METHODS: LICHEN CHEMISTRY

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Reagents

Meltzer's Reagent

Dissolve 0.5 gm iodine in 1 gm potassium iodide,
Mix with 20 ml distilled water and 200 gm chloral hydrate

Identification of Substances

Filter Paper Method

Put fragment on filter paper, add acetone drop by drop. Test rings around fragment with spot reagents. (Santesson 1967).

For Lichens on Soil

When testing lichens on soil, with KOH for atranorin, turn lichen clump upside down so cortex reacts with KOH before soil discolors the solution, and tap the thallus lightly.

Iodine Tests on Asci

Old apothecia may be I (i.e., reddish), but even they will be (K+I)+ blue. A lower concentration of iodine gives a stronger blue (with Lugol's Solution, 10%. added to a dry preparation = "high conc."; to a wet preparation = "low conc."; let the preparation dry out, then add iodine, so you know the concentration. (Hertel, 1986, pers. comm.).

Tests on Epihymenium

HCl turns some from green to blue, others not. (Hertel, 1986, pers. comm.).

Spot Testing with Reagents

Brodo (1984) used a drawnout micropipette (ca. 6 cm long, 1.3 mm inside diameter).

Spot tests should always be made on pieces removed from the thallus so they can be discarded and not contaminate the rest of the specimen.

The pieces can be placed on a microscope slide or piece of filter paper on top of a white background and viewed under a stereomicroscope.

Some tests should be made on sections of specimens, in order to see reactions occuring in tissues such as the inner cortex or inside parts of the apothecia.

Some reactions, e.g., the C+ changes found in some species, are best seen in thin sections under a compound microscope.

It is a good idea to leave a note giving the results of the spot tests, to avoid depletion of material by unnecessary repetition of the tests (Dahl & Krog, 1973).

Some reactions , especially with Pd, may take up to a half a minute or more (Dahl & Krog, 1973).

Tests should usually be carried out on young, fastgrowing parts of the thallus (Dahl & Krog, 1973).

Spot TestsCrowquill Pen

Put a few crystals of Pd on crowquill pen, hold over paper, add drops of alcohol. Do tests in situ, then remove the tested piece.
Use crowquill pen for tests with KOH or Pd, but not C. (Hertel, 1986, pers. comm.)

THIN LAYER CHROMATOGRAPHY METHODS FOR LICHEN SUBSTANCES

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Dec. 1987

Factors affecting Rf values (Alston & Turner, 19).

temperature

equilibration time

exact method of equilibration

shape of chamber

number of plates run at same time

extraction procedures

Plates

Glass

Aluminum

Plastic

Solvent Systems

Standard Systems (and Modifications)

(A) toluene: dioxiane: acetic acid (180:45:5)

(B) hexane: diethyl ether: formic acid (120: 90: 20)

(B') hexane: methyltert.butyl ether: formic acid ( )

(C) toluene: acetic acid (200:30)

Other Systems

E.A. ether: acetic 200:2 (ml)

E.H. ether:hexane 3:1

Detection of Fatty Acids

Fatty acids and terpenoids, which cannot be seen by UV fluorescence or absorbtion (prior to charring), can be detectedusing translucent (glass or plastic) plates sprayed with water (or aqueous solutions such as 10% sulfuric acid). When the plates are sprayed until they are visibly wet, and then allowed to slowly dry in air, these substances are rendered visible as rather opaque spots before the rest of the plate dries out. Brodo (1984) recommended observing the plates against a black background; holding the plates up to the light can also be useful. These spots can then be marked with a dotted outline. Care must be taken to distinguish such spots, which are regular in shape and clearly within "lanes", from various artifacts, which are more irregular and not lined up. Different substances show up at different stages in the drying process, some only appearing at the last minute, and the sizes of the spots change during the drying. Other hydrophobic substances, such as placodiolic acid, will also produce such spots but are usually visible in ultraviolet light before charring and often have characteristic colors in visible or ultraviolet light after charring. Sometimes hydrophobic spots are superimposed over or within nonhydrophobic spots.

Terpenoids and fatty acids can be identified, even without using the above method, by their rather distinctive pale orangewhite fluorescence after charring (Elix, 198). Terpenoids can generally be distinguished from fatty acids because the former appear purplish or brownish after charring and usually form bands rather than round spots (with some exceptions, such as leucotylin).

