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Short communication

Quantitative determination of protolichesterinic- and fumarprotocetraric acids in *Cetraria islandica* by high-performance liquid chromatography

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Abstract

A quantitative reversed-phase HPLC method for the analysis of protolichesterinic- and fumarprotocetraric acids in *Cetraria islandica* (L.) Ach. is described. The compounds were extracted from dry plant material with acetone. HPLC analysis was performed using a LiChrosorb RP-8 column, isocratic elution and UV detection. The method was applied to the quantification of protolichesterinic- and fumarprotocetraric acids in plant material collected in various locations within Iceland. Results showed protolichesterinic acid content of *Cetraria islandica* to vary between 0.1–0.5% dry weight and fumarprotocetraric acid content to vary between 2.6–11.5% depending on collection site.

Keywords: *Cetraria islandica*; Protolichesterinic acid; Fumarprotocetraric acid

1. Introduction

Protolichesterinic acid, an α -methylene- γ -lactone (Fig. 1), and fumarprotocetraric acid, a β -orcinol depsidone (Fig. 1), are considered to be the major biologically active secondary metabolites in the lichen *Cetraria islandica* (L.) Ach., otherwise known as Iceland moss [1].

Antibacterial activity has been attributed to protolichesterinic acid [2] as well as to fumarprotocetraric acid [3]. Protolichesterinic acid has furthermore been found to exhibit in vitro and in vivo activity in a number of biological assays [4–6].

In light of the current use of Iceland moss as a herbal remedy and the enhanced quality requirements widely being implemented in Europe for raw materi-

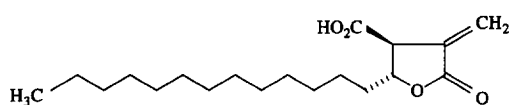
als used for the manufacturing of herbal products [7], a sensitive method for the quantitative determination of protolichesterinic- and fumarprotocetraric acids in *Cetraria islandica* was developed using reversed-phase high-performance liquid chromatography (HPLC) with isocratic elution and ultraviolet detection. The method was subsequently used for determining the concentration of these compounds in lichen samples collected at different sites within Iceland.

2. Experimental

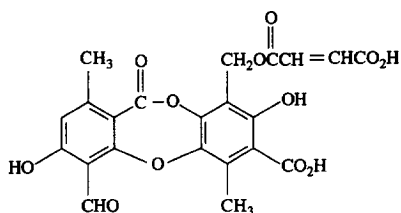
2.1. Plant material

Samples of *Cetraria islandica* (L.) Ach. were collected at the following locations in Iceland.

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A



B

Fig. 1. Chemical structures of (A) protolichesterinic acid and (B) fumarprotocetraric acid.

Northwest Iceland: Fljot (samples No. 1–4), Grims-tunguheiði (No. 5), Blönduos (No. 6), Audkuluheiði (No. 7). *Northeast Iceland:* Flateyjardalsheiði (No. 8), Bardardalur (No. 9), Fljotsheiði (No. 10), Mel-rakkasletta (No. 11). *East Iceland:* Jökuldalsheiði (No. 12 and No. 13). *South Iceland:* Keldnaholt (No. 14), Hellisheiði (No. 15). *West Iceland:* Kaldidalur (No. 16), Klambrafell (No. 17). The samples were dried at room temperature and foreign matter was removed prior to grinding. Voucher specimens are deposited at the Department of Pharmacy, University of Iceland.

2.2. Standards and solvents

Methanol and acetonitrile were of HPLC grade from Rathburn (Walkerburn, UK). Acetone and ortho-phosphoric acid were analytical-reagent grade from Merck, Darmstadt, Germany. As protolichesterinic acid and fumarprotocetraric acid are not commercially available, standard material was used that had been isolated in our laboratory from *Cetraria islandica*, recrystallised and identified by TLC, melting point, optical rotation, IR, UV and NMR spectra. ^1H NMR and ^{13}C NMR spectra of protolichesterinic- and fumarprotocetraric acids were recorded respectively in CDCl_3 and $\text{DMSO}-d_6$ on a Bruker AC 250 instrument (^1H : 250.1 MHz, ^{13}C :

62.9 MHz). Spectral data were comparable to those reported earlier [5,8].

2.3. Apparatus and operating conditions

HPLC analyses were performed using a Hewlett-Packard 1050 chromatograph series consisting of HP 79851A isocratic pump, HP 79853C UV variable-wavelength detector and HP 3395A integrator. The column used was a Hibar LiChrosorb RP-8, 250×4 mm I.D., 10 μm , from Merck, Darmstadt, Germany. The mobile phase consisted of 90% acetonitrile in water containing 1% 0.03 M ortho-phosphoric acid. Injection volume was 20 μl , flow-rate 1.0 ml/min and column temperature was set at 28°C. The wavelength used was 210 nm.

2.4. Standard solutions

A 5.0-mg amount of protolichesterinic acid was weighed and dissolved in 5.0 ml of methanol. A volume of this stock solution was diluted with mobile phase before injection to the HPLC system. Because of poor solubility of fumarprotocetraric acid the maximum obtainable concentration of the standard was 1.0 mg/l of the mobile phase.

2.5. Method validation

Calibration curves for protolichesterinic acid and fumarprotocetraric acid were obtained with six samples of various concentrations using linear regression analysis. Each sample was measured in duplicate. Precision was determined according to Ref. [9]. Recoveries were determined by adding known amounts of protolichesterinic acid and fumarprotocetraric acid to the lichen samples at the beginning of the extraction.

