

## Supercritical Fluid Extraction of Usnic Acid from Lichen of *Cladonia* Genus

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**Abstract**—The process of carbon dioxide supercritical fluid extraction (SFE) of secondary metabolites from the lichen of *Cladonia* genus is studied. The yield of solid extract during SFE with carbon dioxide is significantly higher than during the extraction with acetone, ethanol, and petroleum ether on the Soxhlet apparatus. The maximum content of the target component—usnic acid (UA)—in the extract (91%, yield—2.5% of absolutely dry raw material) is obtained under pressure of 35 MPa, temperature 40°C, and duration of the process of 40 min. Introduction of cosolvents (acetone, ethanol, methylene chloride) to carbon dioxide increases the yield of the target product to 3%.

**Keywords:** supercritical fluid extraction, secondary lichen metabolites, usnic acid, *Cladonia* lichen genus, parameters of extraction process

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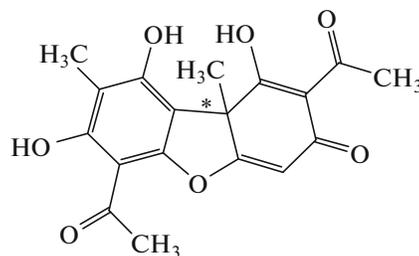
### INTRODUCTION

Lichens are symbiotic associations of fungi (mycobiont) and microscopic green algae and/or cyanobacteria (photobiont or phycobiont) which have acquired the properties of a fundamentally new organism in the course of evolution. Their biochemistry is quite versatile and, as a result of joint coexistence of partners, is characterized by the presence, among others, of compounds of aromatic structures synthesized exclusively by the mycobiont, with part of these compounds being specific to lichens [1, 2]. At the present time, approximately 500 of these compounds have been identified, with 75 of them being specific. It has been shown in a number of publications [3–5] that the secondary phenolic metabolites from lichens exhibit biological activity (antitumor, antimicrobial, antioxidant, and others), which has enabled their successful application for treatment of conditions of different etiologies.

Usnic acid (UA) (Fig. 1)—a derivative of dibenzofuran (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)dione)—is one of the best-studied and most abundant lichen metabolites, first isolated in the mid-19th century. It exhibits a wide spectrum of antimicrobial, antiviral, and anti-inflammatory effects. It exists in two enantiomeric forms differing in the location of the angular methyl group at the C(9b) chiral atom. Due to its high antimicrobial activity towards multiple pathogens of viral, bacterial, and fungal nature, UA finds application in cosmetology, agriculture, the pharmaceutical industry, dentistry, and other

areas of medicine [6–8]. The lichens of the *Alectoria*, *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Everina*, and some other genera are considered as promising sources for the production of UA [9, 10].

Extraction with organic solvents (chloroform, diethyl ether, petroleum ether, acetone, and others) is commonly used for isolation of UA from lichens [11]. Extraction with supercritical medium (carbon dioxide) has been reported only in a few publications [12–14]. However, the conditions (process parameters) for isolation of the target component (UA) from different genera and species of lichens have not been optimized. Such technological parameters of the extraction process as temperature, pressure, duration, and introduction of additives affect the yield of the extract and the concentration of the target component in it. Supercritical fluid extraction (SFE) allows one-step isola-



**Fig. 1.** Usnic acid formula.

**Table 1.** Yield of extracts and UA content in them during the extraction with organic solvents in the Soxhlet apparatus

Solvent	Yield of extract, % of a. d. raw material	Content of UA in the extract, %
Petroleum ether	0.20	12.6
Acetone	0.35	56.0
Ethanol	0.62	13.6

tion of biologically active compounds (BAC) of different nature from plant-derived materials without the use of organic solvents and acidic and/or alkaline reagents [15]. Moreover, there is no need for purification of BAC from the traces of solvents, which significantly simplifies the technological process and favorably affects the application of these extracts in the pharmaceutical industry, for example.

The objective of this study was to study the effect of the conditions of supercritical fluid extraction of lichen on the yield of solid extract and the content of the target component—usnic acid—in it.