Miscellaneous Tips

Some substances produce only very faint spots; if there is doubt as to whether a spot really represents a substance, this can be checked by running several samples of the same specimen in adjacent lanes; if the spot really represents a substance, a horizontal row of several spots should form across the lanes.

TwoDimensional TLC

Several authors, e.g., Maas (1975) have described the use of this method.

TLC METHODS

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Dec. 1987

(This is one of several files of miscellaneous thoughts on TLC methods, which will eventually be merged and rearranged)

TLC Annotations

Annotation slips briefly indicating the chemical contens of were placed in the packets of all borrowed specimens that were sampled for chemical analysis. The primary purposes of these annotations were: 1) to indicate that a sample had been used for TLC, to avoid unnecesary depletion of the specimen by further removal of fragments, and 2) To provide myself and other workers at least some idea of the chemical constituents, as a basis for further investigation... Because of the large number of specimens that were analyzed and annotated, the numerous unknown substances, and many changes in tenchniques and interpretations over the course of the study, it was not feasible to spend an excessive amount of time in preparing or revising these annotations. In many cases, these annotations were preliminary and incomplete (especially with regard to most terpenoids, fatty acids and unknown substances).

The Rf classes and other characteristics given for the unknowns were often only quick estimates, and were sometimes erroneous for various reasons. Descriptions of postcharring flourescence and various other characteristics of the unknowns were often not given. Work on identification of the unknowns has been a gradual process, and is still continuing.

The annotation slips cite the three solvent systems as being the "A", "B" and "C" of Culberson (1970, 1972). However, it should be pointed out that in all of my TLC, toluene was substituted for benzene in Solvent A (as recommended by _____, 19__), and methyltert.butyl ether was substituted for diethyl ether (as recommended by _____, 19__). These changes, made for safety reasons, do have some affects on the Rf values of some substances. Elix (1987) refers to the modified B solution as B'.

In several cases the names given for the substances on the annotations are older descriptive synonyms rather than the correct chemical name (e.g., "consporomic acid"

=2'0demethylpsoromic acid).

Usually the only indication of relative quantity of the substances given was the qualifier "trace" used when the presence of a substance was indicated only by a very faint spot.

More complete and updated descriptions of the unknown substances, and a certain amount of additional and revisedinformation on the known and unknown constituents of many of the individual specimens (especially types) will be given in my publications. As further progress is made in identifying or at least better characterizing the unknowns, information on the chemical constituents of particular specimens will be made available to workers with a special interest in lecanoras or their chemistry.

Record Keeping

When large numbers of plates are being run (especially in critical where there are many unknowns), it is necessary to assign a unique, unambiguous number to each spot sample. In my study I have used a combination of a "set" letter (A through Z, AA through ZZ) marked on each of a group of plates, and consecutive numbers (to 3 digits) for each spot in that set. This kind of combination can be continued using AB, AC, etc., BA, BC, etc., and can be convenient for keeping and updating records, and for locating and reexamining stored plates. These "Sets" of four to seven plates were often run developed and (partially) interpreted together on a single day and thus should be relatively comparable.

Solvent Systems

Problems with Hygroscopic Plates

Fluorescence in Longwave Ultraviolet

To clearly see and distinguish the different colors of fluorescence of spots in longwave ultraviolet light (before or after charring), three things were found essential: 1) using a sufficiently intense uv light source (give specs. of one in Dennis's lab), 2) working inside a booth kept completely dark by black curtains, and 3) wearing greenish tinted goggles (give

specs), which also protect the eyes.

I learned the above rather late in my studies...(how do I phrase that?).

Fluorescence After Charring

The color and intensity of the postcharring fluorescence of many substances change over time, and are affected by a number of variables in the running and development of the plates...

MICROCRYSTAL TESTS

Brodo (1984) described a method for observing the formation of crystals in the epihymenium in response to Pd...(see article for complete description).

STAINS, REAGENTS, AND MOUNTING MEDIA

Water

Sections prepared for temporary observation should be mounted in distilled water. Measurements, and observations on color and granulation, should be made in water.

Glycerin

Bland (1971) recommended mounting sections in 10% glycerin, which is closer to the refractive index of the tissues and makes it easier to see structures clearly.

Application of Reagents, etc. to Microscopic Preparations

Replacement of one mounting medium, stain or reagent can be accomplished simply by adding a drop or two of the new substance to the edge of the cover slip and drawing it under by placing a square of filter paper, paper towel or "bibulous paper" (which is made for this purpose) at the opposite edge.

