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Concentration data of (+)-usnic acid enantiomer from some European and African samples of *Flavoparmelia caperata* (L.) Hale (Parmeliaceae, lichenised Ascomycota) – results of a preliminary study

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Couverture / *Cover*:
Flavoparmelia caperata (L.) Hale from Tanzania (VBI 6169, sample 20)

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ABSTRACT
The usnic acid, cortical pigment, is generally known to protect lichens from extreme radiation. According to earlier studies, the parmelioid lichen *Flavoparmelia caperata* (L.) Hale contains (+)-usnic acid. Since the production of lichen secondary metabolites – like other physiological activities – is influenced by environmental conditions, we assumed that macroclimatic differences in temperature, radiation and humidity in European and African habitats resulted in differences in the concentrations measured in samples of this species from various geographical areas. Therefore, we analysed samples representing populations in Hungary (17), Serbia (three), Kenya (eight) and Tanzania (two). Using a chiral chromatographic method, the presence of (+)-usnic acid was confirmed in thirteen of the aforementioned specimens, with concentrations ranging from 5.08 to 26.43 mg g^{-1} in European samples (12) and 20.27 mg g^{-1} in a sample from Kenya. The usnic acid content (mg g^{-1} dry weight) was measured by HPLC-PDA. The content of usnic acid shows a substantial variation in both continents, ranging from 5.21 to 19.23 mg g^{-1} in Europe and from 6.15 to 23.54 mg g^{-1} in Africa. The comparison between continents did not result in significant differences. This result can be explained by the supposedly similar microclimatic conditions of the habitats (within macroclimatically different sites) that are probably consistent with the specific niche requirements of *F. caperata*.

KEY WORDS
Hungary,
Kenya,
Serbia,
Tanzania,
chiral properties,
chiral HPLC,
HPLC-PDA,
lichen-forming fungi,
lichen secondary
metabolite content.

RÉSUMÉ

Concentration de l'énantiomère de l'acide (+)-usnique dans des échantillons européens et africains de Flavoparmelia caperata *(L.) Hale (Parmeliaceae, Ascomycota lichénisé) – résultats d'une étude préliminaire.*

L'acide usnique, pigment cortical, est généralement connu pour protéger les lichens des radiations extrêmes. Selon des études antérieures, le lichen parmélioïde *Flavoparmelia caperata* (L.) Hale contient de l'acide (+)-usnique. Étant donné que la production de métabolites secondaires du lichen – tout comme d'autres activités physiologiques – est influencée par les conditions environnementales, nous avons supposé que la température, le rayonnement et l'humidité macroclimatiquement différents dans les habitats européens et africains entraînaient des écarts de teneurs dans les échantillons de cette espèce en provenance de diverses zones géographiques. Par conséquent, nous avons analysé des échantillons collectés dans des populations en Hongrie (17), en Serbie (trois), au Kenya (huit) et en Tanzanie (deux). En utilisant une méthode de chromatographie chirale, la présence d'acide (+)-usnique a été confirmée dans treize des échantillons susmentionnés avec une concentration allant de 5,08 à 26,43 mg g^{-1} dans des échantillons européens (12) et 20,27 mg g^{-1} dans un échantillon du Kenya. Cette teneur (mg g^{-1} poids sec) a été mesurée par HPLC-PDA. La teneur en acide usnique montre une variation substantielle dans les deux continents, allant de 5,21 à 19,23 mg g^{-1} en Europe et de 6,15 à 23,54 mg g^{-1} en Afrique. La comparaison entre les continents n'a pas abouti à des différences significatives. Ce résultat peut s'expliquer par les conditions microclimatiques supposées similaires de ces habitats (dans des sites macroclimatiquement différents) et qui sont très probablement compatibles avec les exigences spécifiques de niche de *F. caperata*.

MOTS CLÉS
Hongrie,
Kenya,
Serbie,
Tanzanie,
propriétés chirales,
HPLC chirale,
HPLC-PDA,
champignons lichénisants,
teneur en métabolites
secondaires du lichen.

INTRODUCTION

Lichens, consisting of an exhabitant fungus and one or more extracellular photosynthetic partners, live in association with an indeterminate number of other microscopic organisms (Hawksworth & Grube 2020). They are known to produce at least a thousand lichen secondary metabolites (LSMs) almost unique to this group (Stocker-Wörgötter 2008; Elix 2018). Their biological activities are so far insufficiently known (Molnár & Farkas 2010; Goga *et al.* 2018), however, the related dibenzofuran usnic acid (UA) occurring in the cortex of the beard lichen species (belonging to the large and widely distributed genus *Usnea s.l.*) has been the subject of several studies (Ingólfsdóttir 2002; Cocchietto *et al.* 2002; Çetin Çakmak & Gülçin 2019). It has been known since 1967, that UA is a chiral molecule and occurs in two forms (enantiomers) in lichens in nature (Bendz *et al.* 1967; Kinoshita *et al.* 1997), mentioning that *Flavoparmelia caperata* (L.) Hale produces exclusively (+)-UA. According to previous research referred in the review Galanty *et al.* (2019) there are fields where the (+)-UA ("right-handed" form) is reported to have higher efficacy, while in other applications the (-)-UA ("left-handed" form) is more effective, therefore the bioactive potential of the enantiomers needs further studies.

The cortical pigment UA is generally known as protecting lichens from extreme radiation (Solhaug & Gauslaa 2001; Solhaug *et al.* 2003; Veres *et al.* 2022b). Furthermore its biological activities were also widely studied (Ahmad *et al.* 2017; Luzina & Salakhutdinov 2018; Dieu *et al.* 2020; Muhoro & Farkas 2021; Kulinowska *et al.* 2023). It has been assumed that macroclimatic environmental conditions (Solhaug *et al.* 2003; Veres *et al.* 2020, 2022a, b; Singh *et al.* 2021), temperature (annual mean 0-20°C in the temperate region, 25-27°C in the tropics), radiation (much higher in the the tropics due to the angle of 90-66.5°) and humidity (500-1000 mm in the temperate region, over 2000 mm near the Equator) in European and African habitats (Richards 1952; Ricklefs 2008; Olou *et al.* 2019) have resulted in different concentrations measured in European and African samples of this species naturally occurring in these geographical areas, since the production of lichen secondary metabolites – similarly to other physiological activities – is influenced by the aforementioned conditions. Our aim for a preliminary study was to measure the concentration range roughly and also to confirm the chiral properties of UA in some populations of the widely distributed *F. caperata* (Swinscow & Krog 1988; Verseghy 1994; Brodo *et al.* 2001; Nash & Elix 2002; Divakar & Upreti 2005; Awasthi 2007; Smith *et al.* 2009; Wirth *et al.* 2013; Brodo 2016) based on LC-UV analysis of 20 samples from Central Europe and ten from East Africa. Thirteen of these samples have been analysed with a chiral chromatography method to ascertain the enantiomeric form produced.

