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Standard Paper

Visualizing usnic acid with anisaldehyde reagent

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Abstract

The pale yellowish tint of usnic acid in a lichen thallus itself is a commonly used character in identification keys, particularly in the genus *Cladonia*. Furthermore, the presence of usnic acid is phylogenetically significant in numerous groups of lichens. While the distinctive colour of usnic acid is readily visible when present in high concentrations, it is commonly problematic to discern when in low to moderate concentrations. We explored the use of an anisaldehyde reagent for visualizing usnic acid. Using both usnic acid-containing *Cladonia* samples and pure usnic acid, this reaction quickly yields a bright magenta colour on HPTLC and TLC plates after heating and directly with crude acetone extracts on glass slides heated with a lighter. The same magenta product was observed whether or not the usnic acid was accompanied by barbatic, fumarprotocetraric, psoromic, squamatic or thamnolic acids, each of which alone did not produce any colour with anisaldehyde reagent. However, the merochlorophaeic acids in *C. albonigra* also produced a red reaction. Analysis by high resolution LC-MS of the reaction mixture between anisaldehyde and usnic acid revealed several ions at *m/z* 477.1586 ($[M+H]^+$, $C_{27}H_{25}O_8$) and 463.1385 ($[M+H]^+$, $C_{26}H_{23}O_8$), respectively, consistent with aldol condensation of usnic acid and *p*-anisaldehyde.

Keywords: aldol condensation; *Cladonia*; HPTLC; lichen; spot test; TLC; usnic acid

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Introduction

Two of the most common extracellular cortical metabolites in lichens are usnic acid and atranorin. The functional importance of these substances is well known, for example as protection against UV and microbial infection (Cocchietto *et al.* 2002). Furthermore, those of us who identify lichens to species level frequently attempt to determine the presence of these substances, because they are informative both phylogenetically and for identification.

Determination of the presence of usnic acid is routinely used and is phylogenetically significant in the large and difficult genus *Cladonia* P. Browne (Stenroos *et al.* 2018). Anyone who tries to key out *Cladonia* will often find cases where the colour of the thallus is ambiguous for usnic acid, necessitating thin-layer chromatography (TLC) for a conclusive answer. For example, in Ahti *et al.* (2013) and McCune & Geiser (2023), quite a few steps in the identification keys include couplets similar to: 'Squamules yellowish tinged (usnic acid present)' versus 'Squamules not yellowish tinged (usnic acid lacking)'.

Since usnic acid is a pale yellow pigment, at moderate to high concentrations, a practised eye can easily detect its presence. It varies so much in concentration, however, in relationship to light (BeGora & Fahselt 2001) or temperature (Hamada 1991; Neupane *et al.* 2017), that we all struggle with detecting usnic acid visually. At low concentrations the yellowish tint can be absent or nearly so. A spot test is sometimes useful in these cases: 'KC' consists of a drop of 10% KOH followed by a drop of NaOCl on a lichen fragment. This produces a brief intensification of yellow if usnic acid is present but the results of this test are often ambiguous, especially when usnic acid is in low concentrations or it is accompanied by other substances that have a yellow reaction to the KOH (e.g. stictic acid or thamnolic acid).

We were therefore excited to discover that a reagent in common use when developing TLC plates for plants (e.g. Agatonovic-Kustrin *et al.* 2019) gave a magenta colour in reaction with usnic acid. This reagent contains two acids and *p*-anisaldehyde as a key ingredient; it has only rarely been applied to lichens (e.g. Vu *et al.* 2015; Gerlach *et al.* 2017, 2018), and then only as a derivatization technique for chromatography in the genera *Stereocaulon* Hoffm. and *Usnea* Dill. ex Adans. The use of *p*-anisaldehyde as a visualizing agent for TLC and high-performance thin-layer chromatography (HPTLC) was not mentioned by two standard references on TLC methods in lichenology (Culberson 1972; Orange *et al.* 2001). It was, however, extensively used by Schreiner & Hafellner (1992) with a formulation from Randerath (1965).

For lichens, Gerlach *et al.* (2017, 2018) and Agatonovic-Kustrin *et al.* (2019) emphasized the utility of the acidic *p*-anisaldehyde reagent in producing different colour reactions for different classes of compounds post-HPTLC. Specifically, phenolic molecules often appear violet; a blue or red colour may indicate aldehydes, amines, carbohydrates, esters and ketones; green indicates allylic alcohols. Terpenoids can also be differentiated to some extent: monoterpenes, triterpenes and steroids usually appear blue, purple and grey, and diterpenes appear brown.

