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Permeability testing of the vitamins A + E complex contained in emulsions using a Franz diffusion cell

Objective — to test whether active substances, such as vitamin A and vitamin E, contained in emulsion will penetrate the membrane imitating skin.

Materials and methods. Two derivatives were the analysed active compounds: retinol palmitate and tocopherol acetate. Permeation studies were conducted using Franz-type diffusion cells made of borosilicate glass. The collected samples were subjected to determination by the MALDI-TOF MS method and the analytical HPLC technique. The studies were in vitro tests, which allow for testing of active substances outside a living organism while representing the effects of these compounds on the organism.

Results and discussion. The permeability testing shows that some of the compounds contained in the w/o type of emulsion pass through membranes, which was confirmed using selected methods of HPLC qualitative analysis and MALDI-TOF MS. In addition, apart from the analysed complex, other active ingredients included in the emulsion formula also managed to get through.

Conclusions. In the test carried out at a temperature of (37 ± 0.5) °C in a diffusion cell derivatives of vitamins A and E were detected much faster than in the tests carried out at laboratory temperature of (22 ± 0.5) °C. The temperature of 37 °C corresponds to the transdermal systems used on the skin.

Key words

Vitamin A, vitamin E, in vitro permeability, Franz diffusion cell.

Vitamins are organic compounds necessary for the proper functioning of the organism and the biochemical processes occurring in the body. They are not produced by the organism therefore they have to be provided from the outside. Cosmetic preparations contain a lot of different vitamins. Vitamins which are significantly used as active ingredients of cosmetic products, either individually or in a complex, are vitamins A and vitamin E [1].

Vitamin A

Vitamin A is a group of organic compounds belonging to retinoids. The term «retinoids» was introduced in 1976, it includes retinol (vitamin A) and its natural and synthetic analogues. They play an important role in the mature organism and at the same time are of fundamental importance in the processes of embryonic development. They are characterized by the fact that they affect biological processes. Among other things, they regulate apoptosis, differentiation and proliferation of cells. They affect biological processes by changing the activity of the genes, which act like hormones, through nuclear receptors, which were discovered in 1987. There are two types of receptors: RAR's (retinoic acid receptors) — a, p, y, and RXR's (retinoid X receptors) — a, p, y. Both RAR's and RXR's regulate expression by a positive feedback, by binding to the promoter region of the target gene, they may also have a negative feedback by enhancing the activity of other transcription factors, such as AP 1, which activates cell proliferation and inflammatory processes [2, 3]. The crucial structure of retinoid particles comprises the cyclic terminal group and the polyene side chain which terminates in a polar group. A system of conjugated double bonds of a polyene chain influences the colour of retinoids, which occurs from yellow through orange to red, therefore many of them exhibit chromophore properties. The structural changes that occur in the polyene side chain and in the cyclic terminal group make it possible to create retinoids with different structures and properties [3, 4].

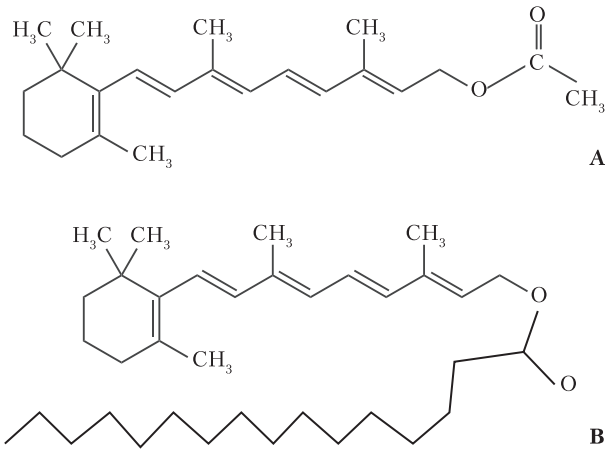


Figure 1. **The structural formulas of the esters of vitamin A (8)**

The most common forms of retinoids used in cosmetic formulations for skin care are:

1) tretinoin — otherwise known as retinoic acid; in chemical terms it is more stable than retinol; the compound stimulates the growth of epithelial cells and increases the synthesis of glycoproteins, which influence the transport of oligosaccharides by the cell membrane; it is normally used externally in the treatment of acne because it reduces inflammation and opens sebaceous glands by stimulating cell proliferation [5];
2) isotretinoin — or 1.3-cis-retinoic acid; is a cis isomer of retinoic acid; it is often used orally in more severe forms of acne; it binds strongly to blood proteins and has a long-term effect [5].

Furthermore, vitamin A is present in cosmetics in the form of esters: retinol acetate A or retinol palmitate B. The structures of the compounds are shown in Figure 1.

Retinol is a cyclic polyene alcohol, which is composed of trimethylcyclohexane (β-ion), which has an 11-carbon side chain with four double bonds. The presence of these bonds makes vitamin A unstable and easily oxidized. It dissolves in ethanol, in petroleum ether and in fats [6,13].

