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Identification and quantification of squalene in Europaean spindle seed oil (*Euonymus europaeus* L.) by optimized high performance liquid chromatography (HPLC) procedure

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Abstract
In Europaean spindle seed oil (*Euonymus europaeus* L.) was identified squalene. The oil from the seeds was extracted with hexane. The procedure by reverse phase high-performance liquid chromatography with UV detection (RP-HPLC UV) was optimized for identification and quantification.Chromatographic separation was carried out in isocratic mode, a mobile phase of acetonitrile: tetrahydrofuran (90:10) on a column of Luna® Omega Polar C18 100 Å, 250 mm × 4.6 mm, 5 µm at 30 °C. The retention time of squalene in the test sample was 9 minutes. The content of squalene in the studied oil was 1.84 mg / ml ± 0.05 mg / ml. The high content of squalene in combination with carotenoids, tocopherols and unsaturated fatty acids can be the cause of the effectiveness of spindle tree oil in the treatment of dermatological diseases.

Keywords: European spindle, *Euonymus europaeus* L., seed oil, squalene, HPLC-UV

Introduction
European spindle is a deciduous shrub or small tree, native for most European countries. Various parts of this plant have been used in folk medicine, having antimicrobial, antiparasitic, insecticidal effects, which is confirmed by data from experimental studies [1, 2].
According to the literature data, plant seeds are rich in fatty oil and carbohydrates, contain 0.1-0.3% alkaloids, cardenolids (0,38% evonosid), and also lectins and chitin-binding peptides. [2-4]
Spindle oil in folk medicine is used as a remedy for treating dermatological diseases of humans and animals, in particular, can be used in the treatment of dermatomycosis and eczema [5]. European spindle seed oil and hexane extract contains a large number of unsaturated fatty acids, carotenoids, and tocopherols, and its action in the treatment of non-allergic contact dermatitis on the model of this disease in rats was confirmed [6].
In addition, the seeds of this plant also have a large number of terpenic and steroidal compounds which, according to the biochemical concept of biosynthesis, are formed from squalene, so the probability of its presence in the lipophilic fraction of the seeds is high. Although data on its content in the European spindle seeds in the literature was not found. Furthermore, in the lipophilic fraction of the bark of this plant by gas chromatography - mass spectrometry squalene was determined and the content of which was sufficiently high [7].
Squalene (2,6,10,15,19,23-hexamethyl-tetrakoza-2,6,10,14,18,22-hexaene) - a viscous oily liquid without odor and color, used as a medication for lowering cholesterol, as adjuvant in vaccines, as well as in dermatology [8, 9].
Squalene is a component of subcutaneous fat of mammals. Acording to the literature data the highest content of squalene in human tissues is found in lipids of skin (about 500 µg/g) and adipose tissue (≥ 300 µg/g) [10], where it plays an important physiological role. It easily penetrates through the skin inside the body, and is a powerful immunostimulant, while it is non-toxic and safe. The softening and hydrating properties of squalene and its biocompatibility with human skin are the reason for its introduction into a range of cosmetic products [11]. As part of the cosmetics, it acts as an effective wound healing agent, as well as protects the skin from free radical damage and prevents its aging, which occurs through the peroxidation of lipids under UV exposure [11].
Squalene in oils is determined by chromatographic methods, in particular by gas (GC) or high performance liquid (HPLC) chromatographia alone or in combination with mass spectrometry.

Gas chromatography has a number of disadvantages compared to HPLC, because it requires special preparation of oil samples for analysis [12, 13]. Quantitative determination of squalene in samples with high oil content is realize to use liquid chromatographs, sensitivity of which is provided by UV detection. We have used the HPLC method which was described by Lu *et al*. [12], however, for our chromatographic system, we used a different length of column and manufacturer and a combined mobile phase of acetonitrile-tetrahydrofuran, which made it possible to achieve high selectivity and effectiveness of the technique.

The purpose of present work was to optimize the method of HPLC-UV in studying the quantitative content of squalene in European spindle oil obtained from the seeds of *Euonymus europaeus* L. collected on the edge of the deciduous forest (Lviv region, Ukraine) during the period from September to October 2016.

Materials and Methods

Collection and extraction

Fruits of spindle *Euonymus europaeus* L., was collected on the outskirts of the city of Lviv (Ukraine) during their full ripeness (September-October 2016). The seeds were separated from the pericarp, dried in a drying cabinet at + 50 °C and grinded to a particle size of 1-3 mm. The resulting seed powder (0.1 kg) was extracted with hexane (0.5 L) under continuous stirring on a mechanical stirrer. After 30 minutes, the extraction procedure was repeated by adding twice the pure solvent to the raw material pressed in the same ratio with the subsequent 30 minute stirring. The hexane extracts were combined, centrifuged for 10 min at 3000 g, filtered and the solvent was distilled to a small volume (30-40 ml), after which the residue was dried in a drying cabinet at + 60 °C in a pre-weighed porcelain cup. The resulting oil was used to detect and quantify squalene.

