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Enzyme Immunoassay of Usnic Acid in Lichens

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Abstract—An enzyme immunoassay for usnic acid in lichens was developed, the sensitivity of which was 0.1 μg/g of air-dried material (0.00001%). Polyclonal rabbit antibodies against bovine serum albumin conjugated to (+)-usnic acid under the conditions of formaldehyde condensation made it possible to determine the analyzed substance in solutions at concentrations from 1 ng/mL when it interacts with immobilized gelatin conjugate homologous in the binding mode. Usnic acid in 2–26600 μg/g (0.0002–2.6%) amounts was found in all 236 studied samples of lichens belonging to 53 species and 8 families.

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INTRODUCTION

Usnic acid (UA) (2,6-diacetyl-7,9-dihydroxy-8,9*b*-dimethyldibenzofuran-1,3(*2H,9bH*)-dione) is one of the lichen metabolites that possess a wide range of antimicrobial, antiviral, and anti-inflammatory activities [1]. For many years, UA was used for the treatment of dangerous infections, and today, despite the proven allergenic potency and toxicity, it is still recommended as a prophylactic agent in dietetics and cosmetology [2]. Biological products on the basis of lichens are usually prepared from thalli extracts, so they always contain a complex set of structurally similar and completely physiologically different active substances. Many of them can have a direct or indirect adverse effect on the body, so control over the component composition in such drugs is necessary.

At present, researchers have at hand several methods for UA determination based on gas and liquid chromatography with ultraviolet, photodiode, and mass spectrometric detection, which provides lower limits of measurements from 50 to 400 ng/mL [3–6]. However, an immunochemical analysis method, which would not be inferior to chromatographic methods in selectivity, would have much greater sensitivity, and would require minimal sample preparation, has not yet been developed for UA determination.

The purpose of the present study was to develop and apply an enzyme immunoassay (EIA) for the quantitative determination of UA among extractive substances from lichens of different species and genera.

MATERIALS AND METHODS

We used (+)-UA (cat. no. 329967, Aldrich, Switzerland); emodin (cat. no. E7881, Sigma, United States); formaldehyde and dimethylformamide (DMFA) (Fluka, Germany); and bovine serum albumin (BSA), ovalbumin (OA), and gelatin (Gel) (Biokhimreaktiv, Russia). An antispecies enzyme conjugate was obtained according to [7] from horseradish peroxidase (EC 1.11.1.7) (Sigma, United States) and donkey antirabbit gamma-globulin serum (Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences). Atranorin was obtained at the Chair of Mycology and Algology (Moscow State University). EIA was performed in polystyrene plates of a high binding capacity (#9018, Costar, United States); the measurements were performed in an AKI-Ts-01 photometer (Russia). The UV spectra were recorded on a Hitachi-557 instrument (Japan).

Protein–UA conjugates were synthesized by formaldehyde condensation. To obtain BSA–UA(10), i.e., a conjugate with a tenfold molar excess of UA, as well as BSA–UA(30) and BSA–UA(100), 24, 72, and 241 μL of 10 mg/mL UA solution in DMFA (241, 723, and 2410 μg of UA, respectively) were added to solutions containing 5 mg (0.7 μmoL) of BSA in 1.5 mL of 0.1 M aqueous sodium acetate. OA-UA(30) and Gel-UA(30) and OA–UA(30) were obtained by adding 1032 and 516 μg of UA to solutions containing 4 mg (0.1 μmoL) of OA or 8 mg (0.05 μmoL) of Gel in 1.5 mL of 0.1 M aqueous sodium acetate. Then, 300 μL (3690 μmol) of 37% formaldehyde was added to each mixture, and, after 6 h of stirring at 30°C, the reaction mixtures were dialyzed against a 1000-fold volume of

