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RP-HPLC determination of betulin in oil-in-water emulsion-based feed additives

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Abstract

Betulin, a lupane-type triterpenoid, is of growing interest as an active component of veterinary and feed formulations. For such complex matrices, robust and traceable analytical methods are required to control the betulin content and ensure product quality. In this work, a simple and rapid reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitative determination of betulin in an oil-in-water emulsion-based feed additive. Chromatographic separation was performed on a C18 column using an acetonitrile–water (80:20, v/v) mobile phase in the isocratic mode at 35 °C, with a flow rate of 0.35 mL/min and UV detection at 210 nm; the total run time does not exceed 3 min. Quantification was carried out by external calibration using a certified betulin reference over the concentration range relevant to the declared product content. The method was validated in accordance with national metrological guidelines with respect to selectivity, linearity, accuracy, precision, range, and robustness. The results demonstrate excellent linearity, satisfactory precision, and recoveries close to 100%, as well as the absence of matrix interferences. The developed RP-HPLC procedure is suitable for routine quality control of the novel feed additive and can be adapted to other betulin-containing products.

Key findings

- A simple isocratic RP-HPLC method for determining betulin in an oil-in-water emulsion feed additive with a 3 min run time was developed.
- The sample preparation uses small-volume liquid–liquid extraction and yields chromatograms free from significant matrix interferences.
- The method was validated according to national metrological guidelines for selectivity, linearity, precision, accuracy, and robustness.
- External calibration in the working range ensures reliable quantification of the declared betulin content food additive.
- The developed procedure is suitable for routine quality control and can be transferred to related betulin-containing formulations.

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1. Introduction

Betulin is a lupane-type pentacyclic triterpenoid isolated from the outer bark of birch trees. It is the primary component of the birch bark extract, often making up 20-30% of the dry weight. It is also found in smaller amounts in more than 300 plant species and is particularly abundant in traditional medicinal plants, including jujube (*Ziziphus jujuba*) fruit, rose hip (*Rosa canina*) fruit, and Indian persimmon (*Diospyros peregrina*) fruit [1].

Nowadays, betulin has attracted sustained interest as an active component in pharmaceutical, cosmetic, and veterinary feed formulations due to its broad biological (anti-inflammatory, antimicrobial, and anticancer) activities with relatively low systemic toxicity in preclinical models [2–3]. One of the most important applications of betulin is in animal husbandry. Studies have shown that betulin exerts preventive and therapeutic activity in immunodeficient, viral and bacterial diseased cattle [4]. This helps to reduce the use of antibiotics, and, consequently, reduce their toxic effects in farm animals. Thus, the ongoing reduction in the duration of the clinical stage of the disease, the absence of relapses and the prevention of the development of chronic disease can decrease the cost and improve the efficiency of veterinary care.

Structurally, betulin consists of a fused pentacyclic lupane framework (four six-membered and one five-membered ring) bearing a secondary hydroxyl group at C-3 in the A ring (Figure 1) [2, 5, 6]. This architecture confers high crystal stability, negligible volatility, and extremely low water solubility. Betulin is relatively soluble in boiling alcohols, ether, chloroform, dichloromethane, benzene, pyridine, tetrahydrofuran and dimethyl sulfoxide [6]. In this regard, aliphatic and aromatic hydrocarbons, alcohols, acetone, and chlorine-containing solvents have been proposed as extractants for birch bark, with the latter being preferable for its efficiency and rate of betulin extraction [7, 8].

The poor solubility of betulin in polar solvents combined with its high lipophilicity poses challenge regarding its bioavailability and technological aspects during formulation of drug/dosage forms [6]. Thus, to achieve therapeutically significant concentrations, approaches are required to develop colloidal systems such as nanoemulsions or nanocrystals. A practical example of such an oil-in-water emulsion system is the feed additives, in which betulin is present in relatively high concentrations [9,10]. Betulin in the feed additive has a particle size comparable to the size of nanosuspensions due to the introduction of betulin in the form of a solution in ethyl alcohol, and an aqueous solution of auxiliary additives made it possible to obtain a stable suspension of betulin, convenient for feeding animals and poultry. The additive is used to prevent metabolic disorders and non-infectious diseases, improve the immunity and natural resistance of farm animals and birds, and improve the productivity of farm animals. This preparation is an emulsion with a normalized betulin content of 5 g/dm^3. The additive contains ethyl alcohol, food emulsifier tween-80, citric acid, emulsion stabilizer Agar, glycerin and water.

