THE PAPER CHROMATOGRAPHIC IDENTIFICATION OF COMPOUNDS USING TWO REFERENCE COMPOUNDS*

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INTRODUCTION

R_F values are subject to significant variation if the experimental conditions are changed in a non-reproducible manner from one experiment to another. Well known reasons for such variations are changes in the dimensions of the apparatus, the grade of paper and its slight heterogeneity, the direction (ascending, descending, or horizontal) and the length of the solvent flow, the duration of development, the equilibration time, the temperature, the composition of the solvent travelling along the paper, the foreign ions and impurities in the solvent or on the paper, the volume of solvent used, the concentrations of the substances being chromatographed, the nature of the mixture being chromatographed and previous mode of treatment (type of desalting, etc.), the distance between the starting point and the source of solvent, the losses of solvent from the tank, the irregular travelling of the solvent along the paper, the incorporation of any other liquid or vapours for special purposes, and many other variables.

It is therefore generally accepted that the R_F values are not sufficient for the identification of a substance. In order to establish with certainty the identity of a substance, it has to be chromatographed simultaneously with an authentic sample of the suspected compound; but this entails the necessity of having a large collection of chemical substances, which is obviously a great disadvantage especially in cases of investigations on substances not commercially available, unstable compounds, etc.

In their classical communication on paper chromatography CONSDEN, GORDON AND MARTIN[1] pointed out that there are variations in the R_F values of a substance even when duplicate runs are made simultaneously in the same chamber. However, since then an enormous number of publications have dealt with the nature and the extent of the variations of R_F values caused by each of the above-mentioned conditions as well as their unforeseen changes during the development. As a result of these studies information has been obtained about the factors which may change the R_F values, and the way of controlling them in order to obtain reproducible results. Although an R_F variability of less than $\pm$ 0.02 has been achieved[2,3] in a few cases, such precision is not usually obtained in practice, so that the main problem *of identifying a substance without having to chromatograph simultaneously an authentic sample* remains unsolved, since all of the aforesaid publications approach this problem in a qualitative way.

An attempt to solve this problem quantitatively is described in the present report.

The introduction of relative values, R_K, given by the ratio

$$R_K = \frac{\text{Distance of the studied substance X from the start}}{\text{Distance of the reference substance K from the start}}$$

can obviously be considered as a first step. R_K values are generally considered to be more reliable criteria for the identification of a substance than its R_F values; *i.e.* not the individual R_F values themselves, but *the pattern* of a chromatogram is more important for characterizing the components of a mixture.

EXPERIMENTAL

Procedure

A number of compounds belonging to different classes of organic substances were submitted to a series of paper chromatographic separations as follows:
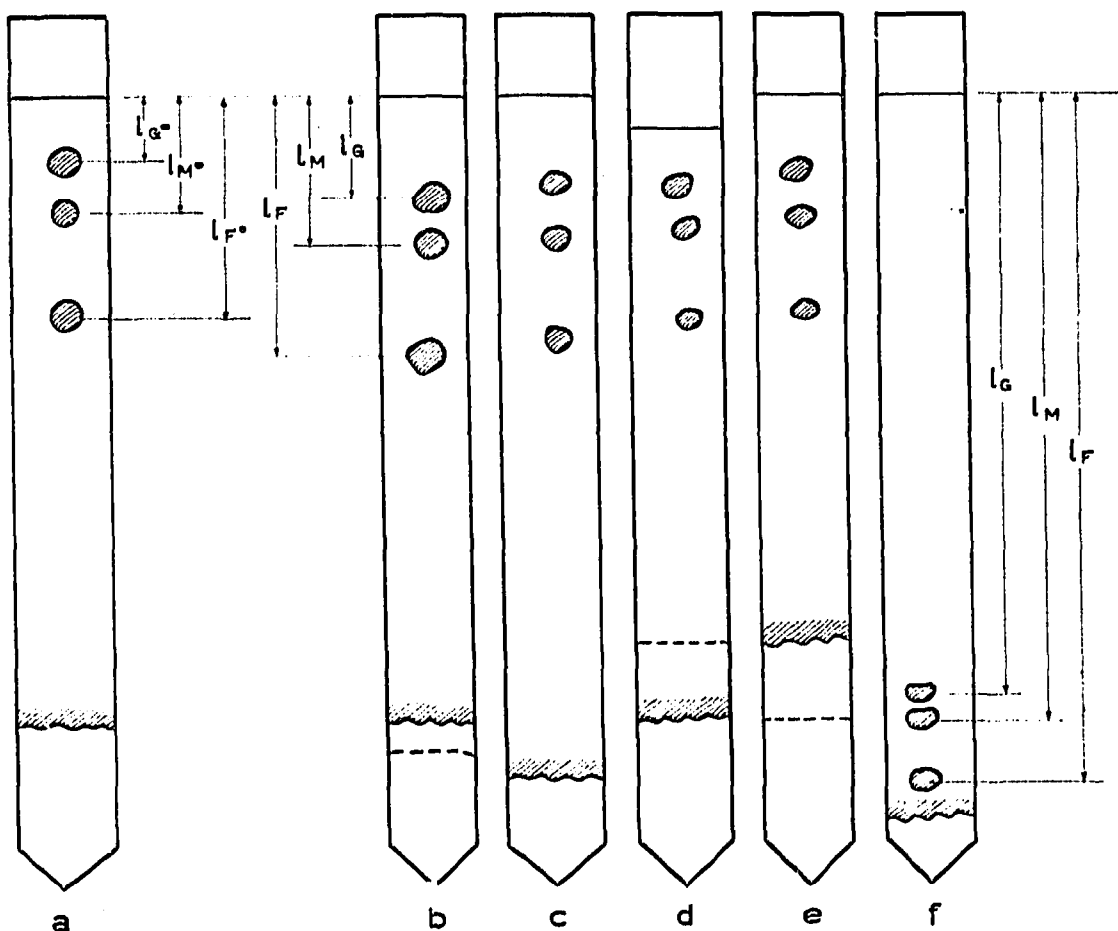

