IMPROVED CONDITIONS AND NEW DATA FOR THE IDENTIFICATION OF LICHEN PRODUCTS BY A STANDARDIZED THIN-LAYER CHROMATOGRAPHIC METHOD

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SUMMARY

A standardized method for the identification of compounds by thin-layer chromatography uses three solvent systems and R_F classes coded on punched cards. This method, applied to the study of secondary products of lichen-forming fungi, has been improved and expanded to include easily prepared hydrolysis and O-methylation products. The data reported allow the confirmation of many substances previously difficult to identify and the proposal of structures for certain types of new compounds extracted from fragments of herbarium specimens. New data are given for 220 compounds and derivatives chromatographed in three standard solvent systems.

INTRODUCTION

The standardized method for the identification of lichen products described in 1970[1] has been used successfully for routine analyses of lichen fragments for taxonomic and phytochemical studies[2-7]. For this method, unknown substances are chromatographed under controlled conditions in three standard solvent systems. The resultant spots are assigned to one of eight R_F classes, which are determined on each chromatogram by the R_F values of a control mixture of two common lichen products, atranorin and norstictic acid. Each compound will have a set of three R_F classes determined by its chromatographic behavior in the three standard solvent systems. The R_F classes are reproducible under considerably varying conditions and over a reasonably large range of actual R_F values. A deck of punched cards, keyed by R_F classes and containing a card for every compound that is potentially identifiable, is sorted to give all known substances that could possibly correspond to the unknown. The cards for the compounds have additional data that allow the initial possibilities to be narrowed. Although this method was developed for the identification of lichen products, the use of R_F classes, standard solvent systems and punched cards could be applied to any chromatographic problem involving the identification of a large and ever-increasing number of compounds.

Many of the lichen compounds are esters of phenolic acids and are easily hydrolyzed with concentrated sulfuric acid. This reaction has been used extensively for proofs of the structures of depsides and tridepsides and was adapted to a micro-

scale, paper chromatographic procedure by WACHTMEISTER[8]. Acidic hydrolysis permits the identification of a number of compounds that are difficult to distinguish. As the hydrolysis products chromatograph very well in the standard solvent systems used to identify the unhydrolyzed compounds, it is possible to assign R_F classes to these compounds and to incorporate punched cards for them directly into the original deck prepared for the unhydrolyzed lichen products. In the same way, cards can be included for O-methylated compounds, which are easily obtained on a small scale by using diazomethane. Finally, acidic hydrolysis of these O-methyl derivatives can provide further evidence of the identity of compounds as well as establish the order of linkage of phenolic acid units in simple depsides.

This paper describes improved conditions for the chromatography of crude lichen extracts and reviews useful methods for preparing hydrolysis products and O-methylated derivatives. Chromatographic data and R_F classes are given for 149 lichen products, 37 hydrolysis products of depsides, 24 O-methylated derivatives and 10 miscellaneous derivatives of non-depside lichen compounds determined by thin-layer chromatography (TLC) in the three standard solvent systems.

MATERIALS AND METHODS

Sources of compounds

Pure lichen substances were used whenever they were available. Otherwise, standards consisted of micro-extracts of herbarium specimens. Hydrolysis and O-methylation reactions were studied only for compounds that were available in a reasonably pure state and for some simple mixtures for which the results could be easily interpreted.

Chromatographic materials and solvent systems

Chromatograms are developed in standard Brinkman tanks to a height of 10 cm on Merck pre-coated Silica Gel F_{254} TLC plates (layer thickness 0.25 mm) that are stored in a desiccator over $CaCl_2$–NaOH but not activated. The plates are cut to a size of 20 × 12.5 cm, and the starting line is 2 cm from the bottom (20 cm) edge of the plate. With 230 ml of solvent in the tank, the distance from the solvent surface to the origin line is 1 cm.

The compositions of the standard solvent systems have been modified so as to give a greater spread of R_F values. The new solvent systems are: (A) benzene–dioxane–acetic acid (180:45:5, 230 ml); (B) hexane–diethyl ether–formic acid (130: 80:20, 230 ml); and toluene–acetic acid (200:30, 230 ml). The organic solvents were purified as previously described[1] but, within reasonable limits, the purity of the solvents does not affect the R_F classes observed for the compounds. The solvent systems were used repeatedly until the R_F values of the atranorin and norstictic acid controls drifted appreciably beyond the ranges observed on the chromatograms used for the original determinations of the R_F classes and of other data stored on punched cards.

In the present study, the tank containing solvent A had a filter-paper liner extending 8.5 cm above the level of the solvent, and plates were run with the SiO_2 layer facing the filter-paper. For solvents B and C, no paper was used, and the chromatographic layer faced the center of the tank. No more than one plate was run in

a tank at the same time. The development time was 45–55 min in all three solvent systems. All tanks were insulated from temperature fluctuations, which are the principal cause of uneven solvent fronts and fluctuating R_F values along the width of the plates. As the control mixture was chromatographed at three positions on every plate[1], it was easily determined that, under the present conditions, R_F values usually varied by less than two $R_F \times 100$ units along the width of any plate.

The chromatograms were viewed in short and long wave length UV light, sprayed with 10 % H_2SO_4 and heated to 110° for 15–30 min to make the spots of aromatic and alicyclic compounds visible. Spots of fatty acids were located by wetting the plates in water and observing opaque spots as the plates dried on the black surface of a slide-warming table.