For complex matrices such as oil-in-water emulsions, reliable control of betulin content requires chromatographic procedures that are not only selective and precise, but also suitable for routine quality control. Its high melting point, together with temperature depended heat capacity in the range of 80–350 K, implies a compact crystal packing with limited lattice mobility and a low tendency to undergo solid–solid phase transitions under ambient conditions [2,4].

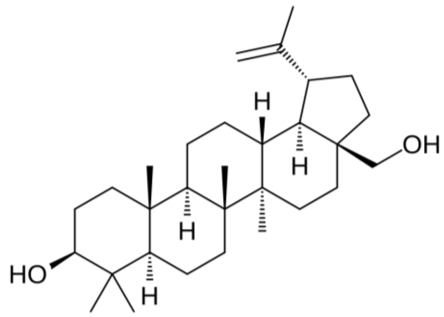

Figure 1 Structural formula of betulin.

The crystalline stability simplifies preparation of solid reference standards, as the compound can be weighed and stored without rapid sublimation or amorphous phase formation. However, this also leads to direct analytical implications, as betulin is essentially non-volatile under conventional gas chromatography conditions and usually requires derivatization (for example, silylation or acylation of the hydroxyl groups) to generate volatile, thermally stable derivatives [6].

Reverse-phase high-performance liquid chromatography (HPLC) with spectrophotometric detection is the most widely used method for the chromatographic analysis of betulin [11–16]. The chromatographic behavior of betulin in this system is impacted by its molecular properties, such as hydrophobicity and weak chromophores. Betulin shows strong retention on C18 phases, necessitating mobile phases with high organic solvent content, usually acetonitrile or methanol. Since the molecule does not have an extended conjugation system, detection at higher UV wavelengths is inefficient. Therefore, effective detection of betulin often requires monitoring at lower UV wavelengths (210–215 nm), or using an evaporative light scattering or mass spectrometric detector [17-19].

Most published analytical methods describe the determination of betulin after its extraction from plant matrices or in biological samples. However, the determination of betulin in oil-in-water emulsion systems are not described in the literature. The developed new feed additive based on such an emulsion with a nominal betulin concentration of 5 g/L is a practically important example of a system in which betulin is present in relatively high concentrations in the emulsion matrix. To support quality assurance and regulatory compliance, this product requires a simple, fast, and reliable reverse-phase HPLC method for quantifying betulin. The aim of this work is to develop and validate an RP-HPLC procedure that meets these requirements, using a sample preparation protocol compatible with the feed additive matrix.

2. Materials and Method

2.1. Chemical and reagents

96% purity betulin was produced by boiling birch bark in isobutyl alcohol before treating with alkali washing with

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water and separating the water, dividing the formed two-phase system by sedimentation, washing and concentrating the extract, and obtaining betulin from the purified extract by crystallising according to [20]. It was supplied to us by Tyumen Tuber Chemical Corporation LLt. The emulsion matrix components such as 95% ethanol, glycerol, Tween 80, sodium chloride, xanthan gum and citric acid as an acidity regulator were purchased from Khimreaktivsnab company (Russia). Betulin (98.0%) was purchased from Sigma-Aldrich (Steinheim, Germany). Water (for GC, HPLC and spectrophotometry grade, Honeywell, Burdick, and Jackson, Muskegon, USA), acetonitrile (HPLC-gradient grade, Panreac, Barcelona, Spain) and dichlormethane (≥99%, EKOS-1, Moscow, Russia) were used for UHPLC analysis.

