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**IDENTIFICATION AND QUANTITATIVE DETERMINATION OF CAROTENOIDS
IN THE WOUND-HEALING OINTMENT WITH SEA BUCKTHORN OIL**

Actuality. Carotenoids are known as important bioactive components of Sea Buckthorn's oil, which ensure its wound-healing activity. These agents are known as active substances of various wound-healing compositions, as they provide a complex treatment for damaged tissues, contributing to their fast recovery and protecting the affected skin from inflammation and infection. Reliable scientific methods for the determination of bioactive compounds should be developed to ensure high quality of the extemporaneously-made medications with due respect to their cost-efficiency and technical accessibility of lab control devices. This work focuses on the elaboration of such a method for qualitative and quantitative determination of carotenoids in extemporaneously-made ointments with Sea Buckthorn oil.

The aim of the study is to develop a method for identification and quantification of carotenoids in a wound-healing ointment with Sea Buckthorn oil, which can be used for quality control of drug stores' extemporaneously-made compositions.

Material and methods. This investigation was conducted using an extemporaneously-made wound-healing ointment produced at a "Harmony-2000" drug store in Chernivtsi, Ukraine. The identification and quantitative determination of the carotenoids was performed using VIS-spectrophotometry and thin-layer chromatography.

Research results. It has been shown that a single-wave direct light-absorption spectrophotometry with an external standard can be used for quantification of the content of total carotenoids in terms of β -carotene in a Sea Buckthorn oil-containing ointment. Since additional components of the ointment do not absorb light within the spectral range used in the present investigation, they do not interfere

with the accuracy of the analysis. The original sample processing method reported in this article ensures a complete statistical uncertainty of $\pm 0,725\%$. The study revealed that the total carotenoid content in the ointment in terms of β -carotene was $0,1897 \pm 0,00075$ mg/g.

Conclusion. An economically expedient method of quantification of total carotenoids in terms of β -carotene in a wound-healing ointment with Sea Buckthorn oil has been developed for the conditions of extemporaneous production. The validity of the method in terms of linearity was verified and confirmed. It was found that the total content of carotenoids in terms of β -carotene in a series of wound healing ointment made by "Harmony-2000" was $0,1897 \pm 0,00075$ mg/g.

Key words: carotenoids, Sea Buckthorn ointment, identification, quantitative determination.

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ІДЕНТИФІКАЦІЯ ТА КІЛЬКІСНЕ ВИЗНАЧЕННЯ КАРОТИНОЇДІВ У СКЛАДІ РАНОЗАГОЮВАЛЬНОЇ МАЗІ З ОБЛІПИХОВОЮ ОЛІЄЮ

Актуальність. Серед арсеналу біологічно активних речовин, які зумовлюють фармакологічну активність обліпихової олії, значна роль належить каротиноїдам. Застосування обліпихової олії у складі ранозагоювальних мазей забезпечує комплексну дію на ушкоджені тканини, сприяє швидкому відновленню шкірного покриву, зменшенню запалення та захисту від інфекцій. Для стандартизації лікарських засобів необхідні науково обґрунтовані методики визначення біологічно активних речовин, тому розробленню методик ідентифікації та кількісного аналізу каротиноїдів у складі мазей з обліпиховою олією присвячена ця робота.

Мета дослідження – розробити методики ідентифікації та кількісного визначення каротиноїдів у складі ранозагоювальної мазі з обліпиховою олією, які придатні для контролю якості серій мазі, виготовлених екстемпорально в умовах аптеки.

Матеріал і методи. Об'єктом дослідження слугувала мазь ранозагоювальна з олією обліпихи, виробництва аптеки «Гармонія-2000» (місто Чернівці), предметом дослідження – ідентифікація та кількісне визначення каротиноїдів у мазі з обліпиховою олією. Для ідентифікації та кількісного аналізу каротиноїдів у досліджуваних зразках (мазі й олії) застосовували

спектрофотометрію, у видимій області спектра, та тонкошарову хроматографію. Отримані результати обробляти статистично з використанням Microsoft Office Excel 2003.

