A METHOD FOR THE PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PHENOL DERIVATIVES, MOULD METABOLITES AND RELATED COMPOUNDS OF BIOCHEMICAL INTEREST, USING A "REFERENCE SYSTEM"

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When dealing with the problem of correlating the production of different metabolic compounds, found in various strains of *Penicillia*, with their general biosynthetic pathways, great difficulties are encountered in the identification of these compounds, especially when they only occur in minor amounts. The pioneering work on mould-metabolic products as carried out by RAISTRICK and his colleagues (for reviews see Refs.[1,2,3]) led to the isolation and identification of a great number of compounds, such as phenol derivatives, tetronic acids, tropolones, etc., from large scale cultures of appropriate moulds by chemical methods.

In order to compare the production capacity of a number of different strains under various conditions, such as addition of antimetabolites or isotopes, it was found favourable, at least in preliminary experiments, to cultivate the moulds on a relatively small scale (100–500 ml of media). On the basis of preliminary paper-chromatographic studies of medium extracts from some *Penicillia* strains (*e.g. P. urticae* Bainier, *P. brevi-compactum*, *P. griseo-fulvum* etc.), EHRENSVÄRD[4,5] showed that 2–3 times as many compounds of "aromatic" character could be detected, as had been reported previously.

It was felt that when substances were found in the culture media (or in mycelia) even in very small amounts compared with the "main" products, these could, after proper characterization or identification, provide additional information regarding the possible precursors or reaction sequences in the biosynthesis of accumulated compounds.

As a basis for the investigations, a suitable "reference system" was worked out for comparative purposes. Since, as previously stated, mould-metabolic products are mainly related to the phenol or phenol-carboxylic acid type of compounds, the reference system had to cover a large number of the most simple phenol derivatives so that possible degradation and decarboxylation products could be detected. Furthermore, by courtesy of Prof. RAISTRICK, it was possible to include a large number of well-known mould metabolites. It was also found useful to collect a large group of non-aromatic compounds, mainly related to the TCA-cycle, and some of the more common natural products of vegetable origin, using the same procedure. The choice

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of substances was, of course, somewhat arbitrary, within the limits of their availability. In many cases the heterogeneity of the compounds proved to be of help in determining the group character of certain types of compounds.

The common paper-chromatographic procedure had to be adapted both to the investigation of the reference substances and to that of the extracts from culture media of moulds. This was done according to the following principles:

The chromatograms were run in six different solvent systems. Each of these was treated as a standard with ten spraying reagents. The limited choice of solvents and reagents was conditioned by the need for obtaining, within a reasonable time, adequate information on the chemical nature of the compounds involved.

To avoid the tedious and time-consuming task of spraying only one chromatogram at a time, a set-up was constructed that allowed six chromatograms at a time to be sprayed with ten standard reagents within a relatively short time (Fig. 1). The principle of this system is to apply the extract along a line on each of the six chromatographic papers and then develop them in six solvents. After drying, they are sprayed vertically with the ten reagents in adjacent strips (about 10 mm wide). When the level to which the substance has travelled is crossed by the vertical reagent strip, a roughly circular spot appears. From this the R_F value can be read off directly by means of a graduated elastic band. At the same time the shades of the colours produced by the action of the different reagents are recorded in numbers. For these colours, an arbitrary

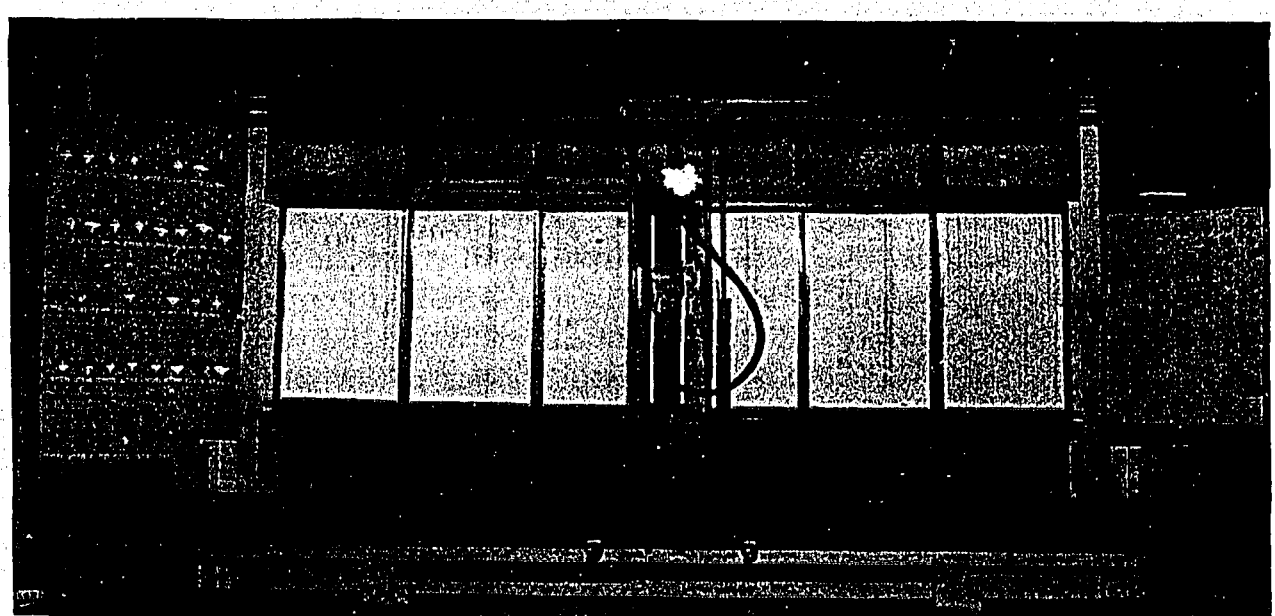

Fig. 1. Over-all picture of the spraying arrangement, placed on an 80 cm high table. Six papers from chromatographic solvent systems can be inserted simultaneously against an illuminated glass screen. The first section from the right shows that it is possible to spray at least 22 strips of reagents, vertically, on each paper of size 24 × 55 cm. The frame shown in the middle of the figure can be moved both horizontally and vertically. It is equipped with two retouching air brushes and is counterbalanced so as to allow an easy up and down movement when spraying. Between the air brushes and the paper there are two slits, 8 × 500 mm, which can only be moved horizontally together with the frame. At both sides of the plate where the slits are fixed, a graduated elastic band is attached, which is protected against sprinkling by an acrylate plate. The R_F values are read off directly and recorded after the band has been properly adjusted, by means of a screw, to the solvent front on the chromatogram.

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standard has been chosen according to the "Derwent" colour pencil shades for rapid recording (see the colour index given for the tables). When the colours are fully developed, the variation of the R_F values of a single component, which are represented horizontally by similar colour sequences in the six solvents, can easily be studied against an illuminated screen. An over-all idea of the results can thus be obtained during spraying. This was found to be advantageous in many cases when checking only the "typical" production picture of a single strain. For a complete record of the numerical data, consisting of R_F values and of a numbered code of colour reactions, the reader is referred to the experimental part of this work.

This system of characterizing unknown substances before attempting to isolate them in a crystalline form, has hitherto resulted in the identification of several new metabolites. For instance, GATENBECK[6] studied the biosynthetic background of anthraquinones and reported the presence of 3-hydroxyphthalic acid in *Penicillium islandicum* Sopp. In the culture media of *P. griseo-fulvum*[7] I detected, among other substances, orcinol and orsellinic acid, which have not been reported previously either in the case of *Penicillia* species or of any other moulds. Penicillic acid, which had earlier been found in several other species of *Penicillia*, was also shown to occur in this species. After isolation and chemical analysis of the three above-mentioned compounds, their identity was confirmed. LYBING AND HAGSTRÖM[8] described an interesting application of this method in an investigation of secretion products in egg water from sea urchin eggs.

GENERAL PROCEDURE

Extracts or mixtures of several compounds, which were subjected to paper-chromatographic investigation were run on Whatman No. 1 filter papers, 24 × 55 cm, in six different solvent systems designated as F, E, A, B, C and D. By drawing a funnel-shaped pen (Fig. 5) along a ruler, various substances in ethanolic solution were applied uniformly on the papers along a line 20 cm in length and 10 cm from the bottom of the paper. The line was dried and the procedure was repeated until the desired concentration of substances was obtained. On both sides of the line on each paper, 2 spots of a "standard" substance were applied in order to maintain a continuous control of the quality of the paper and the constancy of the solvent systems. Usually an extract of about 1 mg dry weight, containing up to 6 components was applied on one paper. However, in most cases it was advisable to test several concentrations in order to get the best separation. In the case of one substance only, the procedure was simplified and a line chromatogram was run in one solvent only. In the remaining five solvents, the substance was applied and run as a spot and developed using the best detection reagent, which was found by spraying a line chromatogram with 10 standard reagents. When there was less than 0.1 mg substance, the procedure could be modified so as to comprise spot-test reactions on filter paper and estimation of the R_F values for 6 spots, one for each solvent system.

The chromatographic papers were hung up for 1 h in the chromatographic jars for equilibration before the solvents were applied. After the chromatograms had been

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run for approx. 4 h in solvents F, E, A, and for 5 h in solvents B, C and D, the solvent front had reached a distance of about 40 cm from the starting line; the solvent front was then marked by a soft pencil. The papers were air-dried overnight at room temperature. This gave the best results when the indicator reagent was used for detecting acidic components. Spraying could even be performed after 2-hours' drying, but in order to get a positive response with the indicator reagent, this should be applied 3 times, followed by ammonia and $CuSO_4$, similarly to the procedure recommended for solvent system E. In the case of the other reagents, the drying time had no noticeable effect.

The six chromatograms were placed in the order F, E, A, B, C, and D against an illuminated screen (as shown in Fig. 1), where they remained during spraying and evaluation. After examining the chromatograms in U.V.-light, they were sprayed with 10 different reagents, and the colours were recorded. For detailed descriptions see the sections describing spraying technique, and colour estimation and recording respectively.

THE SPRAYING APPARATUS

The spraying apparatus was built from L-profile aluminum and ½ inch aluminum rods. The dimensions of the frame were: length 200 cm, height 120 cm and depth 35 cm. An earlier model constructed mainly from laboratory clamps and rods also proved satisfactory. In the middle of the frame, 6 separate sections were set up, corresponding to the size of the papers used, each provided with a glass plate. The sections were arranged so that they could be taken out for cleaning, by pushing them sideways. Each section consisted of a rectangular unit, made from L-profile stainless steel, on the inside of which the above-mentioned glass plate was fitted. Between the inside border of the L-profile unit and the glass a gap of about 1 mm is obtained when the glass plate is pushed backwards. The chromatogram was fastened by pushing the paper between the glass plate and the overlapping (10 mm wide) L-profile border surrounding the unit. No special clamps or tape had to be used to hold the chromatogram upright. This applies also to papers of other sizes, but of the same length.

Behind these sections two daylight lamps, each 1.5 m long, were built in, so as to avoid corrosion of the lamps. In order to secure maximum light intensity and a uniform light source (whereby colour photographs could also be taken), the lamp section was coated on the inside with aluminum foil. The upper part of the spraying apparatus was insulated against fluids and packed with absorbent filter paper in order to collect the rinsing-spray fluids. It is also recommended to connect this with a ventilation device.

At the front of the spraying apparatus there is a mobile rack carrying two air brushes, as shown in Fig. 2. For spraying, this is moved vertically; it can also be shifted horizontally from one paper to another, at a constant distance from the paper. The tips of the air brushes are fixed 9 cm from the surface of the paper. A plate coated with filter paper and provided with two adjustable slits, 8 × 500 mm, was placed between the chromatogram and the two air brushes, 1 cm from the paper surface. This could be moved since it was connected with the rack.