MATERIAL AND METHODS

The research object

The common greenshield lichen, *Flavoparmelia caperata* (L.) Hale (Parmeliaceae, lichenised Ascomycota) is a medium to large, pale yellow green foliose lichen growing on the bark of trees (Fig. 1A, C-E), and less frequently on rock (Fig. 1B). In Europe it is relatively frequent, but sensitive to acidic air pollution (Verseghy 1994; Smith *et al.* 2009; Wirth *et al.* 2013). While it is found usually in lower elevations than 2000 m above sea level in the temperate region, in tropical East Africa (Fig. 1C-E) it is found in higher elevations (at

Fig. 1. — Thalli of *Flavoparmelia caperata* (L.) Hale, in their habitats in Hungary: **A**, on bark of *Quercus* sp. (sample 4); **B**, on andesite rock (near sample 11). Herbarium specimens: **C**, from Kenya (VBI 6044, sample 19); **D**, **E**, from Tanzania (VBI 6169, sample 20; VBI 6251, sample 23). Photographs taken by E. Farkas. Scale bars: 1 cm.

c. 1500-3600 m, Swinscow & Krog 1988). It occurs also in America (Brodo *et al.* 2001; Nash & Elix 2002; Brodo 2016) and Asia (Divakar & Upreti 2005; Awasthi 2007). Its rounded lobes are of 3-8(-13) mm wide (for East African specimens 5-10 mm is indicated, Swinscow & Krog 1988), usually covered by patches of granular soredia arising from pustules in the middle part of the lamina (illustrated in Figure 1 of Farkas & Muhoro 2022). The lobes of the thallus may be smooth, but often wrinkled in older specimens. The lower surface is black with a brown, naked marginal zone, otherwise with simple, slender rhizines, often with a white or brown tip. Its cortex contains usnic acid (a dibenzofuran related compound), the medulla (containing also the fatty acid caperatic acid) reacts orange with p-phenylenediamine due to its protocetraric acid (a depsidone) content.

Sample collection and identification

Altogether 30 lichen samples of *Flavoparmelia caperata* were collected in Hungary (by E. Farkas, L. Lőkös, A. M. Muhoro, N. Varga), Serbia (by L. Lőkös), Kenya (by P. M. Kirika, H. T. Lumbsch, G. Mugambi, A. M. Muhoro) and Tanzania (by E. Knox, T. Pócs) from its typical habitats between

TABLE 1. — List of collected specimens of *Flavoparmelia caperata* (L.) Hale with their main collecting data (date of collection, altitude, collecting site) and herbarium number.

