohydrates ES (4.6×250; 5)/ELSD	CH_3CN:H_2O (70:30) / 1 ml/min	95	[90]

the R_f value of 0.75 given in the literature is close to the D-pinitol R_f value [93]. The spots with R_f 0.59 are due to the presence of other substances remaining in the mixture with the isolated pinitol, which are not determined in this work [71].

Gas chromatography. Gas chromatography (GC) is a method of polyols separation and quantification, which provides high resolution and low detection limits (DL). Since polyols are non-volatile substances, derivatization is necessary to convert them into volatile compounds before gas chromatographic determination. Polyols are usually converted to volatile trimethylsilyl derivatives. Silylation reactions include three main components: a silylating reagent, a solvent, and a catalyst. For the polyols derivatization, various silylation reagents are used: hexamethyldisilazane (HMDS) [92, 78], trimethylsilylimidazole [59, 94] and trimethylsilyl oximes [57]. In works [95–97], myo-inositol was setimated as trifluoroacetyl derivatives or hexa-o-trimethylsilyl ethers.

For the studying the Ca^{2+} ions transport, a method of inositol 1,4,5-triphosphate extracting from alfalfa which perform a signaling function in a plant cell was described [98]. The determination of polyols content, including myo-inositol, in leaves, somatic embryos, and ripe alfalfa grains can be performed by GC-MS [58]. A method of derivatization and determination of myo-inositol in excreta of honey aphids that collect juice from alfalfa flowers is described [100].

The mass spectrometric detector is the most convenient for the detection of polyols, given its high sensitivity, selectivity and versatility, its main disadvantage is its high cost. The flame ionization detector (FID) has become widely used in gas chromatography due to a number of advantages, such as a wide linear concentration range, low cost and low maintenance requirements (compared to a mass spectrometer). H. Kallio et al. [72] reported the use of GC-MS and GC-FID for the analysis of chiro-inositol and myo-inositol, which were extracted from sea buckthorn berries. Trimethylsilyl derivatives were obtained using hexamethyldisilazane and trimethylchlorosilane in pyridine.

Trimethylsisyl derivatives analysis by GC method was carried out using a Varian 3300 GC equipped with a flame ionization detector using a 30 m × 0.25 mm × 0.25 μm capillary column (Supelco, Bellafonte, PA) with a nonpolar phase. 1 μl of the sample was injected in the split flow mode (1:20). The temperature mode was: the temperature of −210 °C for the injector, the temperature of −290 °C for the detector, the temperature from 90 °C (2 min) to 275 °C (10 min), the temperature change rate

was 4 °C/min for the thermostat. The average flow of helium carrier gas was 1.0 ml/min.

The same samples were also analyzed using a Shimadzu 17 A gas chromatograph with a QP 5000 MSD (Shimadzu, Kyoto, Japan). A DB-1MS capillary column with dimensions of 30 m × 0.25 mm × 0.25 μm (J&W Scientific/ Agilent, Folsom, CA) was used. The column temperature program was the same as for GC-FID analysis, and mass spectra were obtained in the mass range m/z 40–400 with an energy of 70 eV. Chromatograms were recorded in the monitoring mode of the total ion current (TIC) or in selected ion monitoring (SIM). Chromatograms in SIM mode showed molecular ions 247 and 260, which confirmed the presence of methylinositols [60].

Conde et al. [101] used gas chromatography with a time-of-flight mass spectrometer (GC-TOF) to study the effect of water deficit on the polyols accumulation in grape berries and leaves. Ten polyols were identified and quantified as mannitol, sorbitol, galactinol, myo-inositol, glycerol and dulcitol. After extraction and derivatization, the samples were introduced into the chromatograph in split-flow mode in a cold injection system and analyzed by GC using a Rtx 5SilMS column with dimensions of 30 m × 0.25 mm × 0.25 μm. Helium was used as a carrier gas; the flow rate was 1 ml/min, the temperature ranged from 50 °C (1 min) to 330 °C (5 min), and the temperature change rate was 20 °C/min. Ion source ionization was performed by electron impact ionization at a temperature of 250 °C, and voltage of 70 V [103].

The conditions for the polyols separation by gas chromatography are presented in the Table 4.

