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# DEVELOPMENT OF STANDARDIZATION METHODOLOGY OF ELECAMPANE RHIZOMES AND ROOTS (INULA HELENIUM L.) FOR THE HYDROXYCINNAMIC ACIDS CONTENT

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**Summary:** the methodologies of rhizomes with roots elecampane standardization were developed through the identification and quantification of hydroxycinnamic acids. To identify the raw materials it was proposed to establish the presence of four hydroxycinnamic acids zones, including chlorogenic and chicoric in TLC profile. The results of hydroxycinnamic acids content determination for industrial and wild samples of raw materials are in the range of 1.10–1.35 %. The developed methodologies allow distinguishing two types of materials – elecampane rhizome and roots and chicory roots, which have a high content of inulin and are similar in crushed condition.

**Key words:** Inula helenium, roots and rhizomes, hydroxycinnamic acids, chlorogenic acid, chicoric acid, standardization.

**Introduction.** The elecampane rhizomes and roots are an officinal medicinal plant raw material (MP), which belongs to a group of medicines that stimulate expectoration with indication in the instruction for use – for upper respiratory tract and lungs diseases (bronchitis, tracheitis, catarrh of the upper respiratory tract); diseases of the gastrointestinal tract (gastritis, enterocolitis, lack of appetite, poor digestion). According to the literature about the analysis and standardization of elecampane rhizomes and roots, this usage is associated with high content of inulin and other fruitosans [1–7]. Numerous studies indicate other kinds of activities of MP and its various extracts–antidiabetic, antioxidant, bacteriostatic, antifungal [8–11], which is associated with the content of polyphenolic compounds, flavonoids, hydroxycinnamic acids. Considering insufficient level of elecampane rhizomes and roots standardization – absence of a monograph in State Pharmacopoeia of Ukraine and leading world pharmacopoeias, it is time to revise quality criteria for this raw material.

Therefore, the object of this study was to develop a methodology of elecampane rhizomes and roots standardization for the hydroxycinnamic acids content.

**Materials and methods.** Research of the quality and quantity content of hydroxycinnamic acids was carried out on three industrial series of MP (JSC "Liktravy" Zhytomyr, Ukraine, samples 1–3 correspond to the series 20315, 30915, 10116) and three growing wild samples of MP (samples 4–6 , collected in Ternopil region).

The methods of thin layer chromatography (TLC) and absorption spectrophotometry in the UV and visible region of the spectrum were used in this work. Chromatographic plates Silica gel 60 $F_{254}$ ("Merck", Germany), chromatographic chamber "CAMAG", an instrument for spotting Linomat 5 ("CAMAG", Switzerland), a lamp for observing chromatograms in ultraviolet light "CAMAG" were used for investigations by TLC method.

The study of the quality content of hydroxycinnamic acids was carried out in methanol extracts of raw materials. The study was conducted using mobile phases: formic acid – water – ethyl acetate (6: 9, 90) and anhydrous formic acid – water – ethyl acetate (10:10:80). The chromatograms observation was performed in ultraviolet light with a wave-length of 365 nm after their sequential processing with 10 g/l solution of diphenylboric acid aminoethyl ester and 50 g/l solution of macrogol 400 in methanol.

Standard samples of hydroxycinnamic acids: chlorogenic acid (Fluka), caffeic acid (Fluka), rosmarinic acid (Fluka), chicoric acid (pharmacopoeia standard sample (SPU CRS)), ferulic acid (Sigma); flavonoids: rutin and hyperoside (SPU CRS) were used for identification. Exact masses of standard samples were dissolved in appropriate volumes of methanol.

Quantitative determination of hydroxycinnamic acids was defined by absorption spectrophotometry in the UV and visible region of the spectrum, using Carry-50 spectrophotometer (Varian, USA).

Reagents of cleanliness qualification were used (ethyl acetate, formic acid, water, diphenylboric acid aminoethyl ester, macrogol 400) that meets the requirements of SPU for appropriate methods of analysis, reagent solutions were prepared according to SPU methodologies.