Fig. 1. Changes of the R_F values of carbohydrates by changing each time at least one of the initial experimental conditions. The initial experimental conditions, strip (a), were as follows: solvent, ethyl acetate–acetic acid–water 70:15:15 (v/v/v); method, descending (all glass apparatus, Shandon, 30 × 17 × 55 cm); time of development, 12 h; pre-equilibration time, 4 h; volume of solvent in the tank, 60 ml; carbohydrates studied, galactose (G), mannose (M) and fucose (F); temperature, 20°; paper, Whatman No 1. The conditions changed each time were the following: strip (b), the water content of the solvent (slightly increased). Strip (c), the time of development (16 h). Strip (d), the distance between starting point and solvent source. Strip (e), pre-equilibration of the tank (none). Strip (f), the tank was replaced by another constructed of wood; losses of solvent through cracks of this tank could not be prevented. Development time, 24 h.

A paper chromatogram was first developed under the conditions described by another worker. Next, several chromatograms of the same substance were carried out, but at least one of the initial conditions was changed each time. A comparison of the R_F values thus obtained illustrated the manner in which variations of the R_F values are caused.

The experimental conditions, together with any changes, are given in the legends to the figures and tables.

Results

The results thus obtained were very instructive, although many of them have been described before.

The variations in R_F value for carbohydrates given in Fig. 1 are very similar to those obtained with all hydrophilic substances studied in this laboratory.

The type of variation in R_F values of polar lipids illustrated in Fig. 2 occurs mainly in paper chromatographic runs of all lipophilic substances (glycerides, free fatty acids,
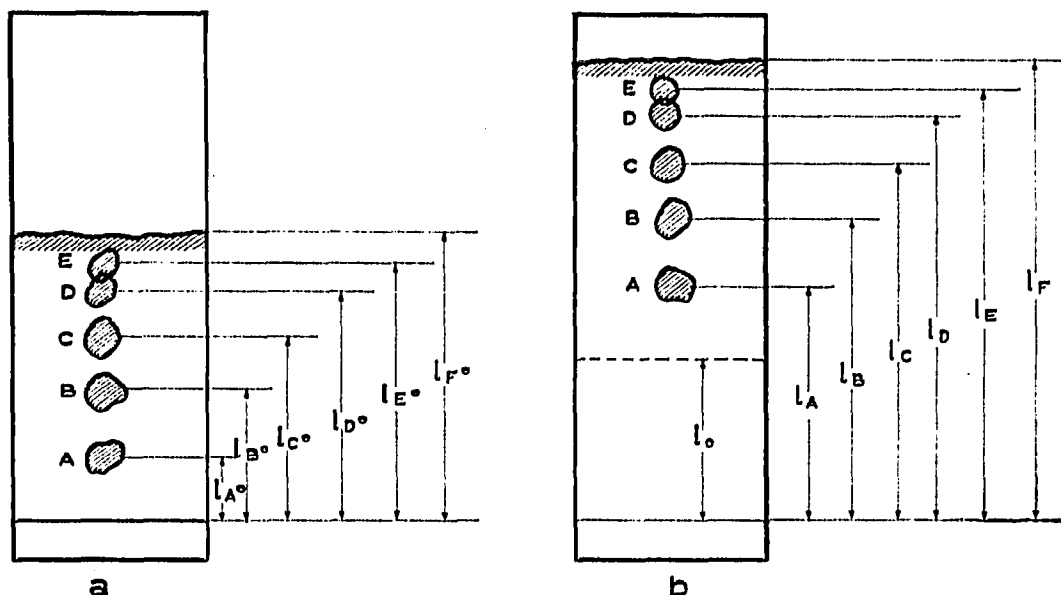

Fig. 2. Effect of the time of development on the R_F values of lipids. Paper, Whatman No. 1 impregnated with silicic acid; solvent, 85 vol. of chloroform–acetone 4:1 (v/v) mixed with 15 vol. of glacial acetic acid–water 9:1 (v/v); method, ascending (all glass apparatus, Shandon); time of development, 3 and 6 h respectively for strips (a) and (b). Lipids studied, sphingomyelin (A), phosphatidylcholine (B), phosphatidylserine (C), phosphatidylethanolamine (D) and cerebroside (E), 30 μg each spot. Volume of solvent in the tank, 200 ml; pre-equilibration time, 1 h; temperature, 20°.

sterols, etc.). However, similar results were also observed during a paper chromatographic investigation of hydrophilic deacylation products of phosphatides, and also in cases in which ideal conditions were not fulfilled (*e.g.* strip (f) in Fig. 1).