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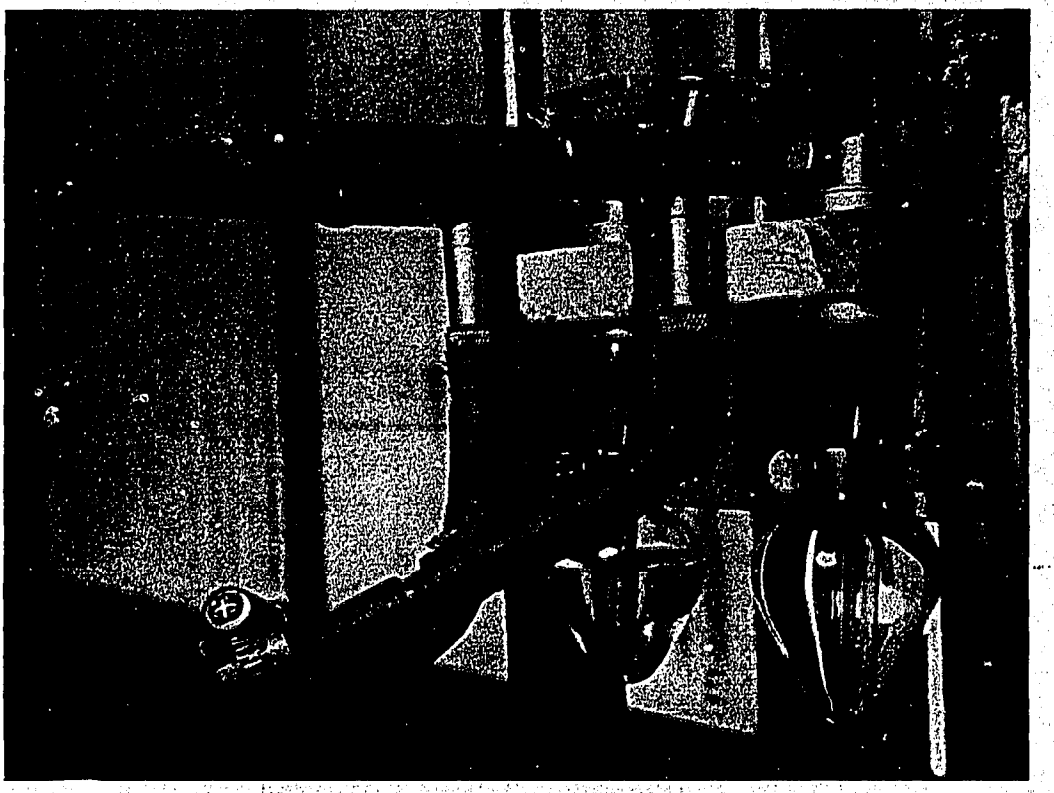

Fig. 2. Detail of that part of the movable frame to which the two air brushes and their reagent flasks are attached on an acrylate plate.

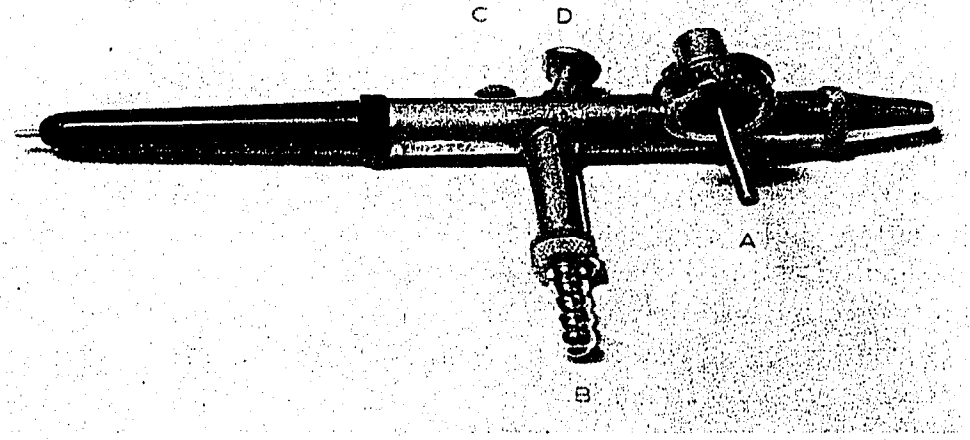

Fig. 3. Retouching air brush, type II B "Grafo", used for spraying the chromatograms. A: Inlet for reagents, which in this special case has been extended to reach the bottom of the reagent flask. B: Inlet for compressed air. C: Screw to regulate the quantity of solution sprayed. D: Push button to start the sprayer.

On both sides of the plate with the two slits, a graduated elastic band for R_F measurements was fixed. This could be adjusted with a screw to follow the variations of the solvent front on the chromatogram.

The R_F values for the centre of gravity of the single spots were read off by moving the rack horizontally adjacent to the spots. In some cases the R_F ordinates were drawn on the chromatograms in pencil.

The spraying screen was provided with a movable ultraviolet lamp. When turning the lights off (in a dark room), the fluorescent spots were outlined in pencil and the colour was recorded.

Fig. 1 shows a rack holding the reagents (left) and a colour index (right).

SPRAYING TECHNIQUE

For spraying, two "Grafo" Retouching Air Brushes, Type IIB were used (Fig. 3). These were operated with compressed air, but compressed nitrogen or carbon dioxide may also be used. The minimum working pressure being 2 atm, the air brushes were operated at 2.4 atm.

The sprayers were fitted on a mobile rack, which allowed horizontal and vertical movement at a constant distance from the chromatographic papers. The reagents were sprayed vertically one after another from the vessels containing 15 ml of the reagent solution. For continuous spraying during 2½ min approx. 15 ml of solution was required (tested with aqueous solutions). This is sufficient to allow the spraying of 60 strips, each 50 cm long, *i.e.* corresponding in all to 30 m chromatogram. The amount of spraying solution can be regulated to a certain extent, as can be seen in Fig. 3. Under normal conditions the spraying cone is adjusted so that about a 20 mm wide line of reagent is applied on the paper. Between the air brushes and the paper there are two slits, 8 × 500 mm, which can only be moved horizontally together with the frame. By this arrangement the reagent zone applied on the paper is reduced from 20 to 10 mm.

When one line has been sprayed on the first paper at a certain position, the sprayer is moved to the next paper and, at the same position, another line is sprayed. After the six chromatograms have been treated similarly the reagent solution is changed. The sprayer is washed for 10 sec with distilled water (or alcohol, depending on the reagent used or the one to be used).

Since the air brushes are precision instruments, the following precautions should be carefully observed:

The reagent solutions must be protected from dust and other visible impurities. Before pouring them into spraying-flasks, they should be filtered. When not in use they should be stored in tightly closed flasks as shown in Fig. 1. If the solutions become turbid, they must be refiltered.

The air brush must be carefully cleaned after use by blowing water of *ca.* 50° through it, especially when ammonia or phosphomolybdic acid have been used. Distilled water should be used for cleaning the brush between sprayings. Ethanol should be used before and after the reagent DB (= 2,6-dibromoquinone-4-chloroimide), otherwise the reagent will precipitate and block the channels in the air brush.

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In order to facilitate successive sprayings with two different reagents within the same strip, the following spraying scheme has proved useful:

Reagents	
Air brush I	*Air brush II*
Bromophenol blue	$CuSO_4$, for paper from solvent E only
—	Permanganate
Ferric chloride	—
Dinitrophenylhydrazine	—
Phosphomolybdic acid	Ammonia
D1, D2, D3 and D4 diazonium reagents	Ammonia
2,6-Dibromoquinone-4-chloroimide	Ammonia

Reagent flasks (15 ml) are hung up by two metal clamps on the rack to the left of the spraying arrangement (Fig. 1). The upper clamp supports the cap-type polyethylene stoppers and the lower holds the reagent flasks, which can thus be easily removed and transferred from the rack to the spraying apparatus and vice versa with one hand only.

SPRAYING REAGENTS

Diazotized sulfanilic acid = D1. 50 mg stable diazonium salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0° and filtered. When not in use, the solution was stored in a refrigerator at the same temperature.

With most phenolic compounds and aliphatic keto acids, this reagent gave a yellow to orange colour—in very few cases a pink colour was produced—on exposing the sprayed strip to ammonia. Sometimes a spot developed even without ammonia.

Diazotized 4-benzoylamino-2,5-dimethoxyaniline = D2. 100 mg stabilized Zn salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0°, filtered and stored in a refrigerator.

With the investigated phenolic compounds this reagent gave purple to red-violet colours—in a few cases yellow and brown colours were obtained—when the sprayed strip was exposed to ammonia.

Diazotized o-dianisidine = D3. 100 mg stabilized Zn salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0°, filtered and stored in a refrigerator.

With the investigated compounds this reagent gave red to blue-violet colours —in a few cases brown or blue colours were obtained—when the sprayed strip was exposed to ammonia.

p-*Nitrobenzenediazonium fluoroborate = D4.* 60 mg dry diazonium salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0°, filtered and stored in a refrigerator.

With the investigated compounds this reagent produced an orange-yellow colour—in some cases brown, violet and red colours were produced—on exposing the sprayed strip to ammonia. Very small amounts of ammonia are required to produce this reaction which is, in most cases, immediate.

2,6-Dibromoquinone-4-chloroimide = DB. 50 mg 2,6-dibromoquinone-4-chloro-

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imide was dissolved in a mixture of 12 ml CaH_2-dried dioxane and 3 ml dry acetone. The solution was filtered and stored in a refrigerator.

With phenolic compounds this reagent gave blue, green and gray colours—in some cases yellow and pink colours were obtained—on spraying the reagent strip with dilute ammonia.

Solutions of all the diazonium reagents and the DB-reagent were stored in pear-shaped flasks of 15 ml on a separate rack, which could easily be moved over to a refrigerator after spraying. The reagent solutions could be used up to 14 days after preparation. However, every day the background colour (blank test) increased in intensity. Dioxane was used in order to dissolve the diazonium salts more easily; cold 50% aqueous ethanol could also be used, but the solutions were then found to lose their activity after a week. Dioxane was purified from peroxides by treatment with CaH_2 for a few days and subsequent filtration. For suppressing the original colour in the D2 and D3 solutions, treatment with active carbon was successful, but afterwards the solutions remained stable only for a couple of days, owing to the change of pH in the solution. Addition of small amounts of acetic acid had no effect on the storing stability of the solutions.

Ferric chloride = Fe. 2% $FeCl_3 \cdot 6H_2O$ in water was used. If storing for longer times is desired, then the stock solution must be at least 6%. The use of saturated solutions of anhydrous ferric chloride in anhydrous dioxane or chloroform may sometimes be advantageous.

With the investigated compounds this reagent gave red-violet, blue or green colours, sometimes also brown, gray and yellow.

2,4-Dinitrophenylhydrazine = DN. 500 mg 2,4-dinitrophenylhydrazine was dissolved in 1000 ml hot 1 *N* HCl. The solution was filtered after a few days.

With aldehydic and keto compounds, this reagent gave yellow to orange colours; in rare cases other substances gave a positive reaction.

Potassium permanganate = Mn. This reagent consists of 1% aqueous solution of potassium permanganate. In some cases the use of slightly alkaline or acid permanganate was preferred.

With the compounds investigated, this reagent gave yellow-brownish spots on a red-violet background. Sometimes the spots were colourless owing to the strong reduction of the reagent to Mn^{+2} ion.

Phosphomolybdic acid = Mo. 2% aqueous solution of $H_3PMo_{12}O_{40} \cdot 29H_2O$ was used. In this case also the strip was sprayed with ammonia in order to develop the colours. Sometimes the colour development was immediate, even if ammonia was not used, and sometimes the colours changed when the reagent strip was exposed to ammonia. With the compounds investigated this reagent gave blue and gray colours; in rare cases yellow or green spots were obtained.

Bromophenol blue = Ind. 300 mg bromophenol blue was dissolved in 500 ml ethanol and 0.25 ml 30% sodium hydroxide was added in order to change the colour of the solution from red to blue.

With acids, this reagent gave yellow spots on a blue background. Chromatograms

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from the solvent system E did not give spots with the indicator reagent owing to the alkali remaining in the paper. In this special case, the bromophenol blue strip was once again sprayed with 2% aqueous solution of $CuSO_4 \cdot 5H_2O$. The background then turned to light blue or gray and the spots obtained, indicating the position of acids, were blue, green or yellow.

Dilute ammonia. Dilute ammonia was used when spraying the diazonium reagents D1, D2, D3, D4 and DB and Mo. In the cases previously mentioned, the dilute ammonia solution was made up as follows: 1 part concentrated aqueous ammonia (25%) and 1 part ethanol.

Copper sulphate. This reagent was used for the detection of acids when bromophenol blue failed to react. The paper was first sprayed with a 2% solution of $CuSO_4 \cdot 5H_2O$ in water and then with dilute ammonia. Practically all aromatic and aliphatic acids gave blue, green or brown spots on an almost colourless background.

Ninhydrin reagent = NH. Amino acids and biogenic amines were detected by spraying with 2% ninhydrin in butanol saturated with water and subsequent heating with an electric dryer.

Additional spraying reagents

For aliphatic acids. Aliphatic acids can also be detected by spraying with 1:4 diluted D3 reagent (*o*-dianisidine-diazotate) and subsequent spraying with 0.1% β-naphthol in 0.02 *N* sodium hydroxide. In this case the spots were almost colourless and the background turned dark violet[9].

The following stabilized diazonium compounds were also tested, both with regard to their usefulness for detecting phenolic compounds and the possibilities they offer for distinguishing between several compounds on paper chromatograms. About 0.7% solutions of diazonium compounds in 1 part dioxane and 2 parts water were used. These were: diazotized 4-amino-2-chlorodiethylaniline $ZnCl_2$ salt, *o*-aminoazotoluene-diazotate, anthraquinone-1-diazonium chloride, diphenylamine-4-diazonium sulphate, 1-diazo-2-naphthol-4-sulphonic acid, NNCD-reagent = 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate (previously described as reagent in paper chromatography[10]) and tetrazotized *o*-dianisidine. The latter shows colour reactions which are similar to those of *o*-dianisidine-diazotate. However, the shades of the colours were deeper.