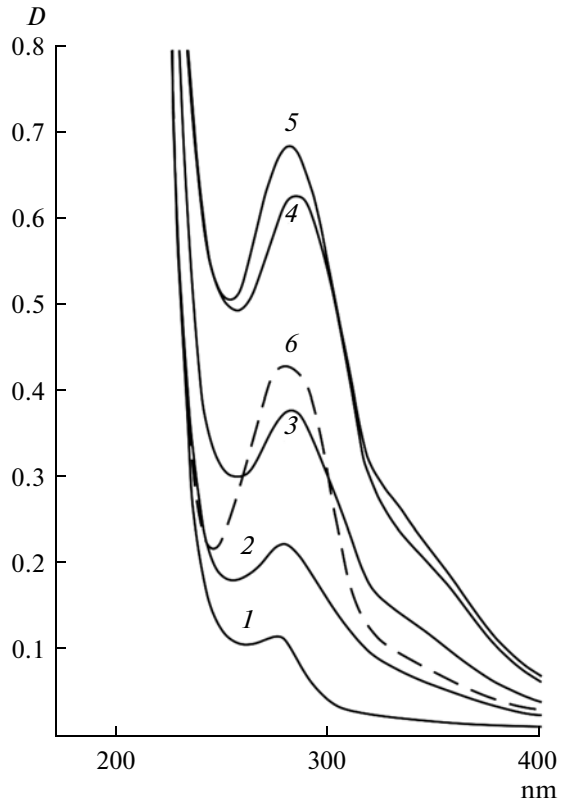

Fig. 1. UV spectra of (*1*) BSA, (*2*) BSA–UA(10), (*3*) BSA–UA(30), (*4*) BSA–UA(100), (*5*) *2* BSA–UA(100/16), and (*6*) UA.

0.5% aqueous sodium chloride with three buffer exchanges. To obtain BSA–UA(100/16), after adding formaldehyde, the reaction mixture was stirred at 30°C for 16 h and then dialyzed. After this, all dialysates were diluted with an equal volume of glycerol and stored at –10 to –15°C. Aqueous solutions of the conjugates with a concentration of 100 μg/mL were used for recording the UV spectra.

Male grey rabbits (2–3 kg) were immunized with the BSA–UA(100/16) conjugate. First, 200 μg of immunogen in Freund's complete adjuvant were injected subcutaneously into 10–15 points on the back of the animals; the second and third injections were made with 300 μg of the immunogen in physiological saline. Repeated injections were performed at one-month intervals. Seven days after the third injection, blood was sampled from the marginal ear vein; serum was separated, diluted with an equal volume of glycerol, and stored at –10 to –15°C.

To perform EIA, the wells of the plates were filled with 0.2 mL of conjugate solutions for immobilization in 0.05 M carbonate–bicarbonate buffer (pH 9.5) and incubated at 4°C for 16 h. Then, the wells were washed 4–5 times with 0.15 M phosphate-buffered saline (pH 7.5), containing Tween 20 (PBS-t), and 0.1 mL of solutions of the analyte and antibodies in the same buffer were added to the wells. After 1 h of incubation, the wells were washed again and filled with 0.2 ml of a working solution of the enzyme conjugate. After 1 h of incubation and washing, 0.2 mL of a substrate solution containing 0.4 mg/mL of *o*-phenylenediamine and 0.005% H_2O_2 in 0.15 M citrate–phosphate buffer (pH 5.0) was added. After 45 min, 50 μl 4 M sulfuric acid containing 0.1 M Na_2SO_3 was added to each well and the optical density was measured at 492 nm.

Working solutions of UA for competitive EIA were prepared from the stock solution in acetonitrile at a 10 μg/mL concentration determined spectrophotometrically (λ = 282 nm, ε = 25350). The calibration curve in "percentage points of antibody binding–UA solution concentration" coordinates was obtained daily or at intervals of 1–2 days under the conditions intermediate precision (n = 10).

Samples of lichen thalli previously used for mycological toxicological analysis [8], as well as epilithic, epiphytic, and epigeal lichens collected in 2010–2011 in the Lapland and Pechora-Ilych Natural Reserves, on the Taimyr Peninsula, and near Petropavlovsk-Kamchatsky and Yakutsk were the objects of the analysis. The method of material extraction did not differ from the one described in [8].

