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ARTICLES

Application of High-Performance Liquid Chromatography to the Determination of the Concentration of Lichen Secondary Metabolites

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Abstract—Preparative isolation of aromatic metabolites from lichens of the *Cladonia* genus (*C. stellaris*, *C. arbuscula*, *C. amaurocraea*, and *C. rangiferina*) growing in Central Yakutia was carried out. Identification by IR, UV, and time-of-flight mass spectrometry has shown that the isolated compounds belong to the group of lichen substances. It was shown that the component composition of the studied lichens corresponds to the previously described one. At the same time, the concentration of perlatolic and barbatic acids in *C. stellaris* and *C. amaurocrae* lichens, respectively, growing in Central Yakutia found by HPLC is higher than in similar types of temperate climatic zones.

Keywords: lichen substances, preparative flash chromatography, HPLC, mass spectrometry, *Cladonia* genus, Yakutia

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The main secondary metabolites of lichens are unique compounds, lichen substances. Most of the open lichen substances are products of the acetate–malonate pathway of biosynthesis [1]. At present, about 800 lichen substances are known, including aromatic (depsides, depsidones, dibenzofurans, and quinones) and aliphatic (fatty acids, γ-lactones, and triterpenoids) compounds [2]. It is known that lichen substances have a wide spectrum of biological activity, including antioxidant, antibacterial, and cytotoxic [3]. At the present time, the production of a number of food biologically active additives, "Yagel'," "Yagel' detox," "Kladosent," etc. is organized in Yakutia and Buryatia based on lichens of the *Cladonia* genus.

A universal procedure for the qualitative determination of lichen substances by thin-layer chromatography was developed in [4]. Later, a standardized HPLC technique was proposed for the determination of the concentration of lichen substances [5]; it was improved in the early 1990s [6, 7]. The first mass spectra of lichen substances (depsids, depsidones, and dibenzofurans) were obtained in the 1960s [8]. In the analysis of *Cladonia stellaris* and *Hypogymnia physodes* lichen extracts by HPLC with tandem mass spectrometry detection (HPLC–MS/MS), molecules of lichen substances underwent electrospray ionization with signals of negative ions were recorded [9, 10]. In the end of the 20th century, most of information about lichen substances known by that time, including the data on their structures, molecular masses, UV, IR, and NMR spectra, were systematized and cataloged in [11].

The aim of this work was to determine the concentration of lichen substances in lichens of the *Cladonia* genus growing on the territory of Central Yakutia by HPLC.

EXPERIMENTAL

Equipment. A Grace Reveleris preparative flash chromatograph (USA). A Varian 7000 FT-IR Fourier transform IR spectrometer (USA). A Shimadzu UV-2600 spectrophotometer (Japan) with a scan range of 190–750 nm. Melting point was determined by differential scanning calorimetry (**DSC**) on a DSC 204 F1 device manufactured by Netzsch Geratebau (Germany). DSC was carried out in the temperature range from 40 to 300°C at a rate of 20 K/min in a helium atmosphere.

An Agilent 1290 high performance liquid chromatograph (USA). An Agilent 6538 UHD quadrupole time-of-flight mass spectrometer (USA) with electro-

Table 1. Secondary metabolites of lichens growing in various types of pine forests in Central Yakutia identified by UV and IR spectrometry