"Genochrome" reagent. 2% aqueous solution of *p*-aminodiethylaniline sulphur dioxide was prepared. This reagent was stable at room temperature for two weeks. When spraying with this reagent aromatic aldehydes immediately gave orange to red spots on a colourless background. The latter turned brown-gray after several hours. Aromatic ketones and phenols do not react under these conditions. Other applications of the "Genochrome" reagent have been described in ref.[11].

Phenolic compounds gave yellow, violet, blue and brown colours when the reagent strip was sprayed the second time with 0.1% aqueous solution of sodium periodate.

Bromophenol blue and hydroxylamine hydrochloride reagent. 150 mg hydroxylamine·HCl was dissolved in 14 ml bromophenol blue solution (Standard Indicator

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solution) and 1 ml water. The pH of the solution was adjusted to 7.2 with a few drops of 2 *N* NaOH. Aromatic aldehydes reacted rapidly and gave yellow spots on a blue background. Ketones failed to react under the prevailing conditions.

p-*Phenylenediamine reagent.* With aromatic aldehydes, freshly prepared 1% aqueous solution of *p*-phenylenediamine gave yellow to orange colours against a bluish-gray background[12].

Azobenzene-phenylhydrazine sulphonic acid. With aromatic aldehydes 0.5% aqueous solution of azobenzene-phenylhydrazine sulphonic acid upon successive spraying with 1 *N* HCl gave after a few minutes yellow to bluish-green spots on an almost light-brownish background[13].

Pyridine and acetic anhydride reagent. This reagent was made up of 7 parts by volume of pyridine and 3 parts by volume of acetic anhydride, according to GODIN[14]. It was found useful in distinguishing between some aliphatic dicarboxylic acids. With the compounds listed in Tables XVIII, XIX, XX and XXI, the following colours were obtained with this reagent:

(1) In U.V. only:	
glyceric, mesoxalic and β-ketoglutaric acids	light blue
citric acid, ketipic acid diethyl ester	blue-green
dl-tartaric acid	green
(2) Visible:	
oxalacetic acid, itaconic acid	red-violet
fumaric acid	yellow
trans- and *cis*-aconitic acid, acetylenedicarboxylic acid	brown

Cyanoacetic acid ester reagent for 1,4-quinones[15]. 1 ml cyanoacetic acid ethyl ester in 14 ml ethanol was sprayed. On subsequent spraying with dilute ammonia the colours obtained with the following compounds were:

thymoquinone, 2,6-xyloquinone, 1,4-benzoquinone	blue
1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone	violet
3-hydroxy-2-methyl-1,4-naphthoquinone	red

For other reagents, which might in some special cases be useful, the reader is referred to the comprehensive treatise by LEDERER AND LEDERER[16].

THE CHROMATOGRAPHIC APPARATUS

The chromatographic apparatus consists of six rectangular glass jars (20 × 30 cm and 60 cm high), for six different solvent systems, designated as A, B, C, D, E, F. Each jar is provided with two glass troughs, each of which takes two chromatographic papers, 27 × 55 cm. However, sheets of Whatman No. 1 filter paper 24 × 55 cm were used throughout the experiments. These were cut from large sheets of Whatman paper, the downward flow of solvent being parallel to the machine-direction of the paper. The troughs were supported and kept fixed in the right position by means of a Pyrex glass-rod frame as shown in Fig. 4.

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For the solvents A, B, C and D a smaller glass dish, about 5 cm deep, containing the water phase of the solvent is placed at the bottom of the jar. In order to keep the atmosphere in the jar saturated with water, two strips of thick paper, approx. 18 cm wide, fixed under the respective troughs, were hung up so as to reach the bottom of the dish. The jar was lined from the bottom to a height of about 10 cm with a strip of thick paper. This was necessary in order to maintain saturation with the organic solvent. The organic solvent covered the bottom of the jar (around the dish) to a height of approx. 1 cm. If, after some time, the level rose to about 4–5 cm the solvent in the jar was entirely replaced. The top of the jar was covered by a glass plate, which was ground so that it fitted tightly; no grease was required. The jars were placed in a

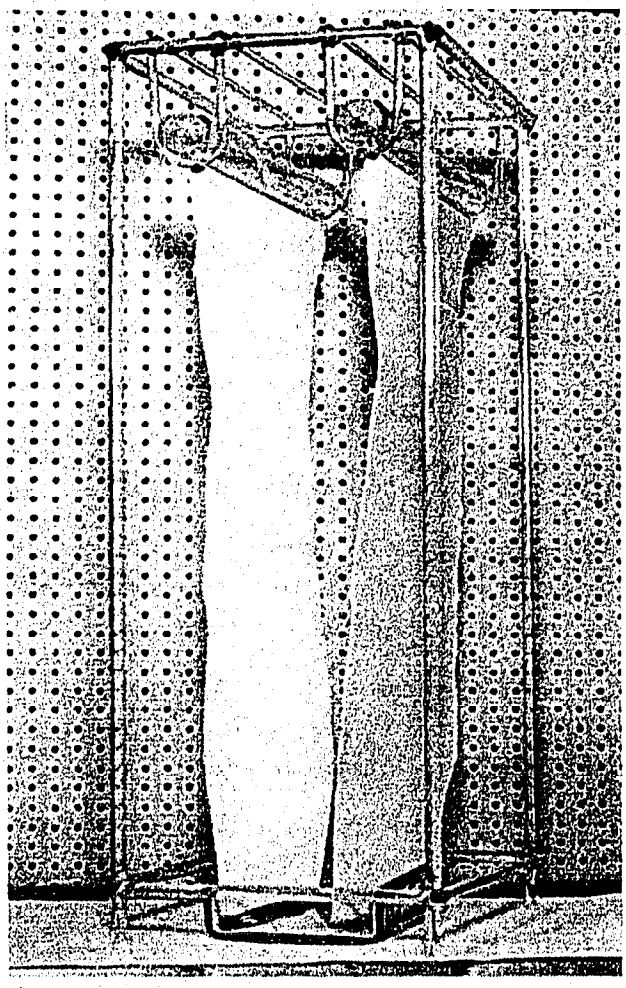

Fig. 4. Pyrex glass frame inside the chromatographic jars. The bottom solution containing the water phase is placed in a rectangular dish.

constant temperature-controlled room at 21°. The solvents were prepared in 10-l portions, which kept well; they were stored in bottles provided with stopcocks, through which the solvents were poured into the troughs.

CHROMATOGRAPHIC SOLVENTS

Six different solvent systems were used in the investigation of reference substances

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and metabolic products from *Penicillium* series. These solvents were originally composed by HÖGSTRÖM[17] for that purpose.

Solvent F. The solvent consisted of a mixture of ethyl methyl ketone 80 parts, acetone 4 parts, water 12 parts and formic acid (100%) 2 parts by volume.

Solvent E. The solvent consisted of a mixture of ethyl methyl ketone 921 parts, water 77 parts and diethylamine (100%) 2 parts by volume.

Solvent A. 1000 ml methyl isobutyl ketone was shaken for 1 h with 100 ml 4% formic acid (4 ml formic acid and 96 ml water). The two phases were separated

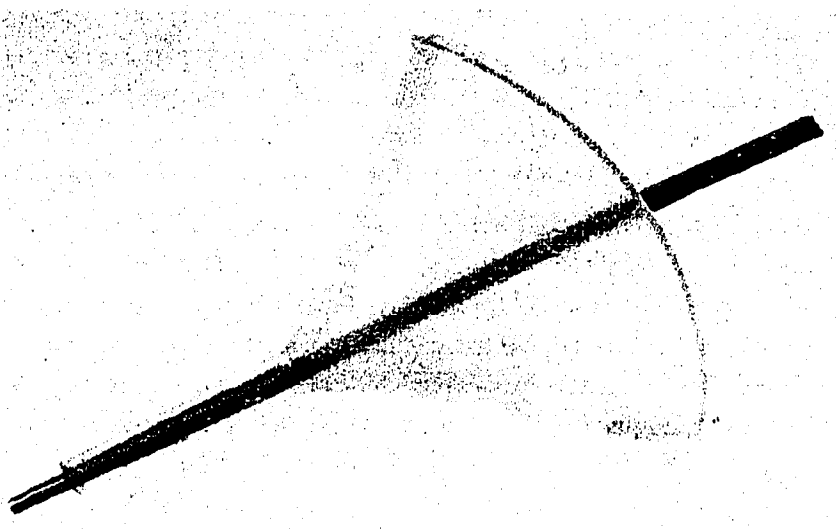

Fig. 5. "Pen" for application of substance solutions in narrow lines on chromatographic paper. The "pen" is made from a polyethylene funnel and a pin of stainless steel and has been devised by Dr. S. LYBING in this laboratory. (Magnification 2×.)

and the clear organic phase was used as mobile phase. The water phase was placed in a separate container at the bottom of the chromatographic jar.

Solvent B. 1000 ml chloroform (stabilized, containing 1% ethanol) was shaken for 1 h with 200 ml solvent of the following composition: 100 parts methanol, 96 parts water and 4 parts by volume formic acid. After separation, the organic phase was used as mobile phase and the water phase was placed in a separate container at the bottom of the jar.

Solvent C. A mixture of 900 ml benzene and 100 ml ethyl methyl ketone was shaken for 1 h with 100 ml 2% formic acid (2 ml formic acid and 98 ml water). After separation, the organic phase was used as mobile phase and the water phase was placed in a separate container at the bottom of the jar.

Solvent D. 1000 ml benzene was shaken for 1 h with 100 ml 2% formic acid. After separation, the organic phase was used as mobile phase and the water phase was placed in a separate container at the bottom of the jar.

All solvents used were C.P. grade and no extra purification was found to be necessary. In the case of both formic acid and diethylamine 100% products were used. Distilled water was used in all preparations.

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Usually 10 l of each solvent were prepared. The solvents were stored tightly closed for 9 months and in our experience storing does not influence their developing power in chromatographic separation. However, especially when using systems B, C, D and E, it was found necessary to change the bottom solvent in the chromatographic jars, as well as the solvent in the troughs every second month. The temperature in the room where the jars were placed was kept as close to 21° as possible by means of thermostatic control.

After mixing the solvents with equal amounts of 90% aqueous ethanol, the pH for each solvent was found to be as follows:

Solvent	F	E	A	B	C	D
pH value	2.7	10.0	3.9	4.8	4.3	5.8

COLOUR ESTIMATION AND RECORDING OF DATA

Colour recording of the chromatograms was performed according to an arbitrary standard colour chart, in which the colours were numbered in 72 shades. This followed the "Derwent" colour pencils index made by Cumberland Pencil Co. Ltd. England. Other colour charts were found to be more complicated to handle for everyday use. These colour shades proved satisfactory for describing the colour reactions employed in this investigation, although many of them were not used owing to the fact that the difference between them was practically indistinguishable.

For comparative purposes, single colours were applied with colour pencils as spots, about one inch in diameter, on Whatman No. 1 paper strips. Within the same spot the colour was applied in such a way that its intensity varied. The best results were obtained by rubbing the spots, after they had been drawn, with cotton moistened with carbon tetrachloride. Six series of 12 spots each were numbered and the whole was fixed between acrylate plates, as seen on the right in Fig. 1.

After each spraying with a reagent, the index number of the colour produced was immediately indicated with a soft pencil on the chromatographic spot. At the same time the spot was encircled. In some cases when the colours altered quickly, within a few seconds, from one definite colour to another, two numbers were indicated in the spot. An immediate evaluation was necessary, since most of the colours produced by the action of different reagents were unstable or were changed by the vapours of other reagents in the neighbourhood. As previously stated, the colours were read off against an illuminated screen. The main difficulty was to avoid subjective evaluation of colours. It is also important not to neglect errors caused by variations in the concentrations of the substances, which influence the colour intensity, or the possible overlapping of another substance or impurity. However, the colour reactions recorded in Tables I–XXI, obtained with approx. 25 γ of substance on an area of 10 × 20 mm, indicate rather good agreement of the colour pattern with that of the structural analogues of the same substance. Even if they are recorded as one of the numbers of a sequence, for instance 30, 31 or 32, 33, this does in practice refer to the same colour. This variation in colour shades occurred during the investigation of reference sub-

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stances, when using on purpose a special numerical code instead of the chemical nomenclature in designating the substances during spraying, in order to avoid subjective colour estimation. At the same time various types of compounds were investigated in order to obtain reliable information concerning the reagents used. Slight deviations in the indicated colours, especially when diazonium reagents were used, as compared later with those of structural analogues, demonstrated the limits of the probable experimental error.

It should be pointed out that in many cases, well-known colour reactions for certain compounds could not be detected with the concentrations used on the paper (for instance ferric chloride produces a colour with resorcinol in a test tube experiment, but here the result was negative). On the other hand, some unusual reactions may be due to impurities, which give mixed colours when they overlap the substances. In these cases, where the impurities had distinctly different R_F values, they could easily be overlooked. Another effect, which is mainly caused by the rate of the reaction, has been observed when spraying the same substance from six different solvent systems. This is obviously connected with the different amounts of acid or alkali remaining in the paper. The colour pattern turns out almost the same, but develops at a different speed.

