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A STANDARDIZED TWO-DIMENSIONAL THIN-LAYER CHROMATO-GRAPHIC METHOD FOR LICHEN PRODUCTS

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SUMMARY

Two-dimensional thin-layer chromatography is useful for microchemical studies on mixtures difficult to resolve by the standardized one-dimensional thin-layer chromatographic method now commonly used for lichen products. A modified two-dimensional technique uses the large body of standardized R_F data already accumulated for these compounds. In addition, correlations of R_F values with chemical structures permit tentative identifications of many trace constituents, including new natural products, resolved from microextracts by the two-dimensional method. The standardized two-dimensional procedure also allows more reliable comparisons of chromatograms and the determination of R_F classes of components of complex mixtures. The method is illustrated for the orcinol-type depsides of two closely related species, *Parmelia loxodes* and *P. verruculifera*.

INTRODUCTION

The relative ease with which most characteristic lichen products can be identified by a commonly used standardized thin-layer chromatographic (TLC) method[1–3] has allowed the identification of major constituents of many species. In addition, TLC can resolve the previously unstudiable minor constituents, and these lesser products may enable the prediction of the total chemical variation within species groups[4]. Consequently, we need a simple TLC method for identifying minor constituents with something of the ease and reliability with which we now survey major products.

Recently Maass[5–8] successfully used two-dimensional TLC to study several species of Stictaceae. It would appear that labile lichen products did not decompose by this method as might have been suspected. Indeed, his results are so impressive that the two-dimensional method should receive wide application in studying chemical variation in lichens.

The principal advantage of two-dimensional chromatography is its ability to resolve compounds poorly separated by one-dimensional methods. The principal disadvantages are (1) that samples cannot be compared on the same chromatogram except by co-chromatography, (2) that artifacts can be introduced if labile compounds decompose before chromatography in the second dimension, and (3) that each anal-

ysis requires an entire plate and is thus more time-consuming and expensive. The present study was undertaken to overcome, insofar as possible, these disadvantages and to relate the one-dimensional standardized method for surveying major products in many individual samples and the two-dimensional method for detecting the associated minor constituents in representative individuals. In addition to its use in identifying known constituents, the method can give tentative structural information for previously unknown natural products. A modified two-dimensional technique that can be correlated with the standardized TLC method is illustrated here by chromatograms of extracts of *Parmelia loxodes* Nyl. and *P. verruculifera* Nyl.

EXPERIMENTAL

Lichen samples and chromatographic materials

The sample of *Parmelia verruculifera* was collected from conglomerate rock in the Svratka River Valley near Veverská Bitýška (alt. 300 m), Czechoslovakia, by Antonín Vězda. The sample of *P. loxodes* was collected from rock at Mosterhavn, Norway, by Johan Havaas. The fragments were extracted first with toluene at room temperature and then with warm acetone.

Two-dimensional chromatograms were prepared on Merck analytical-layer (0.25 mm thick) silica gel 60 F-254 plates (Catalog No. 5765) trimmed to 16.5 cm square. The standard solvent systems[1–3] referred to in this report are: (A) toluene–dioxane–acetic acid (180:45:5, v/v/v); (B) hexane–diethyl ether–formic acid (120:90: 20, v/v/v); and (C) toluene–acetic acid (200:30, v/v).

Procedure for the standardized two-dimensional chromatographic method

For chromatography in the first direction the solution to be analyzed is spotted at position S-1 (Fig. 1) and at position S-1 (1). A comparison sample selected as useful to the analysis is spotted at position S-2 (1). The atranorin and norstictic acid control solution, which defines R_F classes and forms the basis of the standardized one-dimensional method, is spotted at position AN (1). Chromatograms developed in the first direction to a height of 10 cm in one of the three standard solvent systems are dried in a hood with a moderate stream of air for 5 min and viewed under UV light by the same procedure used for the standardized one-dimensional method. The points of maximum concentration of all spots are dotted in pencil, and spots resolved from position S-1 are outlined as well. Chromatograms are next spotted at positions S-1(2), S-2(2), and AN(2) (Fig. 1) with the same samples originally chromatographed and then run in the second direction (to 10 cm) in a different standard solvent system. The finished chromatograms are dried as before and viewed under UV light. Aliphatic compounds not detectable with UV light are marked while the damp, freshly sprayed (10% sulfuric acid) plates dry slowly on a slide warmer. Spots are visualized by charring at 110° for 30 min and outlined in pencil. Trace spots are more easily detected if the plate is illuminated from below. Xerox copies of chromatograms can then be superimposed for comparison, aligning the spots of atranorin and norstictic acid chromatographed on every plate at positons AN(1) and AN(2).