## EXPERIMENTAL

An integral sample of epigeic shrublike lichen from the Cladoniaceae family (mixture of species: *Cladonia stellaris* (Opiz) Pouzar & Ve zda, *Cladonia arbuscula* (Wallr.) Flot., *Cladonia rangiferina* (L.) F. H. Wigg.) collected in June 2014 on Bol'shoi Solovetskii Island (Solovetskii Archipelago, White Sea (the largest archipelago of the White Sea basin, belonging to the northern Taiga subzone)) was used as an object of the study. The location of sampling (62°02' N, 35°43' E) was characterized by fur–pine bushy-lichen forest with the podzol soil type (sandy loam soil in terms of mechanical properties).

The lichen bodies (thalli) were manually separated from admixtures (bark particles, soil, and others) and air-dried without exposure to sunlight. The moisture content of the sample determined with the gravimetric technique was  $9.9 \pm 0.2\%$ ; the ash content determined after dry ashing at 540°C was  $0.53 \pm 0.08\%$ . The lichen sample was stored in dense paper bags in the dark at temperature 5°C. Prior to extraction, the dried material was powdered in an LM 201 rotor-blade mill in nitrogen environment (nitrogen flow rate 50 mL/min, rotor rotation speed 17 000 rpm, duration of milling 20 min with cooling water consumption of 4 L/min and material load of 25 g).

The UA content in the initial sample was determined following extraction with various organic solvents (ethanol, acetone, and petroleum ether) in a Soxhlet apparatus for 24 h.

Supercritical extraction of the lichen was conducted using an MV-10ASFE system (Waters, United States). An aliquot of raw material (~2 g) was placed in a 10-mL volume autoclave and treated with carbon

dioxide at the preset temperature (40, 60, and 80°C) and pressure (10, 20, 30, 35 MPa) for time interval from 20 to 120 min and CO<sub>2</sub> flow rate 5 mL/min (the temperature of the heat exchanger cooling the pump head and flow meter was 2°C). Liquid cosolvents (ethanol, acetone, and methylene chloride) were introduced in an amount of 5% of the indicated CO<sub>2</sub> volumetric flow rate.

Evaluation of the dry residue and yield of the extract with regard to the absolutely dry (a. d.) raw material was determined by drying at 50°C.

The UA content in the extracts was determined quantitatively with the HPLC method using an LC-30 Nexera instrument (Shimadzu, Japan) in the isocratic mode. Samples were dissolved in acetone, filtered, and injected into a chromatographic system (sample loading volume 10 µL). A Zorbax Eclipse Plus C18 column (Agilent, United States) was used for separation; column size 3.0 × 100 mm, particle size 3.5 µm, column temperature 40°C. The used mobile phase comprised 0.5% aqueous solution of formic acid and acetonitrile at a ratio of 20 : 80, flow rate 0.5 mL/min. The spectrophotometric detection technique was used (diode array, wavelength 280 nm). The calibration dependences of the area under the peak on the concentration in the range from 1 to 100 µg/L were constructed with the help of the standard UA sample (Sigma-Aldrich). The graduation dependences were linear in the used concentration range with correlation coefficient 0.99.

The efficiency of the SFE process was characterized with the following parameters: yield of solid extract (% of a. d. raw material), UA content in the solid extract (%), and yield of UA (% of a. d. raw material).

