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Identification of chlorogenic acid in cotyledonous leaves and husks of common sunflower (*Helianthus annuus* L.)

Abstract. The relevance of the study is conditioned by the need to optimise the production process of the sunflower farming ecosystem by increasing environmental sustainability. Among the by-products of processing, the use of which is still not effective, the release of the powerful antioxidant chlorogenic acid has not been sufficiently studied. In this regard, this paper is aimed at revealing the patterns of accumulation and identification of phenolic compounds in cotyledons and sunflower husks. The purpose of the study is to identify phenolic compounds in cotyledonous leaves and husks of various genotypes of common sunflower (*Helianthus annuus* L.) by thin-layer chromatography. In the course of research on this problem, the authors used field, biochemical, and statistical research methods. To identify chlorogenic acid, a solvent system was used: chloroform – glacial acetic acid – methanol – water (60:32:12:8). Chromatography analysis was performed in the SORBFLIL TLC software suite. The object of research is the Auris common sunflower hybrid. It is established that the qualitative composition of the phenolic complex and the number of individual compounds has a pronounced variety specificity. On the chromatogram, this substance has an Rf of ~0.60 (for sunflower husks) and an Rf of ~0.74 (for cotyledonous leaves). It was found that the most effective solvent for chlorogenic acid is aqueous ethanol. Extraction of chlorogenic acid with 40% ethanol provides up to 10 mg/g of chlorogenic acid. In addition to chlorogenic acid, a small amount of caffeic acid was found in the alcohol extract. The materials of the study are of practical value for improving the processes related to the development of the productivity of sunflower crops and the rational use of processing by-products

Keywords: oilseeds, phenols, antioxidants, caffeic acid, chromatogram, photodensitogram

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INTRODUCTION

At the current stage of crop production development as a science, increasing the ecological stability of varieties and plant communities is one of the important factors for intensifying crop cultivation technologies. In the conditions of increasing the number of technogenic means used to optimise the growing conditions of agricultural crops (fertilisers, pesticides, etc.), the role of ecological stability of varieties and agrocoenoses in the implementation of their potential productivity is constantly increasing. The maximum possibilities of plant adaptation to negative environmental factors are determined by the genotype and functional features of the synthesised metabolites, which, in particular, include polyphenolic compounds. Numerous studies show that phenolic compounds perform a wide range of regulatory and protective functions (Pedrosa *et al.*, 2000; Voitsekhivska *et al.*, 2015), directed against various negative environmental factors (infections, insects, UV radiation, temperature stress, etc.) (Levytskyi *et al.*, 2010; Makarenko & Levytskyi, 2013). These properties of polyphenols can determine the varietal characteristics of cultivated plants. The implementation of the stress-protective effect is based on the antioxidant properties of these compounds (Andersen & Markham, 2005; Alexieva *et al.*, 2013).

The biological activity of chlorogenic acid is primarily conditioned by its strong antioxidant effect (Parr & Bolwell, 2000). Its biosynthesis occurs exclusively in plants and, along with phenolic compounds, is widespread in the plant world (Clifford, 1999; Nakatani *et al.*, 2000). Chlorogenic acid is often found in higher dicotyledonous plants, ferns, and many medicinal plants, which are called "plant gold" in China (Liang Wang *et al.*, 2022). It has strong antioxidant, antifungal, antibacterial, and antiviral properties. A number of researchers have established anti-cancer and haepato-protective effects, effects on the regulation of glucose metabolism, and positive effects on blood vessels (Levytskyi *et al.*, 2010; Liang Wang *et al.*, 2022). This property can be used both in medicine, in agriculture, and the food industry. Chlorogenic acid plays an important role in plant respiration, is a regulator of growth processes, occupies an important place in ensuring plant immunity, and performs protective functions. The content of chlorogenic acid depends on the varietal characteristics of plants, natural and climatic factors, and on the method of determination (Makarenko & Levytskyi, 2013; Voitsekhivska *et al.*, 2015; Labeiko, 2021).