Stains

Lactophenol Cotton Blue (LCB)

This stain is essential for clearly seeing the lumina of hyphae, paraphyses, etc. It can also be useful in examination of the walls and ornamentation of spores.

Empty algal cells in a pseudocortex can also sometimes be detected in cotton blue.

According to Brodo (1984), the lactic acid in LCB can act similar to nitric acid, in dissolving some kinds of granular or crystalline inspersions (e.g., many of the large amphithecial crystals that occur in the Lecanora subfusca group and often the small medullary granules).

Lactophenol cotton blue can be cleared from the tissues by drawing a drop of the lactophenol withtout the cotton blue under the coverslip.

Hoyer's Solution

Brodo (1984) used this solution as one of his mounting media, to

help resolve certain types of tissue structures. He reported that it acts like a strong base, dissolving the same types of granules or crystals that KOH does.

Hoyer's solution contains chloral hydrate and therefore is very poisonous.

Chlorzinciodine (CZI)

Empty algal cells in a "pseudocortex" can be detected by staining with "chlorzinciodine", which turns a violet color in the presence of the cellulose walls of the algae, but does not react to the chitinous walls of the fungus (Poelt, 1958 and pers. comm.).

This stain has been used in a number of studies, including those of Galun? (19see "Characters" notebook), Baumgartner (1979), Timdal (1984)

Timdal (1984) recommended a slight modification of the pretreatment described by Baumgartner (1979): the sections should first be stained in chlorzinciodine for a few minutes, then both washed and soaked in Lugol's solution for a few minutes, and finally both washed and stained in chlorzinciodine again. Timdal found that washing the sections with water, as done by Baumgartner (1979) greatly diminished the effect of the pretreatment.

Poelt (pers. comm.) recommends that in species heavily inspersed with granules of usnic acid, it is usually necessary to first flush the cortex with KOH and then water, to remove obscuring granules.

This stain should be stored in bottles made of glass, not polyethylenes.

Other stains

McWhorter (1921) recommended safraninanalinblue stain for seeing cell walls and haustoria.

An 0.1% aqueous solution of phloxine increases contrast by staining cytoplasm more than walls (Rhoades, 1981, unpublished mycology lab notes).

Neutral red and TCC can be used to determine if the algae are alive or not (LeBlanc, et al., 1971).

Some structures (e.g. hyaline epispores) can be seen in black India ink (Mycol. Handbook).

Reagents

The following reagents can be used either for making spot tests or for making various tests or observations in microscopic preparations.

Potassium Hydroxide (KOH or K)

A solution of 10% (2025% according to Taylor, Lichens of Ohio v. 1) potassium hydroxide in microscopic preparations is used to 1)dissolve all granules except the calcium oxalate crystals (i.e., to clear the tissues and facilitate the observation of these crystals), 2) to free and dissociate hyphae and hymenial elements, 3) to make a final attempt at locating spores in a rather sterile apothecium, and 4) to observed any color change in tissues.

The 10% potassium hydroxide solution usually will stay active about a half year if kept stoppered (Dahl & Krog, 1973).

Paraphenylenediamine (Pd or P)

This reagent is prepared by making a saturated solution, using a few crystals in ethanol (7095%). A fresh solution must be prepared every few hours. According to Taylor (19 , Lich. of Ohio v. 1), Pd solution will be effective as long as it is clear, even if it has become colored.

The Pd reagent should not be inhaled.

Material contaminated by Pd should be destroyed, or it will discolor everything in the vicinity.

The formula for Steiner's stabilized Pd (see Thomson, 1967; Taylor, Lich. of Ohio; and article by _____), which will keep at least 6 months, is as follows:

Water	100 ml
Anhydrous sodium sulphite (Photographer's hypo)	10 gm

Paraphenylenediamine	1 gm
Liquid detergent	1020 drops
or	
Wetting agents (Saturated solution of Pril or Teepol)	40 drops

Hypochlorite Solution (C)

An undiluted, liquid commercial bleach preparation containing hypochlorite is used for this reagent.

Sometimes a C+ reaction is best seen if the specimen is pressed with a probe after the C is applied, or if the C is put on filter paper and the sample is squashed and smeared around in the C (Noble, 19 , Dissert.)

Generally a hypochlorite solution is still active as long as it smells strongly of chlorine, which is about a week or two (Dahl & Krog, 1973).