Vitamin A is very often referred to as a growth, anti-infections and anti-aging vitamin. The organism stores it in the liver in the form of ester, or retinol palmitate, which in the process of saponification forms pure vitamin A, which is released into the bloodstream [7, 8]. «Outside the liver retinol is coupled with glucuronic acid, and then it is oxidized to become retinal and retinoic acid, which is excreted in urine and faeces» [6, 9].

Operation of vitamin A is multidirectional. It includes, among others: impact on the development and growth of the organism, impact on the production of hormones, impact on bone formation, impact on the process of keratinization of the epidermis, increase of the protective layer of the skin, it is one of the main compounds responsible for the processes of sight, protection against cancer, impact on the proper development and functioning of skin cells [1, 6, 9].

Vitamin A deficiencies manifest themselves, among other things, through hyperkeratosis, dry skin, brittle nails and weak hair. Other symptoms of the deficiency include the impairment of vision, growth inhibition, and decreased resistance to infection [1, 6]. Vitamin A can also be very easily overdosed, which leads to acute poisoning caused by very high doses, which manifests itself in mental disorders, very high drowsiness, vomiting, and nausea. Normally, the concentration of retinol in blood plasma is 30—60 μg/100 ml, and in the case of acute poisoning it is 150 μg/100 ml [6].

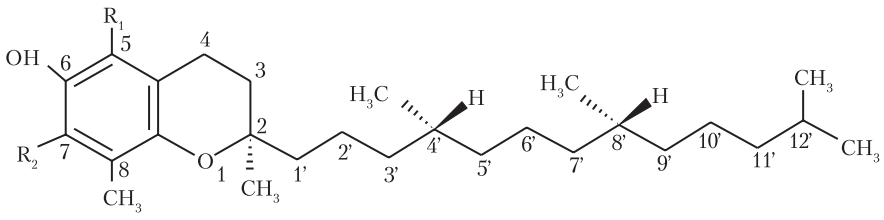
Retinol is included in both products of plant origin (mainly in the form of pro-vitamin A) and animal origin. It is also produced synthetically. Vitamin A is most commonly used in cosmetics in the form of retinol palmitate. It is used, among others, in the treatment of psoriasis, pruritis (in old age), various dermatoses, frostbite, and wounds that do not heal. It is used in various formulations, in the form of tablets and ointments for the treatment of acne. Vitamin A used orally is teratogenic. In the formulations for the skin it is active in the concentration of 0.1 to 2 %.

Vitamin E

Vitamin E occurs most often in the form of tocopherols (Figure 2) and tocotrienols. A common feature of tocopherols and tocotrienols is the bicyclic skeleton of 6-chromanol and a side chain built from three isoprene units. These are oily substances that are soluble in non-polar solvents and oils, insoluble in water. These compounds are resistant to high temperatures, acids and alkalis, they are not saponifiable. Pure tocopherols are sensitive to chemical oxidizing agents, ultraviolet light and oxygen. However, their esters are resistant to these factors, particularly to oxygen. Esterification of tocopherol with selected fatty acids may increase the biological activity of vitamin E [1, 5, 6].

Ester derivatives, whose operating time is extended, are a more permanent form of tocopherols. In medicine and cosmetology tocopherol acetate is most commonly used (Figure 3), which is an ester of α-tocopherol and acetic acid [5, 9—11].

Vitamin E has the following effect: it prevents enzymes, hormones and vitamins from the formation of peroxides and protects the fatty layers of the epidermis, it improves the absorption of oxygen by cells, it improves blood circulation in the skin, it



Tocopherols	R_1	R_2	Chemical name
			3,4-dihydro-2,5,7,8-tetramethylo-2-(4',8',12'-trimethylotridecylo)-2H-1-benzopyran-6-ol
α-Tocopherol (α-T)	CH_3	CH_3	2,5,7,8-tetramethylo-2-(4',8',12'-trimethylotridecylo)-6-chromanol
			3,4-dihydro-2,5,8-trimethylo-2-(4',8',12'-trimethylotridecylo)-2H-1-benzopyran-6-ol
β-Tocopherol	CH_3	H	2,5,8-trimethylo-2-(4',8',12'-trimethylotridecylo)-6-chromanol
			3,4-dihydro-2,5,8-trimethylo-2-(4',8',12'-trimethylotridecylo)-2H-1-benzopyran-6-ol
γ-Tocopherol	H	CH_3	2,7,8-trimethylo-2-(4',8',12'-trimethylotridecylo)-6-chromanol
			3,4-dihydro-2,8-dimethylo-2-(4',8',12'-trimethylotridecylo)-2H-1-benzopyran-6-ol
δ-Tocopherol	H	H	2,8-trimethylo-2-(4',8',12'-trimethylotridecylo)-6-chromanol