Sunflower is one of the main industrial crops in Ukraine. Taking into account processing, it is considered a valuable oilseed crop using by-products of processing, mainly for feed purposes. However, it can be used as a source of production of various types of biofuels, potash fertilisers, paper, hexose and pentose sugar, etc. Common sunflower is also an important source of natural antioxidants and, compared to other crops, its seeds are characterised by the highest potential (Halvorsen *et al.*, 2002). The husk of sunflower seeds contains only 0.7-5.4% of the total amount of its phenolic compounds, while the rest is found in the kernel (Pedrosa *et al.*, 2000; Grana *et al.*, 2017). The predominant

phenolic compound of the sunflower kernel is chlorogenic acid, which is 43-73 % of all phenolic compounds (Weisz *et al.*, 2009) and has strong antioxidant, antifungal, and antibacterial properties (Levytskyi *et al.*, 2010). Scientific studies in most cases relate to the production and use of sunflower protein, and the production of the antioxidant chlorogenic acid from sunflower meal is rather neglected (Labeiko, 2015).

Chlorogenic acid is one of the most valuable compounds that is in great demand among consumers, primarily due to its antioxidant properties. The use of chlorogenic acid is promising in the fat and oil industry as an antioxidant of vegetable oils as a substitute for synthetic substances (tributylhydroquinone, butylhydroxytoluene, etc.) (Shapovalova *et al.*, 2013). However, the use of methanol to produce chlorogenic acid, which in the future can be used in food and supplements, is not desirable. In addition, pre-extraction of chlorogenic acid from meal would allow obtaining plant protein without phenolic compounds and using it for the production of light-coloured food (Labeiko, 2015).

In this regard, the purpose of the study was to identify composite phenolic complexes in the husk and cotyledon leaves of various genotypes of common sunflower (*Helianthus annuus* L.) by thin-layer chromatography.

MATERIALS AND METHODS

The study was conducted during 2017-2018 in the laboratory of biotechnology and cell engineering of the Department of Ecobiotechnology and Biodiversity of NUBiP of Ukraine. Field experiments were carried out in the stationary crop rotation of the Department of Crop Growing in the special subdivision of NUBiP "Agronomic Experimental Station" on typical low-humus chernozems.

The object of research was a hybrid of common sunflower (*Helianthus annuus* L.) Auris (resistant to herbicides based on tribenuron-methyl (SU), listed in the State register of varieties suitable for distribution in Ukraine (2022).

In the course of the study, a field research method was used to investigate the interaction of the research object with biotic and abiotic factors in the field.

Experiment scheme – fertiliser options: 1. $N_{60}P_{50}K_{90}$ (control); 2. $N_{60}P_{50}K_{90} + S_{30}$; 3. $N_{90}P_{75}K_{135} + S_{30}$; 4. $N_{120}P_{100}K_{180} + S_{30}$. Mineral fertiliser application rates were calculated using the balance method for the planned yield, considering the content of the main elements of mineral nutrition in the soil. Plot area 25 m². Repetition – four times. Placement of options is sequential.

The technology of growing sunflower seeds is generally accepted for the forest-steppe zone. Its predecessor was winter wheat. After harvesting the predecessor, stubble ploughing was carried out. In the first-second ten days of October, according to the experiment scheme, 50% of the annual norm of nitrogen fertilisers (ammonium nitrate, 33.4%), 100% of phosphorous and potash fertilisers (double superphosphate, 46%; potassium chloride, 62%) were applied and winter ploughing was

carried out to a depth of 25-27 cm. In spring, when the soil was physically ripe, mulching was carried out using a toothed harrow. On the day of sowing, according to the experiment scheme, 50% nitrogen fertilisers and 100% sulphur (ammonium sulphate) were applied and pre-sowing treatment was carried out with a combined unit. Seeds were sown to a depth of 4-5 cm at a soil temperature of 10°C at a depth of 6-8 cm (second 10 days of April). In the post-sowing period (pre-emergence), a soil herbicide (312.5 g/l S –metolachlor + 187.5 g/l Terbutylazine) was applied. The fungicide (80 g/l Ciproconazole + 200 g/l Azoxystrobin) was applied when the first signs of the disease appeared, and the second application – if necessary with an interval of 18-22 days. All elements of the cultivation technology, with the exception of the subjects, were the same throughout the experiment.