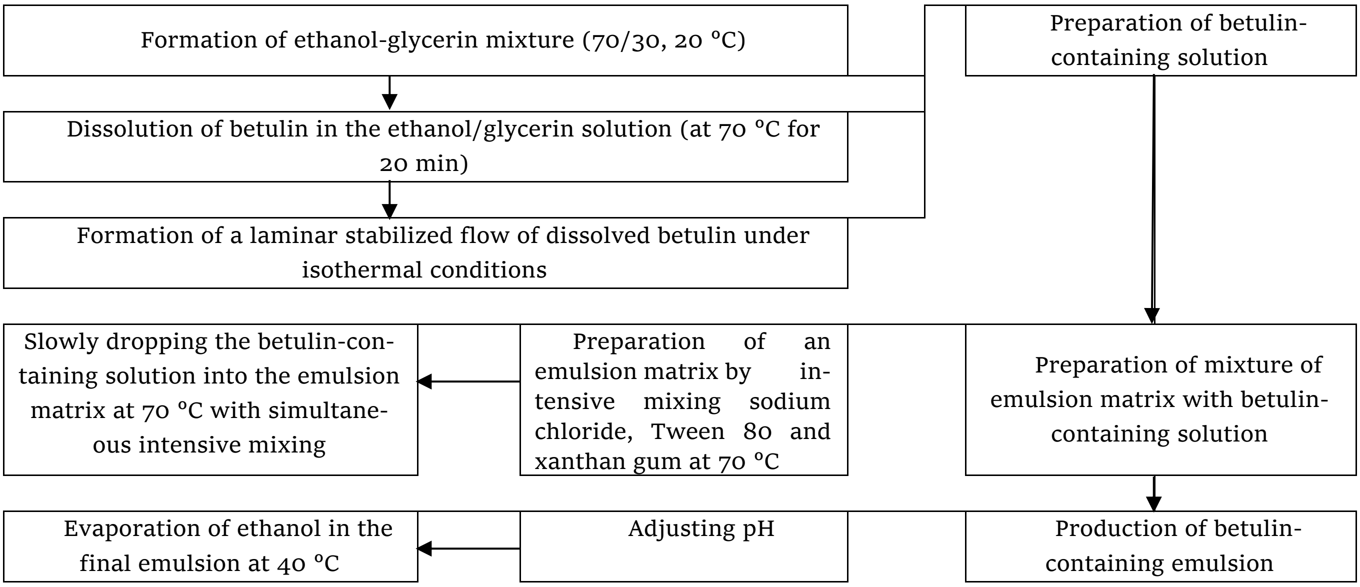
2.2. Preparation of a feed additive based on the oil in water emulsion with betulin

Oil-in-water based-betulin emulsion was prepared according to the method described by Zavorokhina et al. [21] with modifications. Briefly, 4 g of 96% betulin was dissolved in 100 mL of ethanol and glycerol at the 70:30 ratio under continuous stirring at 500 rpm, 70 °C for 20 minutes until completely mixed. The betulin solution was then slowly added, drop by drop, to a separate solution containing sodium chloride as an inhibitor of crystallization, Tween 80 an emulsifier and xanthan gum as a thickener. This incorporation was done using a flow microreactor (Mr-Lab-VS, Germany) at a rate of 0.1 mL per minute. After that, the combined mixture was heated to 70 °C and vigorously mixed using an IKA dispersant T 10 basic Ultra-TURRAX® at 24 000 rpm. Next, the final mixture was adjusted with citric acid up to pH 3.1 and, finally, warmed to 40 °C to remove the ethanol.

The final betulin-containing emulsion contained 7% of ethanol, 3% of glycerol, 1% of betulin, 0.5% of Tween 80, 0.25% of citric acid, 0.2% of sodium chloride, 0.3% xanthan gum, and 85.05% double distilled water. The technological process for the production of the oil-in-water botulin-based emulsion is shown in Scheme 1.

