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Green HPLC Method for Squalene Determination in Dietary Supplement

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The aim of this study was to develop a rapid and environmentally friendly method for determining squalene in dietary supplements. An Agilent 1200 chromatograph was used with Discovery F5 (SiO_2-PFP) and Discovery HS C18 (SiO_2-C18) stationary phases, applying water–ethanol mixtures as mobile phases. To ensure environmental safety, toxic solvents (methanol, acetonitrile) were replaced with ethanol. Detection of squalene at 210 nm in ethanol-based mobile phases proved feasible. Comparison of the chromatographic phases showed that SiO_2-PFP required 10–12% less ethanol than SiO_2-C18 to achieve equivalent retention, improving both efficiency and cost-effectiveness. A validated methodology for quantitative squalene determination was established. Optimal conditions were achieved with a Discovery F5 column (4.6×250 mm, 5 µm), thermostated at 40 °C, and a mobile phase of 85% ethanol, with UV detection at 210 nm. The method met requirements of specificity, linearity, accuracy, and reproducibility. Analysis time per sample was only six minutes, and the use of ethanol ensured safety for both analysts and the environment. Application of the developed method revealed that the tested dietary supplement did not contain squalene. Instead, the undeclared presence of tocopheryl acetate was detected at 5.0 mass percent. This result underlines the importance of stricter regulatory oversight of dietary supplements on the Ukrainian market.

Keywords: HPLC, validation, squalene, dietary supplement, green chemistry

Екобезпечне визначення сквалену в дієтичній добавці методом високоефективної рідинної хроматографії

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Метою роботи було створення швидкої та екологічно безпечної методики визначення сквалену в дієтичних добавках. Для досліджень використано рідинний хроматограф Agilent 1200, стаціонарні фази Discovery F5 (SiO_2-PFP) та Discovery HS C18 (SiO_2-C18), а також водно-етанольні суміші як рухомі фази. З метою екологічної безпеки запропоновано замінити токсичні розчинники (метанол, ацетонітрил) на етанол. Доведено можливість коректного визначення сквалену в етаноловмісній рухомій фазі за довжини хвилі 210 нм. Порівняння хроматографічних фаз показало, що для досягнення однакових характеристик утримування при використанні SiO_2-PFP вміст етанолу необхідно зменшити на 10–12% у порівнянні з SiO_2-C18, що додатково підвищує економічну ефективність методу. Розроблену методику кількісного визначення сквалену валідовано. Оптимальні умови встановлено для колонки Discovery F5 (4.6×250 мм, 5 мкм), термостатованої при 40 °C, з рухомою фазою, що містить 85% етанолу, та УФ-детектуванням при 210 нм. Методика відповідає вимогам специфічності, лінійності, точності й відтворюваності. Тривалість аналізу становить лише 6 хвилин, а використання етанолу забезпечує безпечність для довкілля та аналітика. За результатами аналізу у зразку дієтичної добавки сквален не виявлено, проте зафіксовано наявність незаявленого компонента – токоферолу ацетату у кількості 5,0% (мас.). Це свідчить про необхідність посилення регуляторного контролю ринку дієтичних добавок в Україні.

Ключові слова: ВЕРХ, сквален, дієтична добавка, зелена хімія, валідація

Introduction

In recent years, the dietary supplements have gained considerable popularity as individuals worldwide seek to enhance their overall well-being and support their health through natural means. Among the myriad of compounds found in these supplements, squalene has emerged as a subject of growing interest and research. Squalene, chemically classified as a triterpenoid hydrocarbon, serves as an essential intermediate in the biosynthesis of sterols and other important biomolecules in both plants and animals.

Squalene present in some shark liver oils (dalatias licha, centrophorus uyato) [1], and certain vegetable oils, such as olive oil, wheat germ oil, rice bran oil, and amaranth oil [2]. This compound has been recognized for its medicinal properties, including antitumor, antioxidant, and hepatoprotective effects [3].