The control mixture of atranorin and norstictic acid is obtained by extracting a fragment of *Parmelia perforata* (Jacq.) Ach., *P. acetabulum* (Neck.) Duby, or any other species that contains these compounds and is readily available. This acetone extract can be concentrated and conserved in a capped vial. Approximately equal amounts of the solution are applied to the control spots on each plate. The observable decomposition of the compounds in the solution is too slow to cause problems.

Microhydrolysis of pure compounds and of lichen extracts

Samples (0.1–0.3 mg) of pure compounds or crude lichen extracts are dissolved in concentrated H_2SO_4 (3–6 drops) in a shell vial, and the solution is cooled in a freezer for 15–30 min. Crushed ice is added to the vial and the hydrolysis products are extracted with diethyl ether. Three successive ethereal extracts are drawn off with a pipette and evaporated on a microscope slide. The residue is chromatographed in the three standard solvent systems, using controls of atranorin and norstictic acid to establish the R_F classes of the spots that separate. The unhydrolyzed compound or crude residue is run besides the hydrolyzed material to allow the identification of unhydrolyzed material, but usually hydrolysis is complete if care is taken to dissolve completely all the solid in the concentrated H_2SO_4 before the solution is placed in the freezer. 4-Hydroxy-2-methoxybenzoic acid derivatives are easily decarboxylated and this property may complicate the results. Decarboxylation reactions are minimized by using crushed ice rather than water to dilute the H_2SO_4 solutions before extraction with diethyl ether. The decarboxylation products chromatograph well, however, and punched cards can be included for them in the deck.

Chromatograms of hydrolyzed crude extracts must be interpreted with caution. When the compound of interest is mixed with a significant amount of other hydrolyzable phenolic materials, the substances should first be separated by preparative-layer or high-speed column chromatography[9]. When methyl esters of lichen depsides are hydrolyzed, complete hydrolysis of the depside ester linkage is usually accompanied by partial hydrolysis of the methyl ester linkage. To avoid confusion, the original depside, its methyl ester and the hydrolysis products of the free depside and of the depside methyl ester are chromatographed on the same plates.

O-Methylation of pure compounds and of lichen extracts

Compounds and crude residues (2–10 mg) were dissolved in diethyl ether (0.5–1 ml) in a small vial and treated by a drop-by-drop addition of an ethereal solution of diazomethane until a faint yellow color barely persisted. For simple

orcinol-type depsides, the free carboxylic acid is methylated immediately at room temperature or at 0°, a free 4-hydroxyl group is methylated in about 10 min at room temperature, and free 2- or 2'-hydroxyl groups are methylated only after prolonged treatment at room temperature. In the present study, only methylations of free carboxylic acids and free 4-hydroxyl groups were attempted on crude residues.

At the end of the reaction period, the ethereal solution was evaporated on a microscope slide and the residue was chromatographed. Owing to the extreme toxicity of diazomethane, all of these operations must be carried out in an efficient hood. Ethereal solutions that retain a yellow color or those that contain pigments that mask the color of the diazomethane are treated with a drop of acetic acid just before evaporation of the solvent. Compounds that are only slightly soluble in diethyl ether can be methylated in acetone–diethyl ether solutions.

RESULTS AND DISCUSSION

For the solvent systems used in the present study, increasing the concentration of solvent vapor in the atmosphere of the tank by lining the inside walls with filter-paper leads to decreased resolution and increased tailing of the spots, although the reproducibility of R_F values is increased. As the standardized method uses R_F classes determined on each plate by a control mixture, accurate reproducibility of actual R_F values is not required. For this reason, full advantage can be taken of the improved resolution obtained with lower solvent vapor concentrations. The conditions suggested in the present study allow several pairs of compounds to be distinguished that previously could not be separated by TLC.

Table I summarizes the results obtained under the improved conditions by TLC of many of the most common lichen products of known chemical structure and of a number of "unknown" substances that are easily detectable. The compounds are arranged in the table by R_F classes in the three solvent systems in the order A, then B, then C. Chromatographically similar compounds frequently occur close together in the list, but substances with corresponding R_F classes in solvents B and C and different R_F classes in solvent A are not grouped together in the table. In practice, it is frequently necessary to sort by R_F classes in only two solvent systems because spots may overlap or be indistinguishable in the third solvent. Compounds with the same R_F classes in solvents B and C can be sorted out at once with a deck of punched cards but not so readily with tables of R_F data.

All R_F values are quoted with the corresponding measurements to the norstictic acid and atranorin lines determined by the method previously described[1]. The ranges of R_F values observed for these two control substances is least in solvents A and C. Solvent B must be renewed frequently to maintain the $R_F \times 100$ value of norstictic acid within the range 26–34. Freshly prepared solvent gives higher $R_F \times 100$ values for norstictic acid for one or two chromatograms and then stabilizes at a value near 30. When the $R_F \times 100$ value of norstictic acid falls below 26, the solution is replaced. Similarly, the $R_F \times 100$ values of norstictic acid in solvents A and C are maintained in the ranges 37–44 and 27–34, respectively. These rather large fluctuations in R_F values are not a problem because the R_F classes of the substances being identified rarely change. Compounds that run close to the border between two classes and that