**Results and discussion.** On the chromatogram of methanol extract of the elecampane roots four intensive zones appear, the colour of fluorescence of which indicates that the corresponding to them substances belong to the hydroxycinnamic acids. In particular, on the

chromatogram of the test solution a very intense zone with blue fluorescence and intense with greenish-blue fluorescence appear that are at chlorogenic and chicoric acids levels respectively on the chromatogram of reference solution. The two other zones with intense greenish-blue fluorescence are detected in the chromatogram of test solutions between chlorogenic and chicoric acids zones. Weak zones of blue fluorescence in the bottom of the chromatogram, located below the zone of chlorogenic acid and near the starting line were observed additionally besides the already described.

The samples of raw materials have been generally similar in the composition of hydroxycinnamic acids – the number, position, colour and relative intensity of fluorescence (within one sample) of hydroxycinnamic acids zones are similar for all samples, in particular, for some elecampane samples, and are presented in Figure 1. The chromatogram of the fifth sample of raw materials was slightly different and contained an additional intense zone of blue fluorescence at the bottom of the chromatogram.

Additionally, the chromatography of methanol extract of chicory roots, which are the similar to the biologically active substances composition of elecampane raw materials, has been done. In chicory roots chlorogenic and chicoric acids in significant quantities, caffeic acid are identified. Between chlorogenic and chicoric acids zones there is a zone with weak fluorescence. On the chromatogram of the test solution for all elecampane samples in this area there are two intense zones that are located nearby. Thus, the proposed chromatographic conditions to identify elecampane raw materials allow to establish its identity and distinguish it from chicory roots.

According to the authors [10] in ethyl acetate extract of elecampane roots chlorogenic, caffeic, dicaffeoylquinic, 3-feruloyl-4-caffeoyl-quinic acids were identified, and their content was relatively high. According to the Polish authors [11] in 70 % methanol-water extract of elecampane callus culture chlorogenic and neochlorogenic acids (0.1%), 1.5– and 3.5-di-O-caffeoylquinic acids (0.3 %) were identified; in total, sixteen acid derivatives of caffeic acid, including four dicaffeoyl aldarates (0.085 %), three tricaffeoyl aldarates (0.608 %) and tetracaffeoyl aldarate (0.077 %) were identified. The results of chlorogenic acid identification in the studied samples of raw materials correlate with data [10, 11]. The com-