Lichen acid	t_R, min	T_m, °C	λ, nm (CH_3OH)	ν, cm^{-1} (KBr)
Usnic acid	26.0	207* 204**	232, 282* 232, 282**	1690, 1628, 1538, 1451, 1356, 1334, 1317, 1287, 1221, 1189, 1144, 1118, 1070, 1040, 1024, 992, 958, 840, 819, 802, 701*; 1686, 1630, 1550, 1425, 1360, 1338, 1320, 1290, 1220, 1190, 1142, 1118, 1070, 1042, 1030, 998, 965, 848, 825, 810, 704**
Perlatolic acid	23.5	110* 108**	213, 269, 306* 213, 269, 306**	1659, 1637, 1610, 1461, 1353, 1319, 1301, 1284, 1239, 1210, 1163, 1140, 1074, 1043, 1018, 955, 865, 850, 840, 792, 766, 737*; 1655, 1635, 1610, 1470, 1360, 1320, 1305, 1290, 1250, 1210, 1165, 1150, 1082, 1050, 1030, 970, 865, 850, 810, 775, 750, 720**
Atranorin	22.0	201* 196**	209, 251, 312* 210, 252, 312**	1651, 1583, 1451, 1408, 1378, 1352, 1284 1268, 1236, 1214, 1200, 1163, 1106, 1076, 1028, 1007, 990, 937, 860, 821, 802, 783*; 1652, 1580, 1458, 1410, 1382, 1360, 1285, 1270, 1250, 1210, 1198, 1166, 1108, 1078, 1030, 1010, 990, 960, 870, 830, 810, 790**
Barbatic acid	19.5	189* 187**	214, 275, 305* 218, 276, 310**	1658, 1630, 1574, 1503, 1451, 1399, 1381, 1273, 1233, 1156, 1132, 1079, 1050, 992, 833, 810, 790, 728*; 1730, 1630, 1570, 1495, 1464, 1400, 1320, 1260, 1228, 1180, 1140, 1090, 990, 844, 820, 770, 735**
Fumarprotocetric acid	12.0	260* 255**	212, 238, 314* 212, 238, 314**	2919, 2748, 1741, 1717, 1661, 1562, 1434, 1403, 1352, 1309, 1252, 1217, 1199, 1182, 1127, 1106, 1021, 972, 853, 815, 712*; 3000, 2600, 1720, 1690, 1640, 1568, 1448, 1412, 1360, 1290, 1260, 1230, 1200, 1150, 1125, 1090, 1020, 990, 850, 810, 708**

* Experimental data, ** data of [11].

spray ionization (**ESI**). The equipment was of the resource center "The development of molecular and cellular technologies" of St. Petersburg State University (St. Petersburg). Voltage on the capillary with positive and negative ESI was 2.5 kV, capillary temperature 350°C, atomizing gas pressure 45 psi, dessicant gas (nitrogen) temperature 225°C, drying gas flow rate 5 L/min. Only ions formed in the process of positive and negative ESI were recorded without their additional collision-induced dissociation in the MC/MS mode in the mass range 100–1000 m/z. The resulting chromatograms were processed with the MassHunter WorkStation v. B.04.00 software package (Agilent, USA).

Reagents. Glacial acetic acid (Vecton, Russia), deionized water, ethanol (for preparative flash chromatography). Water "for HPLC" (Panreac), methanol, and formic acid from Merck (for HPLC–UV and MS, respectively) were used.

Aromatic compounds in the lichen composition from the group of lichen substances were isolated by preparative flash chromatography. To prepare the extracts, 20 g of air-dried thalli of lichen (*C. stellaris*, *C. arbuscula*, *C. amaurocraea*, and *C. rangiferina*) were taken and extracted with 200 mL of methanol for 24 h at 20°C under constant stirring. The obtained extracts were evaporated to a volume of 20–25 mL on a rotary evaporator and lichen substances were isolated from them.

The following fractions were obtained from methanol extracts of lichens: *C. stellaris* with retention times (t_R) 22 min (yield 9 mg) and 24 min (31 mg); *C. arbuscula* with t_R 12.0 (10 mg) and 22 min (15 mg); *C. amaurocraea* with t_R 20 (23 mg) and 22 min (14 mg); *C. rangiferina* with t_R 12 (14 mg) and 26 min (25 mg). After the removal of the eluents from the obtained fractions, the dry residue was purified by successive recrystallization from benzene and acetone.

Identification was carried out by comparing melting points, UV and IR spectra of the isolated substances with the characteristics of already known lichen metabolites (Table 1) [11]. To obtain IR spectra in the range 4000–400 cm^{-1}, potassium bromide tablets were prepared. UV spectra were recorded in the range 190–350 nm.