TABLE I

TLC DATA FOR 149 LICHEN PRODUCTS CHROMATOGRAPHED IN THREE SOLVENT SYSTEMS

R_F class			Compound	Example, $R_F \times 100$ values (R_F of X/R_F of N, R_F of A^a)		
A	B	C		A	B	C
1	1	1	Rhodocladonic acid	o	o	o
1	1	1	Consalazinic acid	2/39, 72	1/26, 76	0/27, 77
1	3	2	Fumarprotocetraric acid	1/41, 73	25/28, 75	7/29, 77
1-2	3	1	Unknown Q with hypoprotocetraric acid	3/42, 75	19/32, 77	2/31, 78
1-2	3	2	Protocetraric acid	3/40, 74	19/30, 77	4/28, 78
1-2	3	2	Sublimbatic acid	4/42, 72	23/34, 80	10/30, 79
1-2	3	2-3	Thamnolic acid	3/42, 76	25/32, 79	13/28, 79
1-2	3	3	Hypothamnolic acid	4/40, 75	25/30, 77	18/28, 79
1-2	4	1-2	Caperatic acid	4/41, 78	27/29, 76	6/34, 81
1-2	5	1-3	Unknown red pigment[b]	19/42, 73	38/28, 79	19/27, 78
2	1	1-2	Constictic acid	7/42, 77	3/34, 79	3/28, 80
2	1-2	1	Erythrin	6/41, 76	5/32, 79	1/29, 77
2	1-2	1-2	Unknown in Ochrolechia spp.	8/42, 73	4/27, 78	2/27, 78
2	2	1-2	Porphyrilic acid	9/41, 75	11/30, 75	6/30, 78
2	2	1-2	Unknown PQ-4[c]	15/43, 76	7/32, 77	4/29, 77
2	2	2	Unknown[d]	11/42, 73	5/28, 78	9/27, 78
2	2	2	Salazinic acid	10/39, 72	7/30, 74	4/28, 77
2	2	2	Connorstictic acid	11/39, 72	11/30, 74	2/28, 77
2	2	2	Variolaric acid	18/42, 76	13/30, 77	8/28, 80
2	2-3	2	Unknown S-2 with stictic acid	14/40, 73	14/26, 76	10/29, 79
2	3	3	Decarboxythamnolic acid	5/42, 76	25/33, 79	18/28, 79
2	3	3	Squamatic acid	10/42, 76	25/30, 74	25/29, 78
2	3-4	2	Unknown[e]	6/42, 76	27/30, 77	6/28, 80
2	3-4	2	Pannaric acid	8/42, 77	28/30, 74	9/28, 79
2	4	2	Endocrocin	8/40, 76	33/32, 79	8/29, 77
2	4	2	Echinocarpic acid	11/41, 77	33/32, 79	11/29, 78
2	4	2	Siphulin	14/42, 77	27/27, 77	4/29, 78
2	5	2	Pulvinic acid	4/42, 77	36/27, 76	7/29, 80
2	5	2	Barbatolic acid	7/43, 75	48/33, 77	19/31, 77
2	5	2	Conpsoromic acid	15/41, 75	36/30, 77	12/28, 80
2	5	2-3	Unknown with hypoprotocetraric acid[f]	16/41, 75	36/30, 77	14/27, 79
2	5	3	Physodalic acid	10/43, 77	31/27, 76	19/29, 80
2	5	3-4	Unknown with echinocarpic acid	18/41, 77	38/32, 79	27/29, 78
2	5	4	Haemathamnolic acid	15/40, 74	35/29, 77	27/28, 79
2-3	3-4	2-3	Unknown with protocetraric acid	19/42, 74	32/34, 82	14/28, 78
2-3	3-4	3	Unknown PO-2[g]	22/42, 73	25/27, 76	18/28, 78
2-3	4-5	2-3	Unknown dibenzofuran (?)[h]	22/42, 72	37/34, 80	18/30, 79
2-3	5	2-3	Diploschistesic acid	21/43, 75	39/32, 80	13/28, 79
1-3	1-2	3	Haemoventosin	33/42, 73	2/27, 78	17/27, 79
3	2-3	3	Galbinic acid	29/41, 75	12/29, 77	16/28, 79
3	2	3	Lepraric acid	27/40, 74	11/29, 77	21/28, 78
3	2	3	Stictic acid	32/42, 77	9/27, 77	18/28, 78
3	3	2	Arthoniaic acid	31/37, 73	15/28, 76	13/29, 79
3	3	2	Unknown PQ-2[e]	34/43, 76	27/32, 77	8/29, 77
3	3-4	5	Unknown in Lecanactis spp.	21/42, 76	32/33, 79	41/28, 79
3	4-5	2-3	Alectoronic acid	33/43, 75	34/33, 76	17/31, 77
3	5	3	Unknown PQ-3[e]	25/44, 76	42/32, 77	20/29, 77
3	5	3	Physodic acid	25/42, 74	35/27, 76	18/29, 79
3	5	3	Gyrophoric acid	24/40, 74	42/30, 77	24/28, 79
3	5	3	Hypoprotocetraric acid	25/40, 75	43/29, 77	22/28, 79
3	5	3	Unknown PO-1[g]	29/42, 73	32/27, 76	24/28, 78
3	5	3	Lecanoric acid	28/40, 75	44/30, 76	22/28, 79
3	5	3	Olivetoric acid	34/40, 76	37/28, 76	22/28, 78
3	5	4	Norobtusatic acid	27/40, 76	50/28, 76	30/28, 79

TABLE I (*continued*)