CLASSIFICATION OF THE R_F VARIATIONS

The variations caused by the aforesaid experimental conditions to the mobilities of compounds submitted to chromatography can be classified into the following three groups:

1. Variations which do not change the R_F values themselves, but change the distances of the spots from the starting point, *i.e.* influence the separability of the substances submitted to chromatography (see strips (a) and (c) in Fig. 1). Variations of this type are not contradictory to the postulate of the constancy of R_F values, therefore:

$$R_F^{\circ} = R_F \qquad (1)$$

The symbols R_F° and R_F are used throughout the present report for tabular and measured R_F values, respectively. The term *tabular R_F values* is here used to define any set of R_F values either given in the literature, or uniquely determined in the laboratory, *i.e. tabular R_F values are sets of values taken as models, and which one tries to reproduce.* Any other R_F values of the same group of compounds are here defined as *measured R_F values.*

2. Variations which change the R_F values without changing the ratios of the distances of the spots from the starting point, *e.g.*, $l_G^{\circ}:l_M^{\circ}:l_F^{\circ} = l_G:l_M:l_F$ [see strips (a), (b), (d), and (e) in Fig. 1]. Variations of this type can be expressed as follows:

$$R_F^{\circ} = a \cdot R_F \qquad (2)$$

where a is a coefficient. The postulate of the constancy of R_F values continues to be true, if allowance is made for a transfer of the solvent front (dotted lines in strips (b), (d), and (e) of Fig. 1).

It should be noted that, long ago, PARTRIDGE AND WESTALL[4] pointed out that such a linear variation was caused by temperature alterations. According to these authors, in order to obtain greater constancy between the R_F values observed, the R_F's determined at a temperature t° have to be corrected to the standard R_F's at 20° by multiplying by the factor

$$a = \frac{R_F \text{ of glucose at } 20^{\circ}}{R_F \text{ of glucose at } t^{\circ}}$$

3. Variations which change both the individual R_F values and the ratios of the distances of the spots from the starting line, but still do not change the pattern of the chromatogram if allowance is made for a transfer of the starting line (dotted line in strip (b) of Fig. 2). In such cases the separability of the spots is obviously not affected, *i.e.* the distances between the spots remain constant ($l_B^{\circ} - l_A^{\circ} = l_B - l_A, l_D^{\circ} - l_C^{\circ} = l_D - l_C$, etc.; Fig. 2). Variations of this type can be expressed as follows:

$$(R_F^{\circ})_X = \frac{l_X^{\circ}}{l_F^{\circ}} = \frac{l_X - l_0}{l_F - l_0} = \frac{l_F}{l_F - l_0} \cdot (R_F)_X - \frac{l_0}{l_F - l_0} \qquad (3)$$

where $(R_F^{\circ})_X$ and $(R_F)_X$ are the tabular and measured R_F values of a substance X respectively.

Although an examination of each of the above-mentioned variables is neither possible, nor practically important in ordinary paper chromatographic work, it has been shown that the following general equation:

$$R_F^{\circ} = a \cdot R_F + b \qquad (4)$$

can be applied experimentally.

This equation has been based on the following postulates:

(i) Any kind of R_F variations can be classified in one of the previously mentioned three groups, *i.e.* there are no factors which can influence differently the individual R_F values of the compounds co-chromatographed or, at least, such factors if they exist, cannot cause significant changes in the R_F's.

(ii) All R_F variations are additive properties, *i.e.* the coefficients a and b of eqn. (4) represent in each case the sum of many individual coefficients $a_1, a_2, a_3, \ldots$, or $b_1, b_2, b_3, \ldots$, corresponding to different experimental variables which are not individually of any practical importance.

It is obvious that coefficients a and b can be determined experimentally by measuring in each case the R_F values of two authentic samples:

$$a = \frac{(R_F{}^\circ)_A - (R_F{}^\circ)_B}{(R_F)_A - (R_F)_B} \quad \text{and} \quad b = (R_F{}^\circ)_A - a \cdot (R_F)_A \tag{5}$$

where $(R_F{}^\circ)_A$, $(R_F{}^\circ)_B$ are the tabular R_F values, and $(R_F)_A$, $(R_F)_B$ the measured ones of the two reference compounds A and B.

It should be noted that although eqns. (3) and (4) look very similar, they are actually different, namely, the sum of the two parameters of eqn. (3) is obviously equal to one, while this is not necessarily true in the case of the general eqn. (4).