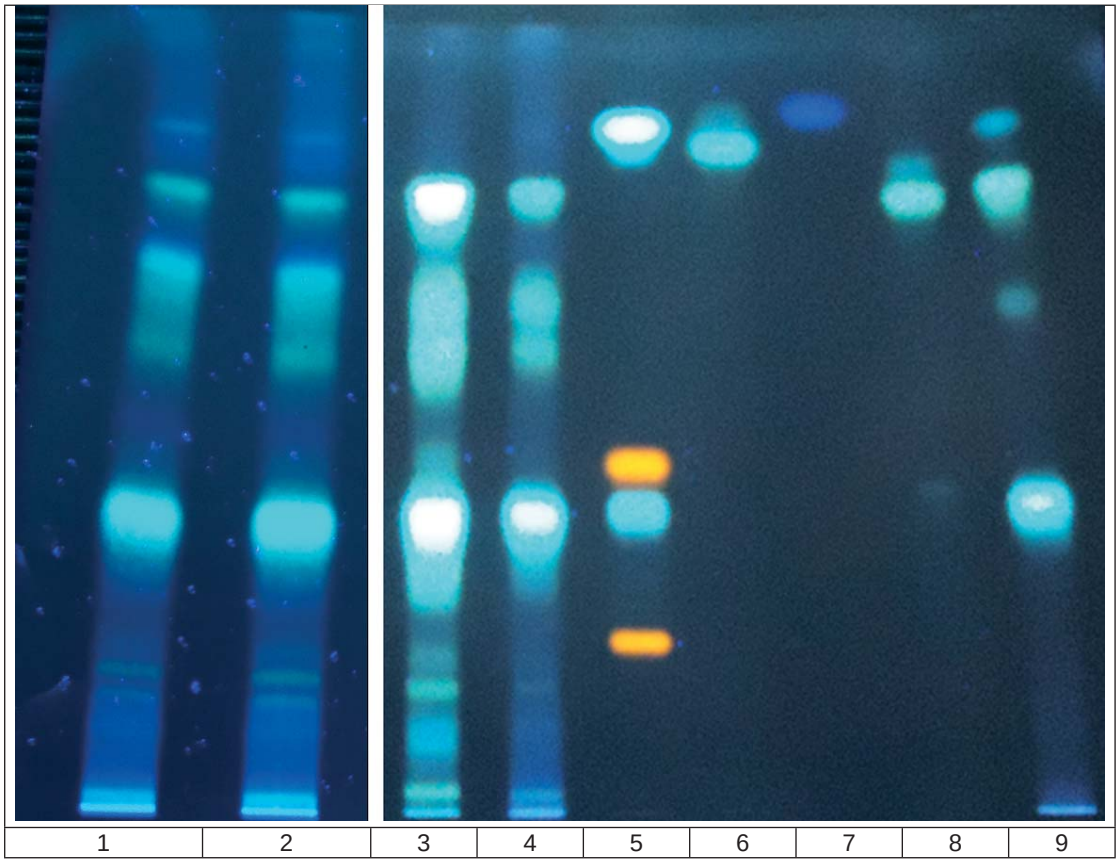


**Fig. 1.** The chromatogram in conditions of hydroxycinnamic acids identification, obtained after sequential processing by solutions of aminoethyl ester of diphenylboric acid and macrogol 400, when viewing in UV light at 365 nm wavelength. Tracks: 1–4 – test solutions for elecampane samples 1, 2, 5 and 6; 5 – reference solution (rutin, chlorogenic acid, hyperoside, caffeic acid – a bottom-up); 6 – reference solution of rosmarinic acid; 7 – reference solution of ferulic acid; 8 – reference solution of chicoric acid; 9 – test solution of chicory roots.

position in local elecampane samples of chicoric acid (dicaffeoyltartaric acid) correlates with the authors' data [11] as for the presence of dicaffeoyl aldarates.

Thus, based on the results of chromatographic researches, taking into account the literature data on the hydroxycinnamic acids composition [1, 5, 10, 11] and biological activity of aqueous-alcoholic extracts of this MP [8] and hydroxycinnamic acids, such as [12–15] it was proposed to identify these substances during the standardization of rhizomes with roots elecampane raw materials.

***The methodology of hydroxycinnamic acids identification in elecampane rhizomes with roots.***

***Test solution.*** Place 1.0 g of the powdered raw material into a 50 ml flask and add 25 ml of methanol. The solution is being refluxed on a water-bath for 45 minutes. Then it is cooled and filtered.

***Reference solution.*** 2.5 mg of the standard samples of chlorogenic, chicoric and caffeic acids is dissolved in 10 ml of methanol.

***Plate:*** TLC silica gel plate.

***Mobile phase:*** formic acid – water – ethyl acetate (10:10:80 V/V).

***Application:*** 30 µL of the test solution and 5 µL of the reference solution, as 10 mm bands.

***Drying:*** in air.

***Distance that a mobile phase should pass:*** 12 cm.

***Drying:*** at the temperature of 100–105° C for 10 minutes in the drying cabinet.

***Detection:*** the hot plate is sprayed with a 10 g/l diphenylboric acid aminoethyl ester methanol solution, dried in air and then sprayed by 50 g/l solution of macrogol 400 in methanol; then it is dried in air for about 30 min and examined in ultraviolet light at 365 nm of wavelength.

***Results:*** on the reference solution chromatogram a fluorescent blue zone corresponding to chlorogenic acid, a greenish-blue fluorescent zone of chicoric acid and a blue fluorescent zone corresponding caffeic acid should be identified (in order of retardation factor ($R_f$) increasing).