R_F class			Compound	Example, $R_F \times$ *100 values* (R_F of X/R_F of N, R_F of A[a])		
A	B	C		A	B	C
3	5	4–5	Lividic acid	32/40, 74	35/29, 77	31/28, 78
3	5	5	Rangiformic acid	29/41, 75	38/29, 76	33/28, 80
3	5	5	Lobaric acid	30/40, 75	46/29, 76	38/29, 80
3	5	5	Picrolichenic acid	38/43, 77	39/27, 76	36/29, 79
3	5	5	Protolichesterinic acid	35/40, 75	47/29, 76	37/28, 81
3	5	5	Psoromic acid	36/41, 73	41/27, 75	41/29, 76
3	5	5	4-O-Methylphysodic acid	37/41, 74	43/28, 76	40/27, 76
3	5–6	4	Alectorialic acid	39/43, 74	48/29, 75	30/28, 77
3	5–6	5	4-O-Methylgyrophoric acid	32/40, 75	56/28, 76	46/29, 80
3	6	5	Virensic acid	26/42, 76	57/30, 77	38/28, 80
3	6	5	Obtusatic acid	35/40, 76	64/29, 76	48/28, 78
3–4	3	3	Strepsilin	41/42, 78	21/27, 77	24/28, 78
3–4	5	5	Baeomycesic acid	39/42, 76	40/32, 79	42/28, 79
3–4	6	4–5	Anziaic acid	40/42, 76	59/29, 74	33/28, 76
3–4	6	5	Evernic acid	38/41, 76	61/32, 79	43/29, 77
3–4	6	5	Grayanic acid	38/40, 75	62/30, 77	44/28, 79
1–4	1–4	1–4	Polyporic acid	43/41, 73	28/27, 75	27/29, 76
4	2	2	Chromone (?) with lepraric acid	39/40, 74	4/29, 77	12/28, 79
4	4	4	Norstictic acid	—	—	—
4	4	4	4-O-Demethylplanaic acid	42/41, 74	32/31, 76	27/27, 76
4	5	4–5	Paludosic acid	39/41, 73	35/27, 75	33/30, 76
4	5	5	Glomelliferic acid	41/41, 73	43/28, 76	42/28, 77
4	5	5	α-Collatolic acid	40/41, 73	32/28, 75	35/28, 77
4	5	5	Miriquidic acid	41/42, 76	48/28, 76	45/29, 78
4	6	5	4-O-Demethylbarbatic acid	39/41, 77	60/32, 79	39/29, 78
4	6	5	Roccellic acid	42/41, 75	60/29, 77	48/28, 80
4	6	5	Colensoinic acid	40/41, 73	73/34, 79	42/28, 80
4	6	5–6	Sekikaic acid	45/42, 78	57/27, 77	51/28, 79
4	6	5–6	Diffractaic acid	44/42, 76	64/32, 80	51/28, 78
4	6	5–6	Barbatic acid	44/42, 77	69/27, 75	52/29, 78
4	6–7	5–6	Divaricatic acid	39/39, 73	75/32, 80	51/29, 78
4	6–7	5–6	Didymic acid	44/42, 75	77/32, 80	52/28, 78
4–5	3–4	5	Schizopeltic acid	42/37, 75	27/30, 74	51/32, 79
4–5	5	3	Rhodophyscin	44/42, 77	32/27, 76	23/29, 76
4–5	5	5	Microphyllinic acid	43/40, 76	35/28, 76	42/28, 79
4–5	6	5–6	Homosekikaic acid	47/42, 72	69/33, 79	56/29, 77
4–5	6–7	5–6	Imbricaric acid	43/42, 76	75/29, 77	48/27, 80
4–5	6–7	5–6	Stenosporic acid	45/42, 77	73/27, 77	54/29, 78
4–5	7–8	5–6	Perlatolic acid	44/43, 73	74/29, 73	52/28, 76
1–5	1–3	1–4	Entothein	47/43, 75	16/29, 76	25/29, 76
5	2–3	3	Yellow-green pigment SV-1[l]	48/43, 75	30/32, 76	21/30, 76
5	4	4–5	Unknown PQ-1[e]	50/43, 76	33/32, 77	32/29, 77
5	5	3	Unknown in *Sticta* spp.	47/42, 72	43/33, 79	24/30, 79
5	5	3	Norlobaridone	50/40, 76	36/28, 76	21/29, 79
5	5	5	2′-O-Methylanziaic acid	46/42, 73	40/28, 73	34/29, 76
5	5	5	Planaic acid	52/43, 73	38/28, 77	50/29, 79
5	5	5	Confluentic acid	54/43, 74	32/28, 76	48/29, 79
5	5	5	Zeorin	55/41, 76	42/30, 77	43/28, 80
5	5–6	5	Cryptochlorophaeic acid	46/42, 77	46/34, 79	37/28, 80
5	5–6	5–6	2′-O-Methylperlatolic acid	55/43, 74	50/28, 73	52/28, 76
5	6	5	Lichesterinic acid	44/41, 75	63/29, 77	43/28, 80
5	6	5	Thiophanic acid	55/41, 73	56/30, 80	52/29, 79
5	6	5	Merochlorophaeic acid	55/40, 75	56/28, 76	61/29, 80
5	6	5	Ergosterol	56/40, 76	66/32, 79	49/29, 77
5	6	5	Emodin	57/40, 77	61/32, 80	39/28, 77

TABLE I (*continued*)