When the total amount of substance was smaller than about 200 μg, the colour reactions were first tested as spots on a filter paper and the R_F values were afterwards estimated by developing one spot for each solvent system with the best detection reagent.

The colour in ultraviolet light was estimated before spraying, the same colour index being used, and the fluorescent regions were drawn by pencil. However, the

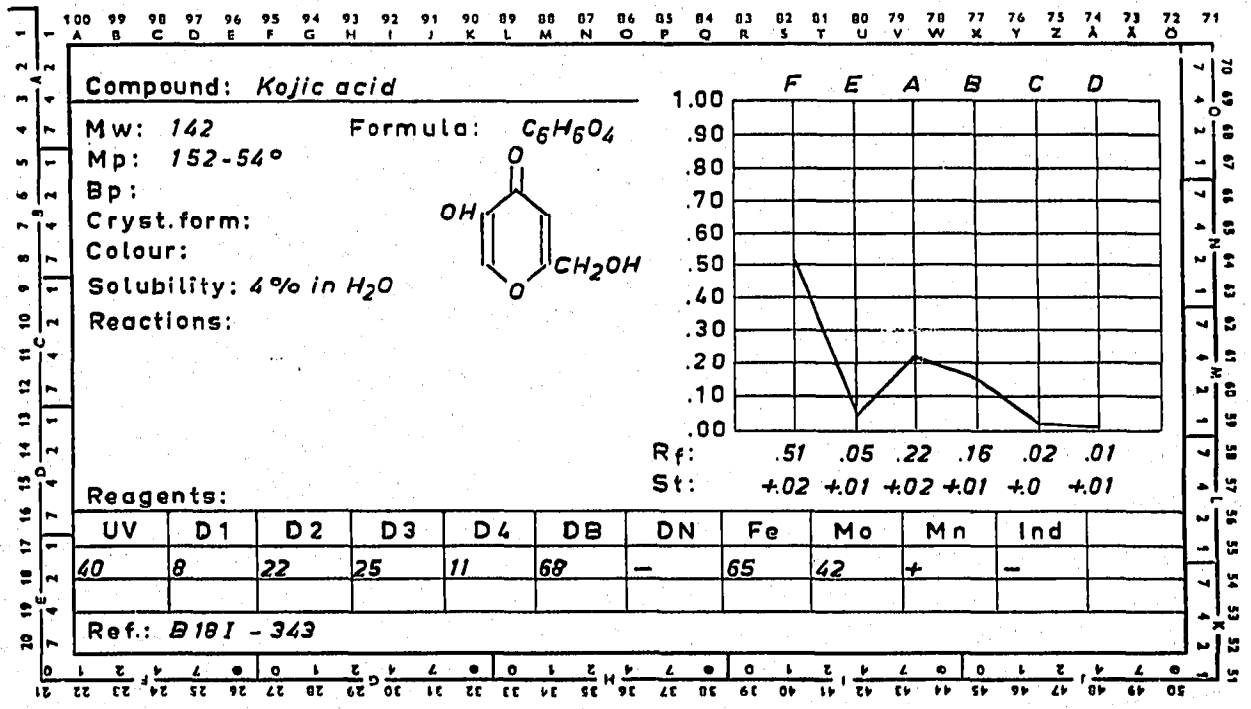

Fig. 6. Sample of record cards for the reference substances.

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accuracy was much less when dealing with strongly fluorescent substances, the brightness of which varied with the pH of the filter paper. Some additional information was obtained when after spraying, the sprayed areas were inspected in U.V. These colour-shifts were not included in the Tables.

Single substances of the reference system were recorded on special punched cards, as shown in Fig. 6. The R_F values were entered on the R_F-network as a diagram. The colours were recorded by numbers on a special section of the cards and were also indicated by means of the colour pencils. A complete record of the numerical data obtained from mixtures, which data consisted of R_F values and of a numbered code of colour reactions, was made with a typewriter on special forms of 22 × 36 cm size. Each form was divided vertically into 6 parts corresponding to the solvent systems used. Each part was divided in turn into 12 vertical columns for the reagents used. Different R_F levels were indicated horizontally. The code-numbered colour reactions were entered horizontally on the forms alongside the appropriate R_F values, representing the colour-reaction sequence for a single substance (or several substances in the case of overlapping). By connecting the middle of the six similar colour sequences, a diagram is obtained, which can be redrawn on the same form on a smaller scale on the R_F-network for comparative purposes. This smaller diagram (of which six on each form were reserved for that purpose) corresponds in size to the R_F-network used for recording the compounds on the special cards of the reference system and can be compared directly as to the type of diagram and mean colour sequence.

When all components of the mixture give almost the same colour pattern, which is often the case when homologous compounds are present, it is sometimes advisable to number the different spots uniformly on 6 chromatograms which appear to belong together. Evaluation is facilitated when the spot areas are connected after the spraying is finished, so that a diagram is obtained. When freshly sprayed, the different spots can be more easily distinguished as to their reactivity and their relative differences in size. Also rapid shifts of colour shades could in many cases be of help in localizing the R_F-position of one and the same substance.

GUIDE TO TABLES I–XXI

The following Tables present information compiled for approx. 450 organic compounds, investigated by the procedure outlined in the previous sections. The R_F values are recorded from six different solvent systems designated by F, E, A, B, C and D. For the composition of these solvents, see the list of abbreviations given below. Under the heading "Detection" (columns 2–11) the colour reactions are recorded for 10 different reagents used for the identification of each compound; in addition, the colour produced in ultraviolet light is indicated in the first column under this heading. The amount of substance used in these experiments was 25–50 γ per spot, which produced different colours when the reagents were applied to them. These colours are referred to by numbers, the explanation of which is found in the colour index. For abbreviations of the reagents, the reader is referred to the following section.

Table I	Monohydric phenols and their derivatives.
Table II	1,2-Dihydric phenols and their derivatives.
Table III	1,3-Dihydric phenols and their derivatives.
Table IV	1,4-Dihydric phenols and their derivatives.
Table V	1,2,3- and 1,3,5-Trihydric phenols, derivatives and related compounds.
Table VI	Naphthalene derivatives.
Table VII	Benzoic acid derivatives.

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Table VIII Aromatic non-phenolic monocarboxylic acids with the COOH group in the side chain and their derivatives.
Table IX Aromatic non-hydroxylated di-, tri- and tetracarboxylic acids, and their derivatives.
Table X Aromatic and heterocyclic amino acids.
Table XI Biologically active nitrogen compounds, *e.g.* biogenic amines, etc.
Table XII Coumarin derivatives.
Table XIII 1,2- and 1,4-Pyrone derivatives.
Table XIV Naturally occurring tropolones.
Table XV Natural products of vegetable origin, *e.g.* pinosylvin, hesperetin, quercetin, etc.
Table XVI Miscellaneous compounds.
Table XVII Metabolic products from moulds and *Penicillium* species.
Table XVIII Unsaturated aliphatic mono-, di- and tricarboxylic acids.
Table XIX Aliphatic dicarboxylic acids.
Table XX Aliphatic hydroxy acids.
Table XXI Aliphatic keto acids and hydroxy- and keto-compounds of biological interest.

ABBREVIATIONS USED IN TABLES I–XXIV

Chromatographic solvent systems:

F = Ethyl methyl ketone–acetone–formic acid–water
E = Water–ethyl methyl ketone–diethylamine
A = Methyl isobutyl ketone–formic acid–water
B = Chloroform–methanol–formic acid–water
C = Benzene–ethyl methyl ketone–formic acid–water
D = Benzene–formic acid–water

For preparation of the different solvents see the section on *Chromatographic solvents*.

Reagents used for detection:

U.V. = Ultraviolet light
D1 = Diazotized sulfanilic acid
D2 = Diazotized 4-benzoylamino-2,5-dimethoxyaniline
D3 = Diazotized *o*-dianisidine
D4 = *p*-Nitrobenzenediazonium fluoroborate
DB = 2,6-Dibromoquinone-4-chloroimide
DN = 2,4-Dinitrophenylhydrazine
Fe = Ferric chloride
Mo = Phosphomolybdic acid
Mn = Potassium permanganate
Ind = Bromophenol blue

For preparation of the various reagents see the section on *Spraying reagents*.

Owing to the lack of space in the Tables, the R_F values have been recorded as 12, 56, 88 but should be read: 0.12, 0.56, 0.88 etc. The colours produced by the reagent are recorded as numbers, and the corresponding shades can be found in the colour index. The — sign means that no reaction was observed under the prevailing conditions. The + sign indicates an uncertain reaction, which was too weak to deserve colour estimation. Reactions with the reagents Mn and Ind are only indicated by the signs: —, + or + +. The + + sign means that a positive reaction was obtained immediately. In the few cases where the colours are recorded by a number placed on top of another number, *e.g.* $\left.\begin{matrix}24\\57\end{matrix}\right|$, this indicates that immediately upon spraying, a violet spot (24 in the colour index) appears which, within a few seconds, turns to light brown (57 in the colour index). Usually most colours are unstable and after some time take on a brownish tone, which is to some extent caused by the chemical influence of other reagents used in the vicinity. This change in colour is not recorded in the tables, nor is there any column for those compounds that, at this low concentration, are visible on the chromatograms because of their own colour.

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COLOUR INDEX FOR THE TABLES

The colours produced by the action of different reagents on the investigated compounds, presented in Tables I–XXIV, have been recorded by number, according to the following code:

01 Zinc Yellow
02 Lemon Cadmium
03 Gold
04 Primrose Yellow
05 Straw Yellow
06 Deep Cadmium
07 Naples Yellow
08 Middle Chrome
09 Deep Chrome
10 Orange Chrome
11 Spectrum Orange
12 Scarlet Lake
13 Pale Vermilion
14 Deep Vermilion
15 Geranium Lake
16 Flesh Pink
17 Pink Madder Lake
18 Rose Pink
19 Madder Carmine
20 Crimson Lake
21 Rose Madder Lake
22 Magenta
23 Imperial Purple
24 Red Violet Lake
25 Dark Violet
26 Light Violet
27 Blue Violet Lake
28 Delft Blue
29 Ultramarine
30 Smalt Blue
31 Cobalt Blue
32 Spectrum Blue
33 Light Blue
34 Sky Blue
35 Prussian Blue
36 Indigo
37 Oriental Blue
38 Kingfisher Blue
39 Turquoise Blue
40 Turquoise Green
41 Jade Green
42 Juniper Green
43 Bottle Green
44 Water Green
45 Mineral Green
46 Emerald Green
47 Grass Green
48 May Green
49 Sap Green
50 Cedar Green
51 Olive Green
52 Bronze
53 Sepia
54 Burnt Umber
55 Vandyke Brown
56 Raw Umber
57 Brown Ochre
58 Raw Sienna
59 Golden Brown
60 Burnt Yellow Ochre
61 Copper Beech
62 Burnt Sienna
63 Venetian Red
64 Terra Cotta
65 Burnt Carmine
66 Chocolate
67 Ivory Black
68 Blue Grey
69 Gunmetal
70 French Grey
71 Silver Grey
72 White = colourless

TABLE I

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MONOHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
91	95	93	91	92	92	Phenol	—	05	08	08	60	38	—	—	—	+	—
90	93	88	93	92	90	Anisole	—	08	14	21	08	32	—	—	—	+	—
93	95	98	97	93	88	*o*-Cresol	—	—	06	—	08	38	—	—	—	+	—
94	94	97	96	93	88	*m*-Cresol	—	05	06	11	08	38	—	—	—	+	—
94	95	96	96	92	87	*p*-Cresol	—	—	06	06	08	72	—	—	—	+	—
95	90	91	93	90	90	4-Chloro-*m*-cresol	33	08	13	60	08	32	—	—	—	+	—
93	91	92	92	92	90	2,3,6-Trichloro-*p*-cresol	—	06	60	17	57	69	—	—	71	+ +	+
90	88	95	89	92	92	2,3-Xylenol	—	08	10	15 | 08	08	25	—	—	30	+ +	—
93	95	94	96	96	88	2,4-Xylenol	—	—	64	64	08	—	—	—	30	+	—
94	94	92	97	96	90	2,5-Xylenol	—	57	06	08	60	30	—	—	30	+	—
95	94	92	97	96	91	2,6-Xylenol	—	—	07	21	—	28	—	—	40	—	—
91	94	91	95	94	89	3,4-Xylenol	—	—	06	—	—	—	—	—	30	—	—
88	93	93	94	93	88	3,5-Xylenol	—	—	07	—	08	67	—	—	—	+	—
89	92	97	96	93	91	2,3,5-Trimethylphenol	—	—	07	08	64	28	—	—	30	+	—
88	86	93	95	93	92	2,4,5-Trimethylphenol	—	07	07	61	64	—	—	—	—	+ +	—
92	90	97	90	92	89	2-Propenylphenol	—	—	—	07	09	38	—	—	30	+	—
95	91	91	94	93	92	4-*tert.*-Butylphenol	—	—	10	19	—	31	—	—	30	+ +	—
94	91	94	95	94	94	4-*tert.*-Octylphenol	—	—	10	19	—	—	—	—	—	+	—
94	96	84	93	92	92	Carvacrol	—	07	08	09	08	33	—	—	30	+ +	—
90	92	97	96	93	92	Thymol	—	—	07	08	60	24	—	—	30	+	—
93	93	86	94	92	92	3-Methyl-4-*tert.*-butylphenol	—	—	07	09	06	25	—	—	—	+ +	—
50	83	42	29	06	00	*o*-Aminophenol	—	06	58	58	64	35	60	63	57	+ +	—
75	91	76	49	13	06	*m*-Aminophenol	71	11	15	61	60	25	—	52	68	+	—