Sample number	Date of collection	Altitude (m a.s.l.)	Collecting site	Herbarium number
1	30.V.2020	250	**Hungary** • Heves County, Mátra Mts (SE side), valley of stream "Hátsó-Tarnóca" at SW side of Mt "Sózó-tető", *c*. 2 km N of Kisnána; on bark (*Quercus petraea*); 47°52'06.9"N, 20°08'54.0"E.	VBI_6029
2	30.V.2020	250	**Hungary** • Heves County, Mátra Mts (SE side), valley of stream "Hátsó-Tarnóca" at SW side of Mt "Sózó-tető", *c*. 2 km N of Kisnána; on bark (*Quercus petraea*); 47°52'06.9"N, 20°08'54.0"E.	VBI_6029
3	30.V.2020	250	**Hungary** • Heves County, Mátra Mts (SE side), valley of stream "Hátsó-Tarnóca" at SW side of Mt "Sózó-tető", *c*. *2* km N of Kisnána; on rock and soil; 47°52'06.9"N, 20°08'54.0"E.	VBI_6030
4	30.V.2020	255	**Hungary** • Heves County, Mátra Mts (SE side), valley of stream "Hátsó-Tarnóca" at SE foot of Mt "Kopasz-hegy", *c*. 2 km N of Kisnána; on bark (*Quercus* sp.); 47°52'05.8"N, 20°08'46.9"E.	VBI_6031
5	05.IX.2021	370	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 4 km NNW of Gyöngyöstarján; on bark (*Quercus cerris*); 47°50'46.4"N, 19°51'05.6"E.	VBI_6032
6	05.IX.2021	370	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 4 km NNW of Gyöngyöstarján; on bark (*Quercus cerris*); 47°50'46.4"N, 19°51'05.6"E.	VBI_6033
7	05.IX.2021	580	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 4 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route; on bark (*Quercus petraea*); 47°51'24.3"N, 19°50'25.8"E.	VBI_6034
8	05.IX.2021	580	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 4 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route; on mossy bark (*Quercus petraea*); 47°51'24.3"N, 19°50'25.8"E.	VBI_6035
9	05.IX.2021	585	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 5.4 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route; on rock; 47°51'24.5"N, 19°50'26.9"E.	VBI_6036
10	05.IX.2021	635	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 5.4 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route; on rock; 47°51'28.9"N, 19°50'28.8"E.	VBI_6037
11	05.IX.2021	680	**Hungary** • Heves County, Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), *c*. 5.6 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route; on rock; 47°51'34.3"N, 19°50'20.0"E.	VBI_6038
12	12.VI.2021	190	**Hungary** • Zala County, Göcsej, Lenti, Mt Cser-hegy (S side), mixed forest in "Szemere-lakosi-dűlő", *c*. 1.5 km ENE of Kerkabarabás; on bark (*Quercus cerris*); 46°40'55.4"N, 16°34'33.0"E.	VBI_6039
13	30.VI.2022	102	**Serbia** • Brnjica, along stream Brnjica; on bark (*Prunus* sp.); 44°38'19.19"N, 21°45'10.03"E.	VBI_6040
14	30.VI.2022	102	**Serbia** • Brnjica, along stream Brnjica; on bark (*Alnus glutinosa*); 44°38'19.19"N, 21°45'10.03"E.	VBI_6041
15	30.VI.2022	102	**Serbia** • Brnjica, along stream Brnjica; on bark (*Prunus* sp.); 44°38'19.19"N, 21°45'10.03"E.	VBI_6040
16	11.VIII.2022	125	**Hungary** • Somogy County, Duna-Dráva National Park, Barcsi-ősborókás, Nagy-Berek, *c*. 3 km NW of Darány; on bark (*Quercus* sp.); 45°59'27.6"N, 17°33'19.5"E.	VBI_6042
17	11.VIII.2022	125	**Hungary** • Somogy County, Duna-Dráva National Park, Barcsi-ősborókás, Nagy-Berek, *c*. 3 km NW of Darány; on bark (*Quercus* sp.); 45°59'27.6"N, 17°33'19.5"E.	VBI_6042
18	11.VIII.2022	125	**Hungary** • Somogy County, Duna-Dráva National Park, Barcsi-ősborókás, Nagy-Berek, *c*. 3 km NW of Darány; on bark (*Quercus* sp.); 45°59'27.6"N, 17°33'19.5"E.	VBI_6043
19	14.VIII.2021	2454	**Kenya** • Nyeri county, at the foot of Mt. Kenya, *c*. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village; from bark, twigs and branches of trees in tropical rainforest; 0°10'25.84"S, 37°9'3.40"E.	VBI_6044
20	24.X.1987	1700	**Tanzania** • Pienaar S Heights, S of Babati, Bereku F. R.; mist effected miombo on the ridge; on bark.	VBI_6169
21	05.IX.2022	134	**Hungary** • Pest County, Vácrátót, Tece, along the 'red line' tourist route (Ág-dűlő), in open sandy grassland; on bark; 47°42'08.5"N, 19°13'27.5"E.	VBI_6045
22	03.IX.2022	114	**Hungary** • Balatonakali, in the forest patch behind the camp "Kapuvári tábor"; on bark (*Quercus* sp.); 46°52'59.8"N, 17°44'16.2"E.	VBI_6055
23	25.X.1988	2350-2500	**Tanzania** • S Uluguru Mts in Morogoro District, in secondary grassland dominated by *Panicum luckwangulense* with scattered *Agauria salicifolia* and *Myrica salicifolia* trees; ramicolous on *Agauria* ct.	VBI_6251
24	21.I.2013	2137	**Kenya** • Baringo County, Eldama Ravine Lembus Forest off Eldama Ravine-Eldoret Road, in a remnant montane forest; on bark; 0°09'36.0"N, 35°45'00.0"E.	dupl EA 2835
25	22.I.2013	2168	**Kenya** • Baringo County, Esageri Hill, 9 km from Esageri Market off Eldama Ravine-Nakuru Road, in a remnant montane forest; on bark; 0°03'00.0"N, 35°48'36.0"E.	dupl EA 2922

Table 1. — Continuation.

Sample number	Date of collection	Altitude (m a.s.l.)	Collecting site	Herbarium number
26	29.III.2014	3008	**Kenya** • Western Kenya, Trans-Nzoia County, Mt. Elgon National Park, in *Olea-Juniperus-Podocarpus* forest; on bark; 1°03'00.0"N, 34°41'00.0"E.	dupl EA 4246
27	10.IV.2014	2022	**Kenya** • Nyeri county, Mt. Kenya, Gathiuru Forest, Naromoru, Bantu lodge area, in disturbed *Juniperus-Podocarpus-Olea* forest; on bark; 0°16'00.0"S, 37°03'00.0"E.	dupl EA 4425
28	22.I.2015	1995	**Kenya** • Nyeri County, Mt. Kenya area, Naromoru, on shrubs and trees in pasture land; on bark; 0°06'00.0"N, 37°56'00.0"E.	dupl EA 4755
29	04.VI.2016	2709	**Kenya** • Elegeyo-Marakwet County, Cherangani Hills, Embobut Forest, montane forest; on bark; 1°05'00.0"N, 35°31'00.0"E.	dupl EA 5123
30	26.X.2021	2462	**Kenya** • Elgeyo Marakwet County, Keiyo South Sub-County, Kaptagat forest, riparian vegetation, dry patch in plantation forest; on bark; 0°26'12.0"N, 35°30'13.0"E.	dupl EA 5671

24 October 1987 and 5 September 2022 (Table 1). The time of collection and the elevation – though otherwise might be important in the process of LSM production – was not considered when samples were selected for the study. Before the analysis by liquid chromatography (UHPLC-PDA-MS and HPLC-PDA – see below), the samples originally identified by their collectors were revised by E. Farkas, L. Lőkös and A. M. Muhoro using a Nikon SMZ18 stereomicroscope (Nikon Corporation, Tokyo, Japan), as well as an Olympus SZX7 stereomicroscope (Olympus Corporation, Tokyo, Japan) and by analysing LSMs applying HPTLC (see below). Voucher specimens are deposited in Lichen Herbarium VBI (Vácrátót, Hungary) and in Herbarium EA (Nairobi, Kenya) (abbreviations follow Thiers 2023). The sites are presented in a map (Fig. 2) of the studied countries, constructed by a computer program for geographical information systems QGIS 3.18.2 'Zürich', released in 2020.

Qualitative analysis of LSMs by HPTLC

Collected samples were prestudied for the presence of UA, protocetraric acid and caperatic acid by high-performance thin layer chromatography (HPTLC) according to standard methods for analysing lichen samples described by Arup *et al.* (1993) and Molnár & Farkas (2011). CAMAG horizontal chamber of 10 × 10 cm (DONAU LAB Kft., Budapest, Hungary), CAMAG TLC Plate Heater III (DONAU LAB Kft., Budapest, Hungary), and 10 × 10 cm thin-layer chromatographic plates (Merck, Kieselgel 60 F254) were used. Solvent system C (toluene : acetic acid, 20:3, v/v) was applied. Compounds were identified by comparing their migration rates with atranorin, zeorin and norstictic acid originating from *Pleurosticta acetabulum* (Neck.) Elix & Lumbsch (norstictic acid) and *Leucodermia boryi* (Fée) Kalb (atranorin, zeorin) control species.