On the test solution chromatogram two fluorescent zones should be detected - at chlorogenic (most intense) and chicoric acid levels on the reference solution chromatogram, corresponding them by color and fluorescence as well as two intense zones of greenish-blue fluorescence between chlorogenic and chicoric acids zones. See below the sequence of zones, present in chromatograms obtained for the reference solution and the test solution (fig. 2). Furthermore, others weak zones may be present in the chromatogram of the test solution.

In electronic absorption spectra of water-alcohol extracts of different series elecampane rhizomes and roots the characteristic for hydroxycinnamic acids absorbance curve with maximum at a wavelength of 325 ± 2 nm was observed. In particular, the electronic absorption spectra of 1, 2 and 4 elecampane samples in conditions of the hydroxycinnamic acids study are presented in Figure 3.

| Top of the plate | |
|---|---|
| Caffeic acid: blue fluorescent zone | |
| Chicoric acid: greenish-blue fluorescent zone | greenish-blue fluorescent zone (intense)<br>greenish-blue fluorescent zone<br>greenish-blue fluorescent zone |
| Chlorogenic acid: blue fluorescent zone | blue fluorescent zone (the most intense) |
| **Reference solution** | **Test solution** |

**Fig. 2.** TLC scheme of chromatogram in conditions of elecampane hydroxycinnamic acids identification.

It should be mentioned that the electronic absorption spectrum appearance for the elecampane rhizomes and roots (Fig. 3) and chicory roots (Fig. 4) are different. This fact may be another additional identification marker when establishing identity of the studied raw materials.

The nature of the spectrum, in particular the presence of clear absorption maximum at 325 nm wavelength, gives for hydroxycinnamic acid determination by direct measurement of absorption in the maximum followed by calculation of content by using specific absorption index. Chlorogenic acid was chosen as the standard for calculating the amount of hydroxycinnamic acids content, despite the fact that it is present in elecampane rhizomes with roots both according to the literature data and to the results of the chromatographic research of industrial and growing wild raw samples.

In developing the methodology of quantitative determination of the hydroxycinnamic acids amount it was found that in order to ensure the fullness of their extraction ethanol should be used as extractant (50 % V / V).

***The methodology of quantitative determination of hydroxycinnamic acids amount in elecampane rhizomes with roots.***

***Stock solution.*** To 1 g (exact sample) of the powdered sample 60 ml of ethanol (50 % V / V) is added. The solution is being refluxed on a water–bath for 45 min. It is cooled and filtered into a 100 mL volumetric flask. The extraction is repeated twice, using 20 and 15 ml of ethanol (50 % V / V) R, refluxing on a water bath for 15 minutes each time. It is cooled and filtered into the same volumetric flask. The filter and the flask are rinsed with ethanol (50 % V/V), combining the filtrate and the rinsing solutions and making the volume of the solution up to 100.0 ml with ethanol (50 % V/V).

***Test solution.*** 2.0 ml of stock solution is made up to 25.0 ml with ethanol (50 % V/V).

***Compensation solution.*** Ethanol (50 % V/V).

The optical density of the test solution at 325 nm is measured relatively to the compensation solution.

The hydroxycinnamic acids amount (X, %) expressed as a chlorogenic acid, is calculated according to the formula:

$$X = \frac{25 \cdot A \cdot 100 \cdot 100}{2 \cdot E \cdot m \cdot (100 - W)},$$

where A – absorbance of the test solution;

m – mass of sample in g;

W – content of wet, in %;

E – specific absorption index of chlorogenic acid at 325 nm (E = 531).

The investigated samples of elecampane rhizomes with roots were analyzed with the help of this methodology, the results are presented in the table.