Sampling of cotyledons in each variant was carried out in the full ripeness phase on 10 plants. The results obtained for each of the indicators were tested for normality according to the Shapiro-Wilk criterion (Razali & Wah, 2011). In the case when the variational series did not correspond to the normal distribution, the number of samples was increased. The seed yield was determined by collecting all seeds from the registered plot and converting the value in t/ha to the basic seed moisture index of 8%.

A biochemical research method was used to determine the content of chlorogenic acid. The qualitative and quantitative composition of phenolic compounds was studied by thin-layer chromatography on sunflower seedlings grown in the laboratory and the husk of sunflower seeds grown in the field. Extraction of phenolic compounds from plant material depends on the type and polarity of the solvent. Ethanol was chosen as the solvent for the isolation of chlorogenic acid. Identification was performed by thin-layer chromatography (TLC) using plates measuring 100×150 mm, sorbent – Silicagel 60 F254 (Merck). Solvent systems were used to identify chlorogenic acid: chloroform – glacial acetic acid – methanol – water (60 : 32 : 12 : 8). To enhance the glow of substances under ultraviolet illumination, UV (365 Nm) plates with chromatographs were treated with 5% EtOH solution AlCl₃ with subsequent heating (5 min at 105°C). The chromatogram was kept for 7-10 minutes at a temperature of 110°C to the appearance of characteristic spots. Photo documentation of the materials was carried out using the AxioVision 40V Carl Zeiss software suite. Digital data processing was performed in the Image Pro Premier 9.0 (Trial version) software suite. Chromatography analysis was performed in the SORBFLIL TLC (Kovalov *et al.*, 2003). To determine the chemical nature of substances, chromatograms were treated with developing reagents. R_f indicators were determined photodensitometrically using *Sorbfil TLC* software suite. The presence of coumarins in methanol (MeOH) extracts was detected by lactone breakdown and qualitative reaction to diazotated sulfonylic acid (Kovalov *et al.*, 2003). The statistical

research method was used to analyse experimental data, determine the reliability of data, and identify dependencies between the studied indicators. Statistical data processing, principal component analysis (PCA), correlation and cluster analysis were performed in the XLSTAT software suite (Addinsoft Inc., USA, 2010).

RESULTS AND DISCUSSION

The qualitative composition of the phenolic complex and the number of individual compounds has a pronounced variety specificity, which is confirmed by the results of the conducted studies.

Based on thin-layer chromatography in methanol leaf extracts by the nature of the glow in the UV region (365 Nm), performed by a colour reaction to a FeCl₃ solution and the R_f value, a substance that can be identified as chlorogenic acid was discovered. On the chromatogram, this substance has an R_f of ~0.60 (for sunflower husks) and an R_f of ~0.74 (for cotyledonous leaves) (Fig. 1). Thus, it is established that secondary metabolites, including oxybenzoic acids, accumulate in the vegetative organs of plants, which play an important role in the formation of their constitutional stability. The general state of the plant organism and its survival strategy depend on the activity of the synthesis of phenolic substances.

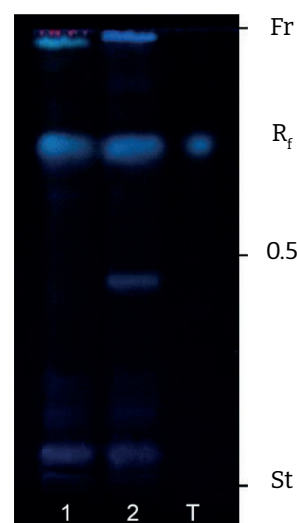


Figure 1. Chromatography of MeOH extracts: 1 – cotyledonous leaves; 2 – husks of Auris sunflower hybrid; T – chlorogenic acid (system: chloroform – acetic acid – methanol – water (60 : 32 : 12 : 8))

The effect of phenolic compounds on plant resistance against negative environmental factors is confirmed by the findings of other researchers (Levytskyi *et al.*, 2010; Gai *et al.*, 2020). Phenolic compounds are crucial for the antioxidant potential of sunflower seeds (Karamac *et al.*, 2012). The possibility of using phenolic compounds obtained from extracts of the aboveground part of sunflower as antioxidants is evidenced by scientific studies by Gai *et al.* (2020).