Результати дослідження. Для ідентифікації β -каротину у складі ранозагоювальної мазі з обліпиховою олією виробництва «Гармонія-2000» запропоновано використовувати спектрофотометричний метод і метод тонкошарової хроматографії. Результатами дослідження підтверджено можливість кількісного визначення суми каротиноїдів у перерахунку на β -каротин у ранозагоювальній мазі з обліпиховою олією, методом прямої однохвильової абсорбційної спектрофотометрії, з наступним розрахунком за методом зовнішнього стандарту. Доведено, що допоміжні речовини не заважають кількісному визначенню суми каротиноїдів у складі ранозагоювальної мазі з обліпиховою олією методом абсорбційної спектрофотометрії, оскільки не поглинають електромагнітне випромінювання в області аналітичного максимуму. Запропоновано методику пробопідготовки, відповідно до якої повна прогнорозована невизначеність кількісного визначення суми каротиноїдів може становити $\pm 0,725\%$. За розробленою методикою встановлений вміст суми каротиноїдів у перерахунку на β -каротин у серії ранозагоювальної мазі з обліпиховою олією ($0,1897 \pm 0,00075$ мг/1 г).

Висновок. Розроблена методика кількісного визначення суми каротиноїдів у перерахунку на β -каротин у складі ранозагоювальної мазі з обліпиховою олією для аптек з екстемпоральним виготовленням лікарських засобів, доведена її валідність щодо лінійності. За розробленою методикою встановлено, що вміст суми каротиноїдів у перерахунку на β -каротин у серії ранозагоювальної мазі з обліпиховою олією виробництва «Гармонія-2000» становить $0,1897 \pm 0,00075$ мг/1 г

Ключові слова: каротиноїди, мазь з обліпиховою олією, ідентифікація, кількісне визначення

Introduction. Actuality. The oil from the fruits of Sea Buckthorn (SB) (*Hippóphaë rhamnóides* L.) is a well-known source of bio-active compounds (BAC) for the production of drugs (Koskovac, 2017; Chen, 2023). BAC of SB are known as suppressors of inflammation mediators, antimicrobial agents, activators of damaged tissues' recovery, and remedies for keloid and hypertrophic scars. Due to such a wide area of activity, SB oil is extensively used in the treatment of burns, general wounds, dermatitis, eczema, and in gynaecology practice (Larmo, 2014; Black, 2020; Dudau, 2021).

As a component of wound-healing ointments, SB oil provides a multilateral activity in the treatment of damaged skin, facilitating its fast recovery and preventing inflammation and secondary skin infection (Ren, 2020). There is a wide variety of medicines, which contain this agent in the Ukrainian pharmaceutical market: "Olasol" by JSC "Stoma", "Mountain Rescuer" and "With Chaga" by LLC "Elixir", "Sea Buckthorn Suppository" by LLC "Phytolick", "Sea Buckthorn Oil" by LLC "Pharmaceutical Factory", "Sea Buckthorn Oil" by PJSC "Kharkiv", and others (State Register of Medicinal Products of Ukraine).

Significant contribution to the pharmaceutical activity of SB oil depends on the content of well-known antioxidant BAC, carotenoids (Jaśniewska, 2021; Rodriguez, 2024). It is a group of tetraterpene organic compounds with a system of conjugated double bonds. Because of such a specific chemical structure, they are intensely colored, absorb primarily in the visible spectral area, and exhibit comparatively high lipophilicity. The antioxidant activity of carotenoids is based on their ability to neutralize active forms of oxygen, which prevents damage to the membrane lipids, proteins, and DNA (Tan, 2019; Black, 2020).

Two basic analytical methods are usually applied to quantify carotenoids in oils or complex emulsions (ointments, crèmes): spectrophotometry (for express-determination of total carotenoids in oils or extracts) and

HPLC (for more accurate quantification of individual carotenoids). The former method is rather simple, expressed and inexpensive, while it is poorly selective and sensitive to many admixtures and carotenoid decomposition products, which can absorb in the working spectral area and cause some over-quantification of carotenoids. Besides, this method is also solvent-sensitive as absorbance peak wavelengths (λ_{max}) and molar absorption coefficients (ϵ) vary for different solvents and, therefore, they require standardization. HPLC is more accurate and selective, and ensures more reliable quantification of individual compounds. On the other hand, this method is more expensive and requires thorough elimination of chlorophylls and metal ions to avoid deterioration of the chromatography columns.