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We first tried this reagent in HPTLC on test samples of *Lobaria* (Schreb.) Hoffm., *Peltigera* Willd. and *Stereocaulon*. Since we observed a strong magenta reaction with usnic acid in *Lobaria* (*L. oregana* (Tuck.) Müll. Arg. and *L. scrobiculata* (Scop.) DC.; nomenclature follows McCune & Geiser (2023)), it seemed promising as a rapid spot test reagent for detecting usnic acid. We therefore sought methods for rapidly obtaining results from this reagent. We conducted tests to see if *p*-anisaldehyde might be useful as a spot test reagent directly on fragments or acetone extracts of lichen thalli. Our goals were to design a rapid, repeatable methodology and to detect other substances that might either interfere with *p*-anisaldehyde use as a diagnostic reagent for usnic acid or provide additional useful colour reactions for other lichen substances. Although we sought a quick tool to identify usnic acid in the field or in the laboratory, the reagent has a largely unrecognized potential to enhance TLC or HPTLC of lichens. For our initial effort, we focused on common substances in the genus *Cladonia* because of the significance of usnic acid to the phylogeny and identification keys in this genus (Ahti *et al.* 2013; Stenroos *et al.* 2018).

Our purposes are therefore to explore the use of acidic *p*-anisaldehyde as a spot test reagent for the visualization of usnic acid with spot tests and HPTLC and to demonstrate the colour reactions obtained from common lichen substances in the genus *Cladonia*. Furthermore, we characterize by liquid chromatography-mass spectrometry (LC-MS) the reaction products of *p*-anisaldehyde with usnic acid.

Material and Methods

Preparation of p-anisaldehyde reagent

We abbreviate this as 'reagent AS' in accordance with its historical use (Wagner *et al.* 1984, p. 299; but note different order of mixing). To prepare reagent AS, first refrigerate 85 ml of methanol for *c.* 30 min (to reduce the heat from the later exothermic dilution of sulphuric acid). Add 5 ml concentrated sulphuric acid slowly and carefully to the cool methanol with gentle manual stirring. Add 10 ml of glacial acetic acid. When the solution is at room temperature, add 0.5 ml of *p*-anisaldehyde (98%, Sigma-Aldrich A88107). Store in a glass bottle in the dark or wrapped with aluminum foil. For use, transfer an aliquot to a brown glass bottle or one wrapped with aluminum foil. Refrigerate both stock solution and aliquot to prolong shelf life. If unprotected from light it will last for around one month, longer if protected. The anisaldehyde reagent should be reprepared when it starts producing a pronounced pinkish background colour on TLC plates.

Caution is needed when using the reagent AS. The compound *p*-anisaldehyde by itself is classified as an 'irritant' and potentially acutely toxic if swallowed. The addition of sulphuric and acetic acids to the reagent exacerbates those hazards.

HPTLC

A high-performance thin-layer chromatography (HPTLC) system (CAMAG), equipped with a TLC-Visualizer 2, Automatic TLC Sampler 4, Automatic Developing Chamber 2, TLC Plate Heater III and a CAMAG® Derivatizer, was used to perform the HPTLC analysis. The instruments were controlled using visionCATS software v. 3.2. HPTLC parameters were in agreement with the United States Pharmacopeia (USP) general chapter 203 (United States Pharmacopeia 2017), with slight modification on the band length to 6 mm. The Universal HPTLC mix (UHM) was used as a system suitability test (SST) and prepared as described (Do *et al.* 2021).

Usnic acid 98% (Sigma-Aldrich) and atranorin ≥ 95% (Cayman Chemical) were prepared at 200 µg ml^{-1} in methanol. Lichens were crushed and extracted in acetone at 100 mg ml^{-1} by sonication at room temperature for 10 min, then centrifuged at 3000 rpm for 5 min. The supernatant was used for further analysis.

Quantities of 2, 3 and 5 µl of the UHM, chemical markers and samples, respectively, were applied to an HPTLC Si 60 F_{254} plate (Merck), then developed with toluene, glacial acetic acid (20:3, v/v). Detection was performed under short-wave UV (254 nm) prior to derivatization and under white light in reflection + transmission (RT) and long-wave UV (366 nm) after derivatization with anisaldehyde reagent (reagent AS).

Reagent AS was sprayed onto the HPTLC plates after development using the Derivatizer with a blue nozzle set at spraying level 3, and the plate was then heated at 100 °C for 3 min; 10% sulphuric acid was sprayed using a yellow nozzle spraying level 4, then heated at the same temperature for the same time.

Tests with acetone extracts

Tests with usnic acid provided a basis for colour comparison with reactions of crude lichen extracts containing usnic acid. We completely dissolved 40 mg of usnic acid in 8 ml of acetone at room temperature, then applied known quantities (1× = 7 µl and 4× = 28 µl) to 25 × 75 mm acetone-cleaned glass microscope slides and evaporated at room temperature. Our '1×' test thus contained 0.035 µg of usnic acid and our '4×' test contained 0.140 µg of usnic acid.