2.3. Sample extraction and preparation

A known volume of emulsion (1 mL) was mixed with an equal volume of dichloromethane in a 2 mL centrifuge tube, vortexed for 2 min and then centrifuged (2400 g for 5 min) to accelerate phase separation. The organic phase, which contained solubilized betulin, was quantitatively transferred into a 25 mL volumetric flask using a Pasteur pipette; the aqueous phase was extracted twice more with fresh portions of dichloromethane, and all organic phases were combined in a volumetric flask. The combined extracts were evaporated to a dry residue at 45 °C. After evaporation, the obtained dry residue enriched in betulin was resuspended in the RP-HPLC mobile phase (acetonitrile–water) with the aid of ultrasonication at 37 kHz for 2 min to break up aggregates and ensure complete dissolution. The volume of the resulting solution was adjusted to the mark and mixed. An aliquot of 5.0 mL was taken from the resulting solution using a pipette with a capacity of 5 mL and placed in a measuring flask with a capacity of 25 mL. The volume of the solution was adjusted to the mark with a mixture of acetonitrile and water in a ratio of 80:20 and mixed. The prepared solution was placed in a vial, covered with a lid and chromatographed. Three parallel samples of the analyzed emulsion were simultaneously prepared for analysis.



Scheme 1 General technological scheme for the preparation of botulin-based emulsion.

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2.4. Preparation of standard solutions

The stock betulin solution with a concentration of 200.0 μg/mL was prepared by dissolving an exact sample of 20.0 mg of betulin standard in a 100 mL volumetric flask in a 70–80 mL acetonitrile-water mixture (80:20) using ultrasound treatment for 2 min at 37 kHz to facilitate dissolution and then adjusted to the volume. A set of five working calibration solutions was prepared from the initial solution by volumetric dilution in 25 mL flasks, obtaining final concentrations of 24.0, 32.0, 40.0, 48.0 and 56.0 μg/ml, respectively. The solutions were prepared immediately before the measurements.

2.5. RP-HPLC-DAD protocol

Chromatographic separation was performed using an Agilent 1290 Infinity II HPLC system(Agilent, USA) in an isocratic mode on the 2.1 mm × 50 mm × 1.8 μm ZORBAX Eclipse Plus C-18 RRHD chromatographic column (Agilent, p/n 959757-902), using a mixture of water and acetonitrile as the mobile phase in the volume ratio of 20:80. The eluent flow rate was set to 0.35 ml / min, and the column thermostat temperature was set to 35 °C. Betulin was detected at a diode matrix detector wavelength of 210 nm and a slit width of 4 nm. The reference wavelength is 360 ± 100 nm. The volume of the administered sample is 2μl. The total analysis time was 3 minutes. The betulin retention rate was 4.2.

2.6. Processing of the obtained results

Chromatograms were processed and the mass concentration (C, g/dm^3) of betulin in the emulsion was determined with the absolute calibration method using the instrument software and data processing according to the calibration dependence, taking into account the degree of dilution of the sample according to the equation:

$$C\left(\frac{g}{L^3}\right) = {x \cdot v_{k1} \cdot v_{k2}}/{V_a} \cdot 1000 = (x \cdot 25 \cdot 25)/(5 \cdot 1000), \quad (1)$$

where x is the value of the mass concentration of betulin in the test solution found by the calibration dependence (μg/mL), V_{k1} is the volume of the measuring flask for the preparation of the initial betulin solution (25 mL), V_{k2} is the volume of the measuring flask for the test betulin solution (25 mL), and V_a is the volume of the aliquot taken from the initial solution during the preparation of the test betulin solution (5 mL).

3. Results and Discussion

3.1. Development of a method for the determination of betulin content in oil-water-based emulsions

The development of a method for the quantitative determination of betulin in a veterinary feed additive using HPLC was carried out in accordance with the requirements of the State Pharmacopoeia of Russia XV [22]. The method of analysis of the new feed additive required a selective and accurate quantitative determination of betulin in the oil-in-water emulsion containing a high volume of lipids, surfactants and preservatives at a nominal betulin concentration of 5 g/L. The procedure was intended to provide a short analytical time, simple sample preparation compatible with routine quality control, and measurement uncertainty that meets the requirements of the State Fiscal Service of the Russian Federation XV on specificity, linearity, correctness, and precision [23].