The thin-layer chromatography combines low cost and expressiveness. A semi-quantitative determination of individual components is possible after elution and spectrophotometry or using densitometry. However, the selectivity of this method is lower than that of HPLC, which can complicate the separation of the structurally similar components and impose the necessity of additional re-extraction of analyte after passing the thin-layer chromatography plate. All these factors can deplete the accuracy of this method (Kotenko, 2012; Maliuhina, 2013; Butnariu, 2016; Duiun, 2022).

A wound-healing medication "Wound-healing ointments with oil of Sea Buckthorn" has been developed by specialists at the pharmacy chain "Harmony-2000" and the authors of this manuscript were tasked to elaborate a method for its standardization. The pharmacy chain owns a measuring lab equipped with a spectrophotometer "ULab-108UV", but it does not have access to HPLC. That is why our work was aimed at the elaboration of a suitable and cost-effective method for quality control that allows quantification of carotenoids in an extemporaneously prepared wound-healing ointment with Sea Buckthorn, using the available lab equipment.

Based on the above, we propose to use cost-effective, rapid thin-layer chromatography for qualitative screening of total carotenoids. Then, spectrophotometry can be used to quantitatively determine total carotenoids, using potassium dichromate as an inexpensive internal standard. Unlike the well-known spectrophotometric quantification of total carotenoids, our approach involves different sample pretreatments. All ointment compositions contain an oil (lipophilic or emulsion), a hydrophilic or gel ointment base. Besides, it consists of emulgators, rheology improvement agents, antibacterial compounds, and other components. All these substances should either be separated at pretreatment or checked for the absence of influence on the quantitative analysis of carotenoids. Since carotenoids are extracted from the ointment using non-polar or low-polar solvents, such as hexane, petroleum ether, chloroform, acetone, or their mixtures, they can be accompanied in the extract by other lipophilic components of the ointment base, the products of interaction between carotenoids and emulgators, and others. All these side-components can cause shifts in the absorbance peaks. We propose non-polar hexane as an extractant. A comparison between the hexane extracts from pure Sea Buckthorn oil and the ointment showed a match in the absorbance peaks positions, which proves that no side components extracted from the oil can affect the spectral characteristics of the absorbance of carotenoids.

The aim of the study the development of a method for the identification and quantification of carotenoids in a wound-healing ointment consisting of SB oil, which can be applied to quality control in extemporaneous production of a series of the ointment.

Materials and research methods. This study was made on the pure SB oil and an ointment with SB oil produced extemporaneously at the drug store "Harmony-2000" in Chernivtsi, Ukraine. The ointment was made of the oil obtained from another side producer.

Qualitative and quantitative analyses of the carotenoid composition in the oil and ointment were conducted using thin-layer chromatography and visible area spectrometry on a SpectroLab UV-1200 spectrophotometer with the standard 10 mm quartz cuvettes. The chromatograms were obtained on the Merck Silica Gel 60 F254 20 cm plates. Besides, an AXIS ANG200C scale, an ultrasonic water bath, and A-class glassware were used for other preparations and auxiliary operations.

Research results and their discussion. *Preliminary preparation of the ointment samples.* 0,550 g of the ointment was placed in a preliminary weighed box, then 5 mL of hexane was added, and the box was placed in an ultrasonic bath. The content was gently stirred with a glass rod until complete dissolution of the ointment, and then poured into a 50 mL graduated flask wrapped in

metal foil to protect its content from light. The box, rod, and other glassware involved in the previous operations were washed with 5 mL portions of hexane three times, and then all washouts were collected in the same flask. Extra hexane was added to adjust the volume to 50 mL. The flask was sealed and left in a dark place for 30 minutes until complete sedimentation of all insoluble ointment components. Then the transparent supernatant was gently taken by a pipette for the next analysis.

Preliminary preparation of the SB oil samples was performed in the same way using a 0,100 g sample of the oil.

Preparation of the spectrometry reference solution: 0,3600 g of potassium dichromate ($K_2Cr_2O_7$) was placed in a 1 000 mL graduated flask, some distilled water was added to dissolve it, and then extra water was added to bring the volume up to the mark. The color intensity of the target solution corresponds to that of a 0,00208 mg/mL hexane solution of β -carotene. An absorbance of the reference solution (A_0) was measured under the same conditions as for the experimental mixtures.