Lichen test materials were a specimen of *Usnea scabrata* Nyl. from Saskatchewan (Table 1) in which TLC had previously shown only usnic acid as a major metabolite, and a selection of *Cladonia* specimens from the herbarium (OSC, Table 1) with previous TLC results. *Cladonia* species were selected to represent many of the most common major metabolites in the genus, with and without accompanying usnic acid.

For the *Usnea* specimen, we made a stock solution from 0.5 g of air-dried *Usnea* in 20 ml of acetone, which stood at room temperature for 1 h before the lichen was removed. For *Cladonia* samples, we placed a small fragment (*c.* 8–10 mm^3 in volume) of each sample on glass slides. We applied four drops of acetone in succession to each fragment, letting each drop evaporate at room temperature before reapplying, then removed the fragment, leaving a test spot of a thin whitish residue *c.* 15 mm in diameter.

Reagent AS was applied in 3 µl droplets with a pipette. Larger volumes spread too widely, while a 3 µl droplet expanded to around the same diameter as the dried acetone extract. We tried various orders of operation, and ultimately chose to apply reagent AS to the dried acetone extract, before baking the slide and reapplying reagent AS two more times.

Heat treatments consisted of 100 °C for 2–6 min in a laboratory oven. We also tested heat treatment with an open flame from a hand-held isobutane Bic lighter, hoping for a simple, fast method that could be applied at a microscopy bench or even in the field. For this we used acetone extracts on glass slides as described above. After applying reagent AS, we waved the slide briefly over the flame until colour started to develop (in the case of a positive test) or charring started to appear (in the case of a negative test).

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Table 1. *Cladonia* and *Usnea* specimens used in *p*-anisaldehyde tests on crude acetone extracts in Fig. 3 and Supplementary Material File Fig. S1 (available online). Specimen numbers are from McCune collections (OSC) except as noted.

Species	Major substances by TLC	Number	Country: State
Cladonia albonigra Brodo & Ahti (Fig. S1)	4-*O*-methylcryptochlorphaeic + merochlorophaeic + cryptochlorophaeic acids	Lesica 2022-2a	USA: Montana
C. albonigra (in Fig. 3)	4-*O*-methylcryptochlorophaeic + cryptochlorophaeic acids	7484	USA: Montana
C. amaurocraea (Flörke) Schaer.	barbatic + usnic acids	9937	Canada: Saskatchewan
C. arbuscula (Wallr.) Flot.	fumarprotocetraric + protocetraric + usnic acids	27599	USA: Alaska
C. bacillaris (Ach.) Genth	barbatic acid	9437	USA: Montana
C. decorticata (Flörke) Spreng.	perlatolic acid	27481	USA: Alaska
C. fimbriata (L.) Fr.	fumarprotocetraric acid	21054	USA: Idaho
C. portentosa Dufour (Coem.)	perlatolic + usnic acids	29922	USA: Washington
C. rei Schaer.	homosekikaic acid	14149	USA: Michigan
C. scotteri Ahti & E. S. Hansen	atranorin	27615	USA: Alaska
C. squamosa Hoffm. var. *squamosa*	squamatic acid	26378	USA: Alaska
C. squamosa var. *subsquamosa* (Nyl. ex Leight.) Vain.	thamnolic acid	30983	USA: Oregon
C. sulphurina (Michx.) Fr.	squamatic + usnic acids	13763	USA: Montana
C. transcendens (Vain.) Vain.	thamnolic + usnic acids	39416	USA: Oregon
Usnea scabrata Nyl.	usnic acid only	26243	Canada: Saskatchewan

Tests directly on lichens

We tested several usnic acid-containing lichens with direct application of reagent AS. Lichen fragments were placed in wells in a ceramic spot plate and the reagent added until the fragment appeared fully saturated. We then heated the samples to 100 °C for 2–6 min, then applied another drop of the reagent. This was repeated for a second cycle of heating and reagent application.

Reaction products

We characterized the reaction products between reagent AS and pure usnic acid by LC-MS^2. Reagent AS (1.6 ml) was mixed with 16 mg of usnic acid. The mixture was then heated at 100 °C for 5 min. The product mixture was filtered using a 0.2 µm syringe filter and diluted 100-fold with acetonitrile. LC-MS analyses were performed using an Agilent 6545 Q-TOF system equipped with a Kinetex C18 column (2.6 µm, 100 Å, 50 × 2.1 mm; Phenomenex). The solvent gradient ranged from 30–100% acetonitrile over a period of 15 min. The blank sample was prepared with 100% LC-MS-grade acetonitrile.