Squalene can be determined by GC or HPLC methods. As for gas chromatography methods, often a multi-step sample processing is necessary, which usually involves the removal of triglycerides, fractionation of non-saponifiable compounds into several classes, and subsequent analysis using gas chromatography [4, 5]. These methods are not only laborious but also potentially problematic due to significant squalene loss during saponification and extraction. As for liquid chromatography methods, sample processing is used, including alkaline hydrolysis [6, 7], extraction hexane or dichloromethane [8], solid phase extraction [9],evaporation [10], fractional crystalisation [11, 12]. The sample preparation step is often one of the most polluting and resource-intensive parts of the analytical process [13]. To decrease pollution risks and reduce the environmental impact of laboratory work, 2-propanol may be used for sample preparation instead of more toxic or persistent organic solvents [14].

Conventional methods for squalene determination often involve the use of hazardous solvents such as hexane or dichloromethane, which can pose a risk to both human health and the environment. Eco-friendly methods that use non-toxic solvents such as ethanol can eliminate this risk.

The consumption of dietary supplements has been steadily increasing in Ukraine each year, reflecting a growing awareness of health and wellness among the population. The use of dietary supplements without strictquality control regulations has raised significant concerns in Ukraine [15]. As a result, it becomes crucial to ascertain the content of squalene in these supplements to ensure both its proper concentration and overall safety. In light of this concern, we propose a novel, environmentally friendly method for the determination of squalene, using ethanol as a solvent in liquid chromatography.

The development of eco-friendly approaches to squalene determination holds promise in contributing to the broader objective of reducing the environmental impact of analytical chemistry. Usage of these green methodologies allows us to curtail the use of hazardous solvents, thereby minimizing waste generation and lowering the carbon footprint associated with conventional analytical procedures.

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Experimental part

Materials and methods

Reagents and reference materials

Dietary supplement: Squalene NATURE'S ORIGIN capsules, For Health By Earth (squalene ≥50%, gelatin, glycerin) bought in internet shop in Ukraine.

Reference material: Squalene, Supelco, cat. number: PHR1009.Tocopheryl acetate, Sigma-Aldrich, cat. number: T3376

Reagents: water for chromatography, obtained using the Simplicity UV system, Millipore, USA; ethanol 96% (v/v) of high purity, manufaurer the State Enterprise "UKRSPYRT"; acetonitrile, gradient grade, Sigma-Aldrich, cat. number: 34851; 2-propanol, Sigma-Aldrich, cat. number: 34863.

Equipment, chromatography conditions

Glassware: volumetric glassware – volumetric flasks and pipettes, class A. Analytical balance: Mettler Toledo XS204, permissible load is 220 g, and the sensitivity is 0.1 mg. Ultrasonic bath: Daihan, Wuc-A010H, the Republic of Korea. UV-spectrophotometer: Agilent 8453, manufactured by Agilent (USA). The method development was carried out by means of high-performance chromatograph Agilent 1200 HPLC manufactured by Agilent (USA). Discovery HS C18 250×4.6 (5 μm) and Discovery F5 250×4.6 (5 μm) columns were used as the stationary phases in the study.

Chromatography conditions for squalene determination in the Dietary supplement: Column – Discovery F5 250×4.6 (5 μm). Mobile phase – ethanol/water = 85/15% (v/v). Column thermostat temperature – 40°C. Wavelength – 210 nm. Flow rate of the mobile phase – 1.0 mL/min. Injection volume – 3 μL.

Solution preparation

Dietary supplement of Squalene. The mass content of 10 capsules has been determined as 6.0112 g; therefore, the average mass of one sachet is: 6.0112 ÷ 10 = 0.6011 g.

Preparation of the solution for testing the suitability of the chromatographic system: 20 mg of squalene + 20 mg of alpha-tocopheryl acetate in a total volume of 100 mL with 2-propanol.

Preparation of the standard solution: 50 mg of squalene in a 50 mL volumetric flask diluted with 2-propanol. Then, 5 mL of this solution to 25 mL diluted with 2-propanol (0.2 mg/mL).