Identification Methods

NMR spectroscopy. The structures of some common polyols, including inositols, can be confirmed using ^{13}C and ^{31}P NMR spectroscopy. Thus, myo-inositol-1,3,4,5,6-pentakisphosphate, extracted from erythrocytes of birds, was identified by these methods after chromatographic separation of inositol polyphosphates on a Dowex 1×2 cm column [105]. Trigalactosylcyclitols, as well as digalactosylmyo-inositol, trigalactosylmyo-inositol and trigalactosyl D-chiro-inositol from buckwheat bran, were identified by 2D NMR on ^{1}H nuclei [105]. The use of the ^{31}P NMR method made it possible to identify the complex of myo-inositol-1,2,6-trisphosphate with Zn(II) ions, as well as to show the influence of the phosphate groups position on the Zn(II) ions coordination [107].

4-Hydroxiphenlyacetate derivatives of inositol were separated and identified by the UPX method using ^{1}H NMR spectroscopy [107]

In [66], prior to NMR spectroscopy for the D-pinitol determination the following sample preparation was performed. *Argyrolobium roseum* dried plant material with a mass of 500 g was kept in 2 liters of 95% ethanol in a percolator for 15 h at the temperature of the environment. The solvent was drained and the process was repeated four times. All portions with a sum volume of 8 l were combined, filtered and evaporated to dryness with a mass of 36 g, which was washed with hexane (4×500 ml) to remove non-polar impurities of extract. After the wash, the dry residue was obttained with a weight of 26 g. HPLC-MS with electrospray ionization (ESI) was performed using a column with silica gel (520 g, 60–120 mesh) and ethyl acetate as eluent. The polarity of the mobile phase increased with the gradient addition of methanol. The obtained fractions in the ethyl acetate:methanol (1:1) system were combined and evaporated on vacuum rotors. In this way, a dry residue weighing 9.5 g was obtained, which was recrystallized in hot ethanol, followed by obtaining a pure compound weighing 5.2 g. Pyrazinamide was added as a reference substance. Pinitol and pyrazinamide were weighed, and a solution was prepared in deuterated water with a concentration of 2 mg/ ml. The signal of the methoxy group in pinitol, integrated as three protons, has a chemical shift of 3.46, and the weak-field proton (H-3) of pyrazinamide with a chemical shift of 9.05 was taken into account for quantification.

Conclusions

Inositols and their derivatives belong to cyclic polyols. They are biologically active compounds with a wide spectrum of action, and therefore there is an urgent need to develop modern methods of their extraction. The analysis of literary sources shows the high interest of scientists in the development of valuable biotechnological processes for the production of polyols, including inositols, from plant raw materials and microorganisms.

Various solvents, mostly polar, are used to extract polyols from vegetable raw materials in a wide range of temperatures and pressures, the maximal temperature is 200 °C, the pressure during extraction can reach 10 MPa.

Table 4

The conditions for the polyols separation by gas chromatography

The name of the plant	Found polyol	Conditions / Derivatizing reagent / Detector	Column (L, m × d, mm), adsorbent layer thickness, μm / thermostat column temperature program	Lite-ratu-re
Soya	Pinitol, myo-inositol, D-chiro-inositol	Trimethylsilyl-imidazole /MSD	RTX 1 (60×0.25) 0.25 / 80 °C (1 min) up to 180 °C, 20 °C/min, up to 240 °C, 3 °C/min, up to 300 °C (1 min), 20 °C/min	[59]
Mulberry leaves	Myo-inositol, galactinol	1. The dried extract was dissolved in pyridine (2.5% with hydroxylamine chloride) at a temperature of 75 °C for 30 min. 2. Silanization with GMDS at a temperature of 45 °C for 30 min /MSD	TBR-1 (30×0.25) 0.25/ 100 °C to 200 °C (15 min), 15 °C/min	[57]
Black-eyed pea, buckwheat, carob pods, chickpeas, pea grass, lentil, soya	Inositols methyl-inositol, glycosyl-inositol	1. The dried extract was dissolved in pyridine (2.5% by hydroxylamine chloride) at a temperature of 75 °C for 30 minutes. 2. Silanization with GMDS at 45 °C for 30 minutes/ MSD	HT5 (25×0.22, 0.1) / 180 °C (10 min) up to 200 °C (15 min), 5 °C/ min, up to 270 °C, 15 °C/ min, up to 290 °C, 1 °C/ min, up to 300 °C, 15 °C / min, (15 min), up to 360 °C, 15 °C/ min (15 min)	[56]
Outer leaves of artichoke	Chiro-inositol, myo-inositol and scyllo-inositol	1. The dried extract was dissolved in pyridine (2.5% with hydroxylamine chloride) at a temperature of 75 °C for 30 minutes. 2. Silanization with GMDS at 45 °C for 30 min / FID	ZB-1MS (30×0.25) 0.25 / 200 °C (14.5 min) up to 220 °C (3 min), 20 °C/min, up to 270 °C (20 min), 25 °C/min	[62]
Carrots	Scylo-inositol and myo-inositol	1. The dried extract was dissolved in pyridine (2.5% by hydroxylamine chloride) at a temperature of 75 °C for 30 minutes. 2. Silanization with GMDS at a temperature of 45 °C for 30 min / MSD, FID	CP-SIL5CB (25×0.25) 0.25 / 180 °C (36 min), up to 300 °C (50 min), 10 °C/min	[103]

