Optimization of usnic acid extraction conditions using fractional factorial design

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Abstract
Usnic acid is a unique lichen metabolite of industrial importance, widely studied to explore its pharmacological potential and valued especially as an antibacterial agent in cosmetics. Although a vast number of papers describe usnic acid extraction from various lichen species, none has so far provided an unequivocal indication of the best extraction procedure for this compound. Thus, the current study was focused on the direct comparison of three commonly used usnic acid extraction methods (heat reflux, shaking, ultrasound-assisted extractions), which were optimized using fractional factorial design. Heat reflux extraction, shaking extraction and ultrasound-assisted extraction were first optimized in a series of experiments using fractional factorial design, with respect to three parameters: the extraction time, the solvent used and the number of extraction repetitions. HPLC was employed for usnic acid quantitative analysis. The best scores for each extraction method were statistically compared and the optimal conditions were indicated. The optimal set of parameters for usnic acid was established to be a single, 60 min heat reflux extraction with acetone. This extraction scheme provided 4.23 ± 0.08 mg g⁻¹ d.w. of usnic acid, while for ultrasound-assisted and shaking extractions the amount was two- or even four times lower (2.33 ± 0.17 and 0.97 ± 0.08 mg g⁻¹ d.w., respectively). The optimal procedure for usnic acid extraction described here may be suitable for effective acquisition of this compound for scientific research purposes, but also for applications in the pharmaceutical or cosmetic industries.

Key words: extraction, fractional factorial design, lichen, optimization, usnic acid

Introduction
Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione) is a structurally unique, yellow, lipophilic metabolite found selectively in lichens. The biological and pharmacological properties of usnic acid are well documented and comprise antibacterial, antiviral, anti-inflammatory, analgesic, antiproteozal or insecticidal activities (Galanty et al. 2019). The compound has been particularly studied for its anti-cancer potential and the results seem to be very promising, with a marked activity reported both in vitro and in vivo (Araújo et al. 2015). Moreover, usnic acid has been extensively used by the cosmetic industry, as an antiperspirant or an additive in toothpastes (Ingolfsdottir 2002). Some recent reports also indicate its photoprotective (Varol et al. 2015) and wound healing (Pagano et al. 2019) properties, when applied externally. Taken together, these attributes of usnic acid, its wide distribution in nature in significant amounts of up to 5% (Galanty et al. 2019), and an easy isolation procedure (Ingolfsdottir et al. 1998; Lohezic Le Devehat et al. 2007; Galanty et al. 2017), make this lichen metabolite an attractive subject of research.

Even though there are many reports describing the extraction of usnic acid from natural sources by different methods (König & Wright 1999; Yilmaz et al. 2004; Roach et al. 2006; Burlando et al. 2009; Einarsdottir et al. 2010; Honda et al. 2010; Brovko et al. 2017; Piska et al. 2018), they do not provide reliable information on the most effective conditions. This discrepancy and the incompleteness of data concerning the extraction parameters for usnic acid have motivated us to explore this issue in more detail.

Fractional factorial designs are important statistical tools which efficiently allow the influence of several relevant factors to be controlled and varied simultaneously on a planned experiment, in order to obtain the assumed goal in the most effective way. These tools are widely used in monitoring experiments in different research fields, as they can significantly shorten the total time of the experiment, as well as indicate the most effective procedure (Gunst & Mason 2009). Fractional factorial experiments have so far rarely been used for the optimization of lichen metabolite extraction, with the exception of lepraric acid, erythrin (Parrot et al. 2015) or diploicine, norstictic and variolaric acids (Bonny et al. 2009).

Thus, the aim of the present study was to optimize the extraction conditions for usnic acid using experiments with a fractional factorial design in order to investigate the influence of a number of parameters on the efficacy of the particular extraction method.

For this purpose, we chose Cladonia arbuscula (Wallr.) Flot. as a lichen material containing usnic acid and compared different types of extractions: heat reflux extraction in a hot water bath,
dynamic shaking extraction and ultrasound-assisted extraction. For each type of extraction three parameters were selected for comparative purposes: the time of extraction, the solvent used and the number of extraction repetitions. Then, the influence of these parameters was investigated and the most effective conditions for each method were compared with each other to establish the optimal recovery of usnic acid from the lichen.