Preparation of squalene solutions for determining the linearity, accuracy, precision of the method: About 80 mg of the Dietary Supplement placebo, 30–75 mg of squalene was placed into 50 ml flasks and diluted with 2-propanol to produce 9 solutions with squalene concentrations in range 0.12–0.3 mg/mL. The flasks

were placed in an ultrasonic bath for 5 minutes, after the solutions were cooled and diluted to volume. Then, 5 ml of this solution to 25 mL diluted with 2-propanol.

Assay sample preparation: about 130 mg of the Dietary Supplement was placed into 50 mL flasks, to which 25 mL of 2-propanol was added. The flasks were placed in an ultrasonic bath for 5 minutes, after the solutions were cooled and diluted to volume. Then, 5 mL of this solution to 25 mL diluted with 2-propanol. Solution was filtered through PTFE membrane filter with pore diameter of 0.45 µm into vial.

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Results and discussion

Eluent selection. Chromatographic columns filled with a stationary phase based on silica gel with attached octadecyl groups (SiO_2-C18) are typically used in pharmaceutical analysis for quantitative determination of squalene. Such a sorbent is hydrophobic and for elution of hydrophobic squalene (logP=11.6) from its surface, 90% or more acetonitrile or methanol concentration in mobile phase is needed [7,10-11]. Such solvents are toxic and modern approaches to method development involve reducing or avoiding the use of such solvents. The degree of eco-friendliness of an organic solvent is evaluated based on its environmental, health, and safety (EHS) criteria and life-cycle assessment (LCA) [16].

Ethanol (EtOH) is one of the most environmentally friendly organic solvents, which makes it particularly desirable for “green” analytical chemistry [17]. Compared to acetonitrile and methanol, ethanol is less toxic and has a lower vapor pressure, leading to less evaporation and therefore less inhalation.

Wavelength selection. Squalene has 6 double bonds in its structure, but these bonds are not conjugated, so UV absorption occurs in the range of 190–220 nm, with a maximum at 202 nm (Fig. 1).

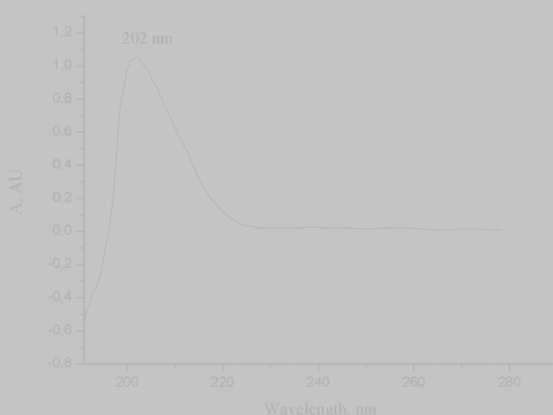

Figure 1. Absorption spectrum of a solution of squalene in ethanol 96% v/v with a concentration of 0.01 mg/mL (blank – ethanol).

Therefore, detection and quantitative determination of squalene in ethanol containing mobile phase in the UV range may be complicated. To address this issue, it was decided to study the absorption of ethanol in the range of 190–220 nm and evaluate the possibility of developing a method for determining squalene with a detection wavelength in this range. As illustrated in Table 1, at a detection wavelength of 210 nm, the absorption of 96% v/v ethanol is 0.4 AU, while at 215 nm, it measures 0.3 AU.

Table 1. Absorbtion of ethanol (96 vol. %) in range of 200–220 nm.

Wavelength, nm	Absorbtion, AU
200	1.6
205	0.6
210	0.4
215	0.3
220	0.2

Squalene exhibits UV-absorption up to 220 nm (Figure 1). Hence, when selecting the detection wavelength, it is essential to consider the spectral properties of both squalene and ethanol. A wavelength of 210 nm may be accepted as suitable for this purpose as ethanol as mobile phase has absorbtion 0.4 which is in an acceptable range, preventing detector overload while allowing reliable detection of squalene.

Column thermostat temperature selection. One of the drawbacks of using ethanol as a component of mobile phases for chromatography is that its mixtures with water give viscous solutions, which results in increased pressure in the chromatographic system. As it was found in the work [18], the use of a thermostat temperature of 40°C is a compromise and significantly reduces the pressure in the chromatographic system.