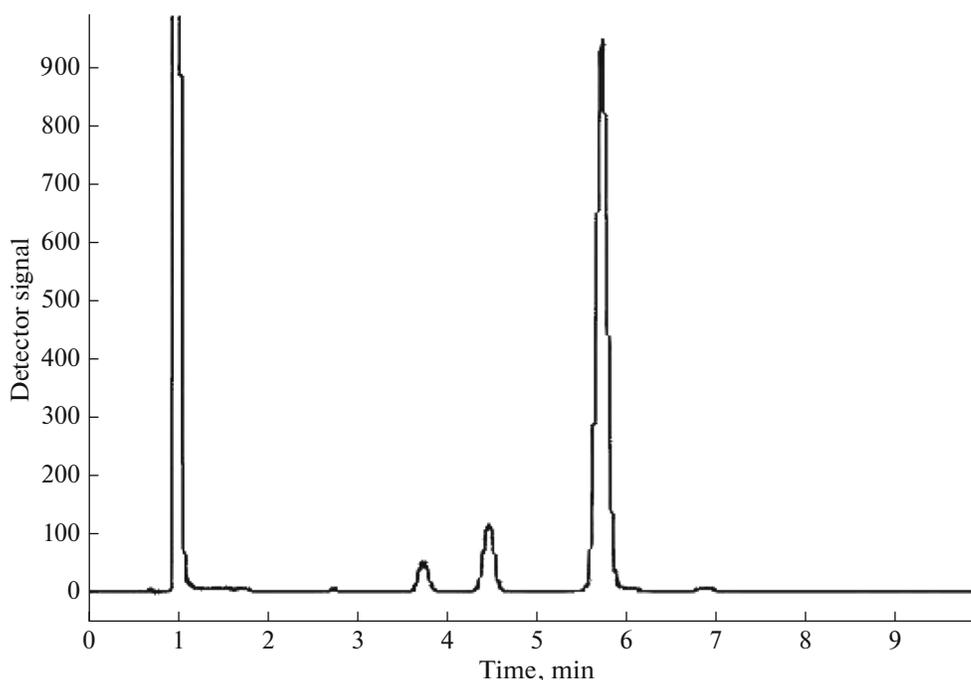
The comparative experiments on extraction with organic solvents (ethanol, acetone, and petroleum ether) were carried out on a Soxhlet apparatus; 10 g of raw material and 250 mL of solvent were used. The number of cycles was 30.

The presented experimental data were obtained by averaging the results of at least three replicates with discrepancy between them of no more than 5%.

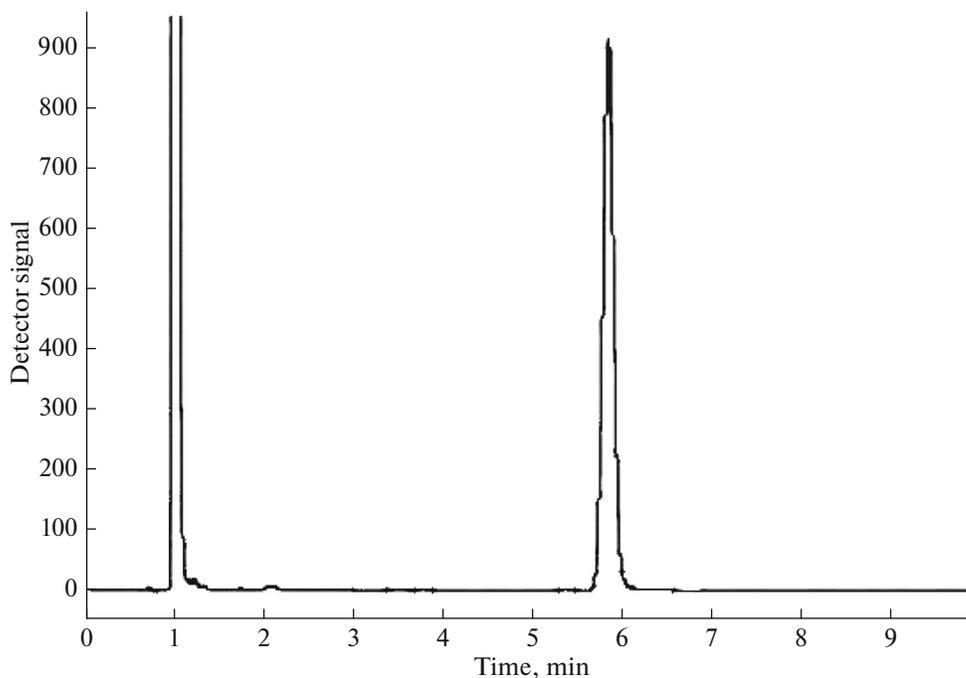
## RESULTS AND DISCUSSION

The results of extraction of the lichen sample with organic solvents on the Soxhlet apparatus are presented in the Table 1. The highest yield of solid extract was obtained with the use of ethanol; however, the content of UA in this extract did not exceed 14%. At the same time, acetone allows extracting 0.35% of solid compounds from lichen with UA content in them up to 56%.