Preparation of standard solution of squalene

Reagents: Certified standard sample of squalene, Squalene analytical standard ≥ 98.0%, Sigma-Aldrich CAS Number 111-02-4); acetonitrile "HPLC" grade (≥ 99.9%, Sigma-Aldrich, USA), and tetrahydrofuran (for HPLC, ≥99.5%, Fluka).

A standard solution of squalene was prepared by dissolving a certified standard sample of 0.10 g (weight loss to 0.0001 g) in tetrahydrofuran. Next, 10 ml of the resulting solution were taken and the volume of the solution was adjusted with acetonitrile to 100 ml, stirred and filtered through a membrane filter with pores in diameter of 0.2-0.5 μm. 1 ml of standard solution contains about 0.0001 g of squalene.

Preparation of the solution of the test sample

The test sample was prepared by dissolving 2,5 ml of European spindle oil in tetrahydrofuran in a volumetric flask of 250 ml capacity. Further, 10 ml of the resulting solution was taken up in a 100 ml volumetric flask and the volume was adjusted to acetonitrile.

The resulting solution was stirred and filtered through a membrane filter with a pore diameter of 0.2-0.5 μm.

Instrumentation and Chromatographic conditions

Identification and quantification of squalene were performed with liquid chromatograph Thermo Scientific Dionex UltiMate 3000 UHPLC. The system consists of an UltiMate 3000 RS pump, an UltiMate 3000 RS autosampler, UltiMate 3000 RS Diode Array Detectors and an UltiMate 3000 RS column compartment (Dionex, Olten/Switzerland).

Chromatographic separation of analyte was performed with a reverse phase column Luna® Omega 5 мкм Polar C18 100 Å 250 mm× 4.6 mm × 5 μm, at 30 °C. The composition of the mobile phase: acetonitrile-tetrahydrofuran (90:10).

Flow rate of the mobile phase -1.0 ml / min; Injection volume - 0.005 ml.

The optical detector (UV-VIS-DAD) (see above), which provides continuous recording of the optical density of the eluent at 205 nm (or at constant scanning in the region of 192-400 nm, provided that the scanning spectrophotometric detector is used)

The results were processed with Chromeleon® Chromatography Data System software (Version 7.2 SR4). Filtration of the mobile phase and samples before the injection into chromatograph was performed using PTFE membrane syringe filters with diameter of 25 mm and a pore size of 0.45 microns by Phenex.

Results

Fatty oil of spindle *Euonymus europaeus* L., extracted with hexane, is a viscous liquid of bright orange color with a specific odor. The yield of oil is 20-28%, which depends on the collection parameters. The density (at 20 °C) is 0.939 g / cm3.

The presence of squalene in fatty oil was determined by liquid chromatography, comparing the peak time of squalene at the chromatogram of the standard sample (Fig 1.) and the peak corresponding to squalene on the chromatogram of the solution of the test sample.