Selection of the chromatographic column and mobile phase. The capacity factor was determined for squalene and alpha-tocopheryl acetate when using SiO_2-PFP chromatographic phase in combination with water-acetonitrile and water-ethanol mixtures as mobile phases.

Differences in retention factor change trends were observed for squalene and tocopheryl acetate when using ethanol and acetonitrile as the organic solvent in the mobile phase (see Fig. 2). As the proportion of ethanol in the mobile phase decreased, the retention of the compounds increased. The difference between the values of the retention factors also increased, leading to better separation between the compounds, which is consistent with the behavior of compounds in reverse-phase chromatography. When using acetonitrile as the solvent, the retention factors for squalene and tocopheryl acetate significantly little differwhen the proportion of the organic solvent is changed, which practically means incomplete separation of the components and thus the impossibility of using such a combination of mobile and stationary phases for developing a determination method.

When using SiO_2-C18 as a stationary phase, due to the higher hydrophobicity of this phase, the retention factors of compounds exceed the corresponding values obtained on SiO2-PFP (see Fig. 3). For example, at

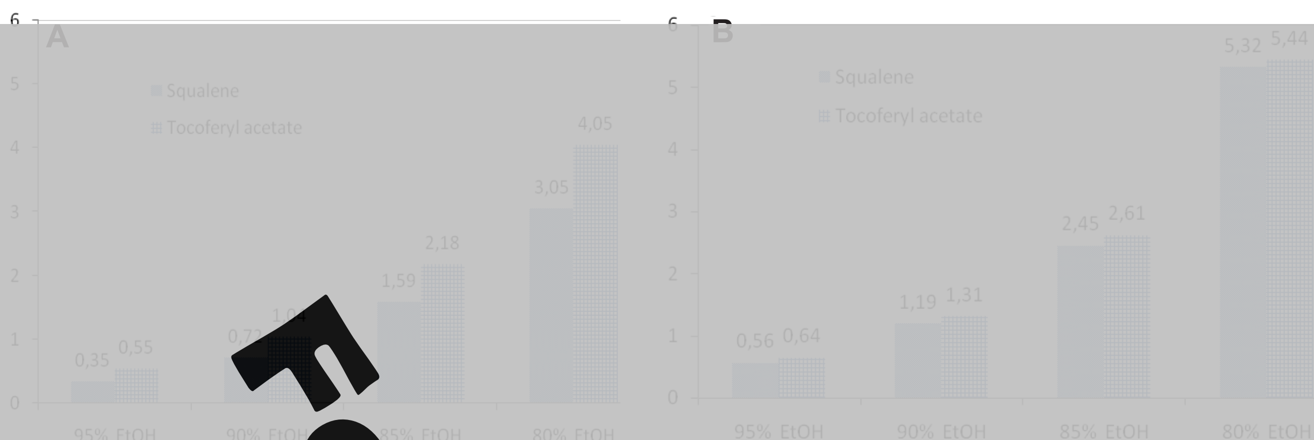

Figure 2. SiO_2-PFP. The dependence of retention factors (k) of squalene and tocopheryl acetate on the ethanol (A) and acetonitrile (B) content in the mobile phase (95–80%).

95% ethanol content, k for squalene on C18 is 3.08, while on PFP it is 0.35. In addition, the elution order of compounds changes, which is related to the different retention mechanisms on these stationary phases. Mainly a dispersive retention mechanism is realizedon SiO_2-C18, while on pentafluorophenyl, dipole-dipole and π-π interactions are additionally present, which enhances the retention of the electron-enriched molecule of tocopheryl acetate.

Based on the obtained data for the chromatographic method, SiO_2-PFP in combination with a water-ethanol mobile phase containing 85% ethanol was selected.

Validation of the squalene assay procedure

Suitability of the chromatographic system

Before starting the validation work, the suitability parameters of the chromatographic system were established and found to be in compliance with them. The chromatographic system is considered suitable if the following conditions are met for the chromatogram of the solution for testing the suitability.

Table 2. System suitability requirements and results.