Qualitative analysis of LSMs by UHPLC-PDA-MS

The compound identity of UA was further verified using ultra-high performance liquid chromatography-photodiode array detection-mass spectrometry (UHPLC-PDA-MS). The Waters Arc UPLC system consisted of a quaternary solvent pump, an autosampler with temperature control at 10°C, a column manager with temperature control at 25°C, a PDA detector and a single quadrupole MS detector. One CORTECS C18 column (4.6 × 50 mm, 2.7 µm) was used for chromatographic separation with a flow rate at 0.5 ml min^{-1}. Solvent A was 0.1% formic acid, and solvent B was acetonitrile. The gradient elution condition was: 0-1 minute, 20% B; 1-8 minutes, linear increase 20-100% B; 8-8.5 minutes, linear decrease 100-20% B; 8.5-10 minutes 20% B. UV spectra were recorded from 220 to 380 nm, and ions in negative ion mode were scanned from 100 to 1000 m/z. Single ion monitoring for UA was set at 343.1 m/z.

Quantitative analysis of LSMs by HPLC-PDA

The amount of UA was measured by high-performance liquid chromatography (HPLC, Alliance e2695, Waters Corporation, Milford, MA, United States) system, including a photodiode array detector (PDA, 2998, Waters Corporation, Milford, MA, United States) in Hungary. Thalli (of 1.5-2 g) were *c*. 4-5 cm in diameter. The pulverised, homogenised material (50 mg of the entire amount 10-20(-30) g) was dissolved in 10 ml pure acetone and placed into an ultrasonic water bath for 20 minutes. The samples of several thalli were then centrifuged for 20 minutes, and the supernatant was filtered through a Cronus Ø 25 mm PTFE syringe filter (0.22 µm). Standard stock solutions (1 mg ml^{-1}) were made from reference standards for UA (Sigma Aldrich Kft., Budapest, Hungary) and for fumarprotocetraric acid (Phytolab GmbH & Co. KG, Vestenbergsgreuth, Germany) dissolved in dimethyl sulfoxide for calibration purposes. The LSMs were quantified according to a five-point (5, 10, 20, 50, 100 µg ml^{-1}) calibration. The chromatographic method based on Ji & Khan (2005) was used. For chromatographic separation, a Phenomenex Luna 5 µm C18, 150 × 4.6 mm column was used, and 10 µl sample volume was injected. There was 40°C in the column oven and 5°C in the sample cooler. For the baseline separation of LSMs, a gradient elution program was used. Solvent A consisted of ortho-phosphoric acid and deionised (Milli-Q ultrapure) water (0.5:99.5, v/v), and solvent B contained ortho-phosphoric acid and acetonitrile (0.5:99.5, v/v). All the chemicals used were HPLC grade. The linear gradient started with a 60% A solvent after the volume decreased to 10% within 20 minutes and then to 0.5% in 30 seconds after which the volume remained constant for 9.5 minutes. The volume of solvent A was changed back to 60% within one minute. The flow rate of solvents was 1 ml min^{-1}. LSMs were detected (n = 5) at 280 nm (UA).

Table 2. — Concentrations (with standard deviation) of usnic acid measured by HPLC-PDA (n = 28) and (+)-usnic acid measured by chiral chromatography (n = 13) in the collected samples of *Flavoparmelia caperata* (L.) Hale (Table 1). Symbols: *, mean concentration measured by HPLC-PDA; **, mean concentration measured by chiral HPLC.

Sample number	Usnic acid* (mg g^{-1} dry weight)	(+)-usnic acid** (mg g^{-1} dry weight)
1	12.35 ± 1.99	–
2	9.03 ± 4.17	–
3	8.66 ± 2.68	–
4	12.95 ± 3.51	–
5	8.47 ± 0.17	17.90 ± 0.43
6	8.78 ± 0.20	18.23 ± 0.19
7	11.48 ± 1.00	9.77 ± 0.07
8	11.22 ± 0.84	19.13 ± 0.79
9	7.71 ± 1.26	15.54 ± 0.79
10	9.04 ± 0.54	15.37 ± 0.45
11	9.28 ± 0.20	13.23 ± 0.22
12	6.83 ± 0.79	5.08 ± 0.28
13	14.43 ± 0.36	17.84 ± 0.47
14	8.92 ± 0.92	9.27 ± 0.42
15	9.92 ± 0.90	–
16	19.23 ± 1.61	26.43 ± 0.24
17	14.73 ± 2.22	–
18	–	13.59 ± 0.50
19	–	20.27 ± 1.44
20	10.62 ± 0.38	–
21	9.33 ± 1.30	–
22	5.21 ± 0.30	–
23	23.54 ± 0.58	–
24	9.55 ± 0.96	–
25	11.94 ± 1.06	–
26	12.89 ± 1.25	–
27	9.96 ± 0.63	–
28	10.30 ± 0.35	–
29	6.15 ± 0.76	–
30	6.92 ± 0.98	–

Determination of usnic acid enantiomers

The contents of UA enantiomers were determined in the Faculty of Pharmaceutical Sciences, University of Iceland, (Reykjavik, Iceland) using a chiral chromatographic method described in details by Xu *et al.* (2022). Twelve samples (nr 5-14, 16 and 18) were collected in Hungary and one sample (nr 19) originated from Kenya. Lyophilized lichen powders (less than 20 mg) were weighed, and LSMs were extracted with acetone in a sonicator. Each specimen was prepared as triplicate (n = 3). After evaporating acetone, dry residues were re-dissolved in 2 ml methanol : acetonitrile (50:50, v/v). Re-constituted extract solutions were subjected to reversed-phase solid phase extraction, prior to chiral high performance liquid chromatography (HPLC) analysis on a Waters Arc HPLC system. Separation of enantiomers was carried out on an Amylose-1 chiral column. Concentrations of UA enantiomers in test solutions were determined using external standard curves. The detection wavelength was set at 280 nm and the flow rate was 0.5 ml min^{-1}.