R_F class			Compound	Example, $R_F \times 100$ values (R_F of X/R_F of N, R_F of A[a])		
A	B	C		A	B	C
5	6	5–6	2-O-Methylperlatolic acid	48/44, 75	60/28, 76	52/29, 79
5	6	5–6	Ursolic acid	59/42, 77	50/27, 77	52/29, 78
5	7	5–6	Sphaerophorin	48/42, 76	76/30, 77	54/29, 76
5–6	5	5	Unknown triterpene[j]	58/43, 75	34/29, 76	42/29, 76
5–6	5	5	Loxodin	58/40, 76	40/28, 76	35/28, 79
5–6	5	5–6	Pseudoplacodiolic acid	60/41, 75	52/34, 80	56/29, 78
5–6	6	5	Evans's substance H	54/40, 75	61/29, 76	48/28, 81
6	3	5	Acetylportentol	65/42, 76	29/34, 79	45/29, 80
6	5	5	Methyl gyrophorate	60/40, 75	44/28, 76	43/29, 80
6	5	5	Triterpene in *Physcia* spp.	64/42, 72	43/33, 80	44/30, 79
6	5	6	Gangaleoidin	64/40, 74	43/29, 77	54/28, 79
6	5	6	Rhizocarpic acid	73/43, 76	41/29, 76	66/29, 78
6	6	5	Ethyl orsellinate	62/41, 71	66/32, 79	44/29, 77
6	6	6	4-O-Methylcryptochlorophaeic acid	60/40, 75	61/28, 76	66/29, 80
6	6	5	Unknown triterpene (?)[k]	67/42, 72	53/33, 79	46/30, 78
6	6	6	Placodiolic acid	67/42, 76	63/26, 77	67/27, 80
6	6	6	Thiophaninic acid	65/40, 73	68/30, 80	65/29, 77
6	6	6	Scrobiculin	69/42, 78	55/27, 77	52/28, 79
6	6	6	Methyl 3,5-dichlorolecanorate	67/40, 75	62/28, 76	66/29, 80
6	6	6	Usnic acid	70/42, 76	70/26, 77	71/27, 79
6	6–7	6	Diploicin	64/41, 76	79/32, 80	66/28, 78
6–7	5	6	Nephroarctin	69/42, 73	53/28, 79	66/27, 78
6–7	5–6	6	Epanorin	74/40, 76	52/32, 79	66/29, 77
6–7	7	6	Vicanicin	73/42, 75	75/30, 77	70/28, 80
6–7	7–8	6–7	Fragilin	73/41, 76	82/32, 79	72/29, 77
7	6	7	Pinastric acid	72/41, 73	57/28, 76	76/27, 76
7	6	7	Pannarin	73/41, 73	68/27, 75	76/27, 76
7	6	7	Leprapinic acid	74/40, 75	59/29, 77	81/28, 79
7	6	7	Lichexanthone	74/40, 74	72/29, 77	78/28, 78
7	6	7	Tenuiorin	77/42, 77	58/27, 77	76/28, 79
7	6	7–8	Parietin	74/41, 73	72/28, 76	77/27, 76
7	6	7–8	Unknown with pannarin[l]	72/38, 73	69/33, 78	75/28, 77
7	6	8	Vulpinic acid	76/42, 77	66/27, 77	82/29, 78
7	7	7	Atranorin	—	—	—
7	7	7	Chloroatranorin	76/42, 77	79/34, 79	80/27, 80
7	7–8	8	Calycin	79/42, 77	81/34, 79	88/27, 80
7	8	8	Pink pigment in *Asahinea* spp.	76/41, 76	84/33, 79	86/30, 78
7	8	8	Pink pigment[m]	73/42, 72	86/34, 80	87/29, 78
7–8	6	6–7	Unknown CS-1[n]	77/41, 75	51/29, 77	76/28, 80
8	8	8	Pulvinic dilactone	77/41, 74	82/28, 75	89/27, 76

[a] The two numbers following the oblique (/) are measurements in millimeters to the norstictic acid line (R_F of N) and the atranorin line (R_F of A) (ref. 2).
[b] In *Melanotheca cruenta* (Mont.) Müll. Arg.
[c] In *Parmelia quintaria* Hale.
[d] In *Parmelia chalybaeizans* (B. Stein. & Zahlbr.) Gyeln.
[e] In *Alectoria virens* Tayl.
[f] In *Parmelia weberi* Hale.
[g] In *Pertusaria panyrga* (Ach.) Mass.
[h] In *Lepraria* spp.
[i] A common accessory pigment in lichens.
[j] In *Dirinaria aegialita* (Ach.) B. Moore.
[k] In *Pyxine caesiopruinosa* (Nyl.) Imsh.
[l] In *Lecanora* spp.
[m] In *Solenopsora candicans* (Dicks.) J. Stein.
[n] In *Cladonia subcariosa* Nyl.

might consequently change in R_F class are keyed on the punched cards for both possibilities.

Comparison of R_F values that are quoted with respect to the corresponding values of norstictic acid and atranorin can be made with considerable confidence. The R_F values of norstictic acid are more important for comparisons of low-R_F compounds and the behavior of atranorin is more important for comparisons of high-R_F compounds, but both controls are always quoted because this gives a better picture of the total character of the chromatograms involved.