Figure 2. **The structural formula of tocopherols (10)**

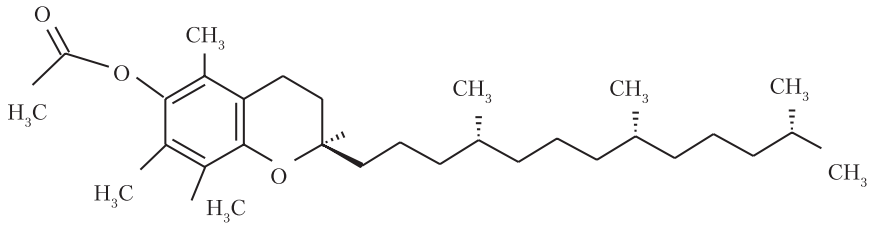


Figure 3. **The structural formula of tocopherol acetate (1)**

strengthens the connective tissue, it stimulates the production of anticoagulants, it has an impact on the stability and permeability of cell membranes.

Vitamin E deficiency can lead to skeletal muscle dystrophy, keratinization disorders, worse wound healing and worse concentration. It can also cause neurological disorders, and decreased vision. Moreover, in men it results in aspermia, and in women the deficiency may lead to miscarriages. For that reason vitamin E is often referred to as fertility vitamin [1, 6, 10].

Vitamin E may be obtained synthetically or naturally from cereal sprouts [1]. A daily intake of this compound for an adult is 15—30 mg.

The most commonly used form, α-tocopherol, is used as an antioxidant in anti-aging, nourishing and regenerating creams. Along with vitamin A and E it is used in the treatment of eczema and acne vulgaris, as well as in wrinkle reduction [1, 5].

It is because of the currently prevailing trend in cosmetology, which recommends the administration of vitamins as both supplements and topically, that the current work characterizes and tests this group of compounds.

AIM

The aim of the experimental work was to develop a formulation of a cosmetic, a preparative of a cosmeceutical with a chosen active ingredient, which was the vitamins A + E complex, and conducting release level tests using a Franz diffusion cells.

MATERIALS AND METHODS

1. Development of a recipe for the cosmeceutical

The cosmeceutical recipe was based on a w/o emulsion, wherein the vitamins A + E complex was placed [12]. The recipe was developed on the basis of a study of the recipes for vitamin A + E cosmetic preparations available on the Polish cosmetics market. Table 1 shows the composition of the emulsion.

Table 1. **The composition of the emulsion**

Common name	INCI name	Quantity [g]
Eucerine	*Eucerini*	12
Castor oil	*Oleum ricini*	4
Distilled water	*Aqua destilata*	44
Vitamin A	*Vitaminum A liquidum*	0.3
Vitamin E	*Vitaminum E liquidum*	0.3

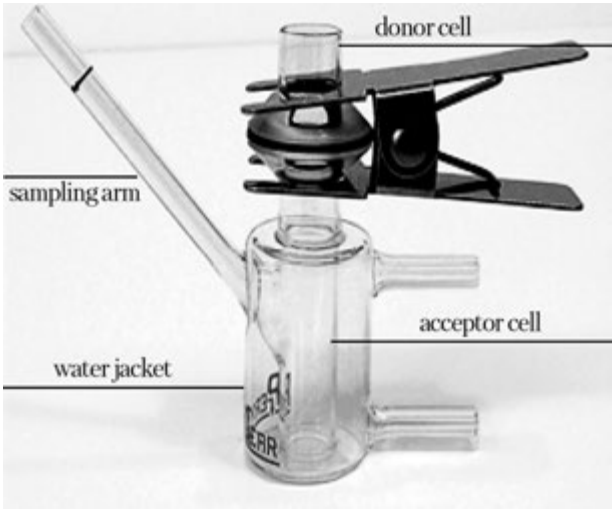


Figure 4. **A single cell for testing permeability**

2. Preparation of the cosmeceutical

12 g of eucerine was weighed on an analytical balance, which was then thoroughly triturated using a pestle in a mortar. Then small portions of previously weighed 4 g of castor oil were added to the eucerine. After thorough mixing of the two components distilled water, previously weighed in a beaker on an analytical balance, was added in small portions. Finally, 0.3 g of vitamin A and the same amount of vitamin E were added to the emulsion. The whole underwent a homogenization process in a homogenizer. 60.6 g of the final emulsion were obtained.

3. Penetration study of the A + E complex

3.1. The permeability testing cell

In the course of the tests the potential for releasing of selected vitamins from the emulsion was tested. The tests were performed using Franz-type diffusion cells, in the Laboratory of Chemistry of Biological Macromolecules at the Faculty of Chemistry of the University of Gdansk.

Franz-type cells are used for permeability testing of active substances by diffusion. The cells used in the test chamber were made of borosilicate glass with the volume of 10 ml. A single test cell is shown in Figure 4.