The chromatogram of the lichen acetone extract is presented in Fig. 2. Other organic compounds absorbing in the UV region of the spectrum are present there in addition to UA.



**Fig. 2.** Chromatogram of acetone extract of lichen produced in the Soxhlet apparatus.



**Fig. 3.** Chromatogram of lichen extract produced with the SFE method (35 MPa, 80°C).

The chromatogram of the lichen extract produced with the SFE method at 35 MPa and 80°C is presented in Fig. 3. The presence of other phenolic compounds in addition to UA was not observed, which indicated high selectivity of supercritical carbon dioxide as an extractant.

Evaluation of the effect of temperature and pressure on the yield of the extract and UA was performed

with the extraction duration of 20 min. Increasing the pressure from 10 to 30 MPa facilitated the increase of the extract yield as a whole (Fig. 4). The increase of temperature from 40 to 80°C at the pressure of 10 MPa resulted in a threefold decrease of the extract yield, while at higher pressure an increase in the extract yield with increasing temperature was observed. This is likely related to the significant drop of CO<sub>2</sub> density

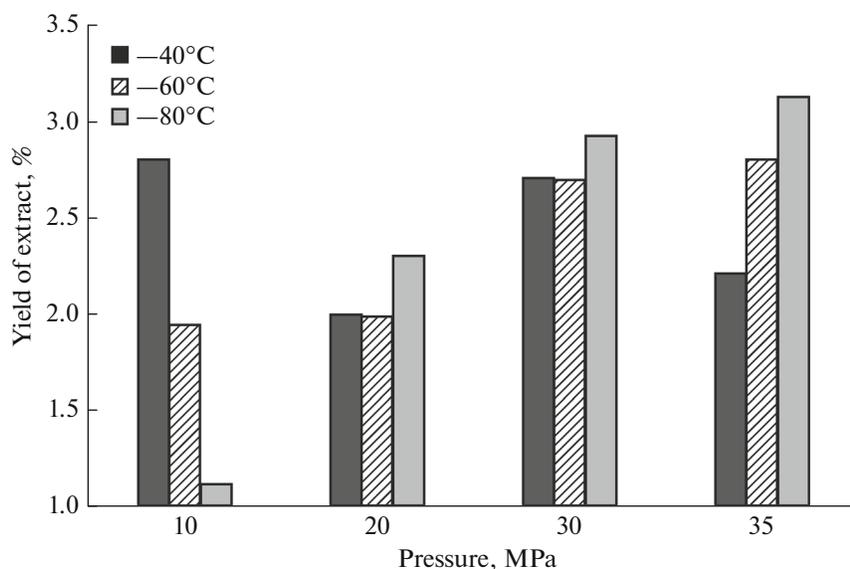


Fig. 4. Yield of solid extract with regard to a.d. lichen thalli at different extraction conditions.

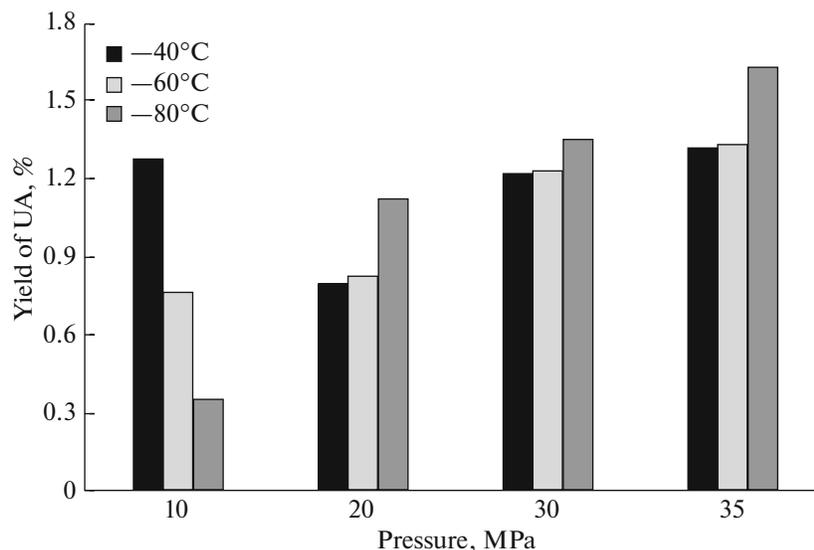


Fig. 5. Yield of UA with regard to a.d. lichen thalli at different conditions of extraction process.