Hexamethyldisilazane, trimethylsilyl-imidazole, trimethylsilyl oximes and trimethylchlorosilane are most often used as reagents for derivatization of polyols followed by their chromatographic separation.

When separating and determining polyatomic alcohols by the GC method, the following detectors are used: evaporative light scattering detector, flame-ionization, and time-of-flight mass detector.

Compared to HPLC, the GC method for the determination of inositols has better sensitivity, but it requires prior derivatization to obtain volatile substances.

Identification of the polyols structure is carried out using the NMR method on ^{13}C, ^{31}P, ^{1}H nuclei.

Author Contributions

“Conceptualization, V.R. and O.V.; resources, O.M. and A.B.; data curation, O.L.; writing—original draft preparation, V.R.; writing—review and editing, N.K.; visualization, A.B. and O.L.; supervision, V.R. All authors have read and agreed to the published version of the manuscript.”

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Conflicts of Interest

The authors declare no conflicts of interest.

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ІНОЗІТОЛИ: БІОЛОГІЧНА РОЛЬ І ЗАСТОСУВАННЯ, МЕТОДИ ДОБУВАННЯ З РОСЛИННОЇ СИРОВИНИ ТА ВИЗНАЧЕННЯ, БІОТЕХНОЛОГІЧНИЙ СИНТЕЗ

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Мета. Провести аналіз методів, які впродовж останніх років застосовують для виявлення, екстрагування та кількісного визначення поліольних сполук з рослинної сировини.

Матеріали й методи. Інозітоли видобувають з рослинної сировини методами рідинної екстракції, під тиском, мікрохвильової екстракції та надкритичної флюїдної ектракції. Розділяють та кількісно визначають інозітоли метолдами високоефективної рідинної, планарної та газової (з попередньою дериватизацією) хроматографії. Структуру деяких інозитолів можна встановлювати методом ЯМР спектроскопії.

Результати. Поліольні сполуки беруть участь у регуляції рівня внутрішньоклітинного кальцію, передавання гормональних сигналів, розщеплення жирів і зниження рівня холестерину в крові, модуляції активності нейротрансмітерів; їх застосовують у виробництві вітамінних препаратів,

Найважливішими біологічно активними поліольними сполуками є інозитоли. Основним джерелом, з якого екстрагують інозитоли, є рослинна сировина — люцерна, паростки пшениці, грейпфрут, фундук та інші. У роботі розглянуто методики екстрагування поліолів органічними та неорганічними розчинниками, у тому числі із застосуванням апарату Сокслета, рідинну екстракцію під тиском, мікрохвильову екстракцію та надкритичну флюїдну екстракцію. Описано процедуру попередньої пробопідготовки та дериватизації поліолів для їх подальшого розділення та кількісного визначення. Проаналізовано сучасні хроматографічні методи якісного та кількісного визначення поліолів. Описано можливість застосування ^{1}H ядерного магнітного резонансу (ЯМР), ^{13}C- та ^{31}P-ЯМР-спектроскопії для ідентифікації структури поліольних сполук, насамперед інозитолів.

Висновки. Інозітоли — біологічно активні сполуки широкого спектру дії, тому існує нагальна потреба в розробленні біотехнологічних процесів їхнього виробництва і добування із рослинної сировини та мікроорганізмів.

Ключові слова: поліоли, інозітоли, екстракція, хроматографія, спектроскопія ЯМР, біотехнологічний синтез.