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A new chemical spot test for miriquidic acid

Within the genus *Stereocaulon*, two northern species, *Stereocaulon alpinum* Laurer and *S. groenlandicum* (Å. E. Dahl) I. M. Lamb, can be especially difficult to distinguish phenotypically. We therefore sought a chemical spot test method to differentiate the two species. The primary distinguishing character is the presence or absence of the substance miriquidic acid. The traditional method for detecting this substance is thin-layer chromatography (TLC), which provides clear identification of miriquidic acid by the presence of a blue-green spot seen on the silica-gel plate after treatment with acid and heat. The dramatic colour change after TLC suggested that a short cut in lieu of the time-consuming TLC process might be possible.

Miriquidic acid was first discovered by Huneck *et al.* (1971), and was later confirmed in *S. groenlandicum* (Lamb 1973) by TLC (Culberson *et al.* 1977). It is also a characteristic substance in many species of the genus *Miriquidica*. The standardized TLC method of Culberson (1972) includes placing spots of an acetone extract of a lichen sample on an aluminum or glass backed silica gel plate, and running the plates in one or more solvent systems, most commonly Culberson's A, B, and C. After the plates have been run they are then observed under UV light and the spots are noted with pencil. Finally the plates are brushed or sprayed with 10% sulphuric acid and baked at 100°C for *c.* 5 min, at which point the characteristic blue-green miriquidic acid spot appears.

Our search for a spot test began with similar reagents to those used by the TLC method. First we conducted macroscopic tests treating acetone extracts of *S. groenlandicum* (containing atranorin, perlatolic, miriquidic and anziaic acids) and *S. alpinum* (containing atranorin and lobaric acid) with several different acids (100% acetic acid, 10% sulphuric acid, 6M nitric acid, and 1M hydrochloric acid), as well as one treatment with the combination of 10% sulphuric acid and TLC solvent A (containing toluene, 1-4-dioxane and acetic acid). In this test, a fragment of each of the two lichen samples were placed into six separate wells on a white porcelain spot plate and each was partially submerged with one drop of acetone. Once the acetone evaporated leaving behind the lichen extracts, the lichen thalli were removed. The two species' extracts were then treated with one of the five treatments detailed previously, leaving the sixth extract of each untreated as a control. After letting the wells dry for 1 h, we then baked the porcelain plate for 20 min and saw a distinctive colour change on two of the *S. groenlandicum* samples: the treatment of the combination of solution A and sulphuric acid, and the treatment of sulphuric acid alone. Surprisingly, the two wells showing a colour reaction developed to pink-red instead of blue-green; however, these results directed all future tests to the use of sulphuric acid exclusively. No pronounced colour changes were seen for the other treatments or extracts.

Hoping for effectiveness of lower sulphuric acid concentrations, as well as to begin to understand the reactions taking place, we similarly tested the original samples as well as additional species using 5%, 1% and 0·5% sulphuric acid applied via a paint brush, air dried for 10 min and then baked for 15 min. The additional samples selected contained different combinations of the compounds which make up *S. groenlandicum*, and would therefore help narrow down whether or not miriquidic acid was the compound now reacting to pink-red. The additional samples tested were *Cetrelia cetrarioides* (Delise) W. L. Culb. & C. F. Culb. (perlatolic acid and atranorin), *Miriquidica scotopholis* (Tuck.) B. D. Ryan & Timdal (miriquidic acid only) and *Lecidea perlatolica* Hertel & Leuckert (perlatolic acid-major). All concentrations

yielded colour changes except the extracts of *L. perlatolica* and *S. alpinum*. The colour changes observed at 0·5% sulphuric acid were gold-orange for *C. cetrarioides*, violet for *M. scotopholis*, and pink-red for *S. groenlandicum*. These colours were persistent, lasting at least 24 h.

Through additional experiments we devised an alternate, faster and equally effective method to perform our new 'S' test. In this method, we extracted a small piece of lichen thallus with one drop of acetone on a glass slide, discarded the thallus and brushed with either 10% or 0·5% sulphuric acid. Then, instead of baking the sample, we wafted the slide with tweezers over a low open flame of a Bunsen burner. Within 30 s we saw the characteristic pink-red colour change on the extract of *S. groenlandicum*. Although the more diluted 0·5% sulphuric acid worked, 10% sulphuric acid gave a more vibrant colour.