Results

HPTLC

The HPTLC fingerprint after derivatization treatment with reagent AS unambiguously detected usnic acid by its magenta colour in white light and deep red colour under UV 366 nm; furthermore, the retardation factor (R_f) for these bands matched that of the usnic acid standard (Fig. 1A). HPTLC with reagent AS also clearly distinguished usnic acid R_f 0.60 from atranorin R_f 0.66, two of the most common cortical lichen metabolites. Results were consistent among runs on different days, as evidenced by good repeatability of R_f values of the UHM under UV 254 nm, and quenching zones R_f 0.03 ± 0.0, 0.41 ± 0.02, 0.48 ± 0.02, 0.81 ± 0.02 (inter-day analysis, $n = 3$).

HPTLC after derivatization with 10% sulphuric acid (adapted from Culberson (1972)) also distinguished usnic acid R_f 0.60 from atranorin R_f 0.66 (Fig. 1B), although the colour difference is less obvious with sulphuric acid than with reagent AS. Both derivatization methods provided useful detailed HPTLC fingerprints for individual samples. However, the colour reactions were quite different under reagent AS and 10% sulphuric acid, providing complementary information under both white light and UV 366. Note, for example, for the *Peltigera* specimens, the striking differences in spot colours between the two derivatization methods.

TLC

Post-treatment with reagent AS after fully developing TLC plates by the traditional Culberson method (Culberson 1972; 10% sulphuric acid + heat) also proved useful. Usnic acid spots with that traditional treatment appear light to dark olive. After painting the spots with reagent AS, and baking for 4 min at 100 °C, a subtle to strong reddish overtone appeared, apparently depending on the concentration of usnic acid or variation in the application of the reagent.

Acetone extracts

Acetone extracts of an usnic acid-containing lichen, *Usnea scabrata*, on glass microscope slides, and of pure usnic acid evaporated

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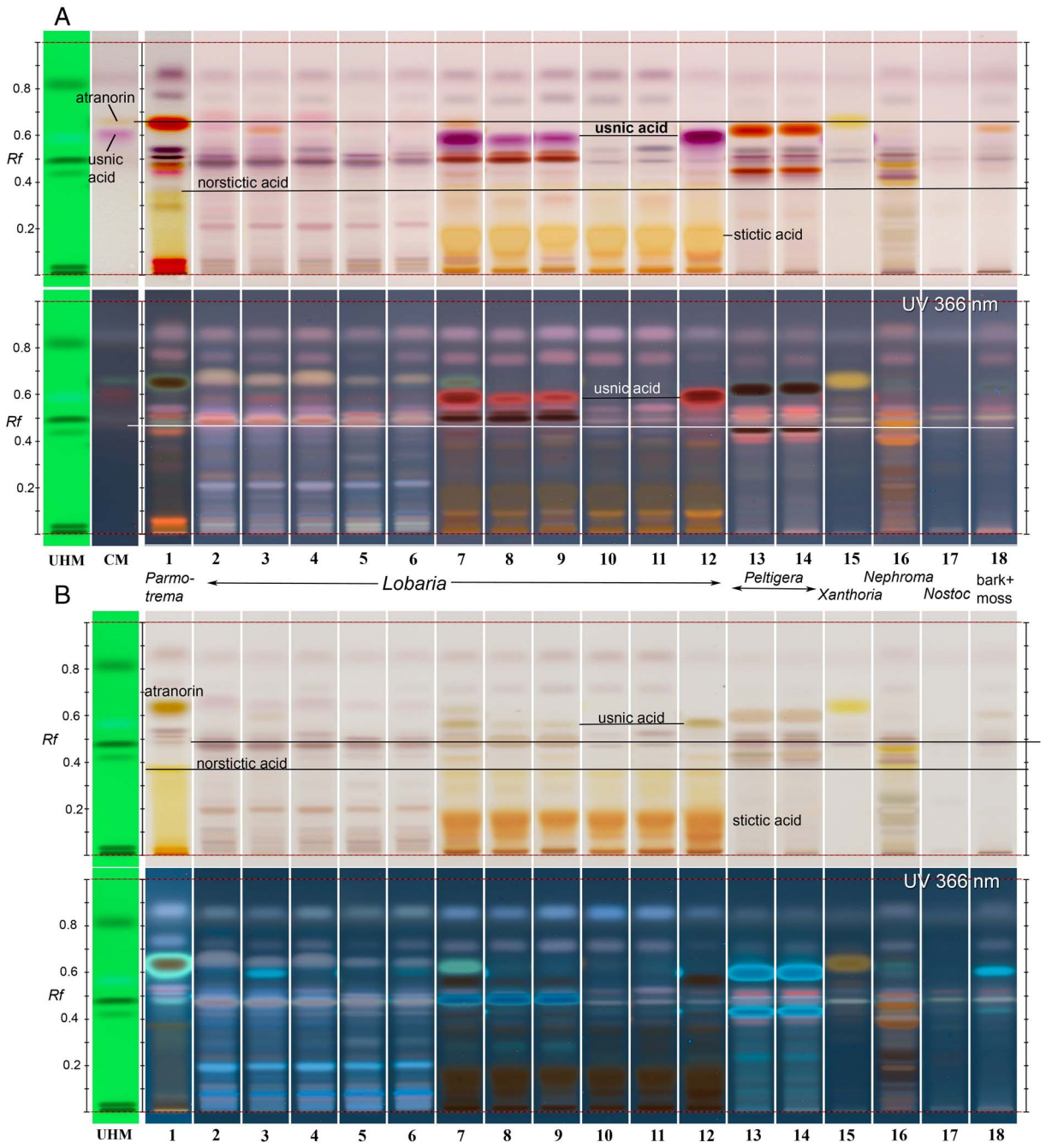