Iodine Reagents (I)

Timdal (1984) recommended studying asci in modified Lugol's solution (with water replaced by lactic acid), which can be used for semipermanent preparations.

The reaction of asci to iodine can be studied both with and without pretreatment with K; the former reaction is denoted the KIreaction, the latter the Ireaction.

Rossman (1981) pointed out that Mycologists dealing with freeliving fungi traditionally use Meltzer's Reagent, while lichenologists use IKI (or Lugol's), and caution must be exercised because in some cases the former may act somewhat differently than the latter two, which lack chloral hydrate. Rossman also pointed out that all Iodine solutions degrade with time and should be used when relatively fresh. A comparison of the compositon of the three reagents are given below.

	Meltzer's	IKI	Lugol's
Chloral hydrate	100 gm		
Potassium iodide	5 gm	1 gm	2 gm

Iodine	1.5 gm	1 gm	1 gm
Distilled water	100 ml	100 ml	300 ml

Meltzer's reagent is very poisonous, and chloral hydrate cannot be purchased without a drug license.

Swinscow (freshwater Verrucaria) measured spores in Meltzer's.

Nitric Acid (HNO3)

Concentrated nitric acid in microscopic preparations is used to 1) test the solubility of granules, 2) dissolve calcium oxalate crystals, and 3) to observe any color change in pigmented structures.

Timdal (1984) used 50% nitric acid.

Sulfuric Acid (H2SO4)

Timdal (1984) used 25% sulfuric acid to dissolve calcium oxalate.

Hydrochloric Acid (HCl)

Some workers recommend hydrochloric acid instead of nitric acid or sulphuric acid.

ChloramineT

Thomson (1967) used a 5% solution of chloramineT in alcohol as a test for usnic acid (which gives a yellow reaction).

Combinations of several reagents

Some workers recommend various combinations of several reagents.

Potassium hydroxide & Hypochlorite solution

According to Taylor (Lich. of Ohio v. 1), not more than a few seconds should elapse between application of K and C

Nitric acid & Potassium hydroxide

Makarevich (1971) recommended examination of tissues first in nitric acid and then in potassium hydroxide.

METHODSMICROSCOPYMISC.

April 1988

Select a full grown but not large apothecium for sectioning. (Sipman 1983).

Soak the apothecia first in 90% ethanol to expell the air, then in tap water. Sipman (1983)

After making measurements and other observations in water and letting the sections air dry, wash away secondary metabolites, by adding and evaporating drops of acetone at room temperature. (Sipman 1983, following the second method described by Thomson 1967, p. 16). Use the acetone extracts for TLC, then resoak the sections in ethanol and then in tap water. Then transport the sections into lactophenol cotton blue.

Observe the semipermanent mounts made in lactophenol cotton blue after one day, and note changes (Sipman 1983).

Moisten the ascocarps with water containing a wetting agent (Harris, 1973; this may be necessary mainly for pyrenolichens).

Asci

Separate asci from paraphyses by gentle pressing with the points of a pair of forceps under the microscope with low magnification. (Sipman 1983; this may be feasible only with large asci).

Cut off about one third of the ascocarp and dissect out a portion of the hymenium, which then may be gently squashed on a slide without the interference of parts of the substrate or carbonized wall fragments (Harris 1973).

Soredia

Examine and measure soredia under a compound microscope at low magnification, with incumbent light and in airdried state. (Sipman 1983).

Crystals

The nature of the crystals of calcium oxalate can be proved by their solubility in concentrated acetic acid and/or diluted

hydrochloric acid. In the first they do not dissolve, in the second they do without effervescence. Experiments to remove the crystals before sectioning, to obtain better sections, are unsuccessful (Sipman 1983).

Stains and Mounting Media

Lugol's Iodine usually provides sufficient contrast to makedetails visible without chemically distorting them. The iodine can then be cleared by pulling 1020% KOH under the coverslip and drawing the iodine out the other side (Harris 1973).

Aqueous phloxine (12% in water) gives good contrast for seeing septation in spores and hyphae, and can be cleared with KOH, leaving only the protoplasm deeply stained (Harris 1973). Spore ornamentation can be seen by staining with acetic cotton blue (Chadefaud, 1969cited by Harris 1973) bu heating just to the boiling point over a flame.