RESULTS AND DISCUSSION

Condensation of UA, taken in 10-, 30-, and 100-fold excesses relative to BSA, in the presence of formaldehyde at 30°C for 6 h resulted in products with quite expectable spectral characteristics. It should be noted that formaldehyde condensation is often accompanied by a change in the absorption spectra of conjugates, which however does not lead to the loss of their immune reactivity as it was in the case of alternariol and tetracycline [9, 10]. The UV absorption maximums of UA at 233 (ε = 34740) and 282 nm (ε = 25350) [8] are overlapped with the BSA absorption at 280 nm; therefore, the presence of hapten in conjugates can only be detected by the growth in the optical density at this wavelength. In all obtained conjugates, we observed increased absorption at 280 nm in comparison with the starting BSA (Fig. 1). Its intensity increased regularly with an increasing amount of hapten in the reaction, and the values of the epitope density in the first three products were 3.1, 7.3, and 14.3. By conjugation of BSA with a 100-fold molar excess of UA under the conditions of a more prolonged (16 h) exposure of the reaction mixture with formaldehyde (Fig. 1), we managed to increase the epitope density up to 15.8. The ratio of the epitope density to the theoretically possible values was on average 21%; it was close to the same value for other physiologically active substances, for example, ciprofloxacin (20%) [11] and

Table 1. Competitive interaction (percentage of antibody binding) of the BSA–UA(100/16) serum with different immobilized antigens

Immobilized antigen and its concentration, μg/mL		Working titer of antibodies	Concentration of the UA solution, ng/mL		
			100	10	1
BSA–UA(10)	0.15	1:3000	42	73	92
BSA–UA(30)	0.15	1:5000	34	77	97
BSA–UA(100)	0.05	1:4000	39	76	93
BSA–UA(100/16)	0.05	1:4000	43	76	95
OA–UA(30)	0.5	1:1000	35	72	91
Gel–UA(30)	0.5	1:1000	33	61	88

cyclopiazonic acid (29%) [12], and was much higher than that of citrinin (8%) [13]. In the protein–UA conjugates, we did not observe the equality of epitope densities to theoretically possible values or their exceeding as it was reported for mycotoxin zearelenone in the same binding procedure [14].

A BSA–UA(100/16) conjugate for immunization provided the preparation of antiserum with working titers 1 : 1000–1 : 5000, which could be used in the competitive analysis of UA at the immobilization of all synthesized conjugates (Table 1). The use of solid-phase antigen Gel–UA(30) made it possible to determine the UA in solutions at a concentration of up to 1 ng/mL. The linearity of the analytical signal remained within the concentration range from 1 to 100 ng/mL, and the values of the confidence intervals calculated for ten determinations were 5–8% (Fig. 2).

It is known that besides dibenzofurans other structural groups, such as depsides, depsidones, and anthraquinones, are present in the aromatic substances of lichens. The antibodies in a competitive analysis did not detect two of them, namely, depside atranorin and emodin—a compound of anthraquinone series—even at a concentration of 1 μg/mL. This pointed to the high specificity of the assay and the ability to use it when working with native extracts. From an immunological point of view, it would be of interest to assess the cross-reactivity of the obtained antibodies against an optical isomer—(–)-usnic acid—and its closest structural analogues, but, unfortunately, we did not have such an opportunity.

Using the developed test system for analyzing stock water–acetonitrile extracts of lichens diluted 10 times with PBS-t, it was possible to determine UA in the amount of 0.1–10 μg/g of dry substance or 0.00001–0.001%. In order to proceed to the determination of UA in lichens, where it is prevalent over other metabolites and is accumulated in amounts ranging from hundredths to tens of percentage points, it was necessary to dilute the extract 100 times or more. By using the chromatographic method, it was previously shown that, among members of the genus *Cladonia* (family Cladoniaceae), UA accumulation of more than 0.05% is common to all studied species of the section *Unciales* [15], most of the *Cladina* species [16], many species of the section *Cocciferae* [17] and the subsection *Foliosae* in the section *Helopodium* [18], as well as individual species of the section *Perviae* [19], section *Cladonia*, and the group Furcatae [20].