Figure 1. HPTLC fingerprints of 18 lichen samples and two control tracks with two methods of derivatization. UHM = universal system suitability test; CM = chemical markers (usnic acid R_f 0.60 and atranorin R_f 0.66). Tracks 1–18: *Parmotrema hypotropum* (1), *Lobaria anomala* (2-4), *Lobaria anthraspis* (5-6), *Lobaria scrobiculata* (7-9), *Lobaria pulmonaria* (10-11), *Lobaria oregana* (12), *Peltigera britannica* (13), *Peltigera leucophlebia* (14), *Xanthoria* sp. (15), *Nephroma laevigatum* (16), *Nostoc* sp. (17) and mossy tree bark (18) as a control for possible contaminants. A, derivatization with anisaldehyde reagent under white light (upper) and UV 366 nm (lower). B, derivatization with 10% sulphuric acid under white light (upper) and UV 366 nm (lower). R_f = retardation factor.

from an acetone solution, invariably produced an initial magenta colour on heating (Fig. 2; video link in Supplementary Material File S1, available online). The colour intensified greatly after reapplying a very small amount of reagent AS to the slide shortly after removal from the oven. Over minutes to an hour the magenta colour tended to gradually convert to a more violet colour (Fig. 2) or sometimes dark blue. This colour persisted on slides for over a week, at which point the test was terminated.

Similar colour changes were obtained with direct heating over an open flame for only a few seconds using a hand-held lighter (Fig. 2). Note that residues on the slide that were overheated became dark blue (arrow in Fig. 2C), while underdeveloped areas were yellowish (arrow in Fig. 2A). Negative controls (no usnic acid, only reagent AS) turned a very pale pinkish after heating, easily distinguishable from the strong magenta to violet reaction from usnic acid.

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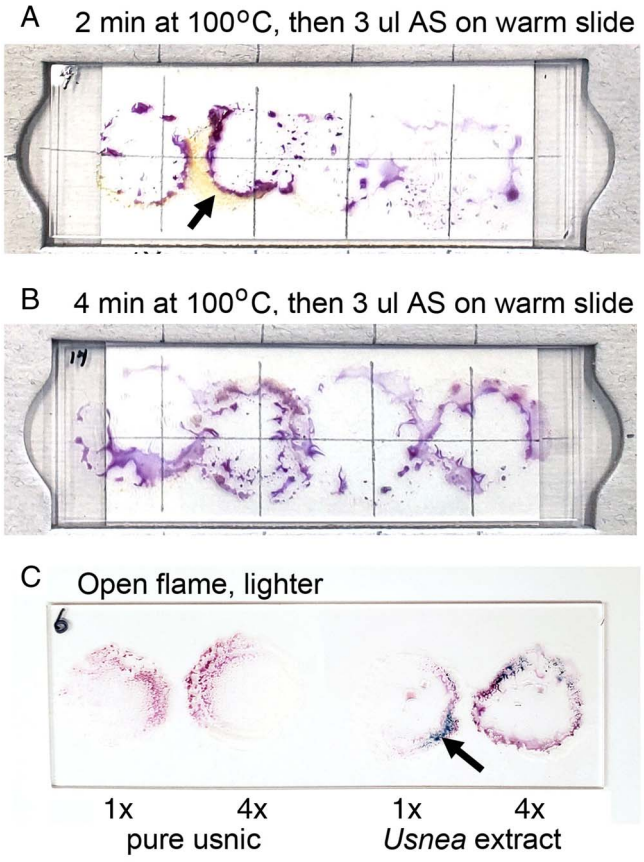

Figure 2. Reactions of usnic acid (both pure and crude extract from *Usnea scabrata*) to the *p*-anisaldehyde (AS) reagent and heat. The starting extractions on each of the three slides were identical. Two amounts of acetone solution were tried, 7 µl ('1x' in photograph) and 28 µl ('4x'). The '1x' of pure usnic acid contained 0.035 µg of usnic acid and '4x' contained 0.140 µg. Reagent AS was applied in two cycles: first 3 µl before heating, then baked in oven or heated on open flame. A, 100°C for 2 min. Arrow indicates yellow tinge where the reaction was not fully developed. B, 100°C for 4 min. C, after direct heating on open flame with hand-held lighter. Arrow indicates dark blue tinge where reaction was overheated.