In all respects we tried to duplicate the conditions of the standardized one-dimensional method[1–3] so that the results would be comparable. We found, however, that the first solvent can alter the properties of the silica gel layer and consequently
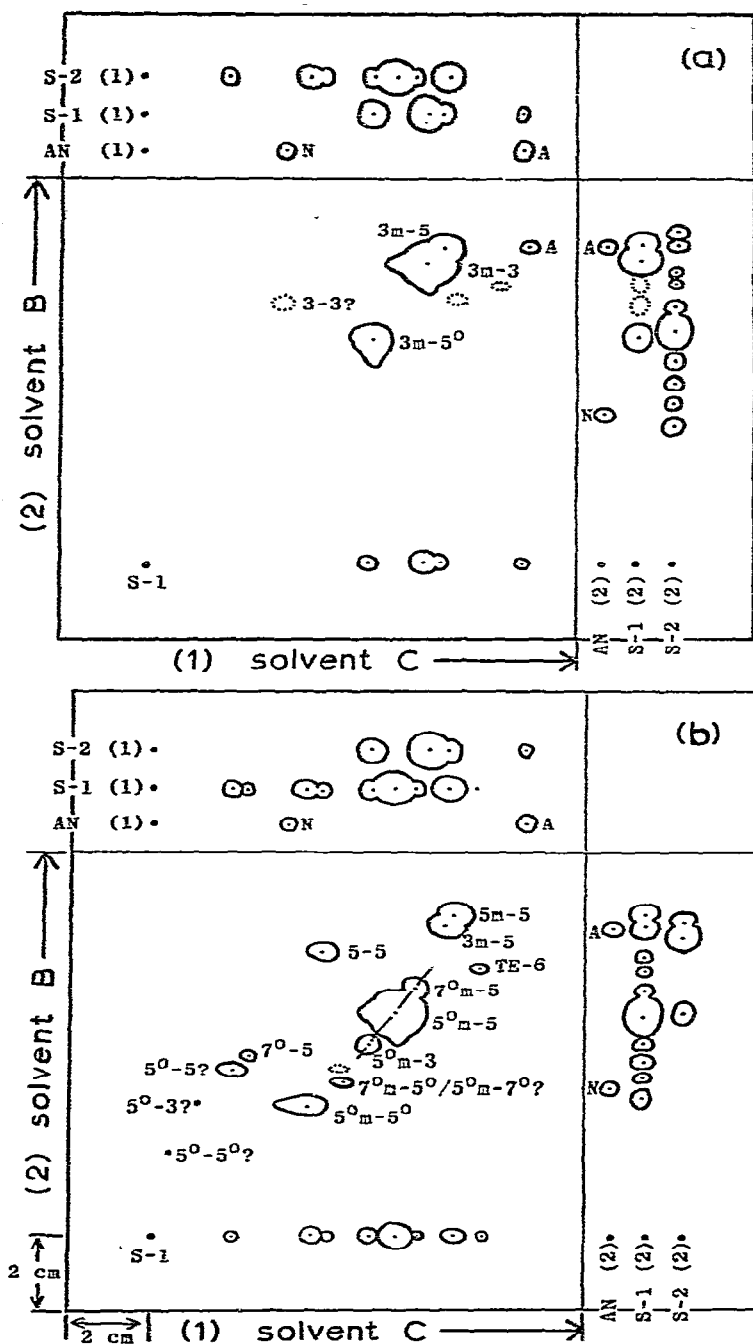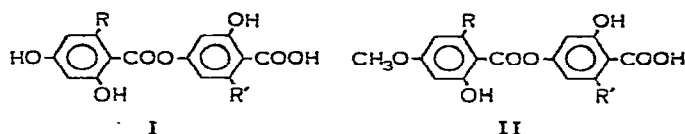
Fig. 1. Standardized two-dimensional TLC of lichen extracts including one-dimensional chromatograms of the sample (S-1), a comparison sample (S-2), and the atranorin (A) and norstictic acid (N) control sample that are run on the same plate. (a), Toluene extract of *P. verruculifera*; (b), warm acetone extract of *P. loxodes* previously extracted with toluene at room temperature. See Table I for the structures of the orcinol-type depsides.

also the R_F values for some compounds in the second solvent system. Such R_F changes are usually obvious when the two-dimensional portion of the plate is compared with the one-dimensional portions. Some R_F changes, caused by solvent or moisture retained on the plate, vary with drying time. R_F changes are more pronounced when solvent B is used for the first direction. They are also greater for certain types of compounds than for others. None of the R_F values[2] for substances (Table I) identified in *P. loxodes* and *P. verruculifera* were seriously altered when chromatographed first in solvent C and then in solvent B. The very slight increase in R_F values in solvent B of certain spots relative to that of atranorin was not observed if the usual formic acid pre-equilibration step[3] was eliminated.