3.2. The membrane

In order to conduct the permeability testing Whatman® membranes, made of cellulose acetate, were used, whose pore size was 0.45 μm and diameter — 25 mm. The pore size corresponded to the size of the pores in human skin.

3.3. Conditions for the release of active ingredients

The permeability testing was performed using two Franz-type cells fitted with a magnetic stirrer, the speed of which was set at 350 rpm.

The tests were performed at (26 ± 5) °C and (37 ± 0.5) °C, which is similar to the transdermal systems applied to the skin. The temperature in the cell was maintained by a thermostat.

The acceptor fluid used in the tests was double distilled degassed water at physiological pH measured at 7.010.

Each time, 1 g of the emulsion was placed on the membrane in the donor cell.

The cell was sealed with parafilm to prevent evaporation and it was secured with a buckle.

3.4. Qualitative analysis

To determine the molecular weight of the compounds released from the emulsion in the acceptor fluid mass spectra were made, which were obtained by mass spectrometry by matrix assisted laser desorption ionization (MALDI-TOF MS) in Physical and Chemical Laboratories of the University of Gdansk. Each time the matrix for the tested compounds was α-cyano-4-hydroxycinnamic acid (CCA) and 2,5-dihydroxybenzoic acid (DHB).

At the same time, to confirm the presence of derivatives of vitamins A and E released from the emulsion in the liquid acceptor, and in order to identify them the analysis technique of high performance liquid chromatography (HPLC) was used in a0 reversed phase system (RP) using a BECKMAN GOLD SYSTEM. The conditions for elution were as follows: samples were analysed directly on RP-18 columns (stationary phase) at UV light detection having a wavelength of 275—295 nm. Methanol and acetonitrile (95 : 5 v/v) were used for the mobile phase.

The applied methods of analysis are simple, repetitive, selective and can be used in routine analyses of commercial cosmetics for the determination of vitamins soluble and insoluble in fat [13].

RESULTS

The first step in the release study was the analysis of the active compounds by the methods described

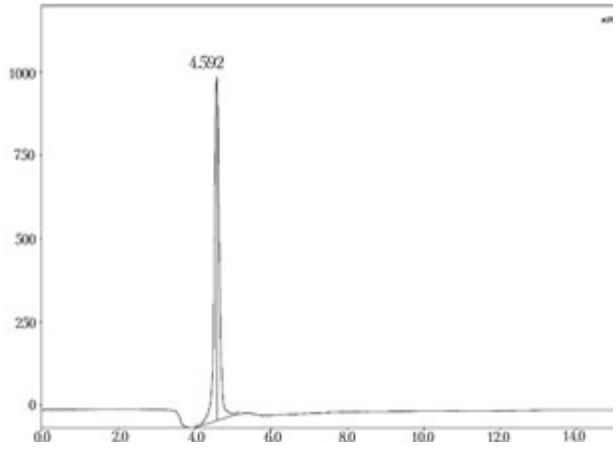


Figure 5. **FAn HPLC chromatogram of retinol palmitate, the peak corresponding to the compound was recorded at a retention time of 4.592 min**

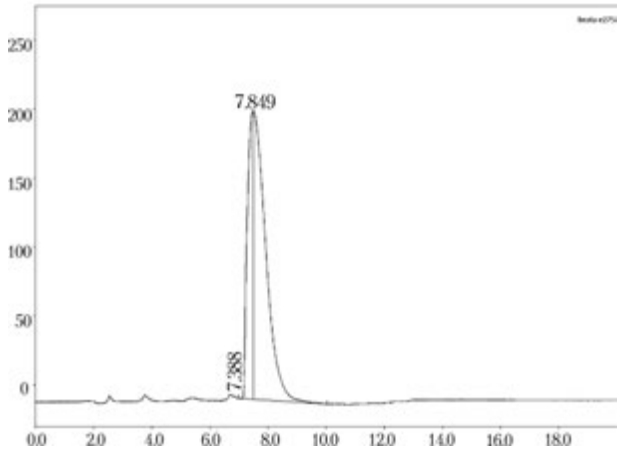


Figure 6. **An HPLC chromatogram of tocopherol acetate, the peak corresponding to the compound was recorded at a retention time of 7.029 min**

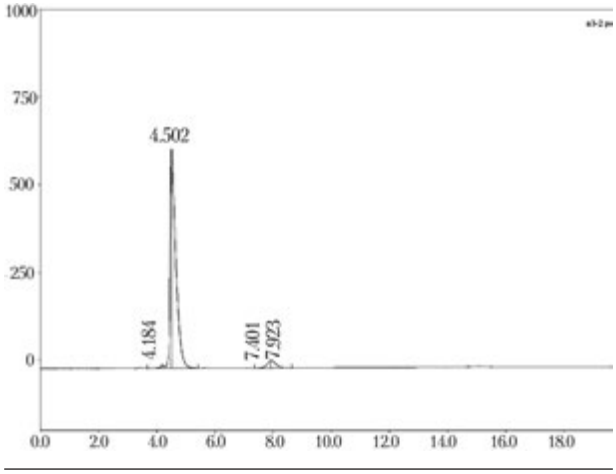


Figure 7. **An HPLC chromatogram of the A + E complex (retinol palmitate and tocopherol acetate), the peak corresponding to vitamin A time was recorded at a retention time of 4.502, and vitamin E at a retention time of 7.923 min**