We analyzed lichens of the family Cladoniaceae. It was shown that significant differences in the UA content were observed in the species *Cladonia*. The content of UA in *Cladonia amaurocraea*, *C. deformis*, *C. stellaris, C. sulphurina*, and *C. uncialis* was 0.5–2.3% (Table 2), which agrees with previously obtained

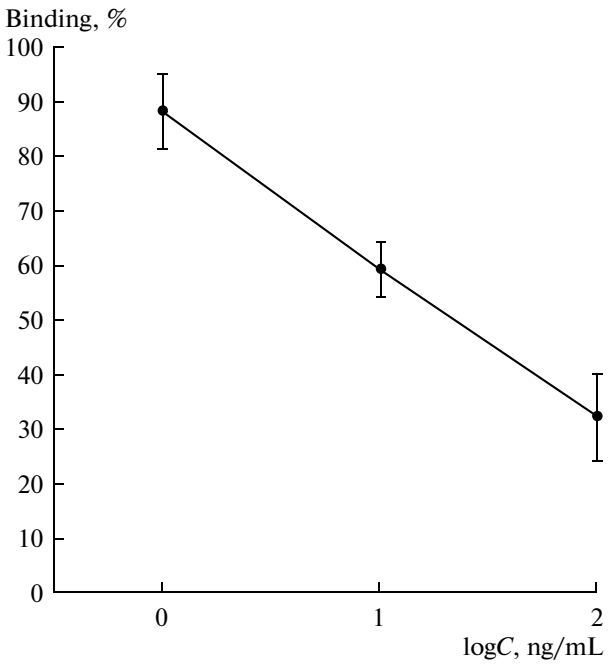

Fig. 2. Calibration curve for an immunoassay with the anti-BSA–UA(100/16) serum and immobilized antigen Gel–UA(30) ($n = 10$) for UA solutions in the presence of acetonitrile.

Table 2. Content of usnic acid in lichens of the family Cladoniaceae

Lichen species (number of tested samples)	UA, μg/g, min–**mean**– max
Cladonia amaurocraea (Flörke) Schaerer (4)	9200–**13100**–20900
C. arbuscula (Wallr.) Flot. (6)	480–**2020**–4100
C. cenotea (Ach.) Schaer. (1)	158
C. cornuta (L.) Hoffm. (7)	5–**63**–200
C. deformis (L.) Hoffm. (3)	13000–**14100**–14700
C. macroceras (Flörke) Ahti (2)	1350, 2500
C. mitis Sandst. (6)	600–**900**–1600
C. rangiferina (L.) Web. (9)	390–**840**–1900
C. rangiformis Hoffm. (2)	4, 5
C. stellaris (Opiz) Pouz. et Vězda (4)	10000–**15600**–23200
C. sulphurina (Michx.) Fr. (9)	9000–**12600**–19600
C. uncialis (L.) Wigg. (4)	5000–**5800**–6400

data [15–17, 21]. In another group—*C. arbuscula*, *C. macroceras*, *C. mitis*, and *C. rangiferina*—the accumulation of this substance was in the range 0.05–0.4% (Table 2). However, it was previously reported that the UA content in two samples of *C. arbuscula* collected in Leningrad oblast and Vorsmi Island (Estonia) was 0.99 and 1.60%, and in two samples of *C. mitis* it was 0.97 and 1.72% [21]. In one sample of *C. macroceras* from Finland, UA was not detected by the chromatographic method with a detection threshold of 0.05% [20], and in two analyzed samples collected in the northern Murmansk region (near Kola) and on the coast of the White Sea near the Arctic Circle its content was 0.1 and 0.2% (Table 2).