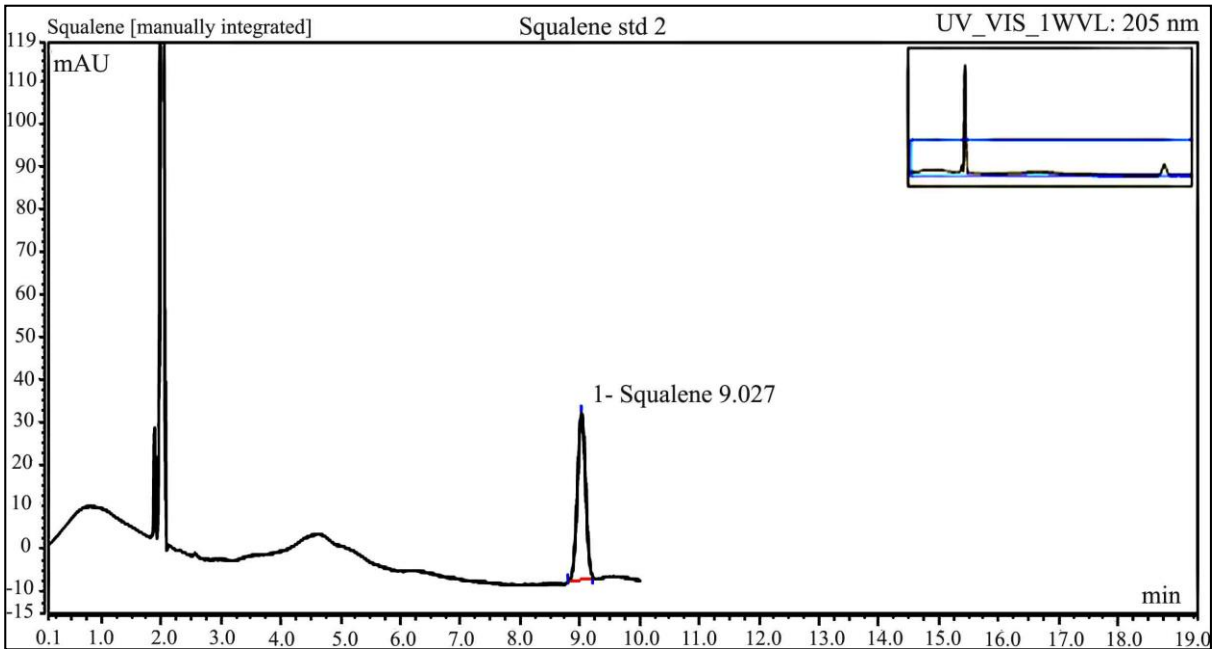

Fig1: Chromatogram of standard solution of squalene 0.1 mg / ml

For the separation of the substances of spindle oil, the parameters of the column and the mobile phase have been tested, as described above (see "Instrumentation and Chromatographic conditions"). This allowed to achieve high selectivity and effectiveness of the methodology. When using the isocratic division mode, the elution time of squalene was 9 minutes.

As can be seen from Figure 2, the tested methodology is characterized by complete chromatographic separation of squalene from other components of the oil, which is a necessary requirement for the effectiveness of the technique. The retention time of squalene of *Euonymus* oil coincides with the retention of the standard sample of squalene (Fig. 1). Under the chosen chromatographic conditions, the symmetry factor of the peaks obtained on a chromatogram for a standard solution and a sample of squalene is close to one and the retention time is 9 minutes.

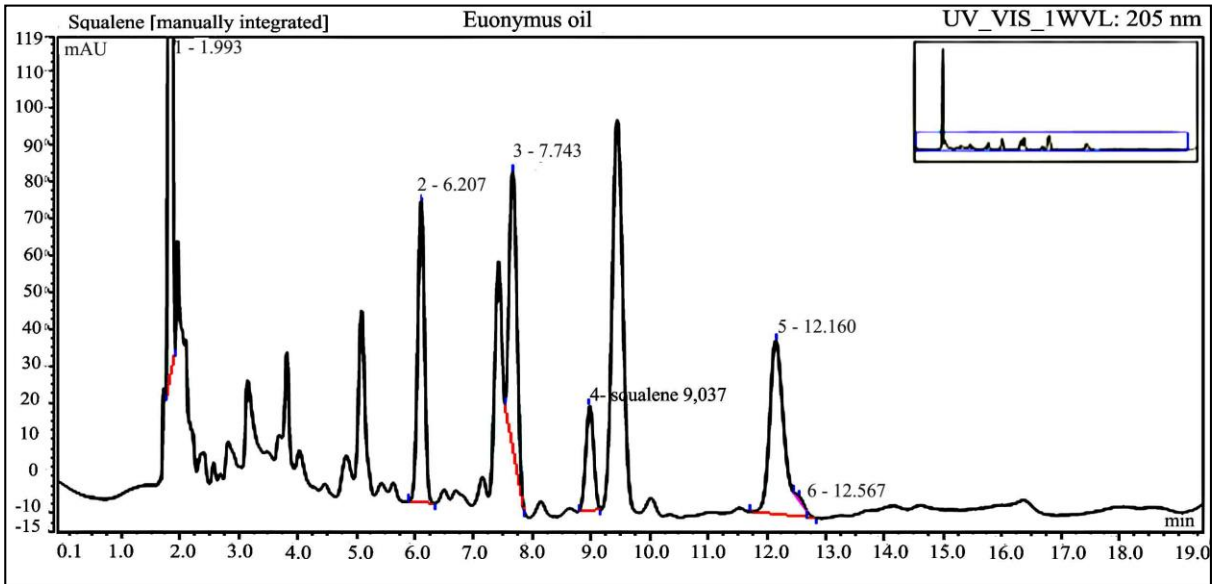

Fig.2: Chromatogram 10 mg / ml of a solution of Euonymus oil (fragment)

Quantitative definition

The construction of the calibration graph of the standard squalene sample was carried out after analysis of five consecutive chromatograms in the concentration range of 1-100 μg / ml (1, 5, 10, 25, 50, 75, 100 μg / ml), the difference in time of release did not exceed the relative standard deviation calculated for a standard sample solution.