When developing a method to reduce analytical time beyond those in previously published protocols [11-16], a short column with a fine-grained sorbent (1.8 μm) was used, facilitating quick analyses, saving solvent consumption, and maintaining high separation efficiency. A typical chromatogram of a betulin-containing emulsion sample solution is presented in Figure 2. The total analysis time was 3 min with acceptable betulin retention factor values of 4.2, resolution from matrix component peaks of more than 2, and chromatographic column efficiency for a betulin peak of more than 5000 theoretical plates. The betulin peak has a fairly good shape with an asymmetry factor of 1.4. As can be seen from the data presented, the chromatographic system is suitable for analysis in accordance with the requirements of the State Pharmacopoeia of Russia XV [22].

The composition of the mobile phase and the detection wavelength were selected taking into account the hydrophobic nature and weak UV absorption of betulin. The isocratic mobile phase, containing 80% acetonitrile, delivers sufficient elution force to separate betulin from the excipients of the preparation in a short time without generating excessive back pressure on the column with particles less than 2 μm. The mobile phase, consisting of a mixture of water and acetonitrile, exhibits a transmission limit below 190 nm; thus, UV detection at low wavelengths (210 nm) offers adequate detection limits and sensitivity for a concentrated analyte, particularly aligning with earlier HPLC methods for betulin and related triterpenoids [11–16].

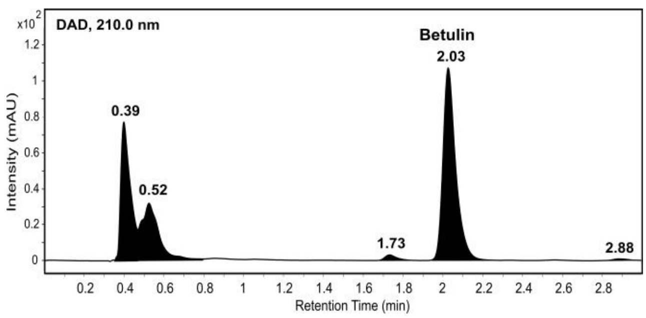

Figure 2 Representative HPLC-DAD profile of the oil-in-water based-betulin emulsion done on the ZORBAX Eclipse Plus C-18 RRHD chromatographic column using a mixture of acetonitrile and water (20:80) in volume as the mobile phase at a diode matrix detector wavelength of 210 nm.

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The strategy for quantification of betulin in an oil-in-water emulsion matrix was based on the external standard approach. The working solution of the betulin standard was gravimetrically prepared and diluted to obtain a series of calibration solutions covering the range from 60 to 140% (24–56 μg/mL) of the nominal expected concentration of the test solution corresponding to the regulated betulin content in the finished emulsion.

The analyzed feed additive was prepared in the form of an emulsion that includes other substances, such as inorganic salts, which can complicate the analysis when working with mobile phases with a high content of organic solvent. Based on the high solubility of betulin in low-polar solvents, the liquid-liquid extraction method was chosen for the extraction of the analyte. The interfering components of the emulsion remain in the aqueous layer of the resulting two-phase system. Based on existing literature [6–8], hexane was initially studied as an extractant for the separation of betulin from the emulsion; however, it was found that betulin was only slightly soluble in hexane. Due to this, the solubility of betulin in other organic solvents suitable for extraction (chloroform, ethyl acetate and dichloromethane) was additionally studied. However, dichloromethane was chosen, as it dissolves betulin well and has a low boiling point, which facilitates rapid subsequent removal during sample preparation. Since the mobile phase is often used as the sample solvent, the solubility of betulin in a mixture of acetonitrile and water (80:20) was also tested. It turned out that the target compound is soluble in this composition to a concentration of 250 micrograms/ml.

Three independent emulsion samples were analyzed for each sample. The areas of chromatographic peaks of betulin were converted to concentrations in the test solutions using a calibration graph. The calculation of the main statistical characteristics was carried out in accordance with the instructions of the RF State Budget [24]. The final mass concentration of betulin in the tested emulsion was taken as the arithmetic mean of three parallel samples, computed after evaluating for significant variances (errors). The uncertainty of the analysis results was assessed by calculating the confidence interval using Student's t-test for three parallel determinations at a 95% confidence probability.