The spectrometry identification of carotenoids in the wound-healing ointment. All absorbance spectra of the ointment samples and the standard working solution of SB oil were recorded vs hexane as a reference solution without adding any extra antioxidants to the experimental samples. The spectra of the above-mentioned solution of potassium dichromate were recorded vs water. All measurements were performed using the 10 mm cuvettes by a SpectroLab UV-1200 spectrophotometer.

All experimental results are shown in fig. 1. As seen from that Figure, both absorption spectra of the solutions of SB oil and a sample of the SB oil-containing ointment exhibit a wide multiplex absorption band extending between 370 and 520 nm and consisting of several narrower overlapping bands. The latter assumption is based on an indistinct saddle between 395 and 410 nm, which can be evidence of partial overlapping between two separate bands. Another absorption band exhibits a more distinct shoulder between 423 and 435 nm with peak absorption at 449–451 nm. This maximum isn't sharp, and rather has a slope, and therefore it can be used for quantitative determination of carotenoids.

Another less intense absorption band can be seen between 470 and 490 nm with a peak at 476 nm. In the absorbance spectra of a solution of the oil, there is a bend point at 495 nm. However, no such bend can be found in the spectrum of a solution of the ointment. The patterns of the oil and ointment spectra are close, and they only vary in the absorption intensity caused by the various weights of the samples taken. This closeness in the spectra patterns evidences that the ointment base and its other components do not affect the spectral characteristics.

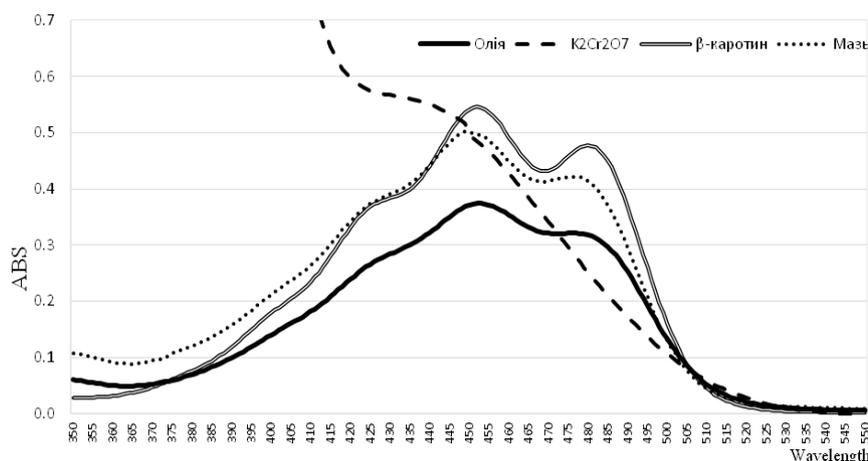


Fig. 1. Absorption spectra of the hexane solutions of the ointment, SB oil, standard solution of β -carotene, and an aqueous solution of potassium dichromate

A preliminarily recorded spectrum of a hexane solution of β -carotene exhibits a similar pattern; hence, β -carotene is a light absorbing component of the oil and ointment, which determines the shape of their spectra, positions, and intensities of all peaks and bend points. The spectrum of an aqueous solution of potassium dichromate is a smooth descending line with a shoulder between 415 and 450 nm, coinciding with the absorption maximum of carotenoids. Therefore, this solution can be used as an external standard for the determination of carotenoids in terms of β -carotene.

Based on the above, the total content of carotenoids in the wound-healing ointment can be determined spectrophotometrically in the visible spectral area, followed by the calculation using an aqueous solution of potassium dichromate as an external standard.

Spectrophotometrical identification of β -carotene in the wound-healing ointment. Spectrophotometrical identification of carotenoids in the ointment was conducted in the spectral area between 350 and 550 nm. The absorption spectrum of an ointment sample corresponds to that of the standard solution of SB oil: it has a wide absorption band between 370 and 520 nm with a peak at 449–451 nm, a less intense absorption band between 470–490 nm with a peak at 476 nm, and a characteristic shoulder between 423 and 435 nm. The ratio between absorbances at 450 and 476 nm ranges between 1,06 and 1,30. Therefore, the spectral characteristics of the hexane solution of the ointment prepared for the quantitative determination prove that it consists of carotenoids, mostly β -caroten.