Statistical analysis for the comparison of concentrations of usnic acid

The concentrations of UA were measured in the 30 samples from four countries in two continents (Table 1; Fig. 2). After checking the outliers, samples with extremely high values (nr 16 and 23) were omitted from the further analysis. The mean values of 21 European (18 from Hungary and three from Serbia) and nine African (seven from Kenya and two from Tanzania) measurements were compared by a one-way Analysis of Variance (ANOVA) in R environment (software version 3.6.3, R Core Team 2020). Normality of data distribution was checked visually by Q-Q plot (quantiles of the residuals are plotted against the quantiles of the normal distribution with a 45° degree reference line) and by Shapiro-Wilk normality test. Our data followed a normal distribution. A level of $p < 0.05$ was considered for a significant difference.

Abbreviations

HPLC-PDA	high-performance liquid chromatography, including a photodiode array detector;
HPTLC	high performance thin layer chromatography;
LSM	lichen secondary metabolite;
UA	usnic acid;
UHPLC-PDA-MS	ultrahigh performance liquid chromatography-photodiode array detection-mass spectrometry.

RESULTS

We analysed samples collected in populations in Hungary (17), Serbia (three), Kenya (eight) and Tanzania (two) (Table 2). The contents of UA enantiomers were determined using a chiral chromatographic method described earlier and the presence of (+)-UA was confirmed in several of the above specimens. The UA content (mg g^{-1} dry weight) was measured by HPLC-PDA. The content of UA shows a substantial variation in both continents, ranging from 5.08 to 26.43 mg g^{-1} in Europe and from 6.15 to 23.54 mg g^{-1} in Africa.

Identification of LSMs by HPTLC

The occurrence of UA, protocetraric acid and caperatic acid was found in each of the samples from Europe and Africa analysed by HPTLC (Fig. 3).

Identification of LSMs by UHPLC-PDA-MS

The UA content was confirmed also by UHPLC-PDA-MS. The UV spectrum and MS spectrum is presented in Figure 4.

Quantitation of usnic acid

The UA content (mg g^{-1} dry weight) was measured by HPLC-PDA in 28 samples (19 from Europe including 16 from Hungary and three from Serbia; nine from Africa including seven from Kenya and two from Tanzania). The content of UA shows a substantial variation in both continents, ranging from 5.21 to 19.23 mg g^{-1} in Europe and from 6.15 to 23.54 mg g^{-1} in Africa (Table 2).

Quantitation of usnic acid enantiomers

Specimens collected in Hungary (10), Serbia (two) and Kenya (one) were analysed by chiral chromatographic method and the presence of (+)-UA was confirmed. The chiral chromatogram (Fig. 5) shows that only the (+)-UA was produced by the lichenized fungus *Flavoparmelia caperata*

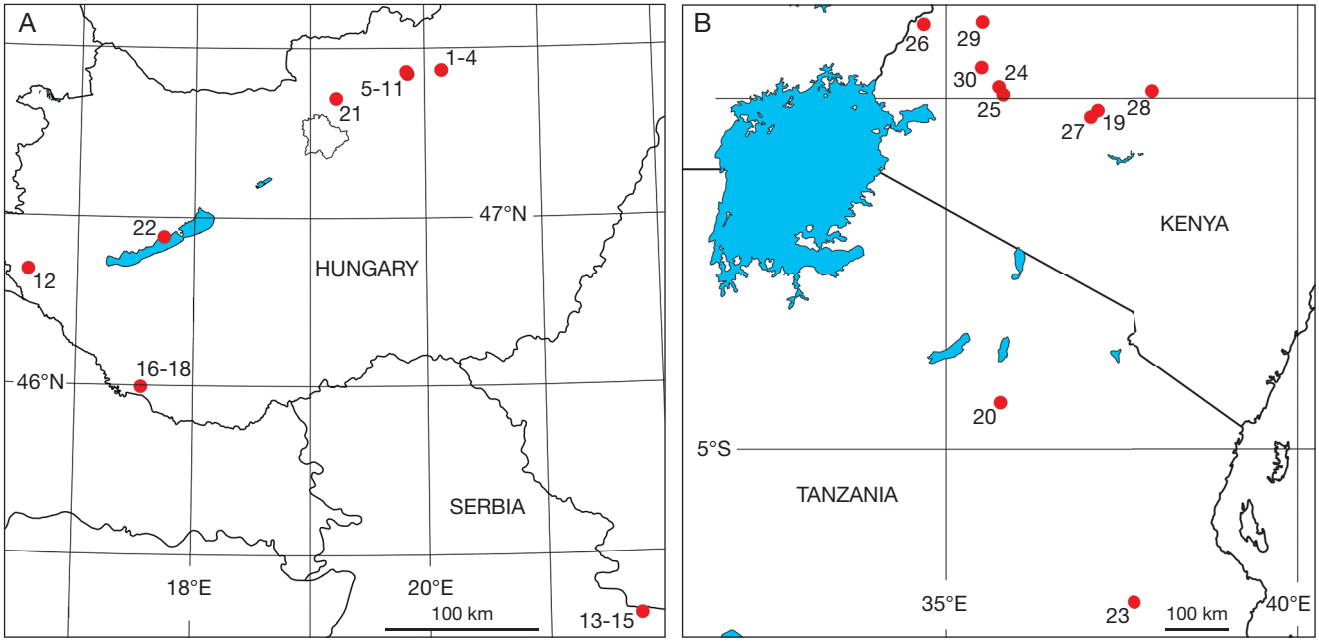

Fig. 2. — Collecting sites of the investigated samples of *Flavoparmelia caperata* (L.) Hale (Table 1): **A**, in Hungary and Serbia; **B**, in Kenya and Tanzania.

in all samples. The (-)-UA was not detected at the current limit of detection (*c.* 18 ng ml^{-1}). In addition, the content of (+)-UA shows a substantial variation, ranging from 5.08 to 26.43 mg g^{-1} lichen dry weight matters, and thus the isomer could account for up to 2.6% lichen dry weight in Europe (Table 2). The single specimen from Africa contained 20.27 mg g^{-1} UA.

Comparison of usnic acid concentrations measured in European and African samples

The values of usnic acid concentration from 28 of the 30 investigated specimens measured by HPLC-PDA were compared by statistical analysis. There was no significant difference ($p = 0.91$) detected in usnic acid concentration between the African (mean = 9.79 ± 2.29) and European (mean = 9.90 ± 2.52) samples (Fig. 6).

