Development and validation of a reliable LC–MS/MS method for quantitative analysis of usnic acid in Cladonia uncialis

Natalia Sveshnikova, Tao Yuan, Jamie M. Warren and Michele D. Piercey-Normore*

Abstract

Objective: The purpose of this study was to develop and validate a specific and sensitive liquid chromatography tandem mass-spectrometry method for quantification of usnic acid concentration in the lichen, Cladonia uncialis, suitable for detection of relatively small fluctuations of usnic acid concentration in response to environmental changes.

Results: The resulting method was fully validated according to international guidelines and demonstrated good selectivity and sensitivity with minor levels of a matrix effect and high accuracy.

Keywords: Liquid chromatography tandem mass-spectrometry, LC–MS/MS, Secondary metabolites, Lichen, Usnic acid, Cladonia uncialis

Introduction

Usnic acid (UA) is one of the most common and most studied lichen-specific secondary metabolites and is taxonomically widely distributed in species of Cladonia, Usnea, Lecanora, Ramalina, Evernia, members of the Parmeliaceae, and other lichen genera [1]. Isolated in 1844 [2] and studied ever since, it is still of interest in industry for its antibacterial, antiviral, antioxidant, anti-inflammatory, analgesic and even anticancer properties [1, 3–5]. The biological role of UA in lichens is considered to be species-specific [6], and may include UV protection for the photobiont [7], from herbivores [8, 9], and from fungal and bacterial pathogens [10, 11].

Under natural conditions, the accumulation of UA in lichens is thought to depend on environmental factors [7, 12–14] with seasonal variation [15, 16]. Methods of extraction and quantification of UA were reported depending on available equipment, research goals and required accuracy of the method, making it difficult to compare across studies. One of the most reliable modern analytical methods is Liquid Chromatography Tandem-Mass Spectrometry (LC–MS/MS), having advantages of both accurate identification and quantification of the substance in question. The use of LC-UV with MS in recent publications [17, 18] was not sensitive enough for detection of subtle variation in UA, was not validated, and were conducted on species with matrix interferences. The development of a standard method to detect UA may help to elucidate its ecological role. The goal of this study was to establish a method for reliable monitoring of subtle changes in concentration of UA, specifically for Cladonia uncialis (L.) F.H. Wigg. [19–21], from the natural lichen thallus and validation according to Bioanalytical Method Validation (US and EU [22–25]).

Main text

Materials and methods

Chemicals

All reagents used were of analytical or higher grade and purchased from Sigma Aldrich (Merck KGaA) unless otherwise stated. Usnic acid (UA) standard stock solution was prepared by solubilising 1 mg of UA in 1 mL of 100% acetonitrile.

Sample preparation

50 mg of the top 10 mm of dry Cladonia uncialis thallus was crushed with mortar and pestle, soaked in 10 mL
of 100% acetonitrile, vortexed for 30 s and agitated at 150 rpm on the shaker (LSE Orbital Shaker, Corning™ LSE™) for 20 min at room temperature. This extraction was repeated four times with the same material to obtain residual UA. All extracts were pooled into one glass tube, and the combined volume was adjusted to 50 mL with 100% acetonitrile.

For the control, an extract from Cladonia ochrochlora (a non-UA-producing species) was prepared in the same manner to emulate the matrix effect.

**LC–MS/MS procedure**

LTQ XL™ Linear Ion Trap Mass Spectrometer (Thermo Scientific™) was used to conduct mass spectroscopy. Filtered (Whatman® Mini-UniPrep® G2, PTFE membrane, pore size 0.2 μm) samples were injected in a volume of 10 μL into a C8 LC column (Phenomenex, Luna® 3 μm C8(2) 100 Å, LC Column 100 × 2 mm) and separated by UltiMate™ 3000 RSLCnano System. Chromatographic separation was performed at a flow rate of 0.2 mL/min using a gradient elution program, starting from 80% of eluent A (water with 0.1% formic acid, v/v) and gradually changing to 5% A and back over 40 min. Exact gradient parameters: 80% of eluent A/20% B (100% acetonitrile acidified with 0.1% formic acid (v/v)) for 5 min, gradual changing to 5% A/95% B over 23 min, 5% A/95% B for 5 min, return to 80% of eluent A/20% B over 7 min.

Mass spectrometry measurements were performed on an LTQ OrbiTrap XL MS (Thermo Fisher). Samples were introduced to MS via electrospray ionisation using the following conditions: sheath gas flow rate, 30 (arbitrary units); auxiliary gas, 5 (arbitrary units); ESI voltage, 4.0 (kV); capillary voltage, −35 (V); capillary temperature, 275 (°C); and tube lens voltage, −110 (V). The collected spectra were scanned over the mass/charge number (m/z) range of 155–2000 atomic mass units (Xcalibur version 4.0). MS spectra were generated by collision-induced dissociation of the metabolite ions at normalized collision energy of 35%.

**Method validation**

The LC–MS/MS method was validated with respect to the specificity, linearity and sensitivity, precision and accuracy, matrix effects and recovery.