Thin-layer chromatography identification of carotenoids in the wound-healing ointment. The 10 mm lines with 30 μ L of hexane solutions of the ointment and SB oil (as a standard) were applied to the starting line of

a Merck Silica Gel 60 F₂₅₄ thin-layer chromatography plate. The plate was dried in air and placed in the chromatography chamber enriched preliminarily with an 8:2 mixture of hexane and methyltertbutyl ether as a mobile phase. After complete passing of the solvents across the plate, it was removed from the chamber, dried in warm air flux until the solvent's odor disappeared completely. Next, a solution of phosphoric-molybdenum acid in 96% ethanol was sprayed on the plate, followed by its air-drying and warming in a drying chamber at 105 °C for 5 min. The plate was inspected in visible light to check for a dark-blue spot with $R_f = 0,56$.

Two spots were found in the chromatogram of the ointment: the lower one, corresponding to the location, color, and R_f to the spot in the chromatogram of the working standard solution of SB oil, and another wider and more indistinct spot, probably associated with other lipophilic reduction agents present in the ointment (see fig. 2).

Therefore, thin-layer chromatography can be applied to the identification of β -carotene in the wound-healing ointment by "Harmony-2000".

The method of quantitative determination of total carotenoids (mg/g) in terms of β -carotene in the wound-healing ointment with SB oil. This determination was conducted using an aqueous solution of potassium dichromate as an external standard. The absorbance of the experimental solutions was determined at 450 nm in 10 mm cuvettes, with hexane as a reference. The total content of carotenoids as mg/g was calculated by the formula:

Based on the above statistical values, an extended uncertainty of the experimental data was calculated as $\pm 0,725\%$. Therefore, it can be concluded that this investigation proved that the mean content of carotens in terms of β -caroten in a series of wound-healing ointment with

SB oil by “Harmony-2000” was $0,1897 \pm 0,00075$ mg/g. Relative uncertainty of the mean content was 0,396%, which doesn't exceed the expected value.

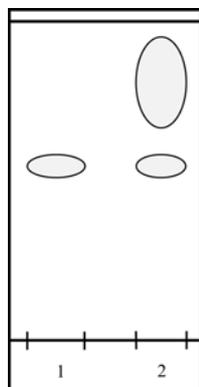


Fig. 2. The structure of a thin-layer chromatogram of the wound-healing ointment: 1 – SB oil; 2 – the ointment

$$X_{\text{MГ}} = \frac{A \cdot V \cdot 0,00208}{A_{\text{СТ}} \cdot m_{\text{H}}}, \text{ where:}$$

A – mean absorbance of a solution at 450 nm;
 A_{st} – absorbance of the reference solution at 450 nm;
 0,00208 – an amount of β -carotene in a solution with the absorbance equal to that of the standard solution of potassium dichromate;

V – a volume of the experimental solution;
 m_{s} – weight of a sample, g.

Table 1

Determination of the quantitative content of total carotenoids in terms of β -carotene in the wound-healing ointment with SB oil (*)

№	Sample mass, g	Absorbance	Content of total carotenoids in terms of β -carotene, mg/g
1	0,5494	0,500	0,191
2	0,5520	0,497	0,189
3	0,5500	0,496	0,189
4	0,5498	0,499	0,190
5	0,5509	0,505	0,192
6	0,5488	0,490	0,187

(*) $A_{\text{st}} = 0,497$.

Table 2

Some metrological values of the experimental data from table 1

Metrological parameter	Value
Mean content, mg/g	0,1897
Variance, S^2	$3,067 \times 10^{-6}$
Standard deviation of a separate value, S	0,001757
Reliability, P, %	95
Student's t-test, t (P,f)	2,57
Confidence interval of the mean content, ΔX_{mean}	0,00075
Relative uncertainty of the mean content, $\bar{\varepsilon}$, %	0,396

All obtained experimental results are shown in table 1, and table 2 represents the statistical values of the experimental data.

Determination of the linearity limits for the experimental method. 9 standard solutions were prepared to evaluate the linearity of the studied spectrophotometrical method for the determination of total carotenoids in terms of β -carotene. Their concentrations were chosen in such a way as to cover the entire practical range of concentrations of the acting component. The State Pharmacopeia of Ukraine requires ensuring a range of 80–120% with a step of 5% for the quantitative determination of an active pharmaceutical component. Based on the experimental data (table 3), a diagram representing the normalized dependence of absorbance on the concentration of a carotene-containing solution has been built (see fig. 3). The linearity of the diagram and its compliance with the requirements of the State Pharmacopeia of Ukraine are represented in table 4 (State Pharmacopoeia of Ukraine, 2025).