**Material and Methods**

**Lichen material, chemicals and instrumentation**

*Cladonia arbuscula* subsp. *squarrosa* (Wallr.) Ruoss was collected in July 2015, from northern Poland in dry, non-coastal Scots pine forests; its identity was verified by one of the authors (AG). The voucher specimen was deposited in the herbarium of the Department of Pharmacognosy JU MC (Ref. no. KFg/2015/L3). All samples used during the analysis were prepared from lichen material collected from the one location. The lichen material was dried in the dark at room temperature and cleaned to remove impurities. Before analysis, whole lichen thalli (both upper and lower parts) were ground into small pieces in a mortar to provide uniformity and homogeneity of the tested material. Chloroform, acetone, methanol and water of HPLC grade were obtained from Sigma-Aldrich (Germany) and orthophosphoric acid (98%) from POCCh Gliwice (Poland). Usnic acid standard of analytical grade was obtained from Sigma-Aldrich (Germany).

A water bath LWT (WSL Poland) and ultrasonic bath Sonic 3 (Polsonic, Poland) were used for heat reflux and ultrasound-assisted extraction, respectively. For dynamic shaking extraction, a laboratory shaker was used (type 3585; ELPAN, Poland).

Usnic acid quantitative determination was performed using a Dionex high-performance liquid chromatography (HPLC) system, with PDA detector, on a C-18 column (5 μm, 250 × 4.6 mm), at 25 °C. The mobile phase consisted of 1% H₃PO₄ A water bath LWT (WSL Poland) and ultrasonic bath Sonic 3 (Polsonic, Poland) were used for heat reflux and ultrasound-assisted extraction, respectively. For dynamic shaking extraction, a laboratory shaker was used (type 3585; ELPAN, Poland).

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**Sample preparation and extraction**

Aliquots of 0.2 g each of the ground lichen thalli were transferred into glass round-bottomed flasks, tightly closed and kept in darkness until needed for further extraction procedures. For a single extraction, 20 ml of solvent was used.

Heat reflux extraction was conducted on a water bath (90 °C). Dynamic shaking and ultrasonic-assisted extractions were conducted at room temperature. Each combination of the experimental parameters (one of nine) was tested in six replicates. For heat reflux and shaking extractions, three different times were applied, namely 15, 30 and 60 min, while ultrasound-assisted extraction times were 10, 20 and 30 min. Acetone, chloroform and methanol were used as solvents. To investigate the influence of the number of extraction repetitions on its efficacy, a single sample was single-, double- or triple-extracted. This was carried out by treating the sample only once with the solvent (1 × 20 ml) or by repeating the extraction twice (2 × 20 ml) or three times (3 × 20 ml).

After extraction, the obtained extracts were transferred into 10 ml volumetric flasks and the usnic acid content was analyzed by high-performance liquid chromatography (HPLC). Before the analysis, extracts were filtered through the 0.45 μm membrane filters into the HPLC 1.5 ml vials.

**Experimental design and statistical approach**

To optimize the extraction procedure for usnic acid, the three different extraction methods mentioned above were compared. Within each chosen method three parameters were considered: 1) the time of extraction, 2) the solvent used, 3) the number of extraction repetitions from a single sample. In order to investigate the impact of the experimental parameters on the effectiveness of extraction, an experimental planning method was used (Dobrowolska et al. 2016). This was achieved by using the fractional factorial design of experiments: 33-1 in which 1/3 of the full 33 design was selected, with 9 different combinations of the chosen factors (parameters), resulting in 9 trials. The scheme of factor coding and the plan of the experiment with the original parameters and their levels are shown in Table 1. We purposefully used different numbers of extraction repetitions from a single sample (1, 2, or 3), treating it as one (potentially) significant factor (i.e., independent parameter on three levels) and according to the plan of the experiment. Similarly, we tested three levels of extraction time, using slightly milder conditions (shorter time periods), for ultrasound-assisted extraction because of the risk of degradation of usnic acid, but the essential scheme of the experiment was always preserved. The amount of usnic acid in each of the tested samples was calculated as mg per gram dry weight (mg g⁻¹ d.w.) of the lichen material used, and then the best factorial set was selected for each extraction method.

First, the outlying values of usnic acid concentration, if they appeared in a particular factor (parameter) combination, were removed as outliers. For this purpose, we used the software ‘Extreme Outlier’ (MP System Co., Chrzanów, Poland), with an algorithm implemented by Shoemaker and based on a robust technique proposed by Tukey (Shoemaker 2018). Altogether we found 10 outliers out of 162 results (6%). Then, the results obtained for each type of extraction were compared using an ANOVA test with a Tukey post-hoc test (using the package STATISTICA v.13; StatSoft, Tulsa, USA) to identify the best (i.e. biggest) two extraction results. The same procedure was applied to ultimately identify the best type of extraction and the best combination of parameters from the six results (two best scores for each tested extraction type) previously obtained. Differences with P < 0.05 were considered to be statistically significant.