As can be seen from these results, the investigated samples contain the hydroxycinnamic acid in an amount of slightly more than one percent. These data are generally lower than reported by the authors [6] – 2 %. The

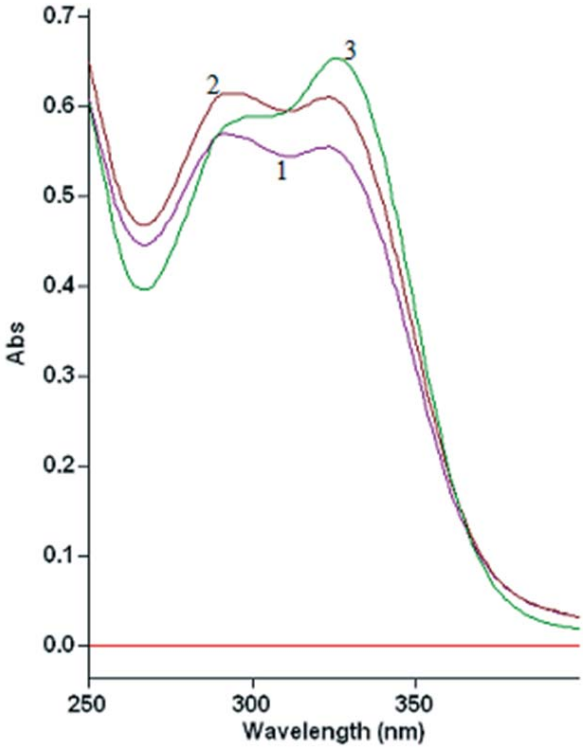


**Fig. 3.** The electronic absorption spectra of the test solution of elecampane rhizomes and roots extracts (curves 1-3 correspond to 1, 2 and 4 samples of raw materials, ethanol (50% V/V)).

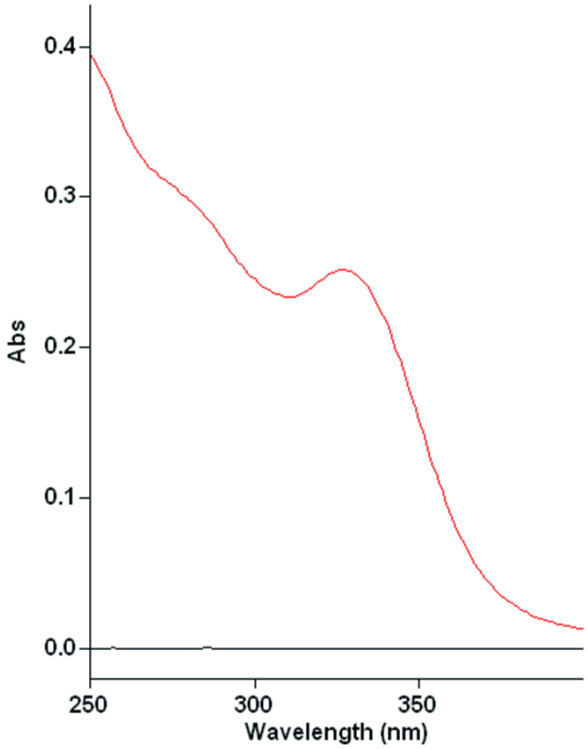


**Fig. 4.** The electronic absorption spectrum of the chicory roots extracts (ethanol (50 % V / V)).

**Table.** The results of determination of the hydroxycinnamic acids amount in elecampane rhizomes with roots

| Sample | Content of hydroxycinnamic acids (%), expressed as a chlorogenic acid and dry raw material |
|---|---|
| Series 20315, JSC "Liktravy" | 1.10 ± 0.02 |
| Series 30915, JSC "Liktravy" | 1.23 ± 0.03 |
| Series 10116, JSC "Liktravy" | 1.30 ± 0.02 |
| Growing wild, Ternopil region, Zbarazh | 1.24 ± 0.03 |
| Growing wild, Ternopil region, Berezhany | 1.35 ± 0.02 |
| Growing wild, Ternopil region, Podvolochisk | 1.20 ± 0.02 |

difference in the content can be explained from different perspectives - the results of hydroxycinnamic acids determination of wild raw growing in central regions of Ukraine are shown in the work [6], but the extractant used, the conditions of sample preparation and method of final determination are not listed. The total content of hydroxycinnamic acids defined in local raw material is comparable to the content of hydroxycinnamic acids and their derivatives, obtained in elecampane callus culture [11].