DISCUSSION

The occurrence of UA, protocetraric acid and caperatic acid was found in each of the investigated *Flavoparmelia caperata* samples from Europe and Africa analysed by HPTLC (Fig. 3) as expected from the literature data (Swinscow & Krog 1988; Verseghy 1994; Brodo *et al.* 2001; Nash & Elix 2002; Divakar & Upreti 2005; Awasthi 2007; Smith *et al.* 2009; Wirth *et al.* 2013; Brodo 2016). The UV spectrum and MS spectrum (Fig. 4) by UHPLC-PDA-MS also confirmed the UA content.

However, the concentration values remained under data measured in another foliose species (*Nephromopsis nivalis* (L.) Divakar, A.Crespo & Lumbsch, synonym of *Flavocetraria nivalis* (L.) Kärnefelt & A.Thell) (Bjerke *et al.* 2005). Bjerke *et al.* (2005) finding 51.1 to 53 mg g^{-1} UA in Arctic, alpine and Patagonian populations reported also large variations among samples. Though the pharmaceutical application and other effects of usnic acid have been widely studied (Solhaug & Gauslaa 2001; Solhaug *et al.* 2003; Ahmad *et al.* 2017; Luzina & Salakhutdinov 2018; Dieu *et al.* 2020; Muhoro & Farkas 2021; Veres *et al.* 2022a, b, Kulinowska *et al.* 2023), the effective amounts (for UA or crude extracts) are usually given and concentration values for comparison are less available. Kulinowska *et al.* (2023) found 9.12 to 13.28 mg g^{-1} UA in the fruticose *Cladonia uncialis* (L.) Weber ex F.H.Wigg. that is comparable amount with values measured by us.

In their review, Galanty *et al.* (2019) present that while *F. caperata* and several species of genera *Cladonia*, *Ramalina*, *Usnea*, *Xanthoparmelia* are characterized by (+)-UA production, other species of *Cladonia* and *Alectoria* contain (-)-UA exclusively. Furthermore, a mixed content occurs in *Cladonia stellaris* (Opiz) Pouzar & Vězda, *Nephromopsis cucullata* (Bellardi) Divakar, A.Crespo & Lumbsch (synonym of *Flavocetraria cucullata* (Bellardi) Kärnefelt & A.Thell), *Nephromopsis nivalis* and *Vulpicida pinastri* (Scop.) J.-E. Mattsson & M.J.Lai.

They also pointed out that various internal and environmental variables may affect qualitative and quantitative differentiation between individual samples of the same species, including the production of (+)- or (-)-UA. For instance, the genetic variety of the fungal component and the geographical origin of the lichen may also induce differences in the production of the LSMs. Though there are differences in activity between the various enantiomers, it is not clear from the literature reviewed whether one of the two enantiomers is more effective than the other in various respects

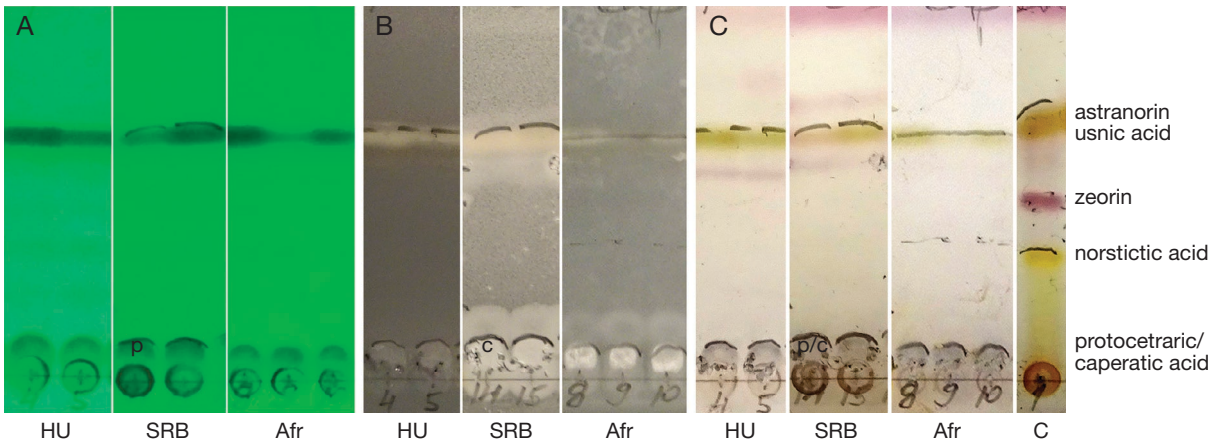

FIG. 3. — Usnic acid, protocetraric acid (**p**) and caperatic acid (**c**) sampled from specimens of *Flavoparmelia caperata* (L.) Hale collected in Europe (**HU**, Hungary; **SRB**, Serbia) and Africa (**Afr**) are presented on developed HPTLC plates (in solvent system C according to Arup *et al.* 1993) with control: **A**, under UV 254 nm; **B**, sprayed with water; **C**, in daylight after spraying with 10% sulphuric acid and charring.

(except for antiviral (Yamamoto *et al.* 1995; Sokolov *et al.* 2012), insecticidal (Emmerich *et al.* 1993) and phytotoxic (Romagni *et al.* 2000) activities). Still it is suggested that not only the lichen species should correctly be identified, but that the specific rotation of UA enantiomers or the ratio of the mixture should also be determined.

In our study carried out by chiral chromatographic method, we were interested in the production of (+)- or (-)-UA. The concentration values obtained by this method (Table 2) were not further analysed for quantitative comparison, as the sample preparation methods were not the same in the various methods used in this study. This resulted in considerable discrepancies between data measured by HPLC-PDA and chiral chromatography for some samples (e.g. nr 7 or 8).