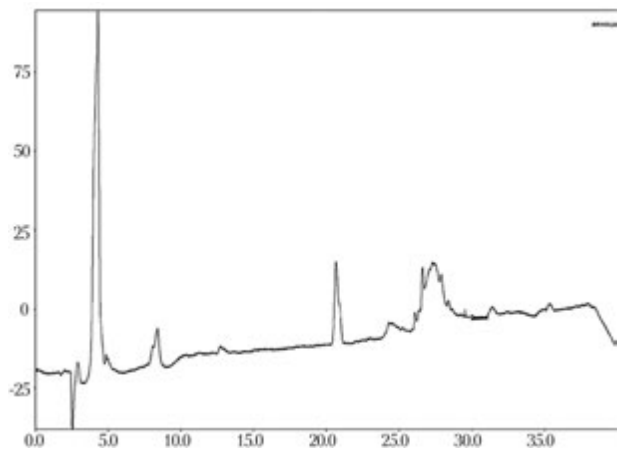


Figure 8. **An HPLC chromatogram of the A + E complex (retinol palmitate and tocopherol acetate) in emulsion, the peak corresponding to vitamin A was recorded at a retention time of 4.507, and vitamin E at a retention time of 7.922 min**

in Section 3.4. To this end, chromatograms and mass spectra of crude vitamin derivatives were obtained: retinol palmitate (vitamin A) and tocopherol acetate (vitamin E), vitamin complex (A + E) and the A + E complex in the emulsion. The chromatograms performed with the use of RP-HPLC are presented in Figure 5—8.

Afterwards the active compounds tested were subjected to mass analysis. To do this, first of all molecular weights of the active compounds were calculated, which were, respectively:

1) retinol palmitate (vitamin A): 526.86 g/mol;
2) tocopherol acetate (Vitamin E): 472.76 g/mol;
3) eucerine ingredients: cholesterol — 386.65 g/mol; cetyl alcohol — 242.44 g/mol; white petrolatum — a mixture of hydrocarbons: docosane 310.12 g/mol and tricosane 324,20 g/mol;
4) the components of castor oil: 80 % ricinoleic acid glyceride — 372.40 g/mol and 7 % oleic acid glyceride 356.11 g/mol, 3 % linoleic acid glyceride 354.68 g/mol, 2 % palmitic acid glyceride 330.56 g/mol; 1 % stearic acid glyceride 358.78 g/mol.

The weights of particular compounds were determined for raw compound samples and on the emulsions. The resulting mass spectra are shown in Figure 9—12.

As the experiment progressed and samples were taken out from the acceptor cell along with the penetrated substances, regular injections of samples using HPLC-RP were carried out. After 16 hours, in the cell connected to a thermostat, the presence of peaks belonging to the examined vitamins was revealed in a sample of acceptor fluid. By contrast,

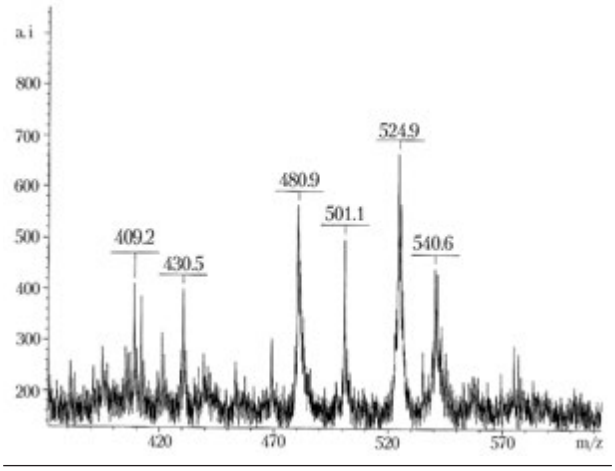


Figure 9. **A mass spectrum of retinol palmitate, measured using MALDI-TOF MS (DHB matrix), the peak of m/z = 524.9 corresponds to the ion (M)+**