(from 14.3 to 5.1 mol/L) with the increase of temperature from 40 to 80°C on the 10-MPa isobar [17]. At higher pressure (20–35 MPa), the drop in density in this temperature range does not exceed 15%, compared to 70% for 10 MPa.

It follows from the data presented in Fig. 3 that the yield of solid extract during SFE is significantly higher than in the case of acetone extraction. The highest yield of extract was 2.93–3.13% at the temperature 80°C and pressure 30–35 MPa.

The main BAC of the investigated lichen is UA; hence, the extraction process was optimized to increase the yield of this component and enrich the produced solid extract with it.

The general trend of the dependence of UA isolation from the lichen on temperature and pressure is similar to the trend of the solid extract yield (Fig. 5). The abnormal temperature dependence of this parameter during extraction at pressure of 10 MPa is likely also related to the drop of the carbon dioxide density. The SFE demonstrated higher efficiency in comparison with organic solvent extraction under all used conditions. From the point of view of the UA yield, it is worthwhile to conduct the extraction process at the temperature of 80°C and pressure 30–35 MPa.

The quality of plant extract is estimated based on the content of the target component. The acetone extract contained 56% of UA and the carbon dioxide extracts contained in general a lesser amount of UA

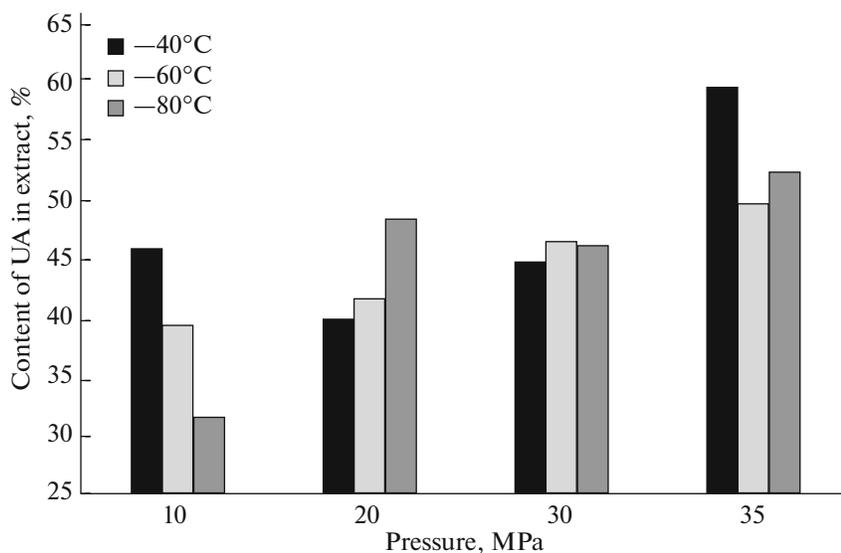


Fig. 6. Content of UA in the extract produced with SFE at different conditions.