The comparison between continents (Europe and Africa) did not result in significant differences ($p < 0.05$) in the concentrations of UA analysed by HPLC-PDA in our samples originated from these areas. Thus that means that our hypothesis formulated in the aims could not be verified. Consequently, the concentration of the produced UA can practically be regarded as having the same range (between the minimum of 5.08 mg g^{-1} and maximum of 26.43 mg g^{-1}) over the investigated natural habitats where the lichen *F. caperata* is able to develop. As UA is a cortical pigment that protects against UV and solar radiation (Solhaug *et al.* 2003; Veres *et al.* 2022a), it can be concluded that the amount of radiation might reach similar levels in the colonised habitats (both for corticolous or saxicolous ones). Furthermore, this result suggests that the macroclimatic differences (Moreau 1938; van Zinderen Bakker 1962) might be less important in this respect and the role of the microclimatic conditions is predominant (Richards 1952; Ricklefs 2008; Heilmann-Clausen *et al.* 2014), as it has long been studied (Scaëtta 1933; Gieger 1957). Bearing in mind that the species is generally found at lower altitudes (below 2000 m a.s.l.) in the temperate region (Verseghy 1994; Smith *et al.* 2009; Wirth *et al.* 2013), than in the tropics (*c.* 1500-3600 m) (Swinscow & Krog 1988), it can be assumed that the habitats at these different elevations are probably characterised by similar climatic and especially microclimatic conditions on both continents and represent the specific niche requirements of *F. caperata*. Under these circumstances the similar UA content of the lichen thalli in these environments is not surprising. However, more detailed studies might reveal further important details about the conditions (e.g. microclimate, elevation, seasonality, genetic background) that modify the quantities of UA produced.

CONCLUSION

Although no significant differences were found in the populations of the geographical areas investigated with regard to the concentration of UA, there were populations and specimens with high amounts of UA in each country and continent. Therefore, if a study on bioactivity of locally available lichen material is to be carried out, the species studied here can be selected for further research in different geographical regions. Given that the chiral nature of the UA found in the studied species has been confirmed here, as (+)-UA, further study with an increased dataset applying HPLC-PDA may clarify other details on production conditions and extraction efficiency.

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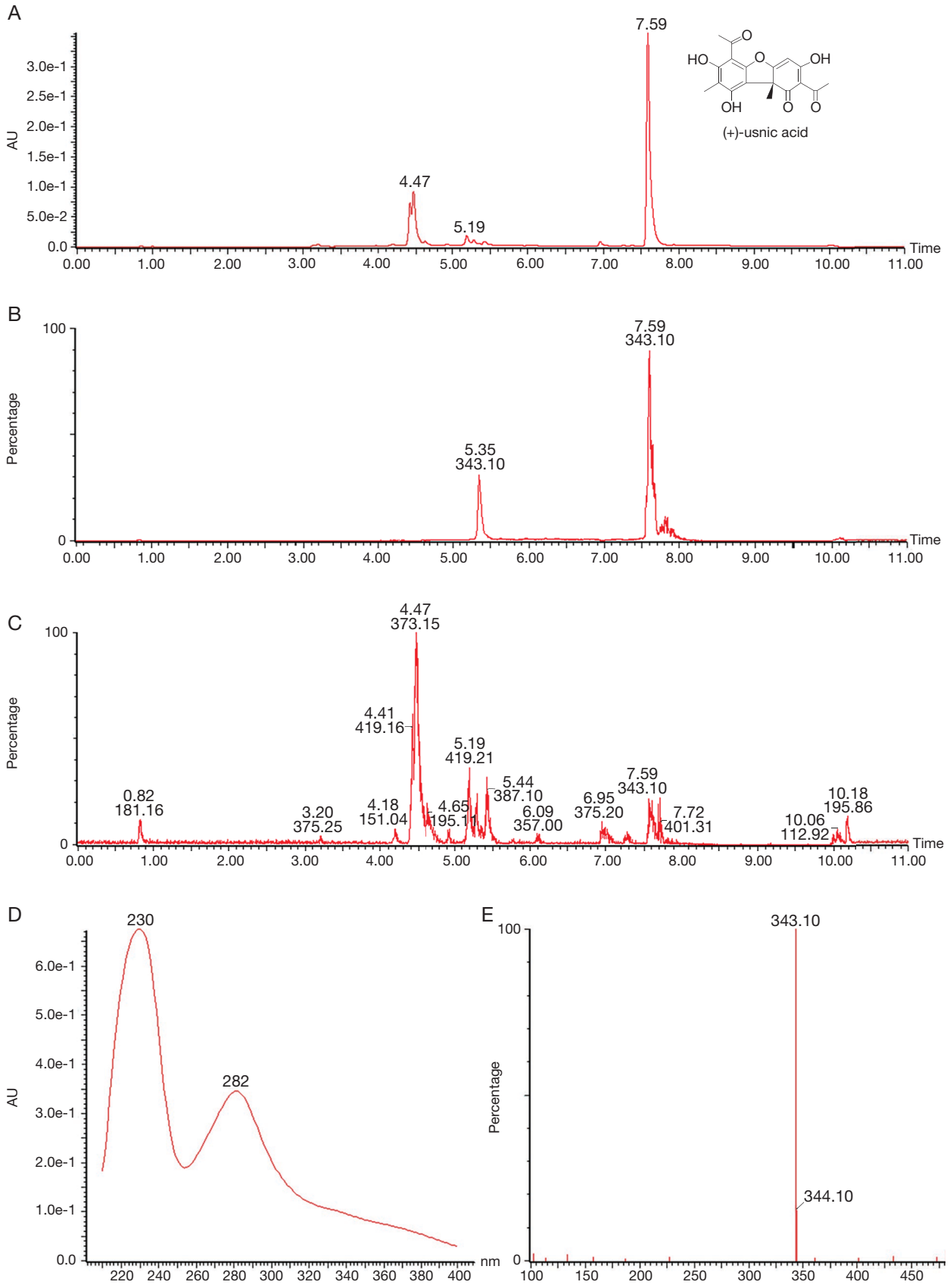

FIG. 4. — UPLC-PDA-MS analysis of acetone extract of *Flavoparmelia caperata* (L.) Hale: **A**, chromatogram showing peaks detected at 280 nm, where (+)-usnic acid eluting at 7.59 minutes; **B**, extraction ion chromatogram of the ion at m/z 343.10 detection in negative ion mode, corresponding to deprotonated usnic acid molecular ion; **C**, total ion chromatogram; **D**, UV spectrum of usnic acid; **E**, mass spectrum of usnic acid.