APPLICATIONS

One-dimensional run

An acidic hydrolyzate of a polysaccharide was submitted to paper chromatographic separation using the isopropanol–water 80:20 (v/v) solvent system. The tabular R_G values of various monosaccharides in this system (Table I) were taken from SMITH[5].

TABLE I

R_G VALUES OF MONOSACCHARIDES ON WHATMAN NO. 4 PAPER, TAKEN FROM SMITH[5]
(Solvent: isopropanol–water, 80:20)

Compound	R_G	Compound	R_G
Rhamnose	1.52	Sorbose	1.02
Ribose	1.37	Glucose	1.00
Fucose	1.35	Galactose	0.80
Xylose	1.30	Glucosamine	0.65
Arabinose	1.12	Muramic acid	0.62
Mannitol	1.10	Galactosamine	0.50
Dulcitol	1.05	Inositol	0.35
Sorbitol	1.05	Glucuronic acid	0.20
Mannose	1.05	Galacturonic acid	0.15
Fructose	1.05		

The chromatogram is illustrated in Fig. 3, where it is evident that this hydrolyzate contains five components, one of which (spot C) is galactose. The measured distances of the spots from the starting line are: $l_A = 4.1$ cm, $l_B = 8.7$ cm, $l_C = 11.0$ cm,
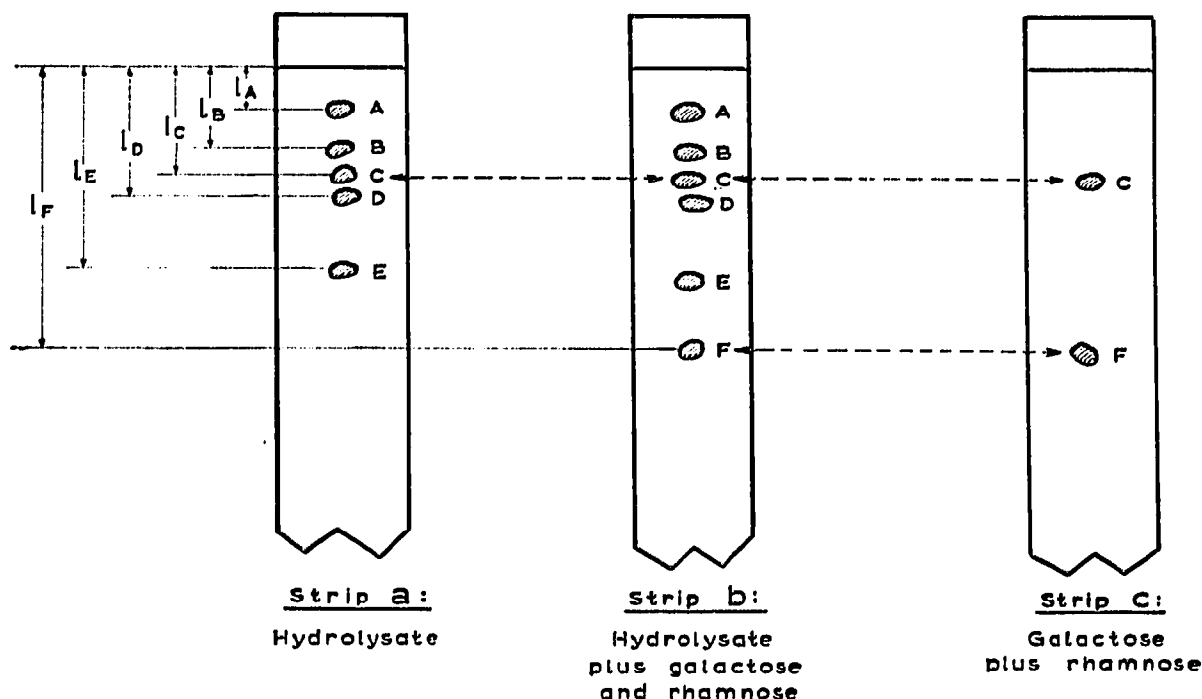
Fig. 3. Paper chromatographic separation of a polysaccharide hydrolyzate using the isopropanol–water 80:20 (v/v) solvent system. Method, descending, 24 h. Paper, Whatman No. 1.

$l_D = 14.1$ cm, $l_E = 18.7$ cm, $l_F = 22.1$ cm. Using eqn. (5) for the two reference compounds, galactose and rhamnose,

$$a = \frac{(R_G^\circ)_F - (R_G^\circ)_C}{l_F - l_C} = \frac{1.52 - 0.80}{22.1 - 11.0} = 0.065 \qquad b = 0.80 - 0.065 \times 11.0 = 0.085$$

Then, according to eqn. (4), the following R_G°'s are calculated:

Spot A: $0.065 \times 4.1 + 0.085 = 0.35$ (Inositol)
Spot B: $0.065 \times 8.7 + 0.085 = 0.65$ (Glucosamine)
Spot D: $0.065 \times 14.1 + 0.085 = 1.00$ (Glucose)
Spot E: $0.065 \times 18.7 + 0.085 = 1.30$ (Xylose)

These calculations have been made using R_G°'s instead of R_F°'s, and l_X's instead of $(R_F)_X$'s in eqns. (4) and (5). Coefficients a and b calculated in this way are different from the ones obtained if the aforesaid replacement had not taken place, but obviously lead to the same R_G° values.