Table 3

Evaluation of linearity of the method for quantitative determination of total carotenoids in terms of β -carotene

№	C, %	C (mg/g)	$C_{i/st}$	Mean absorbance	$A_{i/st}$
1	80	0,1921	100,1	0,404	80,00
2	85	0,1924	100,2	0,430	85,15
3	90	0,1923	100,2	0,455	90,10
4	95	0,1922	100,1	0,480	95,05
5	100	0,1919	100,0	0,505	100,00
6	105	0,1920	100,0	0,530	104,95
7	110	0,1916	99,8	0,554	109,70
8	115	0,1912	99,6	0,578	114,46
9	120	0,1909	99,4	0,602	119,21
Reference	100	0,1919	100,0	0,505	

Table 4

Evaluation of the linearity of the method for experimental determination of total carotenoids in terms of β -carotene

Parameter	Value	Required by Pharmacopeia	Complies
Slope (tangent), b	0,9795	–	–
Standard deviation, S_b	0,0044	–	–
Constant term, a	1,89	$\leq 5,1$	Yes
Standard deviation, S_a	0,448	–	–
Residual standard deviation, SD_0	0,172	$\leq 1,69\%$	Yes
Correlation coefficient, r	0,9999	$\geq 0,99236$	Yes

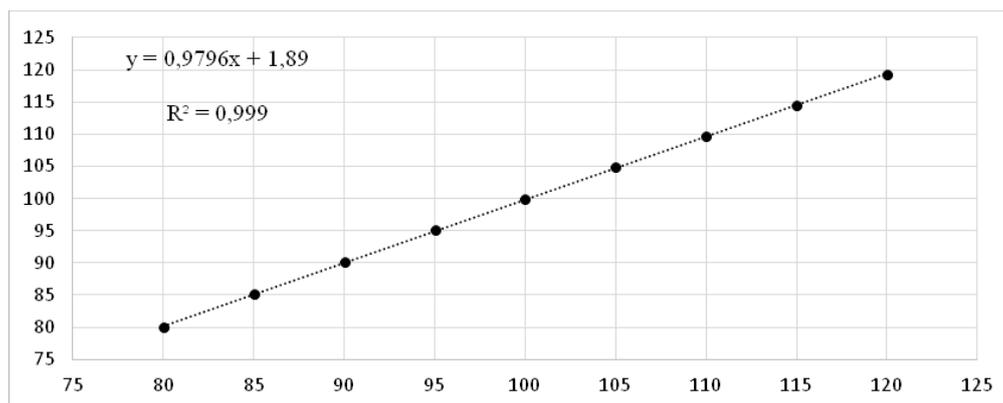


Fig. 3. A normalized dependence of the solution's absorbance on the concentration of an active compound

As seen from the experimental data, the required linearity is maintained across the entire range of concentrations involved in this investigation. Therefore, it can be concluded that the proposed method is suitable for the reliable determination of total carotenoids in terms of β -carotene with a possible deviation of $\pm 20\%$.

Conclusions. 1. Suitable and cost-effective quality control methods were developed to quantify carotenoids in extemporaneously-made ointments with Sea Buckthorn oil.

2. Thin-layer chromatography and spectrophotometry are proposed to identify β -carotene in a wound-healing ointment with Sea Buckthorn oil manufactured extemporaneously by the "Harmony-2000" drugstore chain 2. A spectrophotometric method and a thin-layer chromatography are proposed for the

identification of total carotens in the wound-healing ointment produced by "Harmony-2000".

3. It is confirmed that a direct single-wave absorbance spectrophotometry followed by an external standard processing of the data can be applied to the quantitative calculation of the content of total carotens in the ointment.

4. It is shown that other components of the ointment do not impair the reliability of this photometrical method.

5. The complete predicted uncertainty of the method is approximately $\pm 0,725\%$.

6. Linearity of this method complies with the requirements of the State Pharmacopoeia of Ukraine.

7. It was found that the content of total carotenoids in terms of β -carotene in the wound-healing ointment ranged within $(0,1897 \pm 0,00075 \text{ mg/g})$.

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