TABLE I *(continued)*

R_F values × 100						Compounds	Detection										
'	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind.
2	68	92	88	87	88	*o*-Nitrophenol	65	06	—	—	62	—	—	—	—	+	—
3	86	88	89	89	67	*m*-Nitrophenol	65	06	—	—	09	—	—	—	—	+	—
2	65	87	88	83	47	*p*-Nitrophenol	65	—	—	—	60	—	—	26	—	+	—
2	67	88	95	92	91	2,4-Dinitrophenol	65	—	—	—	60	—	72	—	—	+	+ +
7	64	92	93	93	92	2,5-Dinitrophenol	65	—	—	—	—	—	72	57	—	+	+
4	74	91	93	95	93	2,6-Dinitrophenol	65	—	—	—	60	—	72	—	—	+	+ +
6	83	34	13	36	30	Picric acid	65	—	—	—	—	—	—	—	—	+	+ +
4	73	93	94	92	93	4,6-Dinitro-*o*-cresol	65	—	—	—	—	—	—	—	—	+	—
1	69	93	92	92	78	6-Nitro-*m*-cresol	53	—	—	52	10	—	72	57	48	+	—
2	86	80	29	86	76	2,4,6-Trinitro-*m*-cresol	65	—	—	57	—	—	72	—	—	+	+ +
5	90	78	66	58	24	Saligenin	—	03	13	13	09	33	—	26	—	+	—
4	90	91	80	70	51	5-Methylsaligenin	—	—	—	06	11	—	—	30	30	+	—
4	83	89	90	85	60	2-Hydroxyacetophenone	—	15	—	62	13	38	06	—	—	+ +	—
9	89	83	90	89	71	3-Hydroxyacetophenone	—	06	60	64	12	39	03	24	—	+	—
7	76	87	87	81	42	4-Hydroxyacetophenone	—	—	—	—	13	—	07	—	—	+	—
1	82	89	88	86	58	4-Hydroxypropiophenone	—	07	—	—	09	—	05	—	—	+	—
3	47	90	84	90	64	Salicylaldehyde	33	06	03	06	—	38	06	25	—	+	—
4	92	87	88	83	55	3-Hydroxybenzaldehyde	—	—	—	—	—	38	06	—	—	+	—
4	69	85	85	80	37	4-Hydroxybenzaldehyde	—	03	—	63	—	—	08	—	—	+	—
0	90	93	88	93	89	Anisaldehyde	—	—	—	—	—	32	03	—	—	+	—
4	92	93	93	90	88	3-Methylsalicylaldehyde	—	09	12	65	10	43	06	25	—	+	—
2	51	92	87	86	52	Salicylic acid	33	06	—	—	07	—	—	24	—	+	+
2	11	92	78	88	33	Thiosalicylic acid	33	01	07	07	08	12 21	—	58	30	+ +	+
2	97	91	83	59	17	1,1-Hexahydrophenolcarboxylic acid	—	—	—	—	—	—	—	—	—	—	+
2	38	88	73	79	50	O-Carbomethoxysalicylic acid	34	—	—	—	06	—	—	—	—	—	+
0	37	84	83	92	64	3-Chloroacetylsalicylic acid	33	—	—	—	08	33	—	24	—	+	+ +
4	67	86	87	85	70	5-Chlorosalicylic acid	33	—	—	—	08	—	—	25	—	+	+ +
1	09	86	45	35	01	3-Hydroxybenzoic acid	—	06	—	65 58	07	33	—	—	—	+	+
0	35	72	37	11	00	2-Hydroxymethyl-3-hydroxybenzoic acid	33	08	—	65	12	34	—	+	—	+	+
0	64	72	62	37	08	4-Hydroxyphthalide	33	03	—	64	58	—	—	24	—	+	—
8	08	91	37	28	01	4-Hydroxybenzoic acid	—	06	—	65 58	07	—	—	03	—	+	+
3	93	92	89	87	64	4-Hydroxybenzoic acid methyl ester	—	—	—	—	—	—	—	—	—	+	—
2	13	93	92	87	74	Anisic acid	—	—	—	—	07	—	—	03	—	—	+
3	11	92	39	43	05	4-Aminosalicylic acid	34	11	12	63	12	26	—	65	—	+ +	+
1	21	09	00	00	00	5-Aminosalicylic acid	48	—	62	64	03	53	—	65	68	+ +	+ +
3	68	45	05	15	03	3,5-Dinitrosalicylic acid	69	—	—	—	08	—	—	59	—	—	+ +
3	03	77	20	43	03	3,5-Dinitro-4-hydroxybenzoic acid	65	—	—	—	—	—	—	—	—	+	+ +
2	20	93	89	88	70	*o*-Cresoxyacetic acid	—	—	—	—	—	—	—	—	—	—	+ +
2	18	90	92	84	59	*m*-Cresoxyacetic acid	—	—	—	—	—	—	—	—	—	—	+ +
1	19	88	92	84	59	*p*-Cresoxyacetic acid	—	—	—	—	—	—	—	—	—	—	+ +
4	61	92	92	92	72	3-Methylsalicylic acid	33	06	—	—	07	33	—	24	—	+	+
4	55	92	91	90	67	4-Methylsalicylic acid	33	06	—	—	08	33	—	23	—	+	+
1	46	89	90	89	65	5-Methylsalicylic acid	38	—	—	—	—	—	—	25	—	+ +	+
8	62	78	90	85	68	6-Methylsalicylic acid	33	08	08	62	09	35	—	23	—	+	+
9	10	87	91	82	74	3-Methoxyphenylacetic acid	—	—	—	—	57	—	—	—	—	—	+
7	08	87	91	82	74	4-Methoxyphenylacetic acid	—	—	—	—	—	—	—	—	—	—	+
1	08	88	58	50	05	4-Hydroxyhydrocinnamic acid	—	—	—	—	—	—	—	—	—	+	+
4	18	94	68	52	00	*o*-Coumaric acid	33	08	11	65 07	07	41 35	03	03	32	+ +	+
0	94	96	92	88	58	*o*-Coumaric acid methyl ester	33	06	07	09	62	41	—	—	60	+ +	—
0	27	94	50	74	25	4-Hydroxyphenylpyruvic acid	33	—	06	25	08	50	06	—	—	+ +	+
2	03	80	07	04	00	5-Hydroxy-3-indoleacetic acid	03	14	65	25	62	68	—	—	30	+ +	+
3	00	60	08	05	00	3-Hydroxyphthalic acid	33	—	—	57	09	35	—	65	—	+	+ +
8	04	62	12	07	00	2-Hydroxyisophthalic acid	33	—	—	—	08	—	—	64	—	—	+
6	02	92	24	13	00	4-Hydroxyisophthalic acid	33	—	—	—	—	—	—	65	—	—	+ +

TABLE II

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	In
88	88	86	57	55	10	Pyrocatechol	—	17	70	—	63	35	50	50 67	57	+	—
90	93	89	94	92	91	Guaiacol	—	08	14	22	08	35	—	—	68	+ +	—
89	93	94	94	92	92	Veratrole	—	08	14	21	—	32	—	—	—	+	—
92	79	92	80	79	35	3-Methylpyrocatechol	—	71	17	18	12	35	50	54	57	+	—
93	91	95	82	76	32	4-Methylpyrocatechol	—	12	24	21	62	35	71	52	60	+	—
91	92	87	93	95	91	4-Methylguaiacol	—	17	10	62	24	68	—	58	30	+ +	—
91	92	93	95	93	95	Eugenol	—	—	10	56	64	42 09	—	—	30	+ +	—
89	91	96	96	94	91	Isoeugenol	—	—	—	—	60	07	—	—	30	+ +	—
92	89	88	95	92	93	Isoeugenol acetate	—	05	—	—	—	—	—	—	—	+	—
77	69	72	57	32	17	Vanillyl alcohol	—	—	12	22 12	11	35	—	—	30	+	—
83	77	83	80	58	43	*o*-Vanillyl alcohol	—	09	12	65	25	35	58	56	52	+ +	—
90	85	92	90	86	82	5-Hydroxymethyl-eugenol	—	13	10	13	—	32	03	—	30	+	—
85	81	88	83	74	62	4-Ethylhydroxymethylguaiacol	—	—	13 06	14 08	60	35	—	—	30	+	—
84	67	91	93	92	90	*o*-Vanillin	33	07	10	12	12	41 38	07	68	48	+ +	—
83	85	87	94	92	89	*o*-Veratraldehyde	—	17	—	—	—	32	07	—	—	+	—
92	71	96	93	92	93	*o*-Vanillin acetate	33	08	10	15	10	38	03	71	03	+	—
86	44	88	48	28	01	Protocatechualdehyde	—	—	—	—	59 64	50	07	43	06	+ +	—
83	81	94	89	78	67	Isovanillin	33	07	10	12	12	41	07	68	48	+ +	—
84	44	92	90	84	78	Vanillin	—	—	—	—	62	38	07	—	—	+ +	—
87	89	88	94	92	92	Veratraldehyde	30	06	03	63	10	33	07	—	—	+	—
86	83	86	84	95	92	Piperonal	—	—	—	—	—	—	07	—	—	+	—
88	23	94	94	89	92	4-Acetoxy-5-methoxyisophthal-aldehyde	48	06	—	—	—	—	03	27	04	+	—
88	73	93	89	82	73	Coniferyl aldehyde	38	07	07	07	—	07 27	—	—	—	+	—
88	86	87	93	94	92	3,4-Dimethoxyacetophenone	—	07	65	21	06	33	06	—	—	+	—
88	01	85	61	38	06	3-Methoxy-4-hydroxy-ω-hydroxy-methyl-acetophenone	34	08	—	24	09 60	33	+	—	71	+	—
88	34	84	39	38	04	2,3-Dihydroxybenzoic acid	32	13	—	—	12	34 05	—	30	68	+	+
93	12	93	92	86	78	*o*-Veratric acid	—	—	—	—	—	—	—	—	—	—	+
84	03	67	05	02	00	Protocatechuic acid	—	13	—	—	59	71	—	42	57	+	+
83	04	89	62	42	08	Vanillic acid	—	08	—	65	07	33	—	08	68	+ +	+
80	05	94	91	78	67	Veratric acid	—	—	—	—	—	—	—	08	—	—	+
84	07	87	93	83	93	3,4-Dimethoxyphenylacetic acid	—	06	13	14	57	34	—	—	—	+	+
87	04	89	81	61	23	3-Methoxy-4-hydroxyphenylpropionic acid	—	—	—	—	60	27	—	—	—	+	+
78	02	76	08	03	00	Caffeic acid	40	03	22	58	62	42 53	—	42 67	03	+ +	+
83	02	87	68	41	12	Ferulic acid	33	—	17	25 64	14	25	—	59	40 71	+	+
85	03	85	89	72	49	3,4-Dimethoxycinnamic acid	33	—	—	—	06	—	—	58	—	+	+
93	31	97	92	86	47	2-Methoxy-5-propylphenoxyacetic acid	—	—	—	—	—	—	—	—	—	—	+
92	26	88	92	89	84	5-Methoxy-6-hydroxyisophthal-aldehydic acid	48	—	—	—	—	—	03	—	—	—	+
81	00	81	79	70	23	4-Methoxy-5-hydroxyisophthal-aldehydic acid	33	—	—	—	64	—	06	58	—	+	+
83	00	63	09	06	00	4-Hydroxy-5-methoxyisophthalic acid	30	03	—	—	08	—	—	24	—	—	+