TABLE II

TLC DATA FOR 37 HYDROLYSIS PRODUCTS OF NATURALLY OCCURRING DEPSIDES AND THEIR O-METHYLATED DERIVATIVES

R_F class			substitution						Example, R_F × 100 values (R_F of X/R_F of N, R_F of A[a])		
A	B	C	1	2	3	4	5	6	A	B	C
1-2	2	2	COOH	HO	COOH	CH₃O	H	CH₃	3/43, 75	11/29, 75	7/29,
2-3	4-5	3	COOH	HO	HO	CH₃O	H	C₃H₇	26/38, 73	32/30, 75	22/29,
2-3	6	5	COOH	HO	Cl	HO	Cl	CH₃	20/42, 73	60/27, 76	31/29,
3	3	2	COOH	CH₃O	H	HO	H	CH₂COC₅H₁₁	24/44, 76	21/28, 76	7/29,
3	3	2-3	COOH	CH₃O	H	HO	H	CH₃	27/40, 75	22/29, 77	13/27,
3	4	3	COOH	CH₃O	H	HO	H	C₃H₇	33/40, 74	29/29, 77	20/28,
3	5	3	COOH	HO	HO	HO	H	C₅H₁₁	32/40, 76	49/34, 77	23/29,
3-4	5	3-4	COOH	CH₃O	H	HO	H	C₅H₁₁	40/42, 73	31/28, 77	27/29,
3-4	5	3-4	COOH	HO	H	HO	H	CH₃	40/43, 74	49/30, 77	28/29,
4	4-5	5	COOH	CH₃O	H	CH₃O	H	CH₃	41/40, 75	32/29, 77	44/29,
4	5	3	H	HO	H	HO	H	CH₃	42/41, 76	37/28, 76	17/28
4	5	5	COOH	CH₃O	CH₃	CH₃O	H	CH₃	45/41, 75	48/30, 78	50/29
4	6	4	COOH	HO	H	HO	H	C₃H₇	41/40, 76	58/33, 77	32/29
4-5	6-7	5-6	COOH	HO	H	CH₃O	H	CH₂CH₂COC₃H₅	45/42, 74	74/27, 76	53/29
5	4-5	4	COOCH₃	CH₃O	H	HO	H	CH₃	48/40, 75	31/29, 77	28/29
5	5	5	COOH	CH₃O	H	CH₃O	H	C₃H₇	47/40, 74	41/29, 77	50/29
5	5	5	COOCH₃	HO	HO	HO	H	C₃H₇	51/42, 75	48/29, 76	35/29
5	5	5	COOCH₃	HO	CH₃O	HO	H	C₃H₇	56/42, 75	37/29, 76	45/29
5	5	5	COOCH₃	CH₃O	H	HO	H	C₃H₇	55/40, 74	40/30, 75	35/29
5	5	5-6	COOH	CH₃O	H	CH₃O	H	C₅H₁₁	51/40, 75	46/29, 77	53/29
5	6	4-5	COOH	HO	H	HO	H	C₅H₁₁	45/42, 74	58/27, 76	35/29
5	6	5	COOH	HO	CH₃	HO	H	CH₃	45/42, 73	60/30, 74	42/29
5	6	5	COOH	HO	H	HO	H	C₇H₁₅	44/39, 75	68/33, 77	38/29
5	6	6	COOH	HO	H	CH₃O	H	CH₃	48/43, 74	70/33, 77	56/29
5	7	6	COOH	HO	CH₃	CH₃O	H	CH₃	52/42, 73	75/29, 75	60/29
5	7	6	COOH	HO	H	CH₃O	H	C₃H₇	50/39, 75	75/30, 75	60/29
5-6	5	5	COOCH₃	CH₃O	H	HO	H	C₅H₁₁	56/40, 75	43/29, 77	36/27
5-6	5-6	5	COOH	HO	H	HO	H	CH₂COC₅H₁₁	59/41, 77	54/28, 76	39/30
5-6	7-8	6	COOH	HO	H	CH₃O	H	C₅H₁₁	56/42, 74	78/27, 76	60/29
6	5	3	COOCH₃	CH₃O	HO	CH₃O	H	C₃H₇	62/43, 75	38/29, 76	46/29
6	6	5	COOCH₃	HO	H	HO	H	CH₃	58/39, 73	60/29, 76	45/32
6	6	5	COOCH₃	HO	H	HO	H	C₃H₇	61/41, 75	65/27, 75	45/29
6	6	5	COOCH₃	HO	CH₃	HO	H	CH₃	64/41, 75	70/30, 78	52/27
6	6	5-6	H	CH₃O	H	HO	H	C₅H₁₁	65/42, 74	70/27, 76	53/29
6	6	6	COOCH₃	HO	H	HO	H	C₅H₁₁	64/42, 73	69/27, 76	48/29
6	6-7	5	COOCH₃	HO	H	HO	H	C₇H₁₅	67/42, 75	76/27, 78	48/28
7-8	6-7	7-8	COOH	HO	H	CH₃O	H	CH₂COC₅H₁₁	77/40, 76	71/34, 72	78/29

[a] See footnote to Table I.

Table II gives data for the hydrolysis products of many of the commonest lichen depsides. These data are useful for identifying a number of substances that give very close R_F values. The best example is the series of compounds divaricatic acid (I), imbricaric acid (II), stenosporic acid (III), sphaerophorin (IV) and perlatolic acid (V). The R_F values of these compounds increase slightly as the total length of the side-chains on the rings increases, but the R_F values of compounds having the same total number of carbon atoms in the side-chains (*i.e.*, imbricaric acid and stenosporic acid) are virtually identical under the conditions used here. When these compounds

$$CH_3O-\underset{OH}{\overset{R}{\bigcirc}}-COO-\underset{R'}{\overset{OH}{\bigcirc}}-COOH$$
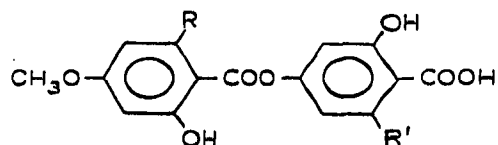
(I) R = R' = C_3H_7
(II) R = C_5H_{11}; R' = C_3H_7
(III) R = C_3H_7; R' = C_5H_{11}
(IV) R = CH_3; R' = C_7H_{15}
(V) R = R' = C_5H_{11}

are hydrolyzed, however, the products are readily distinguishable. For the analysis of crude extracts, it is possible to detect mixtures of such compounds if the proportions of the components are reasonably similar. Naturally occurring mixtures of