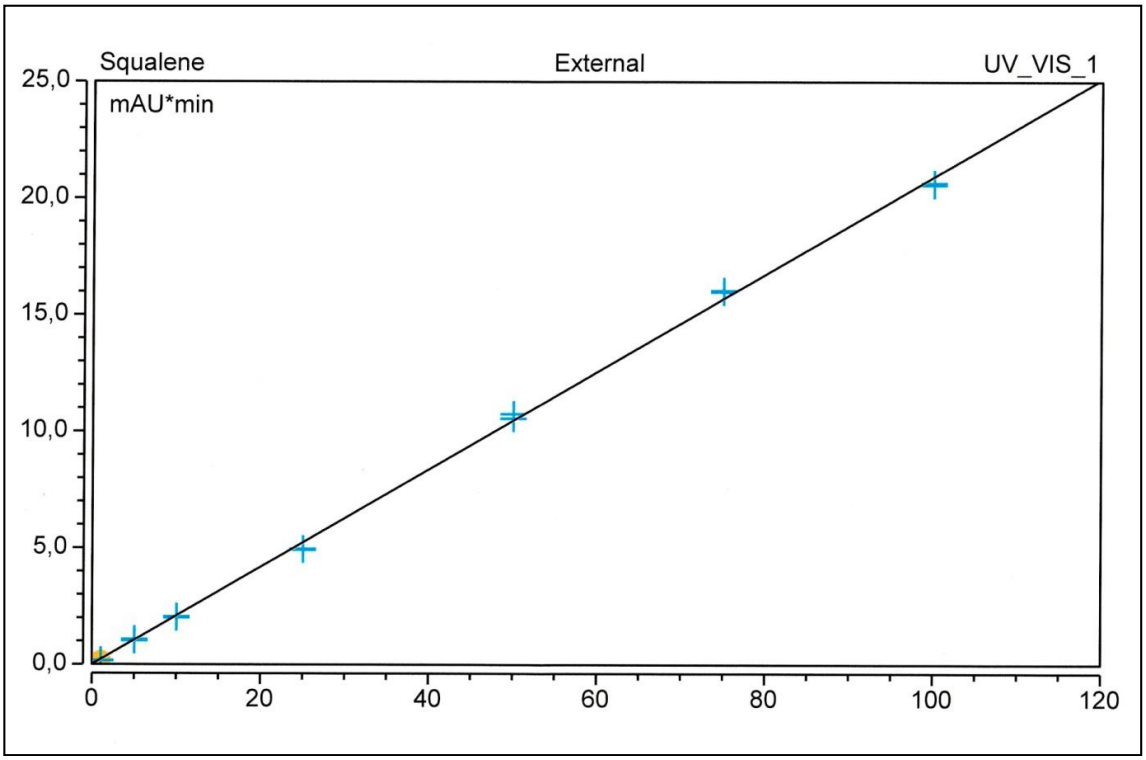

Fig 3: Calibration curve of squalene solution (correlation coefficient R^2 0,9992)

To establish the range of the application of the technique, we performed alternately five chromatographic separations for solutions of the standard sample of each point of the calibration graph (Fig. 3). Experimental data processing was performed using the least squares method for a straight line with two parameters. According to the results of the statistical processing of the calibration curve with the equation y = a + bx, the detection limit was calculated, and the limit of the quantitative determination was 0.29 and 0.88 μg / ml, respectively. The calculated standard deviation of the value of a graph of a gauge graph is 0.01855, which is substantially lower than the lower boundary of the concentration range, which will not affect the accuracy of the analysis and indicates the correctness of the method according to the linearity parameter.

The content of squalene in oil of European spindle was determined from the calibration graph obtained by chromatograms of a standard sample of different concentrations. It was 1.84 ± 0.05 mg / ml (184 mg / 100 ml of oil)

Statistical analysis: Experiments on the determination of squalene in fatty oils were performed five times and average values were recorded. The data were evaluated statistically using Student's t-test, and a value of $p \leq 0.05$ was considered to be statistically reliable.

Discussion

The main source of obtaining of squalene is a fat from shark liver oil, including the liver of deep-water shark *Centrophorus artomarginatus*, where squalene is 25-30% of the weight of the liver [14]. Even higher concentrations of *Centrophorus squamosus* in the liver, containing ≈ 77% of the fatty oil, and the concentration of squalene in it is 79.6% [15], however, taking into account the need of animals protection more environmentally friendly is obtaining the squalene from plant sources.