TABLE I

KNOWN AND TENTATIVE (*) CHEMICAL STRUCTURES OF SOME ORCINOL-TYPE DEPSIDES DETECTED IN MICROEXTRACTS OF *PARMELIA LOXODES* AND *P. VERRUCULIFERA*

Compound	Chemical structure			Symbol
	Formula	R	R'	
Anziaic acid	I	C_5H_{11}	C_5H_{11}	5-5
4-O-Demethyldivaricatic acid[10,]*	I	C_3H_7	C_3H_7	3-3
4-O-Demethylglomellic acid*	I	$CH_2COC_3H_7$	$CH_2COC_3H_7$	5°-5°
4-O-Demethylglomelliferic acid*	I	$CH_2COC_3H_7$	C_5H_{11}	5°-5
4-O-Demethylloxodellic acid*	I	$CH_2COC_3H_7$	C_3H_7	5°-3
Divaricatic acid	II	C_3H_7	C_3H_7	3m-3
Glomellic acid	II	$CH_2COC_3H_7$	$CH_2COC_3H_7$	5°m-5°
Glomelliferic acid	II	$CH_2COC_3H_7$	C_5H_{11}	5°m-5
Loxodellic acid[9]	II	$CH_2COC_3H_7$	C_3H_7	5°m-3
4-O-Methylolivetoric acid[9]	II	$CH_2COC_5H_{11}$	C_5H_{11}	7°m-5
Olivetoric acid	I	$CH_2COC_5H_{11}$	C_5H_{11}	7°-5
Oxostenosporic acid[10]	II	C_3H_7	$CH_2COC_3H_7$	3m-5°
Perlatolic acid	II	C_5H_{11}	C_5H_{11}	5m-5
Stenosporic acid	II	C_3H_7	C_5H_{11}	3m-5

Complications that might arise from hydrolysis of labile ester linkages and oxidation of phenolic compounds prior to chromatography in the second direction were not detected. Such artifacts, however, must be identified if they occur. The incorporation of one-dimensional chromatograms on every two-dimensional plate substantially improves our confidence in the two-dimensional analyses.

RESULTS AND DISCUSSION

Fig. 1a illustrates the separation of stenosporic acid (3m-5) (symbolic notation: see Table I), divaricatic acid (3m-3), oxostenosporic acid (3m-5°), a compound tenta-

tively identified as 4-O-demethyldivaricatic acid (3-3), atranorin, and traces of two unidentified substances from the toluene extract of *P. verruculifera*. For the separation of these components and of related compounds in *P. loxodes*, the choice of solvent C for the first dimension and solvent B for the second dimension is based upon two considerations: (1) solvents B and C separate homologous depsides better than solvent A as determined directly from the R_F tables for the one-dimensional standardized method[2]; (2) the order of the solvents is dictated in the present study by the fact that artifacts are observed more frequently when solvent B is used for the first dimension. Although artifacts may aid the identification of some compounds, they introduce an unnecessary complication here.

The modified two-dimensional method can be used to determine the R_F classes of spots in complex mixtures. The R_F tables[2] for the standardized one-dimensional method can then be consulted for all known substances that could possibly correspond to the unknown compound. For example, in solvent B the spot for anziaic acid (5-5) (Fig. 1b) in R_F class 6 is somewhat below that of stenosporic acid (3m-5). In solvent C it is in R_F class 5, just slightly above that of glomellic acid (5°m-5°). One-dimensional chromatograms of the crude extract are too complex to reveal these correlations, which are easily determined by the standardized two-dimensional method. Similar two-dimensional chromatograms involving solvent A in one direction can then establish R_F classes in this solvent as well.

The positions of the spots on the two-dimensional portions of the chromatograms (Fig. 1) correlate with the chemical structures of the compounds in the following ways:

(1) The spots of homologous compounds lie on straight lines. The series 4-O-methylolivetoric acid (7°m-5), glomelliferic acid (5°m-5), and loxodellic acid (5°m-3) (Fig. 1b) is an example in which each member differs from the next higher one by two methylene groups. A second homologous series is illustrated (Fig. 2) by superimposing the chromatograms of *P. verruculifera* and *P. loxodes* with the control spots of atranorin and norstictic acid aligned. On the resultant composite chromatogram a line passes through the centers of the spots for perlatolic acid (5m-5), stenosporic acid (3m-5), and divaricatic acid (3m-3). This relationship was confirmed by co-chromatography.

(2) The spots for homologous compounds are displaced along the line for that series by a distance approximately proportional to the difference in the number of carbon atoms in the side chains of the aromatic rings. The distances between spots vary with R_F value so that higher spots are less well separated than lower ones, *e.g.*, the separation of perlatolic acid (5m-5) from stenosporic acid (3m-5) is slightly less than that of stenosporic acid from divaricatic acid (3m-3) and much less than that of glomelliferic acid (5°m-5) from loxodellic acid (5°m-3).