TABLE III

TLC DATA FOR 24 O-METHYLATED DERIVATIVES OF LICHEN PRODUCTS

R_F class			Compound	Example, $R_F \times 100$ values (R_F of X/R_F of N, R_F of A[a])		
A	B	C		A	B	C
5	5	5	Methyl hypoprotocetrarate	57/42, 75	48/33, 78	41/30, 79
5–6	5–6	5	Methyl lecanorate	61/43, 75	49/30, 75	42/29, 77
6	4	6	Methyl schizopeltate	68/40, 73	33/32, 79	56/28, 77
6	4–5	6	Methyl 2,2'-di-O-methylevernate	71/42, 76	33/31, 80	56/28, 79
6	5	5	Methyl physodate	60/39, 72	38/27, 77	38/27, 77
6	5	5	Methyl lividate	62/39, 72	33/27, 77	48/27, 77
6	5	5	Dimethyl caperatate	71/43, 75	40/30, 75	49/29, 77
6	6	5–6	Methyl 4-O-demethylbarbatate	68/44, 76	65/29, 77	53/28, 77
6	6	6	Methyl norobtusatate	65/44, 75	57/29, 77	52/29, 78
7	5	6	Methyl 2',4-di-O-methylhypoprotocetrarate	73/42, 75	47/27, 77	64/30, 79
7	5	6	Methyl 4-O-methylphysodate	72/39, 72	47/26, 76	64/27, 77
7	5	6	Methyl 2,2',4-tri-O-methyldivaricatate	76/42, 76	43/31, 80	64/29, 79
7	6	6–7	Methyl diffractaiate	76/41, 75	70/30, 78	77/27, 79
7	6	7	Methyl sekikaiate	76/43, 75	65/29, 76	76/30, 78
7	6	7	Methyl evernate	73/40, 73	72/32, 79	76/28, 77
7	6	7	Methyl 2-O-methylperlatolate	75/39, 73	74/32, 79	76/28, 78
7	7	8	Methyl divaricatate	77/43, 76	77/30, 76	80/30, 78
7	8	8	Methyl imbricarate	77/42, 76	80/26, 76	84/27, 80
7–8	5	6	Methyl planaiate	78/42, 76	50/29, 77	68/28, 78
7–8	6	7	Methyl 2'-O-methylperlatolate	76/40, 73	74/32, 79	75/28, 77
7–8	7–8	8	Methyl barbatate	78/44, 76	80/29, 77	84/29, 77
7–8	8	8	Methyl stenosporate	77/43, 75	80/30, 76	83/30, 78
7–8	8	8	Methyl sphaerophorate	78/42, 76	81/27, 77	85/27, 80
8	8	8	Methyl perlatolate	79/43, 76	82/30, 76	85/30, 78

[a] See footnote to Table I.

TABLE IV

TLC DATA FOR MISCELLANEOUS DERIVATIVES OBTAINED BY CATALYTIC REDUCTION, DECARBOXYLATION OR HYDROLYSIS OF SOME REPRESENTATIVE NON-DEPSIDE LICHEN PRODUCTS

R_F class			Compound	Example, $R_F \times 100$ values (R_F of X/R_F of N, R_F of A[a])			Parent compound type
A	B	C		A	B	C	
I	3	1	Hydrolyzed caperatic acid (= norcaperatic acid)	0/42, 72	26/33, 79	1/30, 79	Fatty acid ester
2	5	2-3	Hydrolyzed hypoprotocetraric acid	15/40, 76	33/30, 77	14/31, 82	Depsidone
3	3	3	Hydrolyzed lividic acid	27/40, 76	27/30, 78	22/30, 81	Depsidone
3	3	4	Hydrolyzed schizopeltic acid (= di-O-methylpannaric acid)	22/43, 75	18/29, 75	32/29, 77	Dibenzofuran ester
3	4	3	Reduced psoromic acid (= hypopsoromic acid)	25/42, 74	32/34, 82	17/28, 79	Depsidone aldehyde
3	4-5	3	Decarboxylated porphyrilic acid (= porphyrilin)	37/42, 72	35/34, 80	21/30, 79	Dibenzofuran carboxylic acid
3	5	3	Hydrolyzed physodic acid	23/41, 76	37/34, 77	10/30, 76	Depsidone
3	5	2	Hydrolyzed lobaric acid	28/40, 76	35/29, 78	27/30, 81	Depsidone
3	5	3	Hydrolyzed grayanic acid	30/40, 76	43/29, 78	33/30, 81	Depsidone
3-4	5	5	Hydrolyzed colensoinic acid	38/40, 76	57/30, 78	36/30, 81	Depsidone

[a] See footnote to Table I.

stenosporic acid with perlatolic acid, imbricaric acid with perlatolic acid, sekikaic acid with homosekikaic acid, and cryptochlorophaeic acid with paludosic acid have been detected by microhydrolysis.