Parameter*	Requirement	Result
RSD, % of peak areas	≤1.0%	0.07%
RSD, % of retention time	≤ 0.5%	0.04%
Efficiency (N)	≥2000	3657
Resolution (Rs)	≥ 1.5	3.05
Symmetry (As)	0.8–1.8	1.29

* for 6 consecutive injections (n = 6).

All the parameters of the chromatographic system satisfy the predefined requirements.

Specificity

In reverse-phase liquid chromatography, the main characteristic of a molecule that correlates with its retention is its hydrophobicity, which can be expressed as logP - a measure of affinity to hydrophilic or hydrophobic environment. To demonstrate that the chromatographic system is capable of separating compounds that are similar in their solubility, the separation of squalene (logP = 11.7) with alpha-tocopheryl acetate (logP = 10.8) was checked, as these compounds have close logP values. Results are presented in Figure 4a.

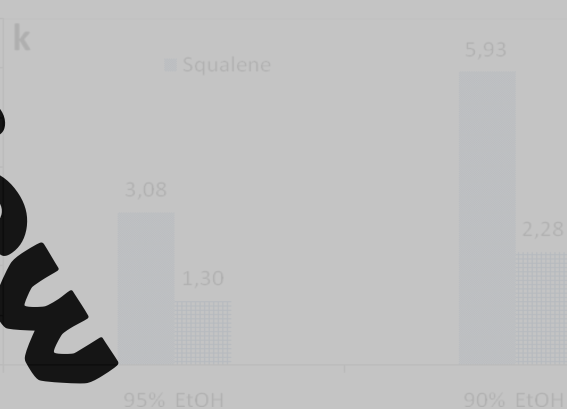

Figure 3. SiO_2-C18.The dependence of retention factors (k) of squalene and tocopheryl acetate on the ethanol content in the mobile phase.

Checking for peaks in the chromatogram of the solvent (in this case – 2-propanol) is an important procedure, as other substances may be present that could mix with the sample components and affect the accuracy and precision of the measurements. Figure 4b shows that there are no peaks on the 2-propanol chromatogram that could interfere with the determination of squalene.

This indicates that the chromatographic system is ready for sample measurements and can provide high accuracy and precision of measurement results. Specificity was established by performing the following conditions:

Separation from alpha-tocopheryl acetate, a compound with close properties has been achieved.

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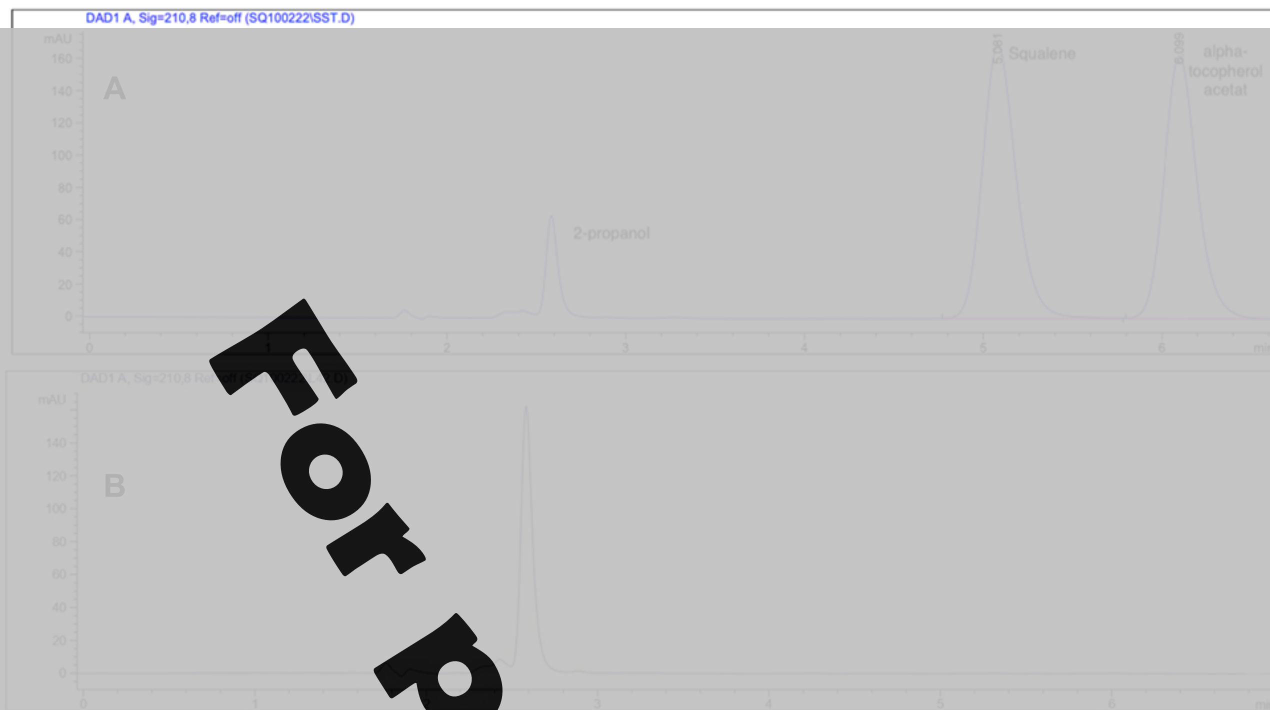