From a systemic perspective, the method developed for the betulin-based oil-in-water emulsion illustrates the adaptation of HPLC to an industrial, non-biological matrix with a high analyte load. The combination of UHPLC hardware, rigorous system suitability testing, grading criteria, and explicit uncertainty budgeting reflected the requirements for veterinary feed additives. The method retains the conceptual simplicity of quantification with an external standard and is readily adaptable to other laboratories equipped with similar equipment.

Compared to the RP-HPLC techniques developed for plant preparations, the method done for the betulin-oil-in-water emulsion has some differences. This method aligns with the techniques for plant extracts that use reverse-phase C18 columns and a high organic component in the mobile phase, but it is optimized for significantly higher concentrations of betulin and emulsified matrices, as opposed to solid or alcoholic extracts. The emphasis on short analytical time is consistent with the need for routine industrial control of high-load drugs and process flows.

3.2. Validation of the developed method for the determination of betulin in the betulin-emulsion

Validation of the developed RP-HPLC method for betulin in the veterinary feed additive followed the general principles of the State Pharmacopoeia of the Russian Federation for the validation of analytical procedures [20], specifically on the determination of the primary normalized active substance. Testing of the suitability and specificity of the system was the first level of validation and was carried out using a calibration solution with a betulin standard concentration of 40.0 micrograms/ml, corresponding to the nominal betulin content in the emulsion, as well as a prepared test sample of the emulsion. The reproducible values of the chromatographic column efficacy, peak asymmetry, and retention factor for betulin validate the suitability of the developed chromatographic method. The specificity of the technique was confirmed through the analysis of chromatograms from both the betulin standard solution and the test solution for peak purity. The purity of betulin peaks was determined by comparing their retention times and UV spectra. An agreement was shown between the retention times and UV spectra in both chromatograms, along with the absence of co-elution of additional peaks from other compounds with betulin in the chromatogram of the tested sample. The betulin peak is adequately separated (resolution exceeded 2) from the peaks of both the uncontrollable substances and the associated impurities. The chromatogram of the sample solvent shows no peaks corresponding to the retention time of betulin, affirming the specificity of the method.

Linearity was evaluated by preparing five calibration points at concentrations ranging from 24 to 56 μg/mL, including a range from 60 to 140% of the nominal betulin content in the emulsion, hence expanding the analytical scope of the technique beyond the requirements of the pharmacopoeia (80–120%). The calibration curve (Figure 3) was constructed using linear regression of the peak area to the corresponding betulin concentration. The measurements were performed three times at each concentration point. The correlation coefficient of the calibration graph is 0.9999, which indicates good linearity.

The accuracy of the method was assessed using three standard solutions containing 60, 100 and 140% of the nominal betulin concentration established in the analyzed feed additive emulsion, which covers the entire range of application of the technique. Each of the solutions was analyzed three times. The accuracy of the results is presented in Table 1.

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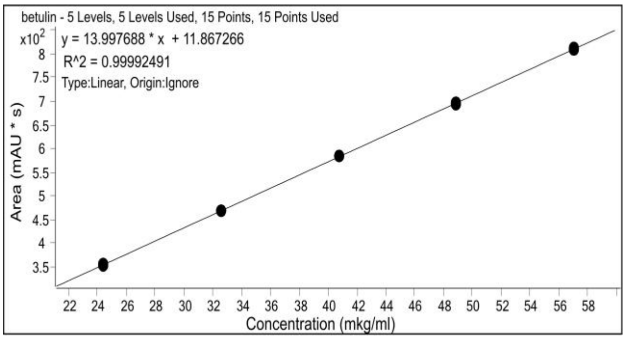

Figure 3 Calibration curve of the standard solutions of betulin for HPLC-DAD profile of the oil-in-water based-betulin emulsion.

Table 1 Accuracy of betulin determination.