Negative results were obtained when the lichens *C. cenotea*, *C. cornuta*, and *C. rangiformis* were analyzed by HPLC with a sensitivity of 0.05% [15, 16]. According to our data, UA is present in the thalli of these species, but in amounts less than 0.02%, and a significant variability, depending on the collecting place, was noted for *C. cornuta* (Table 2).

All these data are directly relevant to the production of biological products, as the main active substance is often claimed to be simply a mixture of species of the genus *Cladonia* (often without concretization and refinements of the conditions of collecting the material).

In order to obtain reliable information for taxonomic studies of the genus *Cladonia*, it is necessary to use large samples. So far, the conclusions about the species peculiarities of UA accumulation were only made based on a few or individual samples. The use of EIA will allow one to obtain further significant knowledge in this area, since the simultaneous testing of tens of samples becomes possible.

Among lichens of the family Parmeliaceae, representatives of the genus *Flavocetraria* (Table 3), as well as the species *Vulpicida pinastri* and *Alectoria sarmentosa* (Table 3), had a high UA content: from 0.7 to 2.6%. The UA content was lower in *A. ochroleuca*: from 0.1 to 0.4%. According to [22], species of the genus *Alectoria* are often a rich source of UA and accumulate it to 6% of a dry sample. The previously reported level of UA accumulation in *Flavocetraria cucullata* (=*Cetraria cucullata* [21]) corresponded to the lower content limit determined by us (Table 3).

Lichens of the genera *Evernia* and *Usnea*, as well as *Cetraria islandica*, ranked lower according to this indicator (Table 3). The UA accumulation levels varied from 0.05 to 0.6% in *E. mesomorpha* and from 0.01 to 0.02% in *E. prunastri*. For two species of the genus *Usnea*, such as *U. dasypoga* and *U. subfloridana*, the ranges of measured contents were similar (0.06–0.1 and 0.01–0.1%, respectively).

In six samples of *C. islandica*, collected in geographically distant places, the amount of UA varied from 25 to 1200 μg/g, which is likely to be due to the conditions of their habitat. The fact that UA in this lichen can be contained at a 0.1% level deserves attention because some pharmaceutical companies produce cold medicines based on *C. islandica* [2]. On the other hand, there are reports that UA could not be detected in these lichens collected in Iceland even in the background content [1]. Levels of UA accumulation of about 0.03% are characteristic of *Arctoparmelia centrifuga* and *Platismatia glauca*, while they were even lower in representatives of the genera *Bryoria*, *Melanohalea*, *Parmelia*, and *Hypogymnia* and the species *Pseudevernia furfuracea* (Table 3).

A low UA content was found in lichens of the families Peltigeraceae and Umbilicariaceae, as well as in four species of other families, such as *Thamnolia vermicularis*, *Lobaria scrobiculata*, *Nephroma arcticum*, and *Xanthoria parietina* (Table 4). The UA content on average composed 0.02% in the species *Lobaria scrobiculata* and *Nephroma arcticum*, and in all other lichens it was below 0.01%. The lowest accumulation