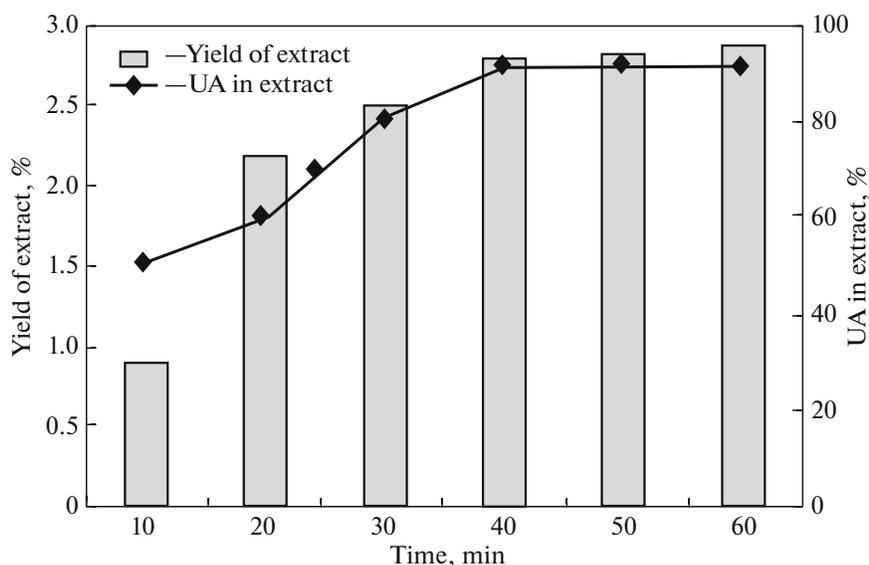


Fig. 7. Yield of extract and content of UA in it depending of duration of SFE.

(45–52%, see Fig. 6). The extract produced at 40°C and 35 MPa contained 60% of UA, but the yield of the extract was only 2.2%. Thus, the next step of the extraction process optimization was the increase of its duration under the same parameters for preparation of the extract enriched with the target component. The obtained results are presented in Fig. 7.

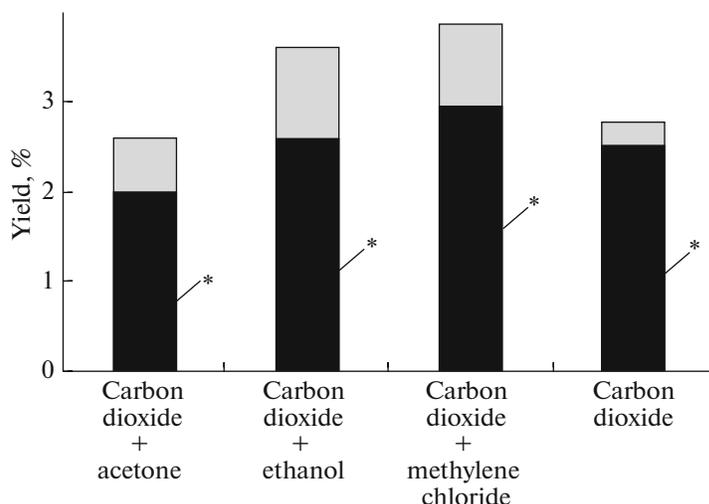
It follows from the presented data that extraction duration longer than 40 min is not advisable. The yield of the solid extract does not exceed 3.5% with practically the same content of UA when the duration of extraction increases from 40 to 60 min.

Thus, the optimal conditions of extraction are as follows: temperature 40°C and pressure 35 MPa with process duration of 40 min. Under these conditions,

the yield of the extract is 2.8% with the content of UA in it of 91%.

The increase of extract yield can be achieved by introduction of cosolvents into the carbon dioxide. We have tested such cosolvents as ethanol, acetone, and methylene chloride. The SFE was conducted at the temperature of 40°C, pressure 35 MPa, and process duration of 40 min (Fig. 8).

Introduction of the cosolvents resulted in the increase of the extract yield to 3.0–3.9%; however, the content of UA in the product decreased to 71–76%. Furthermore, the yield of UA with regard to the initial raw material increased slightly to 2.6–3.0%, compared to 2.5% for the extraction with pure CO<sub>2</sub>. Methylene chloride must be considered as the most efficient among the investigated cosolvents.



**Fig. 8.** Fraction of UA (\*) in the total extract yield (% of a. d. raw material) on addition of cosolvents (acetone, ethanol, methylene chloride) in comparison with pure CO<sub>2</sub>.

## CONCLUSIONS

Supercritical carbon dioxide (either pure or with the addition of cosolvents) is an effective extractant of usnic acid from the thalli of the lichen of *Cladonia* family.

The dependence of the extract yield and its quality on the parameters of the extraction process has been established. The pressure of 35 MPa, temperature of 40°C, and process duration of 40 min can be recommended as the optimum conditions for conducting the SFE process. Under these conditions, the yield of UA is 2.5% of the a. d. raw material. Introduction of cosolvents (acetone, ethanol, methylene chloride) allows increasing the yield of the target product to 2.6–3.0%.

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