**Conclusions.** As a result of the development of standardisation methodology of elecampane rhizomes and roots additional indicators of their quality – the identification and quantification of hydroxycinnamic acids are proposed.

1. It was proposed to identify the investigated raw material comparing TLC profiles of elecampane methanol extract and standard substances solution. Chlorogenic and chicoric acids were selected as identification markers. In the test solution chromatogram two intense zones of greenish-blue fluorescence should be detected between chlorogenic and chicoric acids.

2. The spectrophotometric methodology of hydroxycinnamic acids quantification, calculated into a chlorogenic acid, was developed. The content of hydroxycinnamic acids for industrial raw materials and wild samples is in the range of 1.10–1.35 %. To establish the quality criterion of the hydroxycinnamic acids content results of more samples research for elecampane samples from different areas and different year of growth for taking into account possible effects of environmental factors on their content are needed.

3. The application of the proposed methods of identification and quantification of hydroxycinnamic acids allow to distinguish, by comparison of TLC profiles and electronic absorption spectra, two raw materials that are rich in inulin, identified by its presence, and in the crushed state are similar - rhizomes with roots elecampane and roots of chicory.

**References**

1. Aspects regarding the qualitative and quantitative phytochemical analysis of the *Inula helenium L*. species / Marinela Afemei, Elvira Gille, Irina Boz [et al.] // Analele Ştiinţifice ale Universităţii „Al. I. Cuza" Iaşi s. II a. Biologie vegetală. – 2012. – Vol. 58, Iss. 1. – P. 29–34.
2. Antioxidant activity and fructan content in root extracts from elecampane (*Inula helenium* L.) / Nadezhda Petkova, Radka Vrancheva, Dasha Mihaylova [et al.] // *J. BioSci. Biotechnol.* – 2015. – Iss. 4(1). – P. 101–107.
3. Mitrofanova I.Yu. Opredeleniye summarnogo soderzhaniya polifruktanov i dinamiki ikh nakopleniya v kornevishchakh i kornyakh devyasila vysokogo (Inula helenium L.), proizrastayushchego v Volgogradskoy oblasti / I. Yu. Mitrofanova, A. V. Yanitskaya // Khimiko-farm. zhurn. – 2013. – T. 47, № 3. – S. 45-47.
4. Olennikov D. N. Issledovaniye kolorimetricheskoy reaktsii inulina s rezortsinom v zavisimosti ot usloviy provedeniya / D. N. Olennikov, L. M. Tankhayeva // Khimiya rastitel'nogo syr'ya. – 2008. – №1. – S. 87-93.
5. Chemical Constituents of Inula helenium / Yong-Ming Zhao, Jin Wang, Qing-Wen Shi **//** Chemistry of Natural Compounds. – 2014. – Vol. 49, Iss. 6. – P. 1121–1122.
6. Butko A.Yu. Eksperymental'ne doslidzhennya kil'kisnoho vmistu biolohichno aktyvnykh rechovyn u roslynniy syrovyni omanu vysokoho ta omanu brytans'koho / A. Yu. Butko // Farmats. zhurn. – 2012. – №6. – S. 98–101.
7. Aminokyslotnyy sklad roslynnoyi syrovyny omanu brytans'koho u vehetatsiynyy period / O. K. Yerenko, O. V. Mazulin, P. A. Lohvin [ta in.] // Aktual'ni pytannya farmatsevtychnoyi i medychnoyi nauky ta praktyky. – 2012. – №2. – S. 10-12.
8. Ezhned M.A. Osoblyvosti tsukroznyzhuval'noyi diyi sukhoho ekstraktu z koreniv i korenevyshch omanu vysokoho zalezhno vid dozy / M. A. Ezhned, T. A. Hroshovyy, O. M. Horoshko // Farmatsevtychnyy chasopys. – 2015. – №3. – S. 63-65.
9. Polucheniye sukhogo ekstrakta iz korney devyasila vysokogo i izucheniye yego khimicheskogo sostava / S. A. Matasova, N. A. Mitina, G. L. Ryzhova [i dr.] // Khimiya rastitel'nogo syr'ya. – 1999. – №2. – S. 119-123.
10. Antioxidant and chemical properties of Inula helenium root extracts / Iuliana Spiridon, Constantin Bogdan Nechita, Marius Niculaua [et al.] // Cent. Eur. J. Chem. – 2013. – 11(10). – P. 1699–1709.
11. Hydroxycinnamates from elecampane (Inula helenium L.) callus culture / Anna Stojakowska, Janusz Malarz, Anna K. Kiss // Acta Physiol. Plant. – 2016. – P. 38–41.
12. Jungmin Lee Chicoric acid: chemistry, distribution,