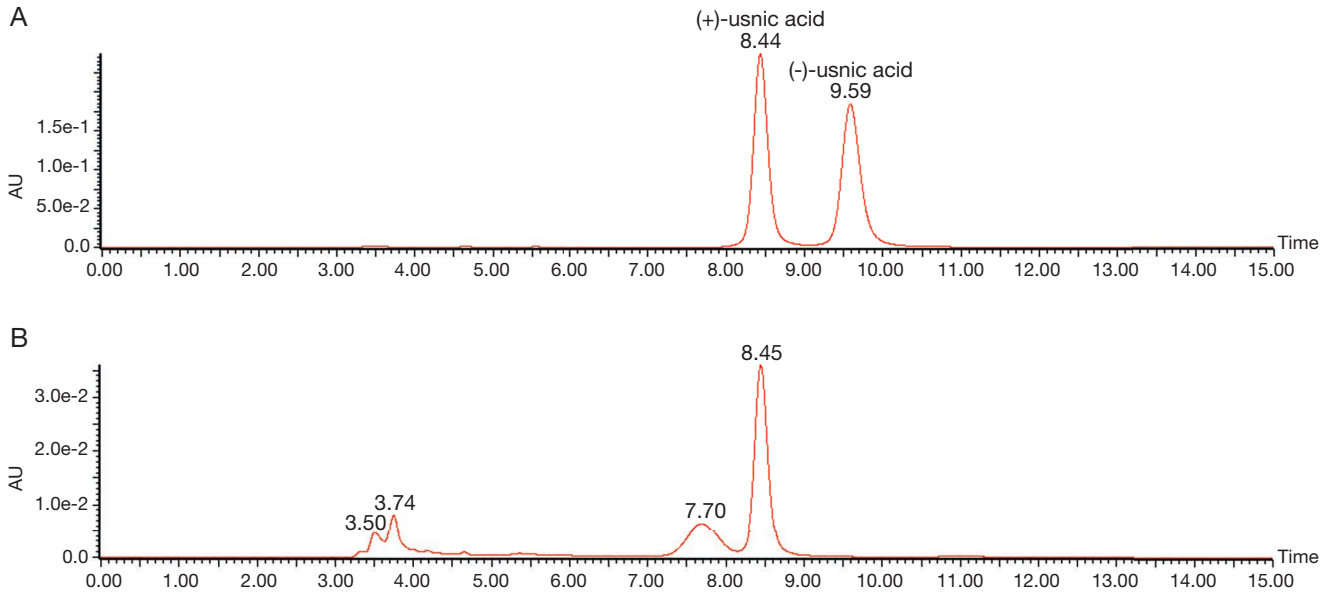

FIG. 5. — Usnic acid enantiomers in the lichen *Flavoparmelia caperata* (L.) Hale: **A**, the chiral HPLC chromatogram showing separation of usnic acid enantiomer standards: (+)-usnic acid eluting at 8.44 min and (-)-usnic acid eluting at 9.59 min; **B**, the chromatogram showing only (+)-usnic acid present in *F. caperata*.

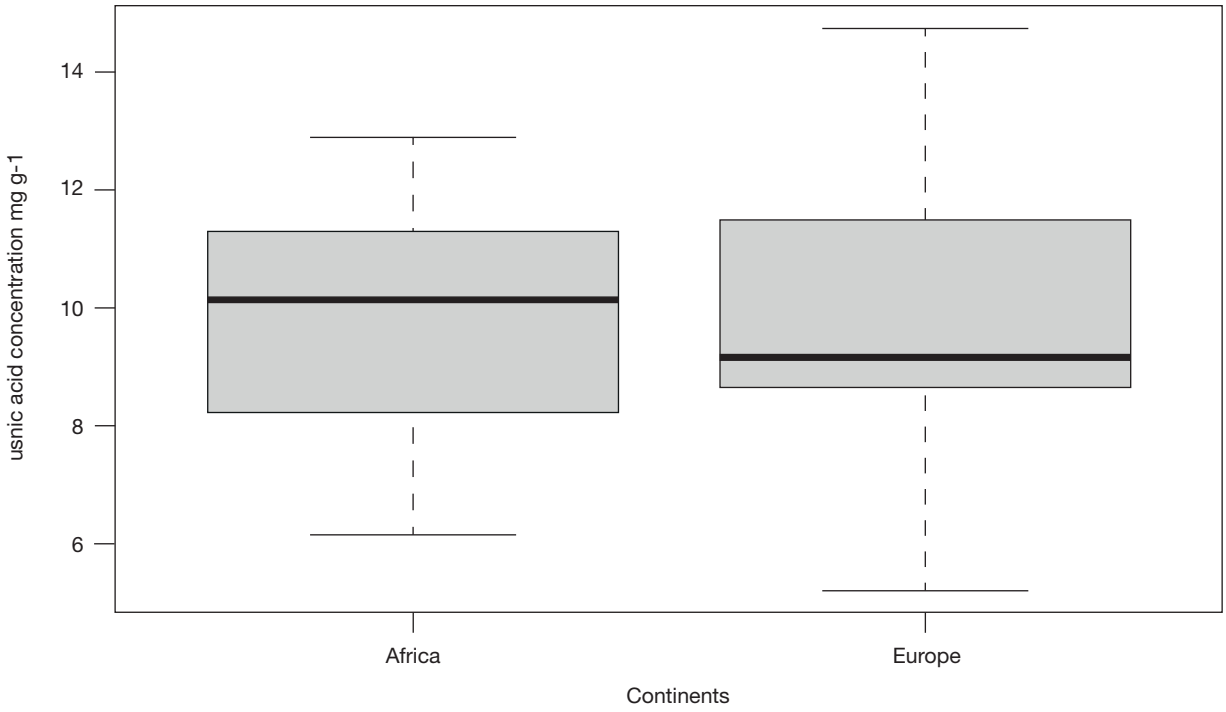

FIG. 6. — Usnic acid concentrations (mg g^{-1} dry weight) in the lichen *Flavoparmelia caperata* (L.) Hale measured in Europe and Africa by HPLC-PDA. The lines represent the minimum and maximum values, the box represents the 25 and 75% of the data, the thick line represents the median. Continents are not significantly different at 95% confidence.

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