Chromatographic separation and detection conditions. The eluents for preparative flash chromatography were a 0.1% aqueous solution of glacial acetic acid (eluent A) and ethanol (eluent B). Gradient elution was used with increasing the percentage of eluent B proportion from 10 to 100% for 30 min at a mobile phase flow rate of 15 mL/min. A 111 × 10 mm cartridge filled with 12 g of C18 silica gel (40 μm) was used for separation. The volume of the injected extract was 3 mL. A fraction collector synchronized with the UV detector was adapted to automatic selection when the absorbance of the solution passing through the mea-

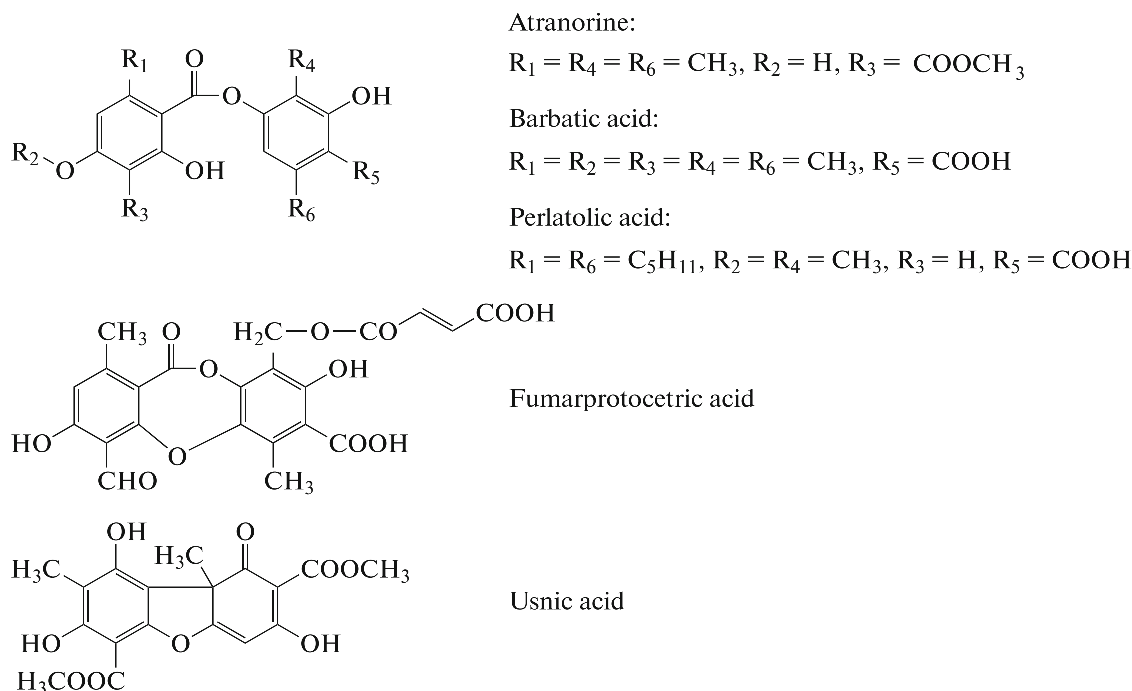

Fig. 1. Structural formulas of the studied lichen substances.

suring cell increased by more than 0.2 rel. units (at a wavelength of 210 nm).

A 0.1% aqueous solution of formic acid was used as mobile phase A and methanol was used as mobile phase B for HPLC analysis; in the gradient elution mode, the percentage of B was increased from 10 to 100% for 40 min at a flow rate of 100 μL/min. Column temperature was 25°C. Separation was carried out on a reversed-phase 150 × 0.5 mm Zorbax SB-C18 (80 Å, 5 μm) column. The volume of the injected sample was 1 μL. Detection wavelength was 210 nm.