Given that the content of chlorogenic acid is quite high not only in cotyledonous leaves, but also in pericarpia, it is justified to consider them as a source of a valuable chemical compound (Table 1). Chlorogenic acid was detected by

thin-layer chromatography in 40% of water-alcohol extraction of cotyledonous leaves and husks, as evidenced by the area of peaks in the fluorescence intensity of individual compounds according to photodensitometry results.

Table 1. Area of fluorescence intensity peaks of individual compounds based on photodensitometry results

Rf Indicators	Peak area, S · 10 ³ (c.u.)									
	0.02	0.07	0.16	0.22	0.45	0.64	0.74	0.88	0.92	0.97
Cotyledonous leaves	27.5	128.4	60.4	56.1	0	35.3	367.5	0	18.8	224.1
Husk	0	133.4	57.4	39.9	111.6	0	370.2	36.2	0	201.7
Chlorogenic acid							104.7			

Obtaining extracts with high antioxidant activity from sunflower seed husks is confirmed by Szydłowska-Czeraniak *et al.* (2011).

Chlorogenic acid is highly soluble in ethanol and can therefore be extracted from the husk, where its content for the Auris hybrid ranges from 10 to 25 mg/g. The content of chlorogenic acid in sunflower husks varied depending on the zonal placement of seeds in the basket, which is a phenomenon of its matrix diversity. The content of chlorogenic acid in the seed husk, which is located in the centre of the flower (within a radius of 50.0 mm), is almost twice as high as in the seeds of its peripheral zone. This is conditioned by the different intensity of its development during certain periods (from flowering, fertilisation of flowers to seed formation) – in the flower, seeds are most intensively formed from the periphery to the

centre. Thus, in the peripheral zone, the seed has a larger weight of 1,000 seeds than in the central zone, which even with the same absolute content of chlorogenic acid, in terms of fractions per unit mass, shows its lower content. This allows considering the possibility of recycling waste that remains during the seed cleaning process.

According to the densitogram, the average content of chlorogenic acid in cotyledon leaf and husk extracts almost did not differ (Fig. 2). The use of ethanol as a solvent does not allow for complete extraction of the phenolic compound, but this method is the most optimal in terms of price/quality. In addition, unlike other solvents, it does not exclude the further use of raw materials, and when used as a substrate for fungi, it improves the effectiveness of sterilisation against pathogenic microflora and spores of competing fungi.