**Results and Discussion**

Quantitative analyses of usnic acid content in various lichen species have been performed using several different extraction methods. The results of these studies are difficult to compare because the analytical conditions vary substantially, often depending on the research goal, and giving inaccurate information on the real content of usnic acid in the given lichen material. Thus, the aim of our study was to explore what would be the most effective, optimal extraction conditions for obtaining usnic acid for its further use in pharmacological studies. For this purpose, we compared three different extraction methods, and within each of these methods the influence of the selected three parameters on the extraction efficacy was investigated. A fractional factorial design was applied for the first time to study usnic acid extraction optimization. The results obtained are shown in Table 2.
For both tested conventional extractions (heat reflux, shaking) the same set provided the best scores, that is 60 minutes acetone extraction, but the results for heat reflux extraction were four times higher (4.25 ±0.08 mg g⁻¹ d.w.) than shaking extraction (0.97 ±0.08 mg g⁻¹ d.w.). Such a large variation is probably a consequence of the different temperature conditions for both extractions. This is an additional important observation, emphasizing the influence of temperature on usnic acid extraction. The highest amount of extracted usnic acid was observed for the sets with a single extraction, which means that the samples obtained by different numbers of repetitions did not differ statistically. This result was surprising as it is a common practice, in many pharmaceutical or ecological studies, to carry out two or three consecutive repetitions of the extraction process.

For ultrasound-assisted extraction the best score was achieved for the set with two repetitions of extraction, 20 minutes each, with acetone as a solvent (2.33 ±0.17 mg g⁻¹ d.w.); however, the effect did not differ significantly from the set with a single, 10 minute extraction using the same solvent (2.27 ±0.13 mg g⁻¹ d.w.). Notably, the amount of usnic acid obtained by ultrasound-assisted extraction was about two times lower (2.33 ±0.17 mg g⁻¹ d.w.) when compared to heat reflux extraction (4.25 ±0.08 mg g⁻¹ d.w.), P < 0.05. The two highest results of each method were finally compared, proving that the optimal conditions were for heat reflux extraction with one repetition and acetone as the solvent (P < 0.05).

The methods so far commonly used by various authors for usnic acid extraction have been mainly conventional techniques, with heat reflux (König & Wright 1999; Piska et al. 2018), Soxhlet (Einarsdottir et al. 2010; Honda et al. 2010) or shaking (Yilmaz et al. 2004; Roach et al. 2006) extractions, while a green technology approach was represented by ultrasound-assisted (Burlando et al. 2009; Brovko et al. 2017) or supercritical fluid (Brovko et al. 2017) extractions. It is interesting to note that these methods are most often used when analytical aspects of usnic acid or its pharmacological activity are of interest, while in the majority of ecologically directed studies, a simple, room-temperature maceration is a common approach (Nybakken et al. 2010; Asplund et al. 2017). As the extraction times applied by different authors varied to a large extent, from several minutes to hours, in our study we opted for 15, 30 and 60 minutes for heat reflux and shaking extractions, and 10, 20 and 30 minutes for ultrasound-assisted extraction. The latter choice was based on

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**Table 1.** Optimization of usnic acid extraction conditions, from *Cladonia arbuscula*, using experiments with a fractional factorial design: parameter values and coded sets of experimental conditions.

| Set number | Time of extraction (min) | Numer of extraction repetitions | Solvent |
|------------|---------------------------|-------------------------------|---------|
|            | parameter value | code | parameter value | code | parameter value | code |
| 1          | HRE, SE: 15 | −1 | 1 | −1 | chloroform | −1 |
| 2          | HRE, SE: 15 | −1 | 2 | 0 | methanol | 0 |
| 3          | HRE, SE: 15 | −1 | 3 | 1 | acetone | 1 |
| 4          | HRE, SE: 30 | 0 | 1 | −1 | methanol | 0 |
| 5          | HRE, SE: 30 | 0 | 2 | 0 | acetone | 1 |
| 6          | HRE, SE: 30 | 0 | 3 | 1 | chloroform | −1 |
| 7          | HRE, SE: 60 | 1 | 1 | −1 | acetone | 1 |
| 8          | HRE, SE: 60 | 1 | 2 | 0 | chloroform | −1 |
| 9          | HRE, SE: 60 | 1 | 3 | 1 | methanol | 0 |