Table III gives chromatographic data for a number of O-methyl derivatives of lichen compounds. The R_F values tend to be high in the standard solvent systems, which are better suited to the more polar parent substances. Many of these O-methyl derivatives chromatograph well and with lower R_F values in solvent systems of toluene or hexane–diethyl ether (9:1), where the organic acid (acetic or formic) used in the three standard solvent systems to prevent tailing of phenolic acid lichen products is omitted. In addition to the substances listed in Table III, methyl gyrophorate and methyl 4-O-methylgyrophorate, which are obtainable from gyrophoric acid by reaction with diazomethane, are known to occur naturally in lichens and are included in Table I.

Table IV lists data for some derivatives of non-depside lichen products. Acidic hydrolysis of orcinol-type depsidones gives a single compound of lower R_F value, but starting material is commonly observed in the product. β-Orcinol depsidones are more difficult to hydrolyze and only highly reduced derivatives, such as hypoprotocetraric acid, gave satisfactory results. The aldehydic depsidones protocetraric acid, norstictic acid and salazinic acid were converted to slightly soluble pigments. Other reactions (Table IV) that are easily adapted for micro-scale analysis of extracts include (1) basic hydrolysis[10] of methyl esters, e.g., schizopeltic and caperatic acids; (2) catalytic reduction[11], e.g., psoromic acid to hypopsoromic acid and protocetraric acid derivatives to hypoprotocetraric acid (Table I); and (3) decarboxylation[12], e.g., porphyrilic acid to porphyrilin.

The method of making the spots visible by spraying the plates with 10% H_2SO_4 and heating them at 110° for 15–30 min is the most general and satisfactory method for routine tests on lichen extracts. Other reagents are useful for particular applications. For example, the sensitivity of this H_2SO_4 spray for the detection of phenolic substances and their O-methyl derivatives is very good, but not so good as the diazonium sprays. These reagents may be useful when (1) trace amounts of phenolic substances must be detected, (2) overlapping or streaking non-phenolic products interfere with the interpretation of phenolic compounds on the chromatogram, or (3) sensitive detection is required for certain aromatic compounds that give pale colors with H_2SO_4.

The colors developed with H_2SO_4 are correlated with the chemical structure of the compounds, and these colors can be helpful in making identifications. Table V summarizes the colors produced for some common types of compounds with specific examples from Table I.

The general method described here could be used for any series of compounds detectable by TLC in more than one solvent system. Naturally, not all compounds to be identified will give ideally round spots and good R_F values in all solvent systems used in such a standardized system, but the method does not require consistently ideal behavior. In fact, some identifications are aided by the knowledge of which of the solvents are "good" for a particular compound. Also, it is not necessary that the control substances for determining R_F classes in the different solvent systems be the same. Any compounds with suitably different R_F values are satisfactory, and if colored substances were chosen for the controls, the plates could be developed until

TABLE V

SPOT COLORS FOR PROMINENT COMPOUND TYPES ON PLATES SPRAYED WITH 10% H_2SO_4 AND
HEATED TO 110° FOR 15–30 min

Color	F_{254}	Compound type	Examples from Table I
Colorless, faint brown or gray	—	Fatty acids	Caperatic acid Lichesterinic acid Protolichesterinic acid Rangiformic acid Roccellic acid
Brown, pink or yellow-brown	—	Alicyclics	Acetylportentol Ursolic acid Zeorin
Deep yellow (± gray rim)	+	Orcinol para-depsides and tri-depsides with reduced side-chains or a 2-keto side-chain on one ring	Anziaic acid Confluentic acid Divaricatic acid Erythrin Evernic acid Glomelliferic acid Gyrophoric acid
Pale green or pale yellow-green	+	Orcinol depsidones with 1-keto or 2-keto A-ring side-chain and reduced B-ring side-chain[a]	Lobaric acid 4-O-Methylphysodic aci Loxodin Norlobaridone Physodic acid
Pale pink	+	Orcinol para-depsides and depsidones with 2-keto side-chains on both rings	Alectoronic acid Arthoniaic acid α-Collatolic acid Microphyllinic acid
Reddish	+	Orcinol depsidones with reduced side-chains	Colensoinic acid Grayanic acid
Brown, brown-purple or purple	+	Orcinol meta-depsides[b]	Cryptochlorophaeic acid Homosekikaic acid Merochlorophaeic acid Paludosic acid Scrobiculin Sekikaic acid
Yellow or lemon yellow (± gray rim)	+	β-Orcinol para-depsides	Atranorin Baeomycesic acid Chloroatranorin 4-O-Demethylbarbatic acid Diffractaic acid Squamatic acid
Uniform dark gray or black	+	β-Orcinol depsidones related to protocetraric acid[c]	Fumarprotocetraric aci Physodalic acid Protocetraric acid
Bright yellow, orange or red	+	β-Orcinol depsidones related to norstictic acid[d]	Constictic acid Norstictic acid Salazinic acid Stictic acid
Blue or blue-gray	+	Dibenzofurans related to pannaric acid	Pannaric acid Porphyrilic acid Schizopeltic acid

[a] The depsone picrolichenic acid gives a similar color.
[b] Similar colors are produced by the benzyl ester alectorialic acid, the β-orcinol meta-depside thamnolic acid and the β-orcinol depsidone psoromic acid.
[c] Similar colors are produced by the chlorinated depsidone pannarin and the β-orcinol depsidone virensic acid.
[d] Several compounds of unknown structure, e.g. connorstictic, consalazinic, echinocarpic and galbinic acids, give similar colors and are probably related chemically to norstictic acid.

the control spots reached a pre-determined height on the plate rather than running the solvent front to a standard height.

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