(3) The lines for each homologous series have different slopes. The values of these slopes are too variable to aid in identifications, but the fact that they differ helps to locate those spots belonging to the same homologous series.

(4) The R_F values for different homologous series with the same O-methylation patterns increase as the number of oxidized side chains decrease. Thus the R_F values are lowest for the glomellic acid (5°m-5°) series, intermediate for the glomelliferic acid (5°m-5) series, and highest for the perlatolic acid series (5m-5).

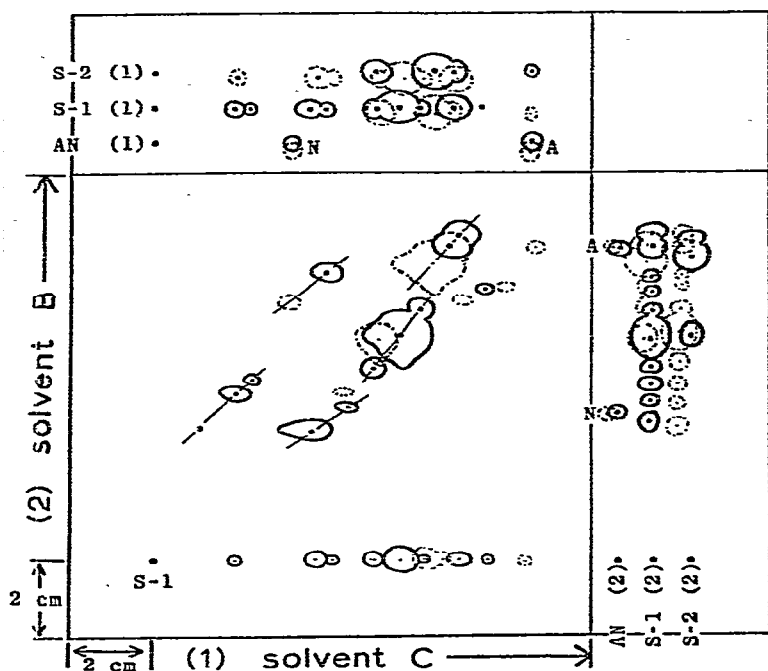(5) The 4-O-demethyl derivatives are displaced to lower R_F values compared

Fig. 2. A composite TLC obtained by superimposing the chromatograms of Figs. 1a and b. The spots of atranorin (A) and norstictic acid (N) on the one-dimensional chromatograms are aligned as closely as possible in order to determine the relative positions of the other spots on the two plates. Spots for homologous compounds lie on straight lines.

to their corresponding 4-O-methylated derivatives. When homologous 4-O-demethyl derivatives are present, new lines are established along which the individuals of each series will be distributed.

These correlations of chemical structure and R_F values allow tentative identifications for several trace constituents studied here. The R_F classes of the small spot labeled 7°-5 (Fig. 1b) were correct for olivetoric acid, and co-chromatography with a known sample of this compound provided confirmatory evidence. In addition, it follows from the R_F behavior of homologous series described above that the larger spot below and to the left of olivetoric acid (7°-5) is probably the new orcinol-type depside 4-O-demethylglomelliferic acid (5°-5). Such 4-O-demethyl derivatives are common in species producing 4-O-methylated depsides[11,12], and these identifications are completely in accord with biogenetic theory.

The spot labeled 7°m-5°/5°m-7° in Fig. 1b lies midway between that of glomellic acid (5°m-5°) and that of microphyllinic acid (7°m-7°) by co-chromatography of an extract of *P. loxodes* and a sample known to contain microphyllinic acid. Therefore, the trace constituent is probably a higher homologue, by two methylene groups, of glomellic acid. The identity of this new depside must be confirmed by other methods because isomers (7°m-5° or 5°m-7°) of this sort cannot be resolved by the standard solvent systems[2]. These compounds can be distinguished by mass spectrometry or by hydrolysis when pure samples are available. Similar comparisons

of R_F behavior suggest that the compound labeled 3-3 in Fig. 1a is the new depside 4-O-demethyldivaricatic acid[1].

The chromatographic behavior of the phenolic acid units of these depsides was also studied so that these compounds would be recognized if they occurred naturally or if they formed by decomposition during analysis. Trace spots occasionally noticed just below those of anziaic acid and glomellic acid on two-dimensional chromatograms heavily loaded with an extract of *P. loxodes* were the only indication that decomposition might have occurred on chromatograms of fresh extracts, but crude acetone extracts stored at room temperature did decompose to give phenolic acid units and unidentified products.

The R_F values reported for compounds chromatographed by the one-dimensional standardized method can now be used to predict the positions of spots of compounds not previously chromatographed by the two-dimensional method. The present technique provides an exceptionally useful link connecting one-dimensional TLC analyses and the two-dimensional method.

ACKNOWLEDGEMENTS

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