Preparation of standard solutions and construction of calibration curves. To prepare calibration solutions, weighed portions of 5 mg of each lichen acid were taken and dissolved in 25 mL of methanol (Merck); solutions with concentrations of 100, 50, 25, 12, 6, and 3 μg/mL were prepared by dilutions of the stock solution. The calibration curves were linear in the studied concentration range and described by the equations $S = 0.217c$ ($R^2 = 0.9755$) for atranorine, $S = 0.4069c$ ($R^2 = 0.9998$) for barbatic acid, $S = 0.2932c$ ($R^2 = 0.9985$) for perlatolic acid, $S = 0.3751c$ ($R^2 = 0.9988$) for usnic acid, and $S = 0.2488c$ ($R^2 = 0.9966$) for fumarprotocetric acid (S are peak areas of the studied analytes obtained at a wavelength of 210 nm; c is concentration, μg/mL).

Sample preparation of lichens. Bushy lichens belonging to the genus *Cladonia* – *C. stellaris* (Opiz) Pouzar & Vězda, *C. arbuscula* (Wallr.) Flot., *C. amaurocraea* (Flörke) Schaer., *C. rangiferina* (L.) F.H. Wigg., growing in Central Yakutia (61°55′ N 129°31′ E), thallons of which are used as raw materials for the production of biologically active additives [11], were used as test samples.

The upper young parts of the air-dried lichens with a length of no more than 1.0 cm were taken for analysis. A crushed portion of a test sample (20 mg weighed portion) was extracted twice with 2 mL of methanol on constant stirring for 24 h and at a temperature of 20–25°C.

All measurements were performed in three biological and analytical repeats. Calculations were carried out using the AnalystSoft, StatPlus software package (statistical analysis program, v.2007).

RESULTS AND DISCUSSION

As was shown by UV and IR spectrometry, lichens *C. stellaris*, *C. arbuscula*, and *C. amaurocraea* contain usnic acid. In addition to usnic acid, aromatic depsides, such as atranorine (*C. rangiferina*), perlatolic (*C. stellaris*) and barbatic (*C. amaurocraea*) acids, were identified in lichen thalli (Fig. 1). As one can see in Table 1, the physicochemical properties of the isolated lichen substances are virtually the same as those listed in the literature, which is indicattive of a sufficiently high degree of chemical purity of the isolated compounds; therefore, the isolated and purified compounds were used as analytical standards for lichen substances in their quantitative determination in the thalli of the lichens by HPLC with UV detection.

Table 2. Characteristics of lichen substances obtained by HPLC and time-of-flight mass spectrometry with electrospray ionization

Analyte	t_R, min	Empirical formula	Ion $[M-H]^-$, m/z	Δ, ppm*	Ion $[M + H]^+$, m/z	Δ, ppm*
Atranorine	31.6	$C_{19}H_{18}O_8$	373.0929	0.03	375.1073	0.38
Barbatic acid	30.1	$C_{19}H_{20}O_7$	359.1172	–3.94	361.1284	–0.69
Perlatolic acid	34.2	$C_{25}H_{32}O_7$	443.2040	7.96	445.2226	–1.17
Usnic acid	31.1	$C_{18}H_{16}O_7$	343.0835	–3.42	345.0975	–1.80
Fumarprotocetric acid	25.1	$C_{22}H_{16}O_{12}$	471.0582	–2.76	473.0704	2.22

* The discrepancy between the calculated and observed masses of ions.

The isolated lichen substances were examined by HPLC combined with time-of-flight mass spectrometry. The data obtained are shown in Table 2. As one can see, all of the studied compounds at positive and negative ESI were characterized by the formation of molecular ions $[M + H]^+$ and $[M-H]^-$, respectively. The discrepancy between the calculated and observed masses of molecular ions did not exceed ±10 ppm.

Mass spectra of standard samples of the investigated lichen substances belonging to the type of depsids (atranorine, barbatinic, and perlatolic acids) are shown in Fig. 2. In the negative ionization mode, in addition to deprotonated molecular ions, we detected peaks of three daughter fragments of ions, the intensity of which for the studied compounds was different (Figs. 3a, 3c, and 3e). At positive ionization, in addition to the protonated molecule of the depsid and the daughter ion of the fragment, the formation of intense peaks of their cationized molecules with sodium was noted (Figs. 3b,3d, and 3f).