S Test Protocol

1. Place a small piece of lichen thallus on a glass slide. (For multiple simultaneous tests, use a well of a porcelain spot plate).
2. Drop acetone on the thallus. Place about enough to partially submerge the thallus (usually one drop of acetone). Let acetone dry.
3. Discard thallus material.
4. Brush 0·5% sulphuric acid over the well or slide with a paint brush, making sure to coat the dried extract and ideally leaving small beads of sulphuric acid as it will dry faster.
5. Rinse brush in clean water then dab on a dry paper towel, between uses.
6. Air dry for 10 min to partially evaporate the water.
7. Gently heat over a flame for about 30 s, or until colour develops. (For multiple simultaneous tests, bake the plate at 100°C for about 15 min.)

If a persistent violet to bright pink colour is visible in the well, then miriquidic acid is present.

To observe what colour a semi-pure extract of miriquidic acid would have when submitted to our 'S' test, we first separated the major lichen substances of *S. groenlandicum* with a TLC plate by running the plate in solvent system C and no further processing. We then separately scraped the silica gel from each individual spot off the plate (atranorin, perlatolic acid and miriquidic acid), placed the silica powder of each in a well previously brushed with 0·5% sulphuric acid, and then baked the plate. Only the well with the silica gel from the miriquidic acid spot had a violet colour reaction, while the wells with the silica gel of the perlatolic acid and atranorin spots had a yellow-gold colour.

The question remained, why were we seeing these colour inconsistencies? Miriquidic acid on a well plate, when treated with our 'S' test, gave a pink-red colour from *Stereocaulon groenlandicum*, and a violet colour from *Miriquidica scotopholis*, but on a silica gel plate using Culberson's (1972) TLC methods, both species gave the standard blue-green colour. An additive colour mixing model could explain the formation of the pink-red colour of *S. groenlandicum*. If we combined the gold-orange colour from our 'S' tests of *Cetralia cetrarioides* (atranorin and perlatolic acid) with violet from *M. scotopholis* (miriquidic acid), perhaps this yields the pink-red colour of *S. groenlandicum*. We confirmed this by experimenting with RGB settings on coloured discs using Microsoft Word. By adding the RGB settings for orange (R:226 G:118 B:0) to the RGB settings for violet (R:81 G:0 B:141), and entering this as the RGB setting for a new disc, one obtains a pink-red colour (R:214 G:80 B:141).

Alternatively, the pink-red colour may be derived from reaction products of a combination of the substances present in *S. groenlandicum*. This presumably occurs due to the molecules being hydrolyzed (by sulphuric acid) or otherwise degraded (by prolonged exposure to acetone) at its ester linkages. Mass spectrometry would be required to identify the specific pigmented products to further elucidate the reaction pathways.

A final test was done to observe the 'S' test on other lichen substances. We selected 12 additional lichen samples that represented some of the most common lichen substances. Only miriquidic acid gave pink-red or violet colour reactions, though the gyrophoric acid complex gave a purple-grey colour (Table 1). Several common substances (atranorin, stictic, norstictic, and salacinic acids) gave orange reactions. Further testing with a wider variety of substances may yield other informative uses for this test.

Table 1. *Reactions of common lichen substances to the "S" test with 10% sulphuric acid, followed by heat.*

Species	Known Substances	S test
Stereocaulon alpinum	atranorin+lobaric acid	none
Lecidea perlatolica	perlatolic acid	none
Cladonia squamosa var. *squamosa*	squamatic acid	none
Cladonia fimbriata	fumarprotocetraric acid	none
Hypogymnia occidentalis	atranorin+physodic acid	none
Cladonia bacillaris	barbatic acid	grey
Usnea longissima	usnic+diffractaic acid	green-grey
Lecidea fuscoatra	gyrophoric acid complex	purple-grey
Miriquidica scotopholis	miriquidic acid	violet
Cladonia carneola	zeorin+usnic acid	slight brown rim
C. squamosa var. *subsquamosa*	thamnolic acid	brown
Parmeliopsis hyperopta	atranorin+divaricatic acid	slight orange
Parmotrema hypotropum	atranorin+norstictic acid	orange
Stereocaulon tomentosum	atranorin+stictic acid complex	orange
Parmelia sulcata	atranorin+salacinic acid	orange
Cetrelia cetrarioides	atranorin+perlatolic acid	gold-orange
Stereocaulon groenlandicum	atranorin+perlatolic acid+miriquidic acid+anziaic acid	pink-red

In conclusion, this 'S' spot test can be used to indicate the presence of miriquidic acid. We recommend the test on an acetone extract on a glass slide, lightly brushing with any concentration of sulphuric acid ranging from 10% to 0·5%, air drying for 10 minutes, then heating over a flame for 30 seconds. A colour change to either pink-red or violet denotes the presence of this substance. This new method could reduce the need for TLC and can be done without the need of a specialized laboratory.

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