and production / Jungmin Lee, Carolyn F. Scagel // Front. Chem. – 2013. – Iss.1. - P. 1–17.
13. Acute hypoglycemic effect and phytochemical composition of Ageratina petiolaris / C. Bustos-Brito, A. Andrade-Cetto, J. D. Giraldo-Aguirre [et al.] // J. Ethnopharmacol. – 2016. – Vol. 5, Iss. 185. – P. 341–346.
14. Kelly L. Johnston Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine / Kelly L. Johnston, Michael N. Clifford, Linda M. Morgan // Am. J. Clin. Nutr. – 2003. – Iss. 78. – P. 728–733.
15. Benalla W. Antidiabetic medicinal plants as a source of alpha glucosidase inhibitors / W. Benalla, S. Bellahcen, M. Bnouham // Curr. Diabetes Rev. – 2010. – Iss. 6(4). – P. 247–254.

## РОЗРОБКА МЕТОДИКИ СТАНДАРТИЗАЦІЇ КОРЕНЕВИЩ І КОРЕНІВ ОМАНУ (*INULA HELENIUM* L.) ЗА ВМІСТОМ ГІДРОКСИКОРИЧНИХ КИСЛОТ

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**Резюме:** розроблено методики стандартизації кореневищ з коренями оману високого шляхом ідентифікації і кількісного визначення гідроксикоричних кислот. Для ідентифікації сировини було запропоновано встановлювати наявність чотирьох зон гідроксикоричних кислот, серед яких хлорогенова і цикорієва, у ТШХ-профілі. Результати визначення вмісту гідроксикоричних кислот для промислових і дикорослих зразків сировини знаходяться в інтервалі 1,10–1,35 %. Розроблені методики дозволяють розрізняти два види сировини, які характеризуються високим вмістом інуліну й є схожими у подрібненому стані – кореневища і корені оману високого і корені цикорію.

**Ключові слова:** Inula helenium, корені і кореневища, гідроксикоричні кислоти, хлорогенова кислота, цикорієва кислота, стандартизація.

## РАЗРАБОТКА МЕТОДИКИ СТАНДАРТИЗАЦИИ КОРНЕВИЩ И КОРНЕЙ ДЕВЯСИЛА (INULA HELENIUM L.) ПО СОДЕРЖАНИЮ ГИДРОКСИКОРИЧНЫХ КИСЛОТ

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**Резюме:** разработаны методики стандартизации корневищ с корнями девясила высокого путем идентификации и количественного определения гидроксикоричных кислот. Для идентификации сырья было предложено устанавливать наличие четырех зон гидроксикоричных кислот, среди которых хлорогеновая и цикориевая, в ТСХ-профиле. Результаты определения содержания гидроксикоричных кислот для промышленных и дикорастущих образцов сырья находятся в интервале 1,10–1,35 %. Разработанные методики позволяют различать два вида сырья, которые характеризуются высоким содержанием инулина и похожи в измельченном состоянии – корневища и корни девясила высокого и корни цикория.

**Ключевые слова:** inula helenium, корни и корневища, гидроксикоричные кислоты, хлорогеновая кислота, цикориевая кислота, стандартизация.

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