In the mass spectrum of fumarprotocetric acid at negative ionization, an intense peak of $[M-H]^-$, peaks of fragment ions $[M-C_4H_4O_4-H]^-$ and $[M-C_4H_4O_4-CO_2-H]^-$ with a relative intensity (I_{rel}) of 82 and 9%, respectively, and also a peak of an adduct ion $[2M-H]^-$ with I_{rel} = 87% were observed. At positive ionization, only a low-intensity peak of a protonated molecule of fumarprotocetric acid $[M + H]^+$ (I_{rel} = 1%) and two peaks of adduct ions $[M + Na]^+$ and $[2M + Na]^+$ with intensities of 100 and 84%, respectively, were recorded. In addition to intense $[M-H]^-$ and $[M + H]^+$ peaks in the mass spectra of usnic acid, at negative and positive ionization we observed peaks of adduct ions $[2M + Na-2H]^-$ and $[M + Na]^+$ with I_{rel} = 37 and 53%, respectively.

It was shown that the composition of lichen substances of the studied lichens corresponds to that described earlier [12, 13]. It is found that *C. stellaris* lichens growing in Central Yakutia belong to chemotypes I (usnic + perlatolic acid), *C. arbuscula* – to chemotype II (usnic + fumarprotocetric acids) [12]. The results of the quantitative determination of the concentration of lichen substances in four geni of lichens of the *Cladonia* genus growing in Central Yakutia and used as raw materials for the production of broad-spectrum biologically active additives obtained by HPLC with UV detection are presented in Table 3. One can see that the concentration of usnic acid in the studied lichens was in the range of 5.5–11.0 mg/g of dry weight, the highest value being noted for *C. arbuscula*. The highest concentration of fumarprotocetric acid was found in lichen *C. rangiferina*. The concentrations of metabolites of the group of depsides, perla-

Table 3. Determination of the concentration of lichen substances in thalli of lichens of the genus *Cladonia* growing on the territory of Central Yakutia ($n = 4$, $P = 0.95$)

Lichen genus	Identified compound	Found*, mg/g of dry mass
Cladonia stellaris	Usnic acid	10 ± 2
	Perlatolic acid	9 ± 3
Cladonia amaurocraea	Usnic acid	6 ± 1
	Barbatic acid	12 ± 2
Cladonia arbuscula	Usnic acid	11 ± 3
	Fumarprotocetric acid	3 ± 1
Cladonia rangiferina	Atranorine	7 ± 1
	Fumarprotocetric acid	13 ± 2

* Average value ± standard deviation.