2.6. Sample preparation

Samples weighing 0.5 g of dried and pulverized lichen material were extracted with acetone in a Soxhlet apparatus for 6 h. The extracts were evaporated to dryness in vacuo and the residue dissolved in 200.0 ml of mobile phase. For analysis of protolichesterinic acid 1.0 ml of this solution was diluted with the mobile phase to 25.0 ml but for

analysis of fumarprotocetraric acid, 0.5 ml was diluted to 100.0 ml with the mobile phase. Solutions were passed through 0.45- μ m filters and injected into the HPLC system in amounts of 20 μ l.

3. Results and discussion

Quantitative analysis of protolichesterinic acid and fumarprotocetraric acid in *Cetraria islandica* was achieved using reversed-phase high-performance liquid chromatography. The plant material was exhaustively extracted with acetone prior to analysis. Due to the poor solubility of fumarprotocetraric acid and the large concentration difference between the two constituents, separate dilutions of the extract had to be made for the quantification of the two constituents. Representative chromatograms are shown in Fig. 2. The retention time for protolichesterinic acid was 3.7 min and for fumarprotocetraric acid 2.3 min.

Over the selected concentration range of 0.125–2.5 μ g/ml for protolichesterinic acid and 0.220–1.1 μ g/ml for fumarprotocetraric acid, the calibration curves were linear. The correlation coefficient for

protolichesterinic acid was 1.0000 and for fumarprotocetraric acid 0.9965.

The repeatability (relative standard deviation) of the method, on the basis of peak-area ratios for six replicate injections was 0.78%. The detection limit was 1 ng. The average recoveries of the compounds from spiked samples were 90% for protolichesterinic acid and 96% for fumarprotocetraric acid.

The method was used to quantify protolichesterinic- and fumarprotocetraric acids in plant material collected from 17 localities in Iceland. Results (Table 1) show that protolichesterinic acid content of *Cetraria islandica* in these samples ranges from 1.4 to 5.3 mg/g plant material. The content of fumarprotocetraric acid ranges from 26.3 to 115.0 mg/g. In both cases a 4–5 fold range is observed between lowest and highest values.

HPLC analysis of lichen extracts and pure constituents was first reported in 1972 [10]. Since then several publications have appeared describing HPLC conditions for qualitative identification of secondary lichen metabolites, often for chemotaxonomic purposes [11,12]. Protolichesterinic acid content of *Cetraria islandica* has not previously been quantitatively determined by HPLC. In previous determinations of fumarprotocetraric acid in *Cetraria islandica* a mean value of 3.4% was obtained for samples collected in Germany and 5.4% for samples collected in Finland [13]. Plant material collected from 31 locations in Finland and the northern part of Norway had a mean fumarprotocetraric acid content of 5.7% [14]. The mean value observed in the present study (7.0%) therefore does not differ greatly from that reported in lichen samples from Scandinavia.

The results do not allow correlation to be made between content of secondary constituents and geographic location at which the plants were harvested.

Due to climatic reasons, it is only realistic to collect lichens in Iceland during the months of June, July and August. In order to have an indication of whether the month of collection affects concentration of constituents, we randomly selected plant material from location No. 15 to study in this respect. No significant difference was observed in the concentration of either constituent over the 3 month period.

Although the sample size investigated in this study was limited, it is obvious that both major secondary constituents of *Cetraria islandica* can vary con-

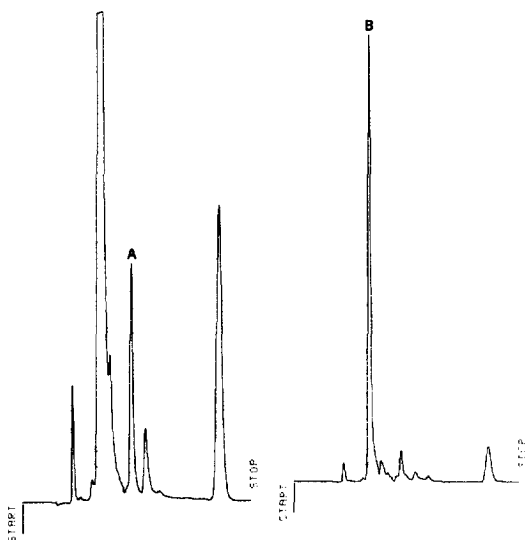


Fig. 2. Analysis of (A) protolichesterinic acid ($t_R=3.7$ min) and (B) fumarprotocetraric acid ($t_R=2.3$ min) from *Cetraria islandica* by HPLC on a Hibar LiChrosorb RP-8 column (250 \times 4 mm I.D.) with 90% acetonitrile in water containing 1% 0.03 M *o*-phosphoric acid and a flow rate of 1.0 ml/min, UV detection at 210 nm.

Table 1

Quantitative HPLC analysis of protolichesterinic- and fumarprotocetraric acids (mg/g dry plant material) in *Cetraria islandica* from different localities in Iceland^a

Region and sample number	Protolichesterinic acid	Fumarprotocetraric acid
<i>Northwest Iceland</i>		
1	4.3	68.8
2	4.0	68.1
3	4.2	72.4
4	4.2	66.4
5	2.9	40.8
6	3.0	66.9
7	2.6	55.0
<i>Northeast Iceland</i>		
8	3.7	100.2
9	2.5	26.3
10	1.8	49.4
11	2.6	55.2
<i>East Iceland</i>		
12	1.9	105.7
13	1.4	33.3
<i>South Iceland</i>		
14	5.3	115.0
15	4.1	81.4
<i>West Iceland</i>		
16	5.3	79.5
17	4.1	98.7

For HPLC conditions and collection sites, see Section 2.

^a R.S.D. <0.1%.

siderably with regard to concentration. The method presented enables accurate determination of these compounds in dry plant material.

Acknowledgments

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