TABLE III

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,3-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
38	91	83	22	28	02	Resorcinol	34	05	15	21	11	65	—	—	—	+	—
38	89	88	18	32	02	*m*-Propoxyphenol	—	08	15	23	12	25	—	—	29	++	—
39	93	89	42	57	08	2-Methylresorcinol	—	05	15	21	12	25	—	—	68	+	—
94	90	91	38	43	05	Orcinol	34	07	15	65	12	26	—	—	68	+	—
88	84	91	60	66	17	4-Ethylresorcinol	—	—	14	22	12	65	—	—	30	++	—
90	91	91	88	88	81	4-Hexylresorcinol = caprokol	—	07	15	23	12	65	—	—	30	++	—
83	79	94	44	59	10	4-Chlororesorcinol	—	06	14	19	12	25	—	—	30	++	—
92	61	91	87	85	42	2,4-Dihydroxybenzaldehyde	—	07	19	22	09	68	07	61	—	++	—
92	73	94	89	90	60	4-Methyl-2,6-dihydroxybenzaldehyde = atranol	65	06	12	65	10	68	03	69	42	+	—
88	62	88	85	83	49	2,4-Dihydroxyacetophenone = resacetophenone	03	03	15	21	09 63	70	+	65	—	+	—
90	82	88	90	90	67	2,6-Dihydroxyacetophenone	—	07 48	19	23	11	25 51	+	70	51	++	—
87	27	83	22	33	01	2,4-Dihydroxybenzoic acid	—	02	15	22	09	24	—	24	68	+	+
94	93	93	94	93	92	2,4-Dimethoxybenzaldehyde	34	06	08	62	—	—	08	—	03	+	—
77	02	77	01	01	00	3,5-Dihydroxybenzoic acid	—	—	15	19	08	25	—	57	25 30	++	+
72	74	61	14	23	04	2,6-Dihydroxybenzoic acid	—	02	15	21	09	29	—	25	—	+	+
93	28	93	47	52	02	Orsellinic acid	—	11	14	22	08	25	—	24	—	++	+
88	81	74	49	68	18	*p*-Orsellinic acid	—	06	19	21	08	24 69	—	28	—	+	+
92	04	83	02	04	00	4-Bromo-3,5-dihydroxybenzoic acid	—	08	19	22	08	28	—	—	30	+	+
89	87	87	83	90	88	2,5-Dimethyl-4,6-dihydroxybenzoic acid methyl ester	—	08	15	21	07	25 51	—	—	51	+	—
92	94	94	93	93	93	2-Methyl-4-methoxy-6-hydroxybenzoic acid methyl ester	—	07	07	23	08	38	—	—	—	—	—
79	00	54	02	00	00	3,5-Dihydroxyphthalic acid	33	07	—	21	10	27	—	65	—	+	+
78	00	62	05	00	00	3,5-Dihydroxy-6-methoxyphthalic acid	33	07	—	19	09	25	—	25 65	01	++	+
79	02	62	79	25	11	Dihydroresorcinol	—	06	15	15	03	56	—	58	71	++	+
86	04	92	82	65	46	5,5-Dimethyldihydroresorcinol	—	—	11	12	01	—	—	62	—	++	+
84	78	84	18	31	02	Hexahydroresorcinol	—	11	14	19	12	25	—	—	30	++	—
92	59	93	95	93	94	2-Nitro-5-methylresorcinol	65	07	19	65	09	69	—	—	—	++	—
94	49	95	91	80	75	4-Nitro-5-methylresorcinol	65	07	19	64	09	69	—	52	—	++	—

RESULTS AND DISCUSSION

This investigation is an attempt to systematize common paper-chromatographic analytical methods in order to make it possible to carry out with a reasonable degree of probability, a preliminary identification of the structure of phenolic compounds derived from biological sources, especially those arising from the metabolism of fungi. It has mainly been based on the comparative study of compounds collected into a suitable reference system, in which six R_F values were presented in diagrams together with their corresponding colour-reaction patterns. The values were compared with similar data obtained when unknown extracts were examined.

References p. 373.

TABLE IV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,4-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
87	92	79	15	20	01	Hydroquinone	—	72	51	72	13	58	—	—	42 70	+	—
74	61	26	16	03	00	Hexahydroquinone = quinitol	—	—	—	—	—	—	—	—	71	+	—
94	93	93	91	95	94	*p*-Butyloxyhydroquinone	—	09	62	62	14	35	—	—	28	+ +	—
89	85	90	35	42	05	Toluhydroquinone	—	56	—	—	11	—	—	—	71	+	—
88	93	94	48	60	16	2,6-Xylohydroquinone	—	—	72	72	—	67	—	—	30	+	—
95	94	97	84	90	72	Thymohydroquinone	—	72	13	72	—	69	—	—	30	+ +	—
75	02	58	03	02	00	Gentisic alcohol	34	52	56	52	51	70	—	+	42 30	+ +	—
50	00	15	00	00	00	2,5-Dihydroxymethylhydroquinone	33	—	—	06	08	69	—	—	30	+	—
97	97	95	82	80	42	Gentisic aldehyde	33	—	—	—	60	70	07	28	30	+	—
88	90	87	89	86	58	2,5-Dihydroxyacetophenone	64	05	06	06	08	54	+	70	01	+	—
91	86	94	93	91	93	2-Hydroxy-5-methoxyacetophenone	03	06	06	06	60	—	—	68	50	+ +	—
83	78	86	15	19	01	*p*-Benzoquinone	—	—	—	—	63	—	—	—	57	+ +	—
88	88	87	28	39	05	Toluquinone	13	72	72	72	57	69	03	—	30	+ +	—
91	92	90	93	86	87	2,6-Xyloquinone	70	—	57	—	—	68	—	—	30	+ +	—
95	93	95	93	92	91	Thymoquinone	70	—	13	—	—	—	—	—	40	+ +	—
29	00	02	00	00	00	2,3,5,6-Tetrahydroxymethylquinone	—	—	—	—	—	—	—	—	—	+	—
31	01	05	00	00	00	Nitranilic acid	59	08	—	—	—	—	—	—	40	+	+
93	dec**	86	94	87	88	2-Methyl-3,5,6-trichloroquinone	—	72	72	72	72	24	—	—	70	+	—
88	36	82	19	25	00	Gentisic acid	38	72	—	—	58	70	—	28	68	+	+
64	04	15	00	00	00	3,6-Dihydroxyphthalic acid	38	72	72	72	14 72	53	—	28	30	+ +	+
89	20	82	10	14	00	2,3-Dicyanohydroquinone	33	72	72	72	72	53	—	29	27	+	—
89	87	93	93	90	88	4,4′-Dihydroxydiphenyl	—	—	—	—	10	—	—	—	—	+	—

* The colour of the compound itself 09.
** Decomposition.

In all about 450 compounds were investigated with regard to their behaviour towards six chromatographic solvent systems and ten colour reagents. About 2,700 R_F values were recorded, as well as some 4,000 colour reactions. Owing to the great number of data obtained from these records it is not possible to discuss them in detail here, and I have, therefore, confined myself to describing the more marked effects of certain types of compounds on the R_F values and colour reactions.

In order to give an over-all picture of the effect that different substituents in the phenolic nucleus have on the R_F values in different solvent systems, a selection of appropriate data is presented in Table XXII (p. 370).

For most compounds listed the approximate R_F ranges are given regardless of the position of the different substituents in the phenolic nucleus. As can be seen from Table XXII, the best separations were obtained with dihydric phenols. For these phenols, a differentiation has been made according to the position of the OH groups: 1,2; 1,3; 1,4. This could serve as a base when taking into account that each additional group, such as alkyl, halogen, aldehyde or acetyl (or ether groups), increases the R_F values to a certain extent, as is the case in solvents B, C, D. An additional carboxyl-

References p. 373.

TABLE V

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2,3- AND 1,3,5-PHENOLS, DERIVATIVES AND RELATED COMPOUNDS

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
82	00	67	08	05	00	Pyrogallol	—	58	65	65	10	60	55	57	57	+	—
86	92	95	95	87	88	1,3-Dimethoxypyrogallol	—	19	21	24	12	35	—	62	70	++	—
88	00	87	44	48	05	4-Ethylpyrogallol	66	08	64	64	06	—	—	70	68	+	—
82	65	60	01	01	00	Phloroglucinol	—	02	65	65	12	70	—	—	68	+	—
87	92	95	95	88	87	Trimethoxyphloroglucinol	—	—	—	—	12	57	—	—	—	—	—
81	00	66	07	07	00	2,4,5-Trihydroxytoluene	—	17	64	64	—	—	57	69	31 25	+	—
88	90	85	94	93	92	Trimethoxygallic aldehyde	—	—	—	—	07	—	06	—	—	+	—
88	31	95	35	32	00	Phloroglucinol aldehyde	—	07	64	65	11	66	07	66	—	++	—
84	89	86	95	80	71	Trimethoxyphloroglucinol aldehyde	33	—	—	—	—	—	07	—	—	—	—
79	07	84	67	48	19	Gallacetophenone	—	06	13	12	06	52	+	52	51	++	—
88	68	96	51	39	00	Acetylphloroglucinol	—	03	65	21	12	69	+	24	30	+	—
77	03	80	04	03	00	Pyrogallol-4-carboxylic acid	34	24	25	25	08	62	—	63	59	++	+
73	00	38	00	00	00	Gallic acid	—	09 70	20	65	09	52	—	68	03 10	+	+
82	02	78	73	29	05	Syringic acid	—	19	19	22	12	35	—	57	68	++	+
81	09	96	92	84	77	Trimethoxygallic acid	—	19	—	—	—	—	—	08	—	+	+
86	87	93	87	92	85	Trimethoxygallic acid methyl ester	—	06	61	62	06	70	—	—	—	—	—
83	04	82	57	34	07	5-Hydroxytoluquinone	—	—	22	22	60	—	03	—	—	++	—
90	00	38	00	00	00	*m*-Digallic acid	—	—	19	17	08	03	—	68	05	++	+
24	00	02	00	00	00	Shikimic acid	—	—	—	—	—	—	—	—	—	+	+
08	09	00	00	00	00	*d*-Quinic acid	—	—	—	—	—	—	—	—	—	+	+

(the effect of which is included in this Table), hydroxymethyl-, hydroxy- or amino-group decreases the value, especially in solvents E, B, C and D. Since solvent E is basic, the presence of a carboxyl group in any structure is demonstrated by a considerable lowering of the R_F value in this solvent. This low value is significant for carboxyl groups and is confirmed by a positive reaction with the indicator reagent. Biogenic amines, coumarins and pyrones could also have relatively low R_F values in solvent E, but they gave negative reactions with the indicator reagent.

For simple monohydric phenols, especially with additional alkyl, halogen or ether groups, the resolving power of the solvents was poor, as was to be expected considering the solvents used. The solvents were originally evolved for investigations of metabolic products from *Penicillium* species, but were later extended to cover a large group of other organic compounds of biochemical interest. In this particular case it was sometimes an advantage to separate rapidly the simple phenols, which have high R_F values in all solvents, from compounds of the phenolic carboxylic acid type. In fact, the latter type predominates in products of mould metabolism. For other metabolic compounds found in moulds (including tetronic acids, pyrones, tropolonic acids, compounds from the TCA-cycle and those with more complex structures, for instance erdin and others), the solvents have proved to give satisfactory resolution, at least solvents E, B and C have with mixtures of up to 10 components.

References p. 373.

TABLE VI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NAPHTHALENE DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	Dr	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
90	92	89	94	90	93	2-Acetonaphthone	33	—	—	—	—	03	03	—	—	—	—
92	18	93	92	88	85	α-Naphthylacetic acid	57	—	—	—	—	—	—	—	—	—	+
92	90	95	94	92	88	1-Naphthol	—	13	22	25	19	36 25	—	13 26	27	+ +	—
92	52	95	87	90	68	1-Hydroxy-2-naphthoic acid	32	09	22 64	65	63	35	—	68	24 71	+ +	+ +
92	91	94	93	92	84	2-Naphthol	—	—	22	25	15	43	—	—	27	+ +	—
94	49	92	79	88	49	2-Hydroxy-3-naphthoic acid	01	12	25	25 35	15	70	—	28	71	+ +	+ +
94	94	92	93	92	91	1,2-Naphthoquinone	13	—	—	—	—	—	—	—	—	+	—
93	82	93	56	63	06	1,3-Dihydroxynaphthalene	—	08	19	23	64	53	—	07	08	+ +	—
92	92	93	58	81	17	1,4-Dihydroxynaphthalene	33	08	23	23	25	68	—	28	28	+ +	—
92	92	88	91	92	92	1,4-Naphthoquinone	65	13	52	26	15	—	—	—	27	+ +	—
91	19	93	93	91	84	2-Hydroxy-1,4-naphthoquinone	63	62	—	62	66	—	01	—	—	+	—
93	93	95	94	96	94	2-Methyl-1,4-naphthoquinone	65	03	—	—	—	—	—	—	34	+ +	—
92	33	85	91	83	87	2-Methyl-3-hydroxy-1,4-naphthoquinone	70	+	+	+	+	—	—	60	+	+	—
86	89	89	52	75	12	1,5-Dihydroxynaphthalene	33	24 15	25	25	12	25	—	—	66	+ +	—
87	88	89	47	66	11	1,6-Dihydroxynaphthalene	69	12	25	25	11	25	—	65	25	+ +	—
95	92	94	54	69	10	1,7-Dihydroxynaphthalene	32	15	65 25	24 35	62	35	—	25 28	45 68	+ +	—
94	92	94	37	60	05	2,6-Dihydroxynaphthalene	32	14	63 25	25 28	65	45	—	48	45 68	+ +	—
92	88	89	39	45	03	2,7-Dihydroxynaphthalene	—	—	23	25	19	67	—	—	43 68	+ +	—

However, interpretation in such cases was rather difficult, but was somewhat facilitated when the R_F data were combined with the results of the estimation of the colour reactions with ten different reagents.