Figure 3. Chromatogram of the suitability of the chromatographic system, where the separation of these compounds is achieved along the baseline, with a separation factor of Rs = 3.05 (A) and blank solution – 2-propanol (B).

There are no peaks on the chromatogram of the solvent that could interfere with the quantitative determination or identification of squalene, as shown in Figure 3.

Linearity and range of application

For linearity, accuracy, presision estimation approach of State Pharmacopoeia of Ukraine was used [19-20]. For this purpose, nine solutions containing a placebo of the dietary supplement and squalene in the concentration range of 0.12–0.3 mg/mL were prepared. These solutions were then quantified against a standard solution without placebo in order to assess the influence of the placebo on the results of the determination. To calculate the criteria and results of linearity, the signals (Ai, Ast) were represented in normalized coordinates according to the following formulas:

Acceptance criteria: The approach is based on the

$$X_i = \frac{C_i}{C_{st}} \times 100\%$$

$$Y_i = \frac{A_i}{A_{st}} \times 100\%$$

principle of insignificance. The confidence interval Δ_2 is significant at the level of p = 5% (and insignificant at the level of 100 - p% = 95%) compared to the interval Δ_1, if the total uncertainty Δ_p exceeds Δ_1 by no more than p%. The inequality is carried out:

$$\Delta_p = \sqrt{\Delta_1^2 + \Delta_2^2} \leq (1 + \frac{p}{100}) \times \Delta_1$$

$$\Delta_2 \leq 0.32 \times \Delta_1$$

Acceptance criterion for the residual standard deviation S_0/b

$$\frac{S_0}{b} \leq \frac{Max\Delta_{As}}{t(95\%, n-2)}$$

b – slope

Acceptance criterion for the correlation coefficient (r):

$$r = \sqrt{1 - \frac{SD_0^2}{SD_{rang}^2}}$$

$$SD_0 = \frac{Max\Delta_{As}}{t(95\%, n-2)}$$

SD_{rang} – the standard deviation is calculated from the calibration point values in percentages from nominal range (60%–150%).

Acceptance criterion for the intercept of the linear dependency (a):

statistical insignificance:

$$a \leq t(95\%, n-2) \times S_a$$

S_a – residual sum of squares deviations

practical insignificance:

$$a \leq \left| \frac{Max\Delta_{As}}{1 - \left(\frac{X_{min}}{100}\right)} \right|$$

X_{min} – the lowest point of the calibration curve.

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If the value of the intercept exceeds the criterion for statistical insignificance, then it is compared to the criterion for practical insignificance. If the value of the intercept satisfies the criterion for practical insignificance, it is considered to meet the requirements for the intercept

During validation, the following data concerning the linearity of the method were obtained and expressed as a linear function presented in normalized coordinates: y=bx+a(Table 3).

Table 3. Characteristics of the calibration curve.