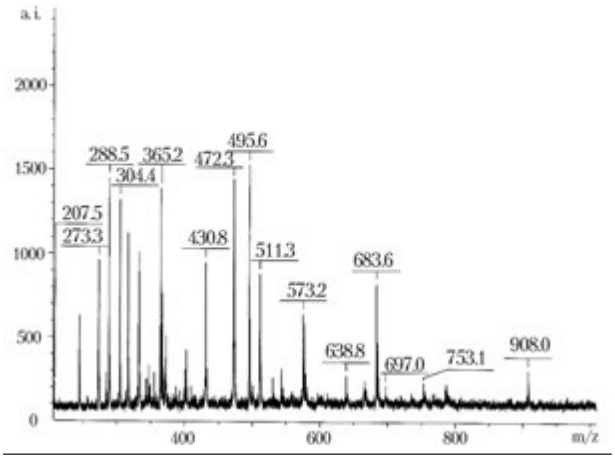


Figure 10. **A mass spectrum of tocopherol acetate, measured suing MALDI-TOF MS (DHB matrix), the peak of m/z = 495.6 corresponds to the ion (M + Na) + peak m/z = 511.3 corresponds to the ion (M + K)+**

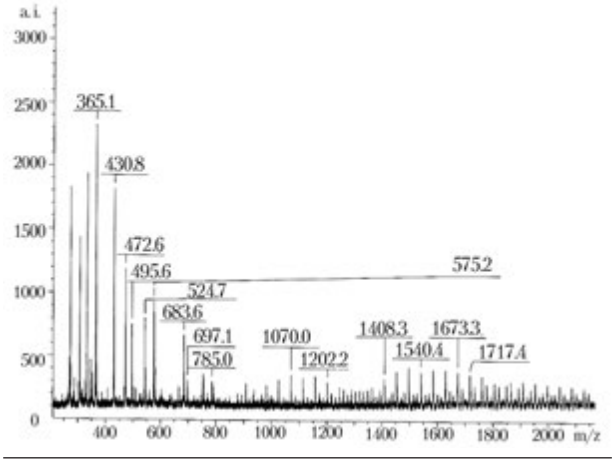


Figure 11. **A mass spectrum of the complex: retinol palmitate and tocopherol acetate, measured using MALDI-TOF MS (DHB matrix), the peak of m/z = 524.7 corresponds to the ion (M)+ of vitamin A, the peak of m/z = 472.6 to the ion (m)+ of vitamin E, the peak of m/z = 495.6 corresponds to the ion (m + Na)+ of vitamin E**

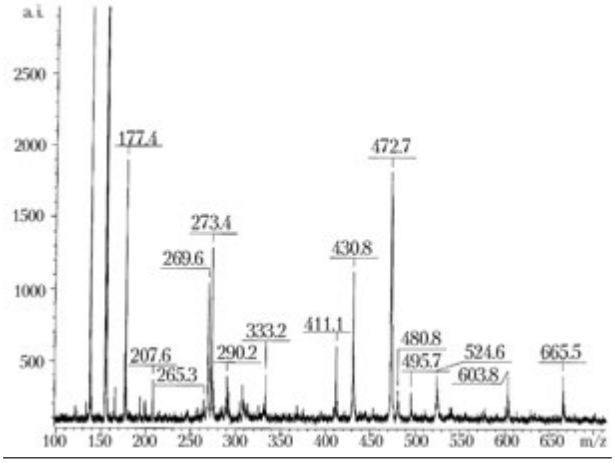


Figure 12. **A mass spectrum of the complex: retinol palmitate and tocopherol acetate in emulsion, measured using MALDI-TOF MS (DHB matrix), the peak of m/z = 524.6 corresponds to the ion (M)+ of vitamin A, the peak of m/z = 472.7 to the ion (m)+ of vitamin E, the peak of m/z = 495.7 corresponds to the ion (m + Na)+ of vitamin E**

in the release testing of the vitamins complex in a cell without a thermostat, on the basis of qualitative studies, the presence of peaks was discovered after 23 hours. The chromatograms are presented in Figure 13 and Figure 14.

The resulting chromatograms differ in the size and intensity of peaks originating from test compounds. This is due to the difference in the conditions of the experiment. The testing carried out in the cell with a thermostat showed more intense signals and a shorter time of penetration.

Then, the same samples, taken from the acceptor cell, were subjected to tests on a mass spectrometer using the MALDI-TOF MS. The mass spectra are presented in Figure 15 and Figure 16. In the mass spectrum obtained after 16 hours for the thermostat samples, a peak derived from vitamin E was observed, corresponding to its molecular weight, a peak corresponding to the weights of ricinoleic acid glyceride, linoleic, docosane, and cetyl alcohol. In the mass spectrum obtained after 23 hours a tiny peak was observed derived from vitamin E, corresponding to its molecular weight. By contrast, the signals corresponding to the molecular masses of castor and linoleic acid glycerides, docosane, and cetyl alcohol were a little more intense. The author of the paper speculates that this was a result of the longer time of permeation. In addition, in both