HRE = heat reflux extraction; SE = shaking extraction; UAE = ultrasound assisted extraction

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**Table 2.** Optimization of usnic acid extraction conditions, from *Cladonia arbuscula*, using experiments with a fractional factorial design: scores for all sets of usnic acid extraction parameters (see Table 1 for parameter values and experimental conditions for each set).

| Set number | Usnic acid content (mg g⁻¹ dry weight), mean ± SD |
|------------|-----------------------------------------------|
|            | heat reflux extraction | shaking extraction | ultrasound assisted extraction |
| 1          | 2.37 ±0.10ᵃ | 0.66 ±0.02ᵃ | 1.89 ±0.16ᵃ |
| 2          | 3.23 ±0.05ᵇ | 0.54 ±0.01ᵇ | 1.79 ±0.06ᵇ |
| 3          | 3.37 ±0.22ᵇ | 0.68 ±0.01ᵇ | 2.27 ±0.13ᵇ |
| 4          | 2.53 ±0.17ᵃ | 0.56 ±0.07ᵇ | 1.96 ±1.3ᵃ |
| 5          | 3.69 ±0.04ᵈ | 0.59 ±0.02ᵇ | 2.33 ±0.17ᵇ |
| 6          | 3.47 ±0.08ᵇ | 0.43 ±0.01ᵇ | 1.83 ±0.14ᵇ |
| 7          | 4.25 ±0.08ᵇ | 0.97 ±0.08ᵇ | 1.05 ±0.05ᵇ |
| 8          | 4.00 ±0.14ᵈ | 0.66 ±0.02ᵃ | 1.30 ±0.21ᵇ |
| 9          | 3.46 ±0.16ᶜ | 0.82 ±0.02 | 0.62 ±0.10 |

When comparing the results, values with different letters (in superscript) differ significantly for columns only.
our previous preliminary investigation, indicating that expression to
sonication exceeding 30 minutes results in usnic acid degradation
(data not shown). As far as the solvents are concerned, data from
the literature indicate that hexane, chloroform, acetone or metha-
nol were used most often (Yilmaz et al. 2004; Bomfim et al. 2009;
Behera et al. 2012), even though the lipophilic nature of usnic acid
together with some experimental data do not support the use of
methanol (Stark et al. 1995; Podterob 2008). The results of our
study also confirmed that methanol is not suitable for effective
usnic acid extraction.

The results of our study, indicating a significant advantage of
heat reflux extraction over the other two tested methods, do not
question the reliability of previously published studies on the con-
tent of usnic acid in different lichen species. The discrepancy in
usnic acid levels determined in our study and those obtained by
other authors may result from many factors, including the differ-
ences in preparation of lichen material (degree of grinding) but
also the geographical site of its collection. For example, samples
of pulverized *C. arbuscula* from Finland, extracted by homogen-
ization with acetone at room temperature, were found to contain
2.1% of usnic acid (Nybakken & Julkunen-Titto 2006), while in a
consecutive study by the same authors on ‘pieces of *C. arbuscula*
collected in Norway, extracted with acetone at room temperature
(2 × 20 min), only 0.3% of usnic acid was reported, which is an
order of magnitude lower (Nybakken et al. 2010). Moreover, in
a study by Falk et al. (2008) on different samples of *C. arbuscula*
collected in Alaska, usnic acid levels ranged from 0.29 to 1.1%; the
lichen material was ground in a mortar and extracted by ultrasound-assisted extraction with acetone, followed by 24
hours maceration at room temperature. In our previous unpub-
lished experiments, a direct comparison of usnic acid yield from
pulverized and ground *C. arbuscula* indicated significant differ-
ences with higher yields from the latter (data not shown).

**Conclusions**

With the support of fractional factorial design, we have shown that the optimal approach for the most effective extraction of
usnic acid from lichen material is a single 60 minute heat reflux
method with acetone. This may help substantially in designing
the best procedure for the acquisition of usnic acid from lichen
material, not only for pharmaceutical research purposes but also
for further applications in the cosmetic industry. A simpler, but
experimentally optimized, procedure should also be designed in the
future for the purposes of ecological experiments. The use of
a comparable extraction protocol would undoubtedly help to
evaluate the results obtained by various authors within each
domain of expertise.

However, further studies are needed to examine other factors
that might influence the effectiveness of usnic acid extraction,namely the degree of grinding of lichen thalli or the temperature
during the process.

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