Table 3. Content of usnic acid in lichens of the family Parmeliaceae

Lichen species (number of tested samples)	UA, μg/g, min–**mean**–max
Alectoria ochroleuca (Hoffm.) Massal. (4)	1200–**2900**–3800
A. sarmentosa (Ach.) Ach. (9)	7900–**11600**–25100
Arctoparmelia centrifuga (L.) Hale (2)	200, 360
Bryoria capillaris (Ach.) Brodo et D. Hawksw. (3)	14–**25**–40
B. chalybeiformis (L.) Brodo et D. Hawksw. (4)	9–**41**–100
B. fremontii (Tuck.) Brodo et D. Hawksw. (3)	6–**39**–98
B. fuscescens (Gyeln.) Brodo et D. Hawksw. (2)	25, 33
B. implexa (Hoffm.) Brodo et D. Hawksw. (4)	8–**13**–16
B. nadvornikiana (Gyeln.) Brodo et D. Hawksw. (1)	51
B. simplicior (Vain.) Brodo & D. Hawksw. (1)	130
B. subcana (Nyl. ex Stizenb.) Brodo et D. Hawksw. (1)	12
Cetraria islandica Ach. (6)	25–**350**–1200
Evernia mesomorpha Nyl. (6)	500–**3000**–6200
E. prunastri (L.) Ach. (5)	100–**150**–250
Flavocetraria cucullata (Bellardi) Kärnefelt et A. Thell (7)	7100–**12600**–26000
F. nivalis (Bellardi) Kärnefelt et A. Thell (9)	7700–**10600**–16500
Hypogymnia farinacea Zopf (1)	164
H. physodes (L.) Nyl. (9)	14–**27**–65
Melanohalea exasperata (DeNot.) O. Blanco et al. (2)	2, 5
M. olivacea (DeNot.) O. Blanco et al. (6)	4–**28**–64
M. septentrionalis (DeNot.) O. Blanco et al. (4)	18–**42**–90
Parmelia saxatilis (L.) Ach. (5)	9–**83**–250
P. sulcata Taylor (4)	10–**62**–138
Platismatia glauca (L.) W.L. Culb. et C.F. Culb. (3)	100–**350**–780
Pseudevernia furfuracea (L.) Zopf (2)	2, 3
Usnea dasypoga (Ach.) Nyl. (3)	580–**900**–1300
U. hirta (L.) Wigg. emend. Mot. (1)	316
U. subfloridana Stirt. (7)	130–**500**–1200
Vulpicida pinastri (Scop.) J.-E. Mattsson et M.J. Lai (5)	11300–**16300**–26600

level (0.0002–0.0005%) was detected in samples of *Melanohalea exasperata*, *Pseudevernia furfuracea* (Parmeliaceae) (Table 3), *Peltigera horizontalis*, *P. scabrosa* (Peltigeraceae), and *Xanthoria parietina* (Table 4).

Thus, UA in 0.0002–2.6% amounts was detected in all 236 studied samples of lichens belonging to 53 species and 8 families. References to a lack or rare occurrence of UA in some types of lichens (for example, *Pseudevernia furfuracea*, *Cetraria islandica*, *Thamnolia vermicularis*, and *Xanthoria parietina*; the genera *Peltigera* and *Evernia prunastri*; and some others) [23–25] are due to the insufficient sensitivity of the used analysis method.

Application of EIA, by which one can obtain exhaustive information on the UA content in extrac-

Table 4. Content of usnic acid in lichens of other families

Family, lichen species (number of tested samples)	UA, μg/g, min–**mean**–max
Icmadophilaceae,	
Thamnolia vermicularis (Sw.) Ach. ex Schaerer (9)	13–**38**–100
Lobariaceae,	
Lobaria scrobiculata (Scop.) DC (7)	85–**240**–530
Nephromataceae,	
Nephroma arcticum (L.) Torss. (9)	80–**177**–320
Peltigeraceae,	
Peltigera aphthosa (L.) Willd. (10)	9–**49**–130
P. canina (L.) Willd. (2)	6, 18
P. didactyla (With.) J.R. Laundon (2)	11, 33
P. horizontalis (Huds.) Baumg. (4)	5–**12**–15
P. leucophlebia (Nyl.) Gyeln. (2)	8, 40
P. rufescens (Weiss) Humb. (3)	6–**9**–16
P. scabrosa Th.Fr. (2)	3, 32
Teloschistaceae,	
Xanthoria parietina (L.) Th. Fr. (7)	4–**15**–39
Umbilicariaceae,	
Umbilicaria deusta (L.) Baumg. (1)	42
U. hyperborea (Ach.) Hoffm. (1)	72
U. proboscidea (L.) Schrad. (2)	30, 132
U. torrefacta (Lightf.) Schrad. (4)	10–**67**–180

tive substances of lichens, will improve the safety and efficacy of dietary supplements and medicinal products based on them and expand the understanding of the biosynthetic capabilities of these symbiontic organisms. This method may also be used for taxonomic purposes.

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