Algae can be seen in endophloic thalli by mounting thin sections in lactophenolcotton blue and warming gently (Harris 1973); the algae will stain deep blue, while the bark cells will be relatively unaffected (Harris 1973). A mixture of aqueous phloxine and 1520% KOH can also be used for this purpose (Harris 1975).

Chlorazol Black (12 percent in water), mixed with KOH on a microscope slid immediately before use is the most satisfactory stain fro the chitinoid ring in the ascus tip of Porina and Trichothelium (Harris 1975).

Use orseillein in lactophenol cotton blue. Orseillin stains the cytoplasm red; when observing with a blue filter the cytoplasm appears dark bluish. Orseillin is a more satisfactory stain than cotton blue and should be used routinely (Anderson 1970).

METHODOLOGY

Descriptions of various techniques and refinements are scattered through a large number of books and articles on lichens or other organisms, and most workers have their own special methods acquired through much experience and trial and error. It is desirable to make a larger portion of this extremely diffused information available in a single place, with comparisons of alternative variations, and to contribute towards eventual standardization of some of the techniques.

EXAMINATION OF LICHENS THROUGH A DISSECTING SCOPE

Wetting the surface of the thallus will often make spermagonia easier to locate. It will also help to see the color and surface structure under pruina.

To examine the undersides of lobes or squamules and determine how they are attached to the rock, carefully detach and lift the structures using a probe needle.

MEASUREMENTS

Measurements given in mm are made under a binocular scope on dry material. Those given in um are made under a compound scope. The latter are usually made in water unless stated otherwise. Timdal (1984) made all microscopic measurements on material mounted in LCB.

Timdal (1984) generally based descriptions of the dimensions of spores or conidia on fifty or measurements (per species). It is desirable (though not always feasible) to measure at least 10 spores (or spermatia, etc.) on each specimen. The largest one and the smallest one should be sought; otherwise the search can be either systematic or haphazard (by moving from each measured one to the closest one to it?).

Measurements can be recorded or expressed in various ways. Timdal (1984) recorded the two extreme values to the nearest 0.5 um (0.5 mm could be used for features measured under the dissecting scope), and calculated the arithmetic mean to the nearest 0.1 um (or less precisely for structures only with mean dimensions under 1.5 um)

STUDYING SPORES

Do not assume that scattered spores belong to the species you are studying, unless similar ones occur in the asci. Also watch out for parasymbionts.

It is important to distinguish between mature and immature spores. In general, immature spores have granules and oil drops; mature ones often do not (Nearing, the Lichen Book).
When measuring thinwalled spores, it is important not to exert pressure on the coverslip, which can increase the spore lenght up to onethird more (Swinscow, freshwater Verrucaria).

MISCELLANEOUS METHODS

Eucortex and Pseudocortex can often be distinguished in rather thick sections if they are in KOH.

To see paraphyses or other tissues clearly, let the sections absorb cotton blue for several hours, then rinse with water (Hertel, pers. comm.).

SemiPermanent Slides

Semipermanent slides, which can be rewet whenever needed, have the advantage of being easy and inexpensive to prepare, and they do not distort hyphae and tissues the way that the permanent mounting media do (Poelt, pers. comm.).

Semipermanent slides can be prepared by simply glueing one or two edges of the coverslip to the slide, and then when a dry slide needs to be reexamined, simply putting water under the coverslip again. Poelt (pers. comm.) uses "UHU Alleskleber" for this purpose; various kinds of model cement or clear nail polish also work well.

Fixing Agents

According to McWhorter (1921), fungal elements fix well in chromacetic acid, while algal elements fix well in hot bichloride of mercury.

A solution of 7% formalin or F.A.A. will preserve lichens (Sass, 1951; recipie on card).

Epihymenium Chemistry

For determination of the actual chemical contents of the

epihymenium, Leuckert (pers. comm.) recommends moistening the apothecia, blotting with a paper towel, scraping with a fine scalpel (carefully avoiding getting the margin) and doing HPLC or (if large numbers of apothecia are available) TLC, on the scrapings.

Reagents

Ferrous (ic?) Chloride ($FeCl_3$)

TLC

Anisaldehyde in ethanol, mixed with sulfuric aciddetects usnic acids, which turn deep violet (Leuckert, pers. comm.).
When gyrophoric acid is present, at least traces of lecanoric acid are usually also present (Leuckert, pers. comm.).

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April 1988

(* = information incorporated into my files on wp)

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