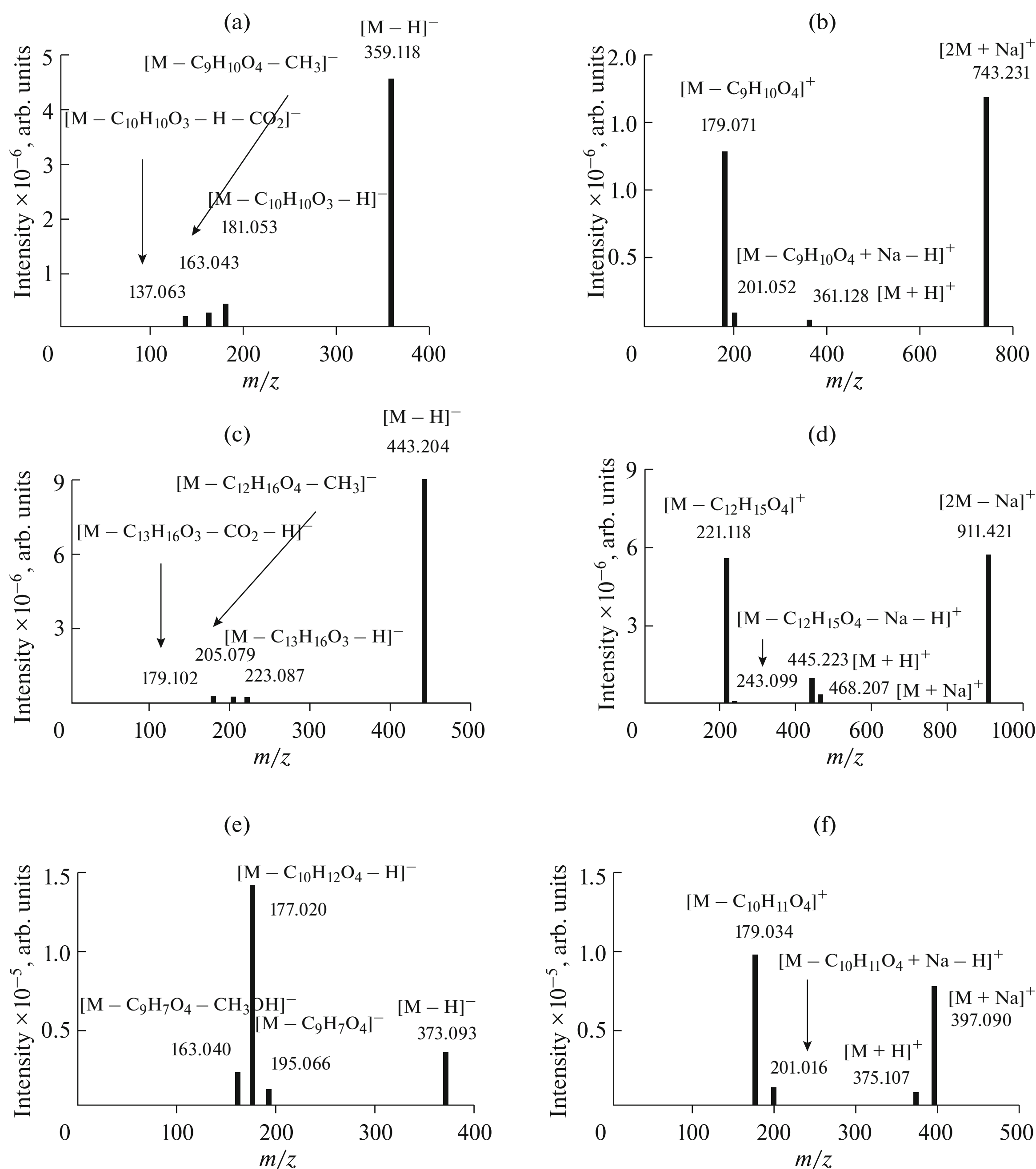

Fig. 2. Mass spectra of barbatic acid, perlatolic acid, and atranorine with electrospray ionization in the detection mode of (a), (c), and (e) negative and (b), (d), and (f) positive ions.

tolic, barbatic acid, and atranorine, in lichens *C. stellaris*, *C. amaurocrae*, and *C. rangiferina* were in the range 6.9–13.0 mg/g of dry weight.

A Comparison of our results and published data showed that the concentrations of atranorine (*C. rangiferina*), usnic (*C. stellaris*, *C. amaurocrae*, and *C. arbuscula*), and fumarprotocetric (*C. arbuscula*, *C. rangiferina*) acids in lichens of Central Yakutia correspond to the values for the similar geni growing in other climatic zones [12, 13].

In lichens *C. stellaris* and *C. amaurocrae* growing in Central Yakutia, the concentration of perlatolic and barbatic acids was on the average 2–3 times higher in comparison with similar geni of temperate climatic zones (Finland, Southern Canada, etc.) [9, 13, 14]. The increased concentration of depsides in the thalli of the studied lichens points to their possible partici-

pation in the regulation of metabolic processes contributing to the formation of the adaptive potential and resistance to the conditions of the sharply continental climate of Central Yakutia.

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