It is evident that the evaluation of paper chromatographic results by this method leads to reliable findings in nearly all cases, whereas the older method of using a single reference compound gives confusing results. For instance, in the case examined above, using galactose (spot C) as the reference compound, the following results are obtained:

$(R_g)_A = 4.1:11.0 = 0.37$ $\qquad$ $(R_G^\circ)_A = 0.37 \times 0.80 = 0.30$ $\qquad$ (?)
$(R_g)_B = 8.7:11.0 = 0.79$ $\qquad$ $(R_G^\circ)_B = 0.79 \times 0.80 = 0.63$ (Muramic acid)
$(R_g)_D = 14.1:11.0 = 1.28$ $\qquad$ $(R_G^\circ)_D = 1.28 \times 0.80 = 1.02$ (Sorbose)
$(R_g)_E = 18.7:11.0 = 1.70$ $\qquad$ $(R_G^\circ)_E = 1.70 \times 0.80 = 1.36$ (Fucose or
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ Ribose)

Here, R_g's are the relative R values measured with respect to galactose. Misleading results are also obtained using rhamnose (spot F) as the reference compound; *i.e.* calculating as described above, the following data are obtained:

$(R_G{}^\circ)_A = 0.28$ (?)
$(R_G{}^\circ)_B = 0.60$ (Muramic acid ?)
$(R_G{}^\circ)_D = 0.97$ (Glucose ?)
$(R_G{}^\circ)_E = 1.28$ (Xylose ?)

Two-dimensional run

A mixture of 10 amino acids was chromatographed by the two-dimensional technique using n-butanol–acetic acid–water 120:30:50 (v/v/v) for the first direction and phenol–water–ammonia 160:40:1 (v/v/v) for the second one. The tabular $R_F{}^\circ$ values of the chromatographed amino acids (Table II) were taken from SMITH[5]. The chromatogram is illustrated in Fig. 4, and the measured R_F values are given in Table II.

TABLE II

TABULAR AND MEASURED R_F VALUES OF TEN AMINO ACIDS IN TWO SOLVENT SYSTEMS

Amino acid		n-Butanol–acetic acid–water 120:30:50 (v/v/v)		Phenol–water–ammonia 160:40:1 (v/v/v)	
		$R_F{}^\circ$	R_F	$R_F{}^\circ$	R_F
A.	Aspartic acid	0.23	0.210	0.17	0.150
B.	Cystine	0.05	0.075	—	0.380
C.	Glutamic acid	0.28	0.250	0.26	0.260
D.	Serine	0.22	0.200	0.35	0.340
E.	Threonine	0.26	0.235	0.48	0.485
F.	Alanine	0.30	0.265	0.58	0.600
G.	Histidine	0.11	0.118	0.72	0.735
H.	Valine	0.51	0.415	0.78	0.805
I.	Phenylalanine	0.60	0.485	0.84	0.875
J.	Leucine	0.72	0.570	0.85	0.880

Now suppose that two of these amino acids, aspartic acid (spot A) and leucine (spot J) were used as the two reference compounds, all the others being unknown. According to eqn. (5) for the first solvent system:

$$a = \frac{0.72 - 0.23}{0.57 - 0.21} = 1.36 \quad \text{and} \quad b = 0.72 - 1.36 \times 0.57 = -0.055$$

Then using eqn. (4), the following $R_F{}^\circ$ values are calculated:

$(R_F{}^\circ)_B = 1.36 \times 0.075 - 0.055 = 0.047$ (Cystine)
$(R_F{}^\circ)_C = 1.36 \times 0.250 - 0.055 = 0.285$ (Glutamic acid)
$(R_F{}^\circ)_D = 1.36 \times 0.200 - 0.055 = 0.217$ (Serine)
$(R_F{}^\circ)_E = 1.36 \times 0.235 - 0.055 = 0.265$ (Threonine)
$(R_F{}^\circ)_F = 1.36 \times 0.265 - 0.055 = 0.305$ (Alanine)
$(R_F{}^\circ)_G = 1.36 \times 0.118 - 0.055 = 0.115$ (Histidine)
$(R_F{}^\circ)_H = 1.36 \times 0.415 - 0.055 = 0.509$ (Valine)
$(R_F{}^\circ)_I = 1.36 \times 0.485 - 0.055 = 0.605$ (Phenylalanine)
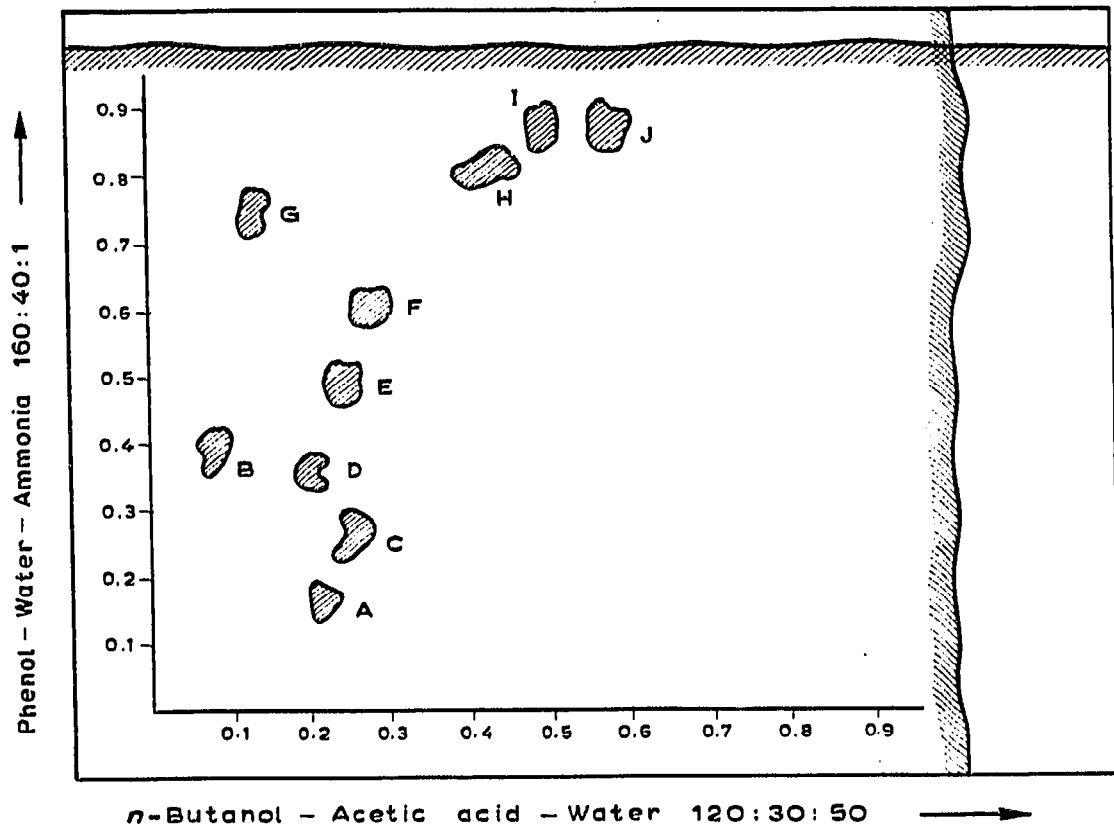
Fig. 4. Two-dimensional chromatogram of ten amino acids.

Similarly, for the second solvent system:

$$a = \frac{0.85 - 0.17}{0.88 - 0.15} = 0.93 \quad \text{and} \quad b = 0.85 - 0.93 \times 0.88 = 0.032$$

$(R_F{}^\circ)_B = 0.93 \times 0.380 + 0.032 = 0.386$ (Cystine)
$(R_F{}^\circ)_C = 0.93 \times 0.260 + 0.032 = 0.274$ (Glutamic acid)
$(R_F{}^\circ)_D = 0.93 \times 0.340 + 0.032 = 0.348$ (Serine)
$(R_F{}^\circ)_E = 0.93 \times 0.485 + 0.032 = 0.485$ (Threonine)
$(R_F{}^\circ)_F = 0.93 \times 0.600 + 0.032 = 0.590$ (Alanine)
$(R_F{}^\circ)_G = 0.93 \times 0.735 + 0.032 = 0.716$ (Histidine)
$(R_F{}^\circ)_H = 0.93 \times 0.805 + 0.032 = 0.782$ (Valine)
$(R_F{}^\circ)_I = 0.93 \times 0.875 + 0.032 = 0.847$ (Phenylalanine)

Multiple development

In multiple development the mobility of a substance can theoretically be calculated as follows:

$$R_1 = R_F$$
$$R_2 = R_1 + R_F \cdot (1 - R_1) = 2 R_F - R^2{}_F$$
$$R_3 = R_2 + R_F \cdot (1 - R_2) = 3 R_F - 3 R^2{}_F + R^3{}_F$$
$$\dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots$$
$$R_n = 1 - (1 - R_F)^n$$

where $R_1, R_2, \ldots R_n$ are the measured R_F values after one, two, $\ldots$, n, developments respectively. The last of the above equations can be expressed as follows:

$$R_F = 1 - \sqrt[n]{1 - R_n} \qquad (6)$$

But owing to the fact that in multiple development the previously described R_F variations are much more marked compared with ones in single development, no attempt until now has been made to calculate R_F values using eqn. (6), $i.e.$ to identify unknown compounds by means of this equation.

However, a theoretical examination of this problem from the viewpoint developed in this paper led to the following very interesting results, which have been experimentally verified.