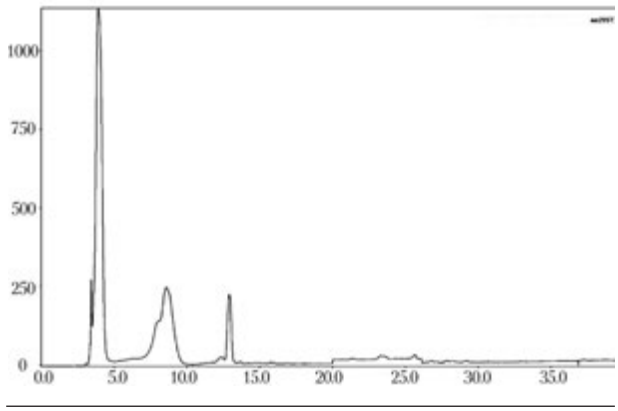


Figure 13. **An HPLC chromatogram of the A + E complex (retinol palmitate and tocopherol acetate) in emulsion after 16 hours, the peak corresponding to vitamin A was recorded at a retention time of 4.707, and vitamin E at a retention time of 7.992 min**

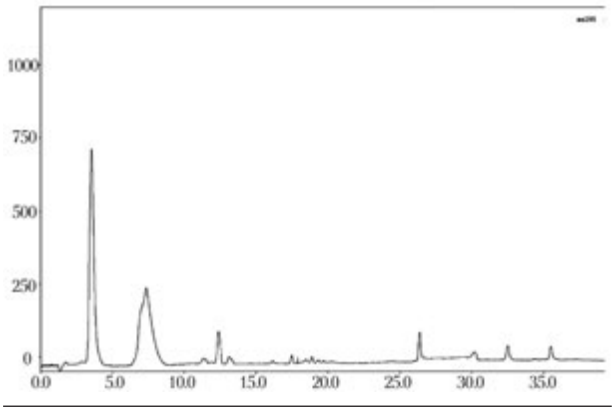


Figure 14. **An HPLC chromatogram of the A + E complex (retinol palmitate and tocopherol acetate) in emulsion after 23 hours, the peak corresponding to vitamin A was recorded at a retention time of 4.597, and vitamin E at a retention time of 7.562 min**

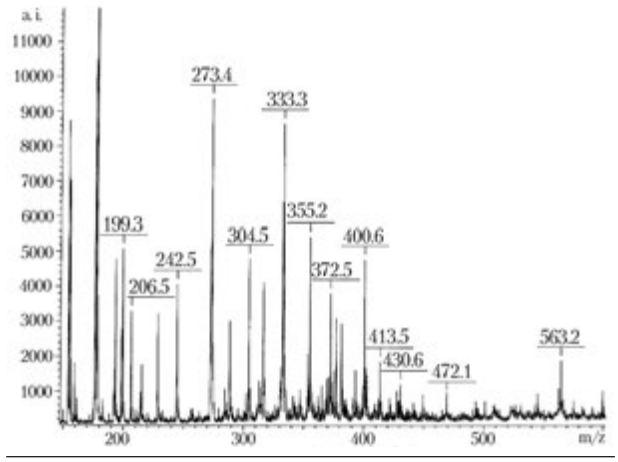


Figure 15. **A mass spectrum of the complex: retinol palmitate and tocopherol acetate released from the emulsion after 16 hours of testing, measured using MALDI-TOF MS (DHB matrix), the peak of m/z = 472.1 corresponds to the ion (M)+ of vitamin E, the peak of m/z = 242.5 corresponding to the ion (M)+ of cetyl alcohol, the peak of m/z = 333.3 corresponds to the ion (m + Na)+ belonging to docosane, the peak of m/z = 355.2 corresponds to the ion (M + H)+ of linoleic acid glyceride, the peak of m/z = 372.5 corresponds to the ion (M)+ of ricinoleic acid glyceride**

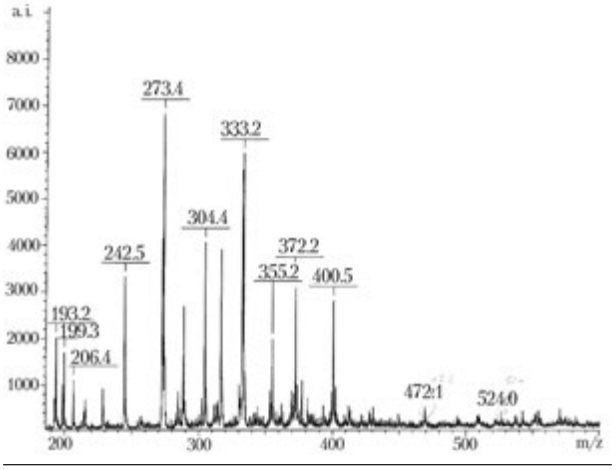


Figure 16. **A mass spectrum of the complex: retinol palmitate and tocopherol acetate released from the emulsion after 23 hours of testing, measured using MALDI-TOF MS (DHB matrix), the peak of m/z = 472.1 corresponds to the ion (M)+ of vitamin E, the peak of m/z = 242.5 corresponds to the ion (M)+ of cetyl alcohol, the peak of m/z = 333.2 corresponds to the ion (M + Na) + belonging to docosane, the peak of m/z = 355.2 corresponds to the ion (M + H)+ of glyceride linoleic acid, the peak of m/z = 372.5 corresponds to the ion (M)+ of ricinoleic acid glyceride**

spectra the signals derived from vitamin A are of very low intensity, bordering on noise, which means that the compound was released in minute quantities.