Unsuccessful separation of simple monohydric phenols could, to some extent, be compensated by carefully studying the colour-reaction patterns, since these are rather informative when it comes to discriminating between several possible structures. The simple phenols also proved useful for the study of miscellaneous colour reactions when correlating structural characteristics with the colour produced by different reagents. On introducing a hydroxymethyl-, aldehyde-, nitro- or acetyl-group into a simple phenol, the R_F values in solvents C and D decrease to a different extent. If an additional carboxyl group is present, the R_F values fall significantly in solvents E, B, C and D. An amino group causes a similar lowering of the R_F values in solvents B, C and D, but in solvent E the R_F remains more or less the same. A simultaneous decrease in solvents F and A should also be interpreted as significant for the presence of an amino group.

References p. 373.

TABLE VII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BENZOIC ACID DERIVATIVES

R_F values × 100						Compounds	Detection						Remarks
F	E	A	B	C	D		U.V.	D4	DN	Fe	Mn	Ind	
88	20	98	92	87	79	Benzoic acid	—	—	—	—	—	+	
95	93	94	92	93	92	Hexahydrobenzoic acid	—	—	—	—	—	+	
90	90	94	92	92	94	Acetophenone	—	—	+	—	—	—	
96	90	97	94	94	93	Benzaldehyde	—	07	06	—	+	—	
88	28	85	88	86	80	*o*-Chlorobenzoic acid	—	—	—	—	—	++	
90	39	95	92	92	91	*m*-Chlorobenzoic acid	—	—	—	—	—	++	
94	92	95	94	93	93	*m*-Chlorobenzaldehyde	—	—	06	—	+	—	Mo-71
94	37	91	92	89	92	*p*-Chlorobenzoic acid	—	—	—	—	—	+	
90	46	95	92	92	84	3,4-Dichlorobenzoic acid	—	—	—	—	—	+	
96	16	88	90	80	29	*o*-Nitrobenzoic acid	70	—	—	—	—	+	
96	45	94	88	85	76	*m*-Nitrobenzoic acid	65	—	—	—	—	++	
95	90	94	93	94	92	*m*-Nitrobenzoic acid methyl ester	65	—	—	—	—	—	Mo-71
91	90	91	89	88	86	*m*-Nitrobenzaldehyde	65	—	06	—	+	—	
96	22	92	88	88	88	*p*-Nitrobenzoic acid	70	—	—	—	—	++	
92	63	92	84	82	26	2-Chloro-3,5-dinitrobenzoic acid	50	—	—	—	—	+	DN U.V.-03
91	10	84	81	72	40	Anthranilic acid	33	09	—	57	++	+	D3-08, DB-41
79	02	69	42	11	02	*m*-Aminobenzoic acid	33	10	—	—	++	+	D3-08, DB-42
85	02	77	42	27	05	*p*-Aminobenzoic acid	—	08	—	58	++	+	
88	92	87	87	76	63	*p*-Aminoacetophenone	—	07	06	—	+	—	DN U.V.-33
91	04	90	43	38	06	2-Chloro-4-aminobenzoic acid	—	07	—	59	+	+	
87	18	89	93	83	82	*o*-Toluic acid	—	—	—	—	—	+	
92	05	85	74	78	28	6-Amino-*o*-toluic acid	38	10	—	—	+	+	D1-08, DB-27
85	25	83	91	78	79	*m*-Toluic acid	—	—	—	—	—	+	
90	15	93	86	81	64	2-Amino-*m*-toluic acid	38	11	—	17	++	+	D1-06, D2-07, D3-64, DB-38, Mo-71
87	34	78	89	83	80	*p*-Toluic acid	—	—	—	—	—	+	
92	91	96	96	93	95	*p*-Tolualdehyde	—	—	03	—	+	—	DB-32
93	54	89	88	89	73	3,5-Dinitro-*p*-toluic acid	65	—	—	58	—	++	Mo-30
88	38	88	92	89	89	*p-tert.*-Butylbenzoic acid	—	10	—	—	—	+	
93	91	95	94	92	93	Cumaldehyde	—	—	13	—	+	—	

Most of the compounds listed in this Table gave no reaction with D1, D2, D3, DB and Mo reagents; for exceptions see Remarks.

At the bottom of Table XXII, several types of aliphatic carboxylic acids are presented. It is interesting to note the wide range of R_F values for solvents F and A, which does not occur when aromatic carboxylic acids are involved. This indicates that when acidic components are found at these R_F levels they very probably belong to an aliphatic series.

In Table XXIII (p. 371), a series is collected of those compounds that were available, in order to demonstrate the effect that *ortho* and *para* substitution in benzoic acid has on the R_F values and on the colour reactions. The decrease or increase of the R_F value when certain substituents are introduced followed the same principle, as demonstrated for dihydric phenols (see above). For instance reference may be made

References p. 373. *(Continued on p. 369)*

TABLE VIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-PHENOLIC MONOCARBOXYLIC ACIDS WITH THE COOH GROUP IN THE SIDE CHAIN, AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection						Remarks
F	E	A	B	C	D		U.V.	D4	DN	Fe	Mn	Ind	
92	92	95	94	92	92	Phenylacetic acid	—	—	—	—	—	+	
81	14	85	63	40	03	*dl*-Mandelic acid	—	—	—	+	+	+ +	
25	02	03	03	04	00	Benzilic acid	34	06	—	—	+	+ +	Mo-71
95	27	95	96	89	88	*trans*-Cinnamic acid	—	—	—	03	+ +	+	
91	12	88	91	84	85	Hydrocinnamic acid	—	—	—	—	—	+	
87	84	92	93	90	87	Cinnamic alcohol	—	—	—	—	+	—	
88	86	91	93	89	87	Cinnamaldehyde	—	—	06	—	+	—	Mo-+
92	16	92	92	84	00	*o*-Nitrocinnamic acid	65	—	—	—	+	+	
94	13	89	88	90	64	*p*-Nitrocinnamic acid	65	—	—	—	+	+	
94	93	89	92	93	90	*p*-Nitrocinnamic acid ethyl ester	65	—	—	—	+	—	
92	03	84	62	32	07	*p*-Aminocinnamic acid	38	06	—	—	+	+	D1-08
87	32	89	81	67	25	*dl*-Phenyllactic acid	—	07	—	51	+	+ +	
88	29	92	54	68	25	Phenylpyruvic acid	—	06	06	42	+	+	D1-17, D2-06 D3-22
87	14	90	73	65	23	α-(3-Indolyl)-acetic acid	—	07	—	—	+ +	+	D3-03, DB-58, 17
92	15	92	83	72	42	β-(3-Indolyl)-propionic acid	—	08	—	—	+	+	D1 U.V.-33
94	17	93	88	83	50	γ-(3-Indolyl)-butyric acid	—	08	—	—	+	+	D1 U.V.-33

Most of the compounds listed in this Table gave no reaction with D1, D2, D3, DB and Mo reagents; for exceptions see Remarks.

TABLE IX

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-HYDROXYLATED DI-, TRI- AND TETRACARBOXYLIC ACIDS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection					Remarks
F	E	A	B	C	D		U.V.	DN	Fe	Mn	Ind	
81	00	62	38	08	00	Phthalic acid	—	—	—	—	+	
46	05	13	15	00	00	Hexahydrophthalic acid	—	—	—	—	+	
88	93	87	95	88	87	Phthalaldehyde	33	03	—	—	—	
82	02	78	44	19	02	Homophthalic acid	33	—	—	—	+	D1-06, D2-17 D3-17, DB-17
82	00	70	32	09	00	Phthalonic acid	33	+	03	+	+ +	
87	03	87	50	26	00	4-Chlorophthalic acid	34	—	—	—	+ +	
90	00	90	90	85	68	3-Nitrophthalic acid 1-monoethyl ester	13	—	—	—	+ +	
80	02	73	36	12	00	4-Nitrophthalic acid	65	—	—	—	+ +	Mo-71
86	03	70	12	15	00	3,5-Dinitrophthalic acid	65	—	—	—	+ +	
83	00	53	00	00	00	3-Aminophthalic acid	38	—	—	—	—	D4-09
84	79	29	01	00	00	4-Aminophthalic acid	71	—	—	—	—	D4-06
89	00	87	50	00	00	Isophthalic acid	—	—	—	—	+	
91	00	89	03	02	00	Terephthalic acid	—	—	—	—	+	
69	00	22	02	00	00	Hemimellitic acid	—	—	—	—	+	
91	00	71	04	01	00	Trimesic acid	—	—	—	—	+	
58	00	18	00	00	00	Pyromellitic acid	—	—	—	—	+	

Most of the compounds listed in this Table gave no reaction with D1, D2, D3, D4, DB and Mo reagents; for exceptions see Remarks.

TABLE X

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC AND HETEROCYCLIC AMINO ACIDS

R_F values × 100						Compounds	Detection										
E	*A*	*B*	*C*	*D*			*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*NH**	*Fe*	*Mo*	*Mn*	*Ind*
00	00	00	00	00	L-Tyrosine		—	08	60	64	63	—	24	—	71	+	—
00	00	00	00	00	Monochloro-L-tyrosine		—	—	—	—	60	—	24	—	—	++	—
00	00	00	00	00	DL-*o*-Tyrosine		34	—	57	13	09	35	64	—	—	++	—
00	00	00	00	00	DL-*m*-Tyrosine		34	—	08	12	09	33	65	—	—	++	—
01	01	00	00	00	3,5-Diiodotyrosine		—	17	—	—	07 23	—	24	—	—	++	—
04	00	00	00	00	DL-Phenylalanine		34	—	—	—	06	—	24	—	—	—	—
01	00	00	00	00	DL-3,4-Dihydroxyphenylalanine		—	06	06	06	61	28	24	42	03	++	—
00	00	00	00	00	DL-Proline		—	72	72	72	02	56	06	—	—	+	—
00	00	00	00	00	DL-4-Hydroxyproline		—	72	72	72	02	56	09	—	—	+	—
00	07	09	01	00	2,2′-Pyrrolidonecarboxylic acid		—	—	—	—	—	—	22	—	—	—	++
03	00	00	00	00	L-Tryptophan		—	—	—	—	06	—	24	—	—	+	—
11	62	86	48	42	Quinaldic acid		33	—	—	—	—	—	—	03	—	+	++
03	09	02	00	00	Kynurenic acid		40	—	—	—	—	—	—	—	—	—	+
01	08	28	04	02	Picolinic acid		—	—	—	—	—	—	+	—	—	—	+
00	31	56	05	01	Nicotinic acid		—	—	—	—	—	—	—	—	—	—	++
00	01	00	00	00	Chelidamic acid		—	—	—	—	—	—	—	03	—	—	++
00	09	00	00	00	Citrazinic acid		44	10	14	22	63	25	—	—	71	—	+

* NH = ninhydrin reagent.
DN reagent gave no reaction with the compounds listed in this Table.

TABLE XI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BIOLOGICALLY ACTIVE NITROGEN COMPOUNDS

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
36	85	00	03	00	00	Ephedrine	—	—	—	60	08	—	—	—	—	+	—
28	73	00	00	00	00	Tyramine	33	12	57	10	06	—	—	—	—	++	—
23	00	00	00	00	00	Adrenaline	—	13	63	65	66	24	—	42	26	+	—
14	00	00	00	00	00	Noradrenaline	—	12	14	14	55	25 69	—	50	28	+	—
92	90	94	93	94	92	Indole	—	06	—	—	10	25	15	—	—	+	—
53	84	01	09	00	01	Gramine	—	—	07	07	11	24	—	—	03	+	—
38	64	00	00	00	00	Serotonine	65	20	24	25	63	35	—	70	28	+	—
92	00	91	84	67	28	Isatin	65	07	08	60	—	—	03	—	—	+	—
24	16	00	01	00	00	Pyridoxine	32	09	24	25	12	35	—	64	71	++	—
16	53	05	31	00	00	Imidazole	—	—	08	12	07	+	—	59	03	+	—
47	00	08	02	01	00	Barbituric acid	34	03	13	15	07	+	—	62	—	++	++
33	02	02	00	00	00	Alloxantin	33	—	—	—	—	—	—	—	71	—	+
08	00	00	00	00	00	Uric acid	—	—	—	—	08	12	—	—	28	—	—
25	12	04	01	00	00	Uracil	—	—	—	—	—	—	—	—	—	+	—
25	14	02	08	00	00	Dehydrouracil	—	—	—	—	—	—	—	—	—	+	—

TABLE XII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME COUMARIN DERIVATIVES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
96	87	84	91	87	89	Coumarin	—	—	—	—	—	—	—	—	03	++	—
93	84	92	97	94	92	3,3-Methylene-bis-4-hydroxycoumarin	—	—	—	—	—	—	—	—	—	+	—
87	62	84	85	73	18	Umbelliferone	44	07	17	64	64	43	—	—	—	+	—
88	66	93	92	76	26	3-Methyl-umbelliferone	44	01	13	65	10	71	—	—	—	+	—
87	63	88	87	72	18	4-Methyl-umbelliferone	33	09	15	21	—	56	—	—	—	+	—
86	62	88	87	73	17	4-Methyl-7-acetoxycoumarin	33	—	—	58	—	—	—	—	—	—	—
87	64	89	88	76	25	4-Methyl-6-acetyl-7-hydroxycoumarin	38	08	57	13	58	70	—	09	—	+	—
89	69	90	90	92	92	4-Methyl-7-hydroxy-8-acetylcoumarin	05	05	—	58	—	03	—	—	01	—	—
90	13	85	85	61	08	4-Hydroxycoumarin	34	06	19	21	08	—	—	—	—	+	—
33	01	03	01	00	00	Esculetin	38	—	—	—	61	51	—	—	—	+	—
81	23	77	53	12	00	4-Methylesculetin	01	02	06	07	64	51	—	45	01	+	—
69	66	55	35	45	39	4,5,7-Trihydroxycoumarin-6-carboxylic acid ethyl ester	32	09	64	65	07	69	—	61	30	+	—