If eqn. (4) (solved with respect to R_F) is used in calculations of the mobility of a substance after a multiple development one obtains:

$$R_1 = a' \cdot R_F{}^\circ + b' \qquad \text{(where } a' = 1/a \text{ and } b' = -b/a\text{)}$$
$$R_2 = R_1 + a' \cdot R_F{}^\circ \cdot (1 - R_1) + b' \cdot (1 - R_1) = 1 - (1 - R_1)^2$$
$$R_3 = 1 - (1 - R_1)^3$$
$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$$
$$R_n = 1 - (1 - R_1)^n$$

By transforming the last equation,

$$R_F{}^\circ = c - d \cdot \sqrt[n]{1 - R_n} \qquad (7)$$

where c and d are coefficients derived from a and b,

$$c = \frac{1 - b'}{a'} = a + b \quad \text{and} \quad d = \frac{1}{a'} = a$$

It is evident that eqn. (7) can be used to calculate the tabular $R_F{}^\circ$ values of unknown substances, $i.e.$ to identify them by their R_n values measured after n developments in a certain solvent system. Two reference compounds are again required for the calculation of coefficients c and d of eqn. (7):

$$d = \frac{(R_F{}^\circ)_A - (R_F{}^\circ)_B}{\sqrt[n]{1 - (R_n)_B} - \sqrt[n]{1 - (R_n)_A}} \quad \text{and} \quad c = (R_F{}^\circ)_A + d \cdot \sqrt[n]{1 - (R_n)_A} \qquad (8)$$

It should be noted that by similar, but more complex, calculations it can be shown that eqn. (7) leads to correct results even in cases where the coefficients a' and b' are not exactly identical in the repeated developments of the same chromatogram. In such a case if $a_i = a' + y_i$ and $b_i = b' + z_i$ (where y_i and z_i are small as compared to a' and b'), factors such as $y_i{}^2 R_F$, $a' \cdot y_i \cdot R_F{}^2$, etc. can obviously be neglected. In this manner an equation similar to eqn. (7) results, in which the coefficients c and d are not equal to $(a + b)$ and a respectively, but still have the same values for all compounds co-chromatographed.

Experimental verification. A mixture of 10 amino acids was chromatographed three times by the ascending technique in the n-butanol–acetic acid–water 120:30:50

(v/v/v) solvent system, for which the tabular $R_F°$ values are given in Table II. The R_3 values experimentally determined are:

A. Aspartic acid	0.505	F. Alanine 0.605
B. Cystine	0.210	G. Histidine 0.320
C. Glutamic acid	0.580	H. Valine 0.800
D. Serine	0.490	I. Phenylalanine 0.860
E. Threonine	0.555	J. Leucine 0.915

Now suppose, as previously, that aspartic acid and leucine were the two reference compounds, all other spots being unknown. Using eqn. (8):

$$d = \frac{0.720 - 0.230}{\sqrt[3]{1-0.505} - \sqrt[3]{1-0.915}} = 1.40 \qquad c = 0.72 + 1.40 \times \sqrt[3]{1-0.915} = 1.335$$

Then, by eqn. (7) the tabular $R_F°$ values of the spots B to I are calculated as follows:

$$(R_F°)_B = 1.335 - 1.40 \times \sqrt[3]{1-0.210} = 0.045 \text{ (Cystine)}$$
$$(R_F°)_C = 1.335 - 1.40 \times \sqrt[3]{1-0.580} = 0.285 \text{ (Glutamic acid)}$$
$$(R_F°)_D = 1.335 - 1.40 \times \sqrt[3]{1-0.490} = 0.215 \text{ (Serine)}$$
$$(R_F°)_E = 1.335 - 1.40 \times \sqrt[3]{1-0.555} = 0.265 \text{ (Threonine)}$$
$$(R_F°)_F = 1.335 - 1.40 \times \sqrt[3]{1-0.605} = 0.305 \text{ (Alanine)}$$
$$(R_F°)_G = 1.335 - 1.40 \times \sqrt[3]{1-0.320} = 0.105 \text{ (Histidine)}$$
$$(R_F°)_H = 1.335 - 1.40 \times \sqrt[3]{1-0.800} = 0.515 \text{ (Valine)}$$
$$(R_F°)_I = 1.335 - 1.40 \times \sqrt[3]{1-0.860} = 0.605 \text{ (Phenylalanine)}$$

DISCUSSION

The present method of calculating tabular $R_F°$ values using two reference compounds seems to be applicable to paper chromatographic separations of any kind of organic substance. This is true even in cases where the results observed ought to be discarded as unreliable, if examined on the basis of the older method of one reference compound.

The only point which needs experimental verification is that the coefficients a and b of eqn. (4) are the same for all compounds co-chromatographed. It can be shown that in every chromatogram these coefficients are indeed the same for all compounds belonging to the same class of organic substances, but this is not true for compounds belonging to different organic classes. Thus, for all carbohydrates (pentoses, hexoses, etc.) the coefficients calculated for any given chromatogram were found to be equal. The coefficients a and b for amino acids were found to be different from the ones for co-chromatographed carbohydrates, although these differences were not of great significance for all the solvent systems. This can possibly be explained by the fact that although amino acids are similar to carbohydrates, as far as polarity is concerned, their separation on paper is also affected by their ampholytic nature; this influence being in some cases strong, and in other cases weak.