Actual concentration, µg/mL	Measured concentration, µg/mL	Recovery, %
24.20	23.93	98.9
24.20	23.91	98.8
24.20	23.93	98.9
40.30	40.32	100.1
40.30	40.01	99.3
40.30	40.01	99.3
56.50	56.56	100.1
56.50	56.59	100.2
56.50	56.58	100.1
RSD, %	0.60	

Table 1 shows that the recovery values range from 98.8 to 100.2%, with Relative Standard Deviation (RSD) below 2.0%. This indicates a strong correlation between the response and the concentration of the compound, signifying the absence of systematic error in the methodology.

Precision was assessed on two levels, as repeatability (convergence) and intra-laboratory precision. To determine the repeatability of the method, 6 samples of the prepared emulsion with a nominal betulin concentration of 5 g/dm^3 were analyzed. The results of the repeatability assessment are presented in Table 2.

To assess for the intra-laboratory precision, a complete repetition of the experiments was carried out to determine the repeatability of the technique by a second analyst on another day. The results of determining the in-laboratory precision of the technique are shown in Table 2. The intra-laboratory precision was confirmed, as the relative uncertainty ε was less than 2.00%, and RSD was less than 1.2%. The validation evaluation of the developed method for the determination of betulin in the emulsion of feed additives has shown that the method is specific, linear in the analytical field, correct and precise. An indirect study of robustness showed that the technique allows for small changes in the flow rate, input volume, or use of other batches of columns of the same type, provided that the betulin retention factor remains close to 4 and all the criteria for the system's suitability for selectivity, peak asymmetry, column efficiency, and RSD are met.

Table 2 Results of parallel determination of betulin in a feed additive emulsion with a nominal mass concentration of the active substance of 5.00 g/L

Number of measurements	Analyst 1, concentration, g/L	Analyst 2, concentration, g/L	Metrological characteristics (P = 95 %, n = 12)
1	4.96	5.06	
2	5.10	4.97	
3	4.98	5.01	
4	5.05	5.10	X_{avg} = 5.03 ΔX = 0.03 S = 0.05 RSD = 1.07% $\bar{\mathcal{E}}$ = 0.6% ($\bar{\mathcal{E}}$ is the relative uncertainty)
5	5.08	4.95	
6	5.04	5.05	
Average value, X_{avg}	5.04	5.02	
Confidence interval, ΔX (P = 95 %, n = 6)	0.06	0.06	
Standard deviation, S	0.06	0.06	
RSD, %	1.09	1.14	

3.3. Comparison with previous methods

A literature search was done to compare the newly developed method to those of existing studies and found that most developed quantitative approaches for the simultaneous determination of betulin primarily used HPLC-UV methods and relied on reversed-phase chromatography (RP-HPLC) due to the highly hydrophobic nature of betulin. As shown in Table 3, a limited number of studies have developed and validated an HPLC method for the determination of betulin. These studies found that the chromatographic separation of betulin was influenced by several factors, including elution mode, mobile phase composition, sample matrix, temperature, and operational parameters [25–28]. In comparison to the previously reported methods, our method possesses shorter retention time and analytical time. In addition, our method was specifically designed to rapidly detect betulin in a matrix with a high concentration of betulin. Our study is the first to design an RP-HPLC method for the quantification of betulin in an oil-water-based emulsion.

4. Limitations

The HPLC procedure described in this paper has been specially developed and validated for the betulin-oil-in-water emulsion and for the range of betulin concentrations corresponding to its declared values. Its direct applicability to other dosage forms, such as powders, tablets, suspensions or premixes, and to matrices with a clearly different composition of lipids, surfactants or co-solvents has not been experimentally verified. Extending the method to such systems would require at least a shortened re-validation focused on sample preparation efficiency, selectivity, and matrix effects.

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Table 3 Comparison of the developed method with the previously reported HPLC techniques for betulin determination.