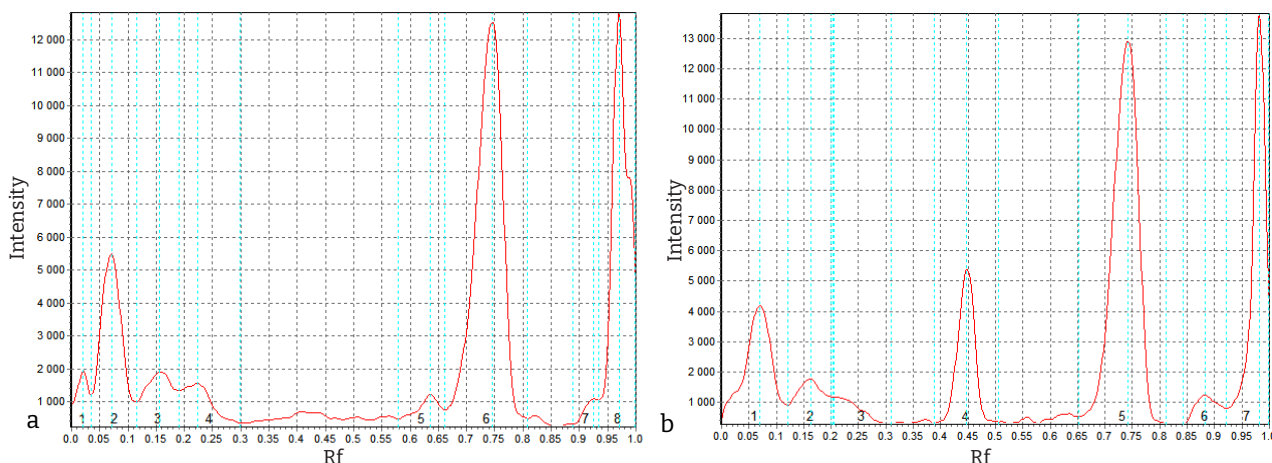


Figure 2. Photodensitogram of MeOH extracts: a – cotyledonous leaves; b – husks of the Auris sunflower hybrid

According to the results of studies, extraction of chlorogenic acid with 40% ethanol provided up to 10 mg/g of chlorogenic acid. Moreover, in addition to chlorogenic acid, a small amount of caffeic acid was found in the alcohol extract (Fig. 3), as well as minor traces of cinnamic, coumaric, ferulic, and sinapic acids.

The confirmed biological activity suggests that the green parts of sunflower plants can be considered a source of natural antioxidants.

Similar results were obtained by Pedrosa *et al.* (2000), where in addition to chlorogenic acid, up to 10 components were identified in the sunflower phenolic complex, including caffeic, cinnamic, coumaric, ferulic, sinapic, traces of vinyl and oxybenzoic acids.

The use of aqueous ethanol as a solvent is confirmed by the results obtained by Kovalov *et al.*, 2008, Labeiko, 2021. However, the use of ethanol as a solvent at concentrations of 40% compared to 50% and 70% in studies by

Labeiko et al. (2019) and Náthia-Neves & Alonso (2021) provides a high yield of this compound and reduces the cost of obtaining it.

Field and laboratory studies have shown that the content of chlorogenic acid varies depending on

environmental factors, varietal characteristics, and cultivation technology. All modern sunflower hybrids are characterised by a high fat content, and therefore, a higher content of chlorogenic acid compared to wild forms, which is confirmed by studies by Pomenta & Burns (1971).

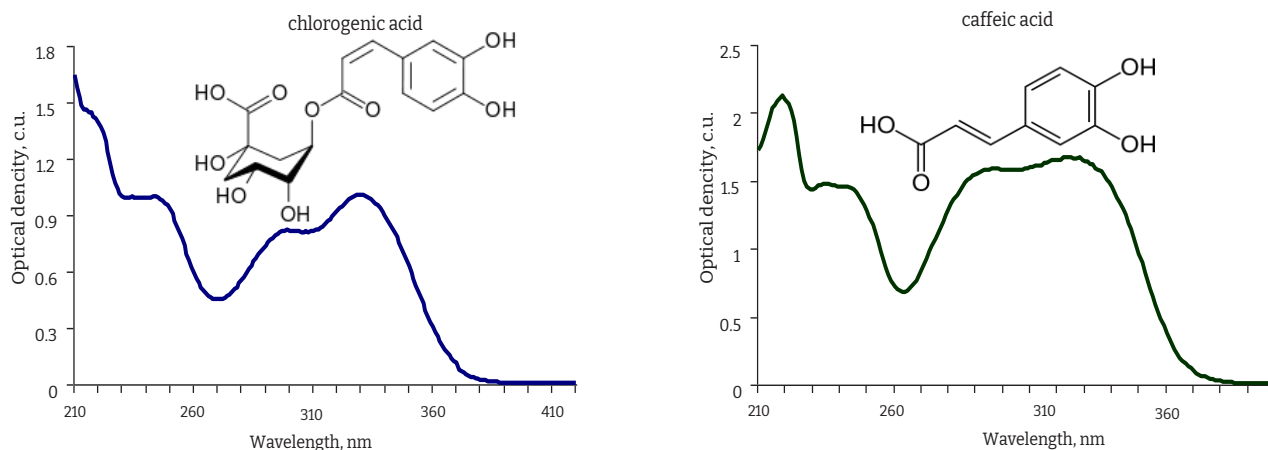


Figure 2. Electronic spectra of chlorogenic and caffeic acids extracted with 40% ethyl alcohol from the Auris sunflower husk