In some cases this difference in the coefficients a and b for co-chromatographed compounds can obviously lead to invaluable deductions about the nature of unknown substances. For instance, during a study on an acidic hydrolyzate of a

fraction of milk lipids, the results illustrated in Fig. 5 were obtained. These indicate that the two constituents of this hydrolyzate (see spots A and B in Fig. 5) are probably not of a purely carbohydrate nature, since their chromatographic properties are rather similar to the ones of lipophilic substances, *i.e.* large negative *b* values, as in the case of Fig. 2.
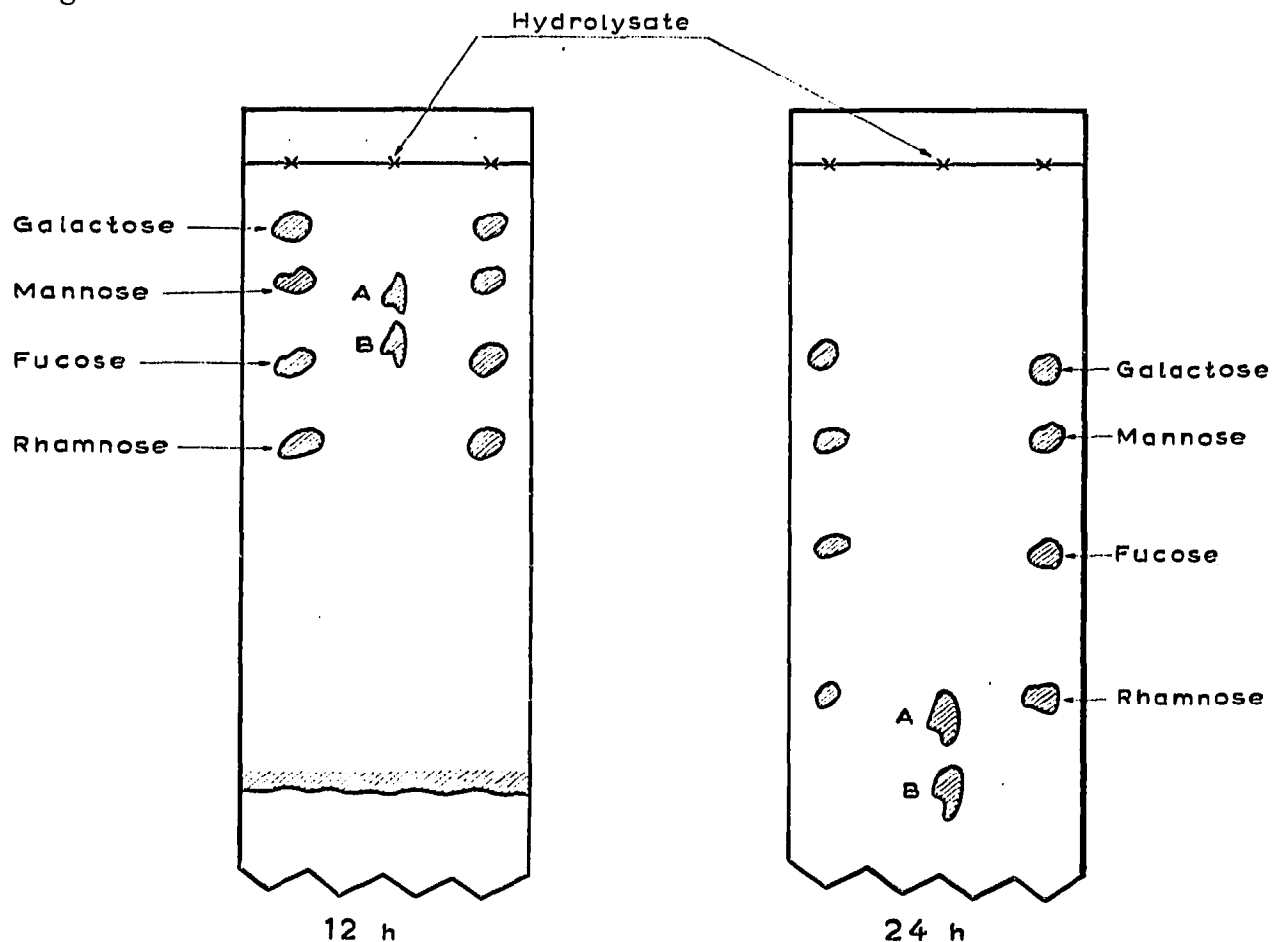
Fig. 5. Paper chromatographic separation of an acidic hydrolyzate of a fraction of milk lipids in ethyl acetate–pyridine–water 2:1:2 (v/v/v). All spots were located by both the silver nitrate and the aniline phthalate reagents.

SUMMARY

A general method using two reference compounds for the paper chromatographic identification of unknown substances is described.

The method devised is based on the fact that any kind of important variations to the paper chromatographic mobility of various substances co-chromatographed caused by different experimental conditions, can be classified into three groups of linear variations.

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