The main sources of squalene of plant origin are amaranth and olive oil with high content of squalene. Although it is contained in sufficient quantities and in a number of other oils, due to a number of reasons, as a source of squalene, they are rarely used (see Table 1).

Table. 1: The content of squalene in the fatty oils of some plants

Source	Extraction method	The content of squalene mg/100 g of oil	Reference
Amaranth seed oil (*Amaranthus cruentus, A. hypochondriacus*)	DI, $ScCO_2$	5000 – 8000	Lozano-Grande *et al*. 2018 [18]; Popa *et al*., 2015 [8]
Camellia seed oil (Camellia oleifera)	DI, P	290 – 7620	Bi *et al*., 2011
Palm oil (*Elaeis quineensis*)	DD, $ScCO_2$	0.1 – 1300	Lozano-Grande *et al*. 2018 [18]; Popa *et al*., 2015 [8]
Olive oil (*Olea europaea*)	DI, DD, P	150 – 1200	Lozano-Grande *et al*. 2018 [18]; Popa *et al*., 2015 [8]
Pomegranate seed oil (*Punica granatum*)	DI	200	Gornaś, Rudzinska 2016 [17]
Watermelon seed oil (*Citrullus lanatus*)	DI	14	Gornaś, Rudzinska 2016 [17]
Apricot kernel oils (15 sorts) (*Prunus armeniaca* L.),	DI	12 – 44	Rudzinska *et al*., 2016 [17]
Corn seed oil (*Zea mays*)		10 -27	Lozano-Grande *et al*. 2018 [18]
Oil of peanut seed (*Arachis hypogaea*)	DI	9.8	Lozano-Grande *et al*. 2018 [18]
Oil of apple seed (*Malus domestica*)	DI	2 – 26	Gornaś, Rudzinska 2016 [17]
Grape seed oil (*Vitis*)	DI, $ScCO_2$	2.7 – 14.1	Lozano-Grande *et al*. 2018 [18]
Sunflower oil (*Helianthus annus*)	DD	2.2 – 2.6	Lozano-Grande *et al*. 2018 [18]

Notes: $ScCO_2$: CO_2 extraction as supercritical fluid, DI: solvent extraction, DD: deodorization distillate, P: pressure extraction [16-19].

Attention is drawn to the fact that different methods of oil extraction give a very different output of squalene in the oils. For example, the content of squalene in Camellia seed oil ranged from 0.29% (acetone extraction) to 2.98% (direct compression) and 7.62% (hexane extraction) [19].

Quantity of squalene in oils also depends on other factors. In a review of Bi *et al*. [19] indicated that the level of squalene in olive oil decreased by 10% - 30% in 270 days storage (depending on the storage temperature and the degree of lighting of the oil).

According to the literature data, squalene has pronounced biological effects. It is one of the predominant components (about 13%) of sebum. It is critical for reducing free radical oxidative skin damage due to exposure to ultraviolet light and other sources of oxidative damage [11]. Squalene is rapidly and effectively absorbed deep into the skin, restoring its healthy elasticity and flexibility.

The presence of squalene in a mixture with carotenoids, tocopherols and unsaturated fatty acids increases the value investigated oil in the treatment of dermatological diseases. This explains the high regenerative and anti-inflammatory properties of spindle seeds oil (*Euonymus europaeus* L.), which were demonstrated on non-allergic contact dermatitis in rats.

The chromatographic separation conditions were optimized for the quantitative determination of squalene in oil extracted from the seeds of spindle tree (*Euonymus europaeus* L.), collected in the zone of deciduous forests of the western region of Ukraine using the reverse phase HPLC method with UV detection (RP HPLC-UV) at a wavelength of 205 nm. We have established that the limit of quantitative determination of squalene is 0.88 μg / ml and the content of oil is found to be 1.84 mg / ml.

Validation characteristics (specificity, linearity, range of application, detection limit, limit of quantitative determination) of the optimized methodology comply with the requirements for the "Quantitative determination" test.

Conclusion

As a result of the present study, squalene was first detected in Europaean spindle oil (*Euonymus europaeus* L.) and its content (1.84 ± 0.05 mg / ml) was determined. An optimized HPLC method was used to b identify and quantify squalene, which is effective for this purpose and at the same time easy to implement. The high content of squalene in combination with carotenoids, tocopherols and unsaturated fatty acids in the spindle oil causes high regenerative and anti-inflammatory properties of this oil.

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