The application of mineral fertilisers has a significant effect on the synthesis of chlorogenic acid: a balanced mineral diet increases its content (Table 2). With an insufficient amount of phosphorus and potassium against the background of high nitrogen application, there is a significant decrease in its content in the husk. Foliar application of growth stimulants in combination with trace elements in chelated form leads to an increase in the content of phenolic compounds in sunflower leaves by 2.3-2.5 times compared to the control variant, which has a positive effect on the accumulation of chlorogenic acid in the achenes. Microelements are an integral part of enzymatic systems, which are biological catalysts that increase the activity of biochemical processes in plants. Due to the synergy of introduced growth regulators and trace elements,

plants are able to use nutrients from the soil more efficiently, and plant resistance to adverse factors increases.

The technology of growing sunflower seeds has a direct impact on the estimated gross yield of chlorogenic acid per unit area: the application of mineral fertilisers significantly affected the gross yield of this phenolic compound.

The highest content and the gross yield of chlorogenic acid was obtained on variants with full mineral fertiliser. Unbalanced mineral nutrition and unilateral application of nitrogen fertilisers against the background of phosphorus and potassium deficiency leads to a decrease in crop yield and a decrease in the antioxidant content in sunflower husks, and as a result – a smaller estimated yield of chlorogenic acid.

Table 2. Crop productivity and estimated gross yield of chlorogenic acid for sunflower cultivation (average for 2017-2018)

Fertiliser options	Yield, t/ha	Huskiness, %	Estimated gross yield of chlorogenic acid, kg/ha
$N_{60}P_{50}K_{90}$ (control)	3.19	21.9	6.71
$N_{60}P_{50}K_{90}+S_{30}$	3.46	21.6	7.47
$N_{90}P_{75}K_{135}+S_{30}$	3.80	22.1	8.31
$N_{120}P_{100}K_{180}+S_{30}$	3.85	22.8	8.60
LSD ₀₅	0.26	1.1	

It was found that the total gross yield of chlorogenic acid depends on the productivity of sunflower seeds. The highest biological yield of the studied hybrid is formed by applying $N_{90}P_{75}K_{135}+S_{30}$ – 3.8 t/ha. A further increase in the rate of application of mineral fertilisers does not lead to a significant increase in crop yield. The

huskiness index of cotyledons, depending on the background of mineral nutrition, is 21.6-22.8 %. Thus, the estimated gross yield of chlorogenic acid for growing Auris sunflower hybrid with the application of mineral fertilisers at a rate $N_{90}P_{75}K_{135}+S_{30}$ is 8.31 kg/ha, $N_{120}P_{100}K_{180}+S_{30}$ – 8.60 kg/ha.

Chlorogenic acid has a stronger antioxidant effect than ascorbic acid, caffeic acid, or vitamin E. The prospects for chlorogenic acid eradication in medicine are confirmed by Liang Wang *et al.* (2022); Cao *et al.* (2020). Preliminary extraction of chlorogenic acid from sunflower processing products expands the possibilities of using plant proteins, which is confirmed by the results of research by M. Labeiko *et al.* (2019); M. Labeiko & Z. Fediakina (2017); W. Jia *et al.* (2022). Even crude chlorogenic acid is of considerable commercial interest, and phenolic compounds extracted from the husk do not reduce, but even slightly improve its properties, which can be used in production: for example, adding to the substrate for growing oyster mushrooms and other edible mushrooms, in medicine, food industry.