Results		Acceptance criteria		
a	0.08	a_{max} (Stat)	2.36	Comply
		a_{max} (Prac)	2.56	
S_a	1.25			
b	1.01			
r	0.999	r_{min}	0.998	Comply
S_0/b	0.97	S_0/b_{max}	1.69	Comply

As seen from Table 3, the requirement for the statistical and practical insignificance of coefficient a is fulfilled. The acceptance criterion for the correlation coefficient (r) is 0.999, with the requirement that it should not be less than 0.998. The residual standard deviation S_0/b compies with acceptance criterion. The obtained linearity results of the developed method meet the established requirements.

Accuracy and precision

Accuracy is calculated by the formula:

$$Z_i = \frac{Y_i}{X_i}$$

$$\delta = |\bar{Z} - 100|$$

Acceptance criterion for accuracy (δ, %):

— Statistical insignificance

$$\delta \leq \frac{\Delta_{As}}{\sqrt{n}}$$

Δ_{As} – uncertainty of results

— Practical insignificance

A systematic error is practically insignificant if it is less than the maximum allowable uncertainty of the analysis

$\delta \leq 0.32 \times Max\Delta_{As}$

If the accuracy value of the methodology exceeds the criterion for statistical insignificance, then it is compared to the criterion for practical insignificance. If the accuracy value satisfies the criterion for practical insignificance, it is considered to meet the requirements for accuracy of the methodology.

Acceptance criterion for precision: The uncertainty of the analysis in the selected range of concentrations does not exceed the maximum allowable uncertainty of the analysis ($Max\Delta_{As}$)

$t(95\%, n\text{-}1) \times S_Z \leq Max\Delta_{As}$

Table 4. Results for Accuracy and Precision.

Results		Acceptance criteria		
Accuracy (δ), %	0.89	δ_{max} (Stat)	0.48	Comply
		δ_{max} (Prac)	1.02	
Precision (ΔAs), %	1.44	Δ_{As}Max	3.2	Comply

The maximum acceptable uncertainty for the squalene determination method is set at 3.2%, which is in line with typical content limits for pharmaceuticals, where the allowed deviation is ±10%. The systematic error (accuracy) of the method was found to be 0.89%, thus fulfilling the requirement for practical insignificance, meaning that its impact on the results is negligible. Additionally, the precision of the method was determined to be 1.44%, well within the established criterion of not exceeding 3.2%, indicating reliable and consistent performance of the method.

Quantitative determination of squalene in the dietary supplement

In the prepared samples of the dietary supplement, we expected a peak corresponding to the retention time of the squalene peak in the standard solution. However, such a peak was not observed. Instead, a peak corresponding to the retention time of alpha-tocopheryl acetate was observed in the sample. The content calculation revealed that the sample contains approximately 5.0±0.2 mass % of alpha-tocopheryl acetate, which was not declared as part of the composition. Squalene has not been found in this dietary supplement.

Conclusion

To ensure the environmental safety of squalene determination, it is offered to replace the toxic component of the mobile phase (methanol or acetonitrile) with ethanol. The possibility of detecting squalene in the ethanol-based mobile phase at 210 nm is shown. A comparison of the chromatographic phases based on the gel modified with pentafluorophenyl (SiO_2-PFP) and octadecylsilyl (SiO_2-C18) groups was carried out. It was found that in order to obtain the same retention characteristics by means of stationary phase SiO_2-PFP, the ethanol content in the mobile phase must be reduced by 10–12% in comparison with SiO_2-C18, which also improves the eco-safety characteristics of the method. On the basis of the conducted research, a methodology

was developed and validated for the quantitative determination of squalene in the dietary supplement. The technique meets the basic requirements for specificity, linearity, accuracy and reproducibility. It is rapid (up to 6 minutes), and the use of non-toxic organic solvents makes it safe for the environment and the analysis. As a result of quantitative determination according to the developed methodology, no squalene was found in the dietary supplement, but the presence of tocopheryl acetate was found in the amount of 5 mass percent. This emphasizes the importance of strengthening the control of the market of dietary supplements in Ukraine.

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