Acetone extracts containing common major lichen substances in the genus *Cladonia* produced a magenta to violet reaction whenever usnic acid was present (Fig. 3, Table 2). The same reaction was observed regardless of whether the usnic acid was accompanied by barbatic, squamatic, thamnolic, fumarprotocetraric, or psoromic acids (Fig. 3; video link in Supplementary Material File S1, available online). All of these are very common metabolites in *Cladonia*, occurring both with and without usnic acid, depending on the species.

Additional common metabolites in *Cladonia* were tested in the absence of usnic acid. The most distinct reaction apart from usnic acid was with the merochlorophaeic acid group (a combination of 4-*O*-methylcryptochlorphaeic, merochlorophaeic and cryptochlorophaeic acids). Tests on acetone extracts from *C. albonigra* yielded a pronounced red reaction. All three of these substances appear to react reddish to red with reagent AS, based on tests with compounds separated by TLC. The reddish colours with these metabolites are similar, but somewhat more intense, than the reddish colours of those spots on TLC plates developed with the standard method of heat + sulphuric acid (Fig. S1 in Supplementary Material File S1). It is worth noting that a number of other lichen substances may give a reddish reaction to AS, such as orsellinic acid, confluentic acid, divaricatic acid, gyrophoric and lecanoric acids (Schreiner & Hafellner 1992), although we have not yet tested these.

No appreciable reactions were observed for atranorin, or psoromic, squamatic and thamnolic acids. Weak reddish brown or brownish reactions were observed for barbatic, fumarprotocetraric, homosekikaic and perlatolic acids. These were, however, barely distinguishable from negative controls containing only the test reagent.

Direct application on lichens

Saturating usnic acid-containing lichen fragments with reagent AS then baking for 3–6 min at 100 °C produced discoloration and darkening in some cases but did not produce a consistent distinct colour reaction. Reapplying reagent AS, which produced the strongest test with the acetone extracts, did not improve the results for direct application.

Reaction products

LC-MS produced two major ions with *m/z* 477.1586 ($[M+H]^+$) and retention times of 5.97 and 7.32 min, followed by four minor ions with *m/z* 463.1385 ($[M+H]^+$) and retention times of 0.32, 4.62, 7.63 and 7.99 min (Figs S2 & S3 in Supplementary Material File S1, available online). The computational MS prediction software SIRIUS (Dührkop *et al.* 2019) confirmed molecular formulas of the major and minor reaction products as $C_{27}H_{24}O_8$ and $C_{26}H_{22}O_8$, respectively. These two molecular formulas and the elution of multiple LC peaks with the same molecular formula but different retention times are consistent with the regioisomeric aldol condensation products of usnic acid and *p*-anisaldehyde, where one set of isomers (*m/z* 477.15) is *O*-methylated.

Discussion

We started with the knowledge that treatment with an acidic *p*-anisaldehyde reagent followed by heating to 100 °C for 3 min on HPTLC plates produced a magenta to purple colour for spots of usnic acid. This inspired us to test for colour reactions with this reagent using pure usnic acid, crude acetone extracts of lichen fragments, and directly on lichen fragments. To seek efficient conditions for simple tests we varied the heat source (hand-held lighter vs oven), heat duration and temperature.

The *p*-anisaldehyde reagent AS proved effective in visualizing usnic acid from crude extracts on glass microscope slides and on silica gel plates from both TLC and HPTLC. TLC has been the most common planar chromatographic technique used as a tool for identification of lichens, although the advantages of HPTLC over TLC are well known (Reich & Schibli 2007). In addition to its common use in lichens, HPTLC has been used for the identification of herbal drugs based on a specific chemical fingerprint that can be correlated with the taxon (Antunes *et al.* 2023).

The colour changes with reagent AS applied to crude acetone extracts of *Cladonia* specimens containing usnic acid were identical to those obtained using pure usnic acid. Although these reactions can be reproduced under controlled heating conditions, we also readily obtained similar results by heating extracts over an open flame from a lighter. We were not, however, successful in producing distinct colour reactions directly on lichens that contained usnic acid. Instead, we observed a general discoloration or darkening of the lichen.