**Specificity**

The Specificity test was conducted by comparing chromatograms of 6 matrix blanks (C. ochrochlora extracts without UA) with a blank spike (UA in 100% acetonitrile) and a matrix spike (C. ochrochlora extracts spiked with UA).

**Linearity and sensitivity**

Two types of calibration standards were used for assessment of linearity and sensitivity of the method: different concentrations of UA in a solvent (acetonitrile) only as blank standards, and the same concentrations of UA in a matrix solution (C. ochrochlora extracts) as matrix standards. The final calibration curves included three replicates per calibration point, and linearity was assessed by linear regression.

The calibration range was narrowed down from a broader initial diapason (chosen based on existing literature) by visual observations of 10 analytical runs. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated using the formulas recommended by the guidelines mentioned above:

\[
\text{LOD} = 3.3 \times SD \\
\text{LOQ} = 10 \times SD
\]

where SD is the standard deviation of the signal at the lowest point of the calibration curve.

**Accuracy and precision**

The intra- and inter-day accuracy and precision measurements were conducted using measurements of three concentrations of UA (within the calibration range) dissolved in a matrix (extract) on a single assay, repeated (with triplicates) three times within 1 week.

**Matrix effect**

The Matrix effect was determined by comparison of the retention time (Rt) and the level of MS signal of the representative blank matrix spiked with a predetermined amount of UA with those obtained for the corresponding amount of UA in the solvent (100% acetonitrile).

To assure the matrix match between C. uncialis and C. ochrochlora, a similar comparison was made using both matrix samples spiked with the same amount of UA. Samples used for measurements contained 20 μL of final extract per mL of acetonitrile.

**Recovery**

The recovery was determined by comparing MS response level of spiked samples pre- and post-extraction, according to SANCO guide, using the average result of 4 replicates. The recovery percentage was calculated by dividing the value for the MS response of spiked pre-extracted sample by that of post extracted sample.

**Results and discussion**

Negative electrospray ionization mass spectrometry has been used for analysis of UA for some time [26,
To test if signal strength was affected in samples of any deviations in peak shape from that of the standard. C. uncialis The un-spiked extract of did not demonstrate chlora extracts without UA) with samples and spiked C. ochro- Specificity, linearity and sensitivity The concentration of the UA sample extracted, using the described procedure with 50 mL final volume of the extract, is expected to be recovered within the quantitative linear range since concentrations of UA in Cladonia species under natural conditions vary between 0.4 and 3.8 in dry weight percentages, corresponding to 80–760 ng/mL in our experimental conditions [17, 30–32]. In the case of the UA concentration exceeding the suggested limits, an appropriate dilution was used. Accuracy and precision Precision was expressed as Coefficient of Variation (CV) and accuracy was expressed as Relative Error (RE), and were evaluated for three concentrations of UA within the linearity range: 50 ng/mL, 200 ng/mL and 500 ng/mL. The resulting fluctuations did not exceed 7% for Intra-assays and 11% for Inter-assays for CV, and 7% for RE calculations in both type of measurements (Table 1).

Although deviations for accuracy and precision were within limits recommended by most of the guidelines (IUPAC, FDA and SANCO, where 15–20% is given as an acceptable level of variation), it is recommended that the calibration samples be included in every sequence in future experiments with re-evaluation of calibration graphs to negate the natural instability of signal in MS.
Fig. 1 Mass spectra fragmentation pattern of UA mixed in or present in a acetonitrile, b C. ochrochlora matrix, c C. uncialis samples
Stability

The matrix solution (extract of *C. ochrochlora*) spiked with UA at the concentration of 400 ng/mL, was kept at room temperature in darkness for 1 month and assessed five times during that period. Inter-assay RE was 2.31%, which is an acceptable level of deviation, demonstrating stability of UA under experimental conditions in this study.

Conclusion

The method suggested in the present article proves to be suitable for accurate measurements of UA concentration in dried field samples of *Cladonia uncialis* under the conditions in this study: sample preparation and storage, maintaining concentration of UA in the extracts between 50 and 500 ng/mL, and regular adjustments of calibration.

Limitations

The method presented in this paper was performed in a single laboratory and the validation was performed on a single species.

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Authors’ contributions

All authors have made substantial contributions to the study. NS and MPN developed the idea for the experiment; NS, TY and JW designed and conducted the experiment; NS, TY, JW and MPN interpreted the data; NS and MPN drafted the manuscript; TY and JW reviewed the manuscript drafts. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article or can be obtained upon request to NS.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The manuscript includes no individual person’s data requiring consent for publication.

Competing interests

The authors declare they have no competing interests.

Author details

1 School of Science and the Environment, Grenfell Campus, Memorial University of NL, 20 University Drive, Corner Brook, NL A2H 5G4, USA. 2 School of Science and the Environment, Boreal Ecosystem Research Facility, Grenfell Campus, Memorial University, 20 University Dr, Corner Brook, NL A2H 5G4, USA.

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