CONCLUSIONS

Permeability of active compounds is a complex process and is influenced by many factors. The permeability test using the in vitro technique with Franz cells is very important in the development of new cosmetics. Using these tests helps to determine the time and the intensity of the permeability of selected substances very precisely, and in this case — vitamins.

The analysis of the results shows that the vitamins contained in the prepared emulsion penetrated the membrane.

The HPLC analysis of the collected samples revealed the presence of vitamin A and E. In the test carried out using a thermostat vitamin derivatives were detected after 16 hours of diffusion, while in the study conducted without the use of a thermostat — after about 23 hours of diffusion.

Tocopherol acetate retention time was 4.5 minutes and that of retinol palmitate — 7.9 minutes.

In the release test not only the studied vitamins penetrated the selected test membrane, but also the compounds that were part of castor oil.

In MS spectra, taken after the samples of the emulsion and the vitamins A and E solution had done the penetrating, peaks were observed corresponding to the molecular weights of these vitamins. The peaks appearing in the spectra are of low intensities because of the small amount of the active ingredients in the emulsion which reached the solution and because of the low concentration of vitamins in the formulation. DHB turned out to be a better matrix for the tested vitamins.

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Визначення проникності комплексу вітамінів А + Е, що міститься в емульсіях, з використанням дифузійної камери Франца

Мета роботи — перевірити, чи активні речовини, такі як вітамін А і вітамін Е, що містяться в емульсії, проникають у мембрану, що імітує шкіру.

Матеріали та методи. Двома похідними були проаналізовані активні сполуки: ретинолу пальмітат і токоферолу ацетат. Дослідження проникності проводилися з використанням дифузійних камер Франца, виготовлених із боросилікатного скла. Зібрані зразки тестували за допомогою методу MALDI-TOF MS та аналітичної методики високоефективної рідинної хроматографії. Дослідження здійснювали в лабораторних умовах, які дають змогу тестувати активні субстанції поза живим організмом і показують вплив цих сполук на організм.

Результати та обговорення. Тестування проникності показує, що деякі зі сполук, що містяться в емульсії типу w/o, проходять крізь мембрани, що було підтверджено за допомогою методу MALDI-TOF MS та аналітичної методики високоефективної рідинної хроматографії. Крім того, поряд із проаналізованим комплексом, інші активні інгредієнти, що входять до формули емульсії, також пройшли крізь мембрану.

Висновки. У тестах, проведених у дифузійній камері при температурі (37 ± 0,5) °С, похідні вітамінів А та Е були виявлені значно швидше, ніж у тестах, проведених при лабораторній температурі (22 ± 0,5) °С. Температура 37 °С відповідає трансдермальним системам, які використовуються на шкірі.

Ключові слова: вітамін А, вітамін Е, проникність у лабораторних умовах, дифузійна камера Франца.

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Определение проницаемости комплекса витаминов А + Е, содержащегося в эмульсиях, с использованием диффузионной камеры Франца

Цель работы — проверить, проникают ли активные вещества, такие как витамин А и витамин Е, содержащиеся в эмульсии, в мембрану, имитирующую кожу.

Материалы и методы. Двумя проанализированными производными активными соединениями были: ретинола пальмитат и токоферола ацетат. Исследование проницаемости проводилось с использованием диффузионных камер Франца, изготовленных из боросиликатного стекла. Собранные образцы тестировали с помощью метода MALDI-TOF MS и аналитической методики высокоэффективной жидкостной хроматографии. Исследования проводили в лабораторных условиях, которые позволяют тестировать активные субстанции вне живого организма и показывают влияние этих соединений на организм.

Результаты и обсуждение. Тестирование проницаемости показывает, что некоторые из соединений, содержащихся в эмульсии типа w/o, проходят через мембраны, что было подтверждено с помощью метода MALDI-TOF MS и аналитической методики высокоэффективной жидкостной хроматографии. Кроме того, вместе с проанализированным комплексом, другие активные ингредиенты, входящие в состав формулы эмульсии, также прошли сквозь мембрану.

Выводы. В тестах, проведенных в диффузионной камере при температуре (37 ± 0,5) °C, производные витаминов А и Е были обнаружены значительно быстрее, чем в тестах, проведенных при лабораторной температуре (22 ± 0,5) °C. Температура 37 °C соответствует трансдермальным системам, которые используются на коже.

Ключевые слова: витамин А, витамин Е, проницаемость в лабораторных условиях, диффузионная камера Франца.

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