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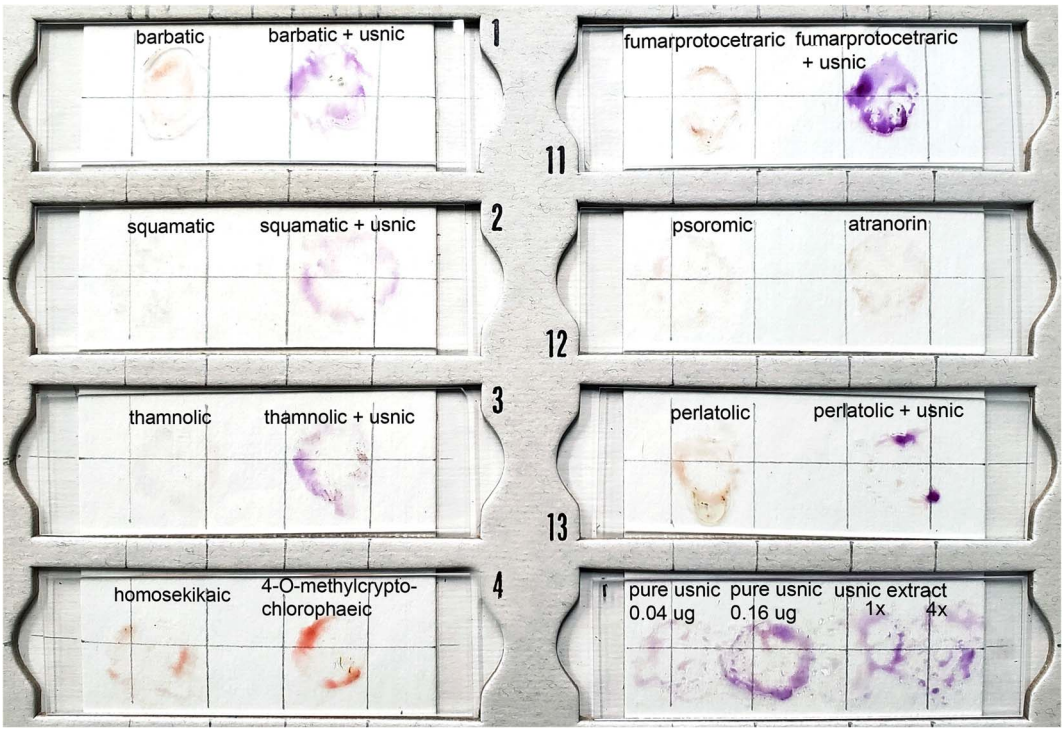

Figure 3. Reactions of common substances in the lichen genus *Cladonia* to the *p*-anisaldehyde (AS) reagent. We applied reagent AS in two cycles: first 3 µl, then 100 °C for 4 min, then 3 µl. Five of the eight slides contrast a single major substance with that same substance along with usnic acid. In each of those cases the reaction of the usnic acid dominated the colour change. The spot labelled 4-*O*-methylcryptochlorophaeic acid from *C. albonigra* also contains merochlorophaeic and cryptochlorophaeic acids as major substances. Bottom right: four spots of usnic-only extracts shown for comparison, the left two spots pure usnic acid and the right two spots from *Usnea scabrata*, as in Fig. 2.

The *p*-anisaldehyde reagent is a universal derivatization reagent and can react with many compound classes (Gerlach *et al.* 2017, 2018; Agatonovic-Kustrin *et al.* 2019). In the case of usnic acid, we hypothesize that *p*-anisaldehyde under acidic conditions at high temperature reacts with either one of the methylketone groups of usnic acid via an aldol condensation to produce regioisomers with *m/z* 463.14 that elute with different retention times (Fig. 4). The major product ions (*m/z* 477.15) are additionally *O*-methylated at one of the usnic acid alcohols. We propose that *O*-methylation of the aldol condensation product occurs due to intense heating in methanol. These conjugated aromatic products possess a magenta colour under acidic conditions. We have also seen a similar magenta reaction in plant species when using HPTLC under white light followed by derivatization with *p*-anisaldehyde, for example in essential oils from *Eucalyptus globulus* and *Eucalyptus grandis* leaves.

Table 2. Reactions of common substances in *Cladonia* to the *p*-anisaldehyde (AS) reagent. Results are based on acetone extracts evaporated on a glass slide, then treated with reagent AS and heat (100°C), followed by retreatment with reagent AS.

Substance	Reagent AS reaction
4-*O*-methylcryptochlorophaeic, merochlorophaeic, and cryptochlorophaeic acids	red
atranorin	negative
barbatic acid	pale reddish brown
fumarprotocetraric acid	pale brownish
homosekikaic acid	pale reddish brown
perlatolic acid	very pale reddish brown
squamatic acid	negative
thamnolic acid	negative
usnic acid	magenta-purple

Surely, we will find other lichen metabolites that show a similar colour reaction to reagent AS. However, of the common substances in *Cladonia* that we tested, only usnic acid yielded the characteristic magenta to violet reaction. The fact that a colour reaction could be produced by different compounds does not negate its utility in lichen identification. For example, the extremely useful P test (*p*-phenylenediamine in ethanol) produces a reddish reaction for quite a few lichen metabolites (e.g. argopsin, fumarprotocetraric acid, protocetraric acid, pannarin, physodalic acid and salacinic acid).