CONCLUSIONS

As a result of the analysis of the current state of research on the identification, content, and influence of phenolic compounds, it was established that the qualitative composition of the phenolic complex and the number of individual compounds has a pronounced variety specificity. Chlorogenic acid was detected in water-alcohol (40%) extraction of cotyledon leaves and husks by thin-layer chromatography. Chlorogenic acid can be extracted from the sunflower husk by 40% ethanol, where its content in plants of the Auris hybrid is 10-25 mg/g.

Seeds placed in the centre of the basket within a 50.0 mm radius contain almost twice as much chlorogenic acid as seeds placed closer to the periphery. The application of mineral fertilisers has a significant effect on the synthesis of chlorogenic acid: a balanced mineral diet increases its content.

The total gross yield of chlorogenic acid depends on sunflower productivity and seed huskiness. The highest biological yield of the studied Auris hybrid is formed by applying $N_{90}P_{75}K_{135}+S_{30} - 3.80$ t/ha.

Extraction of 40% ethanol provides up to 10 mg/g of chlorogenic acid. Estimated gross yield of chlorogenic acid for growing Auris sunflower hybrid with the application of mineral fertilisers at a rate $N_{90}P_{75}K_{135}+S_{30}$ is 8.31 kg/ha, $N_{120}P_{100}K_{180}+S_{30} - 8.60$ kg/ha.

It can be argued that even crude chlorogenic acid is of considerable commercial interest, and phenolic compounds extracted from the husk do not reduce, but even improve its properties.

However, it is necessary to further investigate the dynamics of chlorogenic acid content in different periods of growth and development of sunflower plants and its effect on plant resistance against biotic and abiotic factors. To increase the yield of chlorogenic acid from the products under study, it is necessary to investigate the use of various solvents. Ultimately, the use of ethanol as a solvent does not allow for the complete extraction of the phenolic compound.

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Ідентифікація хлорогенової кислоти в сім'ядольних листках і лушпинні соняшника однорічного (*Helianthus annuus* L.)

Анотація. Актуальність досліджень зумовлена необхідністю оптимізації продукційного процесу агроценозів соняшника за рахунок підвищення екологічної стійкості. Серед побічних продуктів переробки, використання яких дотепер не є ефективним, виділення потужного антиоксиданта хлорогенової кислоти вивчено недостатньо. У зв'язку з цим дана стаття спрямована на розкриття закономірностей накопичення та ідентифікацію фенольних сполук в сім'ядольних листках і лушпинні соняшника. Мета досліджень – ідентифікація фенольних сполук в сім'ядольних листках і лушпинні різних генотипів соняшнику однорічного (*Helianthus annuus* L.) методом тонкошарової хроматографії. У процесі наукових пошуків за цією проблемою застосовували польові, біохімічні та статистичні методи досліджень. Для ідентифікації хлорогенової кислоти використовували систему розчинників: хлороформ – льодяна оцтова кислота – метанол – вода (60 : 32 : 12 : 8). Аналіз хроматографії проводили в програмі Sorbflil TLC. Об'єкт досліджень – гібрид соняшнику однорічного Ауріс. Встановлено, що якісний склад фенольного комплексу і кількість індивідуальних сполук має виражену сортоспецифічність. На хроматограмі дана речовина має $R_f \sim 0,60$ (для лушпиння соняшника) та $R_f \sim 0,74$ (для сім'ядольних листків). Встановлено, що найбільш ефективним розчинником для хлорогенової кислоти є водний етанол. Екстракція хлорогенової кислоти 40 % етанолом дозволяє отримати до 10 мг/г хлорогенової кислоти. Окрім хлорогенової кислоти у спиртовому екстракті у незначній кількості виявлено кавову кислоту. Матеріали статті становлять практичну цінність для вдосконалення процесів, пов'язаних із формуванням продуктивності посівів соняшника, а також раціональному використанню побічних продуктів переробки

Ключові слова: олійні культури, феноли, антиоксиданти, кавова кислота, хроматограма, фотоденситограма