HPLC Column	Mobile phase	Mode of elution	Technique	Selectivity	Run time (min)	Ref.
ACE C18 (150×4.6 mm, 3 μm)	ACN: H2O (89:11) and ACN: MeOH (10:90)	Isocratic	HPLC-DAD	*Vaccinium vitis-idaea*	16	25
SB C18 (100×3 mm, 1.8 μm	Ultra-pure water with 0.1 % formic acid and ACN with 0.1 % formic acid	Gradient	HPLC-DAD	Plant extracts, foods, and nutraceuticals	24	26
EC, NUCLEODUR C18 (100×3 mm, 1.8 μm)	Ultra-pure water with 0.1 % formic acid and ACN with 0.1 % formic acid	Gradient	HPLC-ELSD	Extracts of medicinal herbs	29	27
Diamonsil C18 (250×4.6 mm, 5 μm)	acetonitrile–water (86:14)	Isocratic	HPLC-DAD	Birch bark	20	28
ZORBAX C18 (50×2.1 mm, 1.8 μm)	water–acetonitrile (20:80)	Isocratic	HPLC-DAD	Betulin based oil-in-water emulsion	3	Present study

In addition, validation was performed in the concentration range relevant for routine analysis of the betulin-oil-in-water emulsion and does not consider quantification of trace amounts of betulin in biological fluids or plant samples. The method is based on liquid-liquid extraction with dichloromethane, followed by evaporation and resuspension in the mobile phase. This workflow is well suited for oil-in-water emulsions; however, it may be less efficient or less convenient for matrices with higher viscosity, stronger emulsifying properties, or regulatory restrictions on the use of chlorinated solvents. Potential problems such as incomplete phase separation, emulsion formation, or variable evaporation efficiency were monitored in this study, but they were not systematically investigated in a wide range of sample formulations and laboratory settings. The method can be applicable to polysaccharide and NADES (Natural Deep Eutectic Solvents) nanoemulsions [29,30] which currently find wide application in animal husbandry, drug delivery and food packaging.

Chromatographic detection was based on UV absorption at a single low wavelength, which reduces selectivity compared to mass-selective detection. Therefore, the method may be potentially vulnerable to unknown UV-absorbing components that may co-elute with betulin in other matrices. The current validation was performed in one laboratory, on one type of UHPLC system and one brand and geometry of the C18 column. The resistance to different batches of columns from other manufacturers, as well as to deliberate variations in chromatographic parameters, has only been partially studied and remains the subject of further work, especially if the method is implemented in several quality control laboratories.

5. Conclusion

A simple and rapid RP-HPLC method was developed and validated for the quantitative determination of betulin in a veterinary feed additive based on oil-in-water emulsion. The procedure is based on liquid–liquid extraction of a small volume of emulsion with dichloromethane, evaporation of the organic phase, and reconstitution of the residue in an acetonitrile–water mobile phase. Chromatographic separation on a short sub-2 μm C18 column under isocratic conditions with a high content of acetonitrile provided good selectivity and short analytical time. The method was validated in accordance with generally accepted guidelines for pharmaceutical analysis. With appropriate adaptation of sample preparation and supplementary validation, the approach may be extended to related triterpenoids and to a broader range of industrial and veterinary products containing betulin.

Supplementary materials

No supplementary materials are available.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Conceptualization: V.A.S., A.V.B., E.G.K.
Data curation: V.A.S., F.O.A
Formal Analysis: V.A.S., A.V.B.
Funding acquisition: E.G.K.
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Project administration: E.G.K.
Resources: E.G.K., A.V.B.
Software: V.A.S., A.V.B.
Supervision: E.G.K.
Validation: V.A.S., A.V.B.
Visualization: V.A.S., F.O.A
Writing – original draft: V.A.S., A.V.B.
Writing – review & editing: P.A.V., E.G.K.

Conflict of interest

The authors declare no conflict of interest.

Additional information

AI-assisted tools (ChatGPT, OpenAI) were used only for language editing and formatting advice. All experimental design, data collection, and scientific conclusions were prepared by the authors.

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Ural Federal Agrarian Research Center of the Ural Branch of the Russian Academy of Sciences, https://www.urfanic.ru/ru/.

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