Further tests with reagent AS should include testing a broad sample of substances with post-development of TLC or HPTLC plates. Similarly, testing crude acetone extracts of diverse specimens with known lichen substances would be helpful for increasing the utility of this reagent. Further testing of conditions for the reaction, including the timing and intensity of heating, may produce further improvements in visualizing usnic acid and other compounds. Meanwhile, we have a new tool for easy, rapid detection of usnic acid in *Cladonia*.

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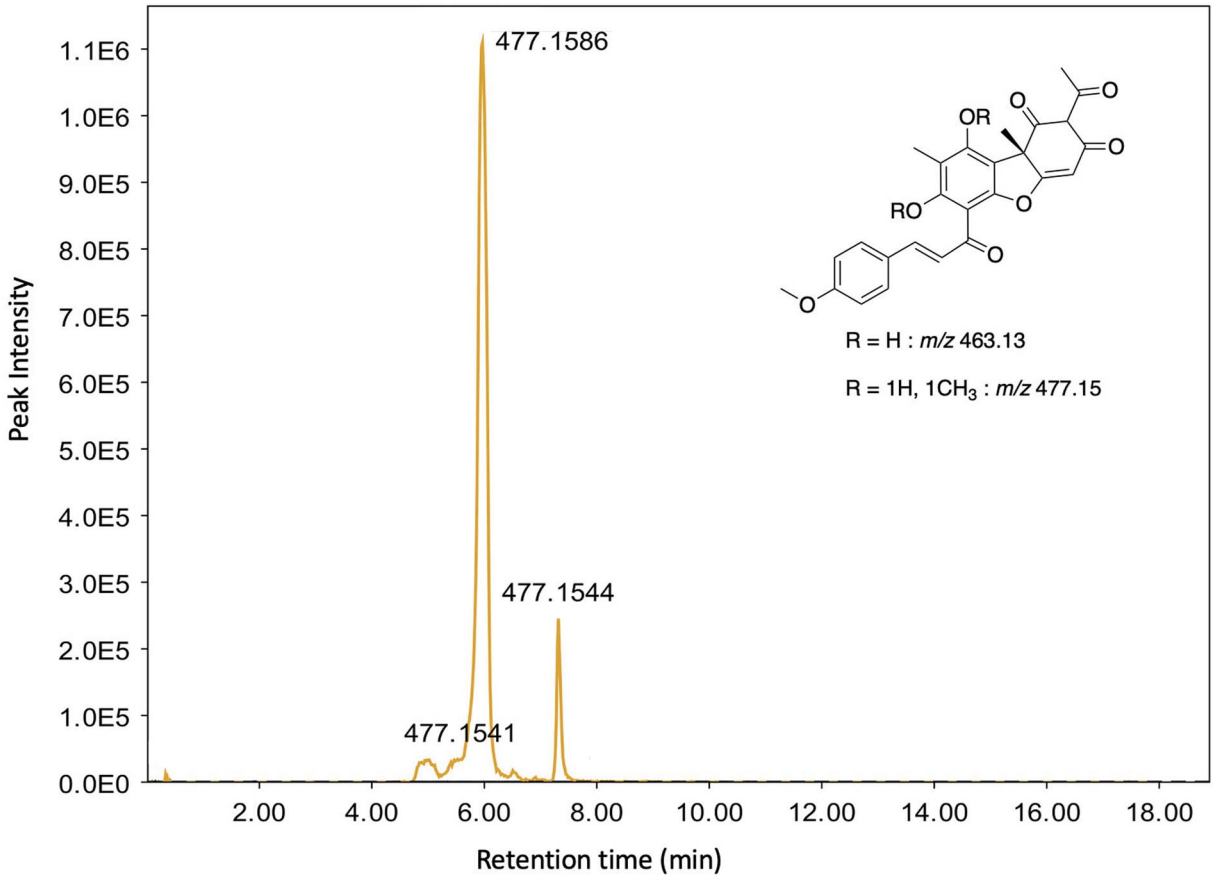

Figure 4. LC-MS profile, XIC (extracted ion chromatogram) of mass range 477.1500–477.1600 of the reaction products of usnic acid and *p*-anisaldehyde reagent AS. See additional chromatograms in Supplementary Material Figs S2 and S3 (available online). Inset diagram: hypothetical structures of the primary reaction products produced by aldol condensation product with *O*-methylation. In colour online.

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Author Contributions. B. McCune, X. Yu: conceptualization, plant material collection and identification. B. McCune, W. Perera, X. Yu: laboratory work. All authors: writing, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Competing Interests. The authors declare none.

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