TABLE XIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2- AND 1,4-PYRONE DERIVATIVES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
84	13	97	97	93	93	Dehydroacetic acid	—	—	—	—	—	—	—	03	—	+	+
87	10	81	77	58	14	Isodehydroacetic acid	—	—	—	65	—	—	—	—	—	+	+
73	49	48	39	75	56	Dehydroacetic-5-carboxylic acid	—	—	63	64	+	—	—	63	71	+	+
77	03	87	83	23	01	Triacetic acid lactone	—	—	—	09	01	69	—	+	—	+	+
51	05	22	16	02	01	Kojic acid	40	08	22	25	11	68	—	65	42	+	—
83	00	78	70	46	13	Patulin	34	08	64	63	03	—	—	—	—	++	—
91	00	83	90	00	00	Coumalic acid	48	—	08	17	+	—	—	—	—	+	+
16	00	03	00	00	00	Chelidonic acid	—	—	—	—	+	—	—	—	—	+	++
95	96	92	95	91	93	Chelidonic acid diethylester	—	—	57	60	+	—	—	—	—	++	—
07	00	03	00	00	00	Meconic acid	34	58	—	24	17	—	—	64	—	++	++

TABLE XIV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NATURALLY OCCURRING TROPOLONES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
96	93	94	97	93	93	α-Thujaplicin	65	11	—	64	15	—	52	51	—	+	+
97	93	93	97	93	94	β-Thujaplicin	65	12	—	65	65	—	—	62 49	—	++	++
92	92	90	97	95	89	γ-Thujaplicin	65	—	—	—	23	—	—	—	—	+	—
93	91	92	97	95	90	Nootkatin	65	—	—	—	—	—	—	—	—	+	—
94	32	94	96	92	92	Thujic acid	39	—	—	—	—	—	—	03	—	+	—
63	00	38	05	02	00	Puberulic acid	—	—	—	—	—	—	—	—	—	+	—
34	02	06	02	02	00	Puberulonic acid	15	58	—	—	08	—	07	53	03	+	—
84	00	50	07	03	00	Stipitatic acid	38	12	65	65	11	—	—	52	—	+	+

TABLE XV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NATURAL PRODUCTS OF VEGETABLE ORIGIN

R_F values × 100					Compounds	Detection										
E	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
85	91	78	79	27	Pinosylvin	33	10	65	23	12	69	02	—	71	+	—
91	92	95	90	90	Pinosylvin monomethyl ether	—	08	65	23	12	38	02	—	—	++	—
87	95	92	90	77	*α*-Conidendrin	34	62	60	56	56	—	—	—	30	++	—
88	95	93	89	79	*β*-Conidendrin	39	62	62	56	60	34	—	—	34	+	—
88	91	96	87	78	Matairesinol	—	13	62	61	65	32	02	—	30	++	—
86	89	97	86	79	Pinoresinol	—	12	62	61	65	35	02	—	30	++	—
91	95	72	74	12	Nordihydroguaiaretic acid	—	14	23	25	64	29	55	51	30 57	++	+
51	42	00	00	00	Haematoxylin	65	07	28	29	12	25 64	17	66 25	54 28	67	—
68	93	87	71	46	Curcumin	10	—	64	64	59	54	—	64	62 23	++	—
01	13	00	00	00	Chlorogenic acid	34	03	56	07	07	42	—	42	32	+	+
02	09	00	00	00	Ellagic acid	70	—	57	57	56	56	—	71	69	+	+
93	92	29	40	00	Phloretin	13	09	23	65	12	66	—	23	28	++	—
23	54	00	00	00	Phlorizin	—	09	23	65	12	66	—	24	28	++	—
12	92	80	76	19	Hesperetin	—	03	13	64	08	28	—	—	—	+	—
03	00	00	00	00	Hesperidin	—	03	13	64	08	28	—	—	—	+	—
02	08	05	00	00	Hesperidin methyl chalcone	48	58	62	64	08	50	—	56	39	+	—
00	00	00	00	00	Rutin	65	06	65	64	11	48	—	50	07 69	+	—
14	91	05	04	00	Quercetin	48	06	63	64	11	48	—	50	03 43	+	—
21	90	05	09	00	Morin	10	60	65	66	11	03	—	51	51	++	—
00	38	00	00	00	*m*-Digallic acid	—	—	19	17	08	03	—	68	03	++	+
18	93	89	82	25	Cetraric acid	13	17 24	—	18	07	—	+	24	34	+	+
67	93	88	73	00	Gyrophoric acid	—	08	22	24	09	25	+	—	71	+	+

TABLE XVI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF MISCELLANEOUS COMPOUNDS

R_F values × 100					Compounds	Detection										
E	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
04	91	91	86	51	3,5-Dihydroxy-2-carbethoxy-4-carboxyphenylacetic acid ethyl ester	33	07	19	23	10	25	—	17	71	—	+
85	93	94	92	92	3,5-Dihydroxy-2,4-dicarbethoxy-phenylacetic acid ethyl ester	33	07	15	22	09	25	—	—	71	—	—
02	96	17	24	00	Aurintricarboxylic acid	65	—	—	—	—	55	—	24	66 71	67	++

TABLE XVII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME METABOLIC PRODUCTS FROM MOULDS AND *Penicillium* SPECIES

R_F values × 100						Compounds	Detection									
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
94	05	78	57	23	07	γ-Methyltetronic acid	—	01	10	12	08	21	—	64	—	+	+
87	23	70	40	35	03	α-Bromo-γ-methyltetronic acid	33	01	15	20	08	22	—	65	—	+	+
27	00	03	03	02	00	Carolinic acid	—	02	—	—	—	—	—	07	—	+	+
54	16	31	71	39	37	Carolic acid	—	—	—	—	—	—	03	07	—	+	+
08	00	03	04	00	00	Dehydrocarolic acid	—	—	—	—	—	—	—	07	—	+	+
33	00	08	17	02	00	Carlic acid	—	02	—	—	—	—	—	06	—	+	+
52	03	19	10	10	02	Carlosic acid	—	—	—	—	—	—	—	07	—	+	+
87	47	84	98	88	87	Terrestric acid	—	06	—	17	—	—	—	06	—	+ +	+
88	85	90	85	71	41	Penicillic acid	—	—	+	+	—	—	—	—	—	+ +	+
28	00	00	00	00	00	Ascorbic acid	—	—	05	05	—	—	+	72	30	+	+
25	01	00	00	00	00	Dehydroascorbic acid	71	—	—	—	12	—	—	—	—	+	—
68	69	42	52	06	01	Terrein	65	—	—	—	—	57	05	—	—	+ +	—
51	05	22	16	02	01	Kojic acid	40	08	22	25	11	68	—	65	42	+	—
81	49	67	80	68	61	Maltol	—	—	—	—	03	—	—	24	71	+ +	—
83	00	78	70	46	13	Patulin	34	08	64	63	03	—	—	—	—	+ +	—
79	00	77	38	65	45	Spinulosin	65	—	—	64	06	—	—	53	—	+	—
75	02	58	03	02	00	Gentisic alcohol	34	52	56	52	51	70	—	+	42 30	+ +	—
97	97	95	82	80	42	Gentisic aldehyde	33	—	—	—	60	70	07	28	30	+	—
88	36	82	19	25	00	Gentisic acid	38	—	—	—	60	70	—	28	68	+	+
88	62	78	90	85	68	6-Methylsalicylic acid	33	08	08	62	09	35	—	23	—	+	+
89	85	90	35	42	05	Toluhydroquinone	—	56	—	—	56	—	—	—	71	+	—
79	00	54	02	00	00	3,5-Dihydroxyphthalic acid	33	07	—	21	10	27	—	65	—	+	+
78	00	62	05	00	00	3,5-Dihydroxy-6-methoxyphthalic acid	33	07	—	19	09	25	—	25 65	01	+ +	+
94	90	91	38	43	05	Orcinol	34	07	15	65	12	26	—	—	68	+	—
93	28	93	47	52	02	Orsellinic acid	—	11	14	22	08	25	—	24	—	+ +	+
34	00	14	03	00	00	*cis*-Ethylene oxide dicarboxylic acid	—	—	—	—	—	—	—	—	—	24	+
83	00	60	08	05	00	3-Hydroxyphthalic acid	32	—	—	57	09	35	—	24	—	+	+
84	26	78	10	03	01	$C_{10}H_{10}O_6$-acid	34	—	—	21	10	27	—	65	—	+ +	+
88	46	85	28	18	00	$C_{10}H_{10}O_7$-acid	71	58	—	18	59	26 70	04	65	01	+ +	+
83	22	78	13	03	00	Ustic acid	33	—	—	21	10	26 52	—	25 65	33	+ +	+
85	36	79	26	11	00	Dehydroustic acid	65	17 58	—	17	10	—	—	25	—	+ +	+
80	04	82	90	25	03	Cyclopolic acid	—	—	—	17	+	—	05	—	—	+	+
91	02	89	93	91	57	Cyclopaldic acid	34	—	—	—	—	—	+	61	—	+	—
89	00	84	35	27	04	3,5,6-Trihydroxy-4-methyl-phthal-aldehyde	65	—	—	—	—	—	08	51	71	+	—
91	17	89	93	92	80	Mycophenolic acid	—	—	—	—	—	—	—	25	—	+ +	+
93	90	88	92	89	82	*d*-Usnic acid	24	—	—	—	08	—	—	58	—	—	+
93	20	92	93	79	23	Erdin	65	—	—	—	—	33	—	70	—	+ +	+
92	91	90	96	95	92	Geodin	65	69	—	—	—	—	—	—	01	+ +	+
86	85	81	93	89	84	Griseofulvin	39	07	—	17 24	—	—	—	71	26	+ +	—
63	00	38	05	02	00	Puberulic acid	—	—	—	—	—	—	—	—	—	+	+
34	02	06	02	02	00	Puberulonic acid	15	58	—	—	08	—	07	53	03	+	+
84	00	50	07	03	00	Stipitatic acid	38	12	65	65	11	—	—	52	—	+	+

TABLE XVIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME UNSATURATED ALIPHATIC MONO-, DI- AND TRICARBOXYLIC ACIDS

R_F values × 100						Compounds	Detection			Remarks
F	*E*	*A*	*B*	*C*	*D*		*Fe*	*Mn*	*Ind*	
90	06	87	84	73	35	Acrylic acid	—	++	++	
93	05	87	88	93	94	Crotonic acid	—	++	+	
89	13	90	88	84	83	Tiglic acid	—	++	+	
91	08	93	89	92	70	β,β′-Dimethylacrylic acid	—	++	+	
92	15	91	91	93	00	Sorbic acid	—	++	+	
70	00	46	18	02	00	Maleic acid	58	++	++	
85	00	68	16	00	00	Fumaric acid	60	++	++	
82	08	74	18	07	00	Acetylenedicarboxylic acid	—	++	++	
92	00	86	31	13	00	Mesaconic acid	—	++	++	
79	00	59	23	03	00	Itaconic acid	18	+	+	DB-70
71	01	46	25	03	00	Citraconic acid	57	++	++	
79	00	58	19	02	00	Glutaconic acid	18	++	++	
78	00	51	02	00	00	*trans*-Aconitic acid	—	++	++	D1-D4 +, Mo-71
85	00	44	01	00	00	*cis*-Aconitic acid	—	++	++	
90	00	75	23	00	00	Muconic acid	—	+	+	
69	00	22	01	00	00	Tricarballylic acid	—	—	++	

Most of the compounds listed in this Table gave no reaction with the following reagents: U.V., D1, D2, D3, D4, DB, DN and Mo; for exceptions see Remarks.

TABLE XIX

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC DICARBOXYLIC ACIDS AND CORRESPONDING METHYL DERIVATIVES

R_F values × 100						Compounds	Detection	
F	*E*	*A*	*B*	*C*	*D*		*Mn*	*Ind*
66	00	30	04	00	00	Oxalic acid	+	++
69	03	32	08	01	00	Malonic acid	—	++
72	07	39	14	01	00	Succinic acid	—	+
78	07	57	34	03	00	Glutaric acid	—	+
79	08	67	55	04	01	Adipic acid	—	+
84	00	71	76	27	02	Pimelic acid	—	+
92	00	88	79	38	01	Suberic acid	—	+
88	00	88	79	68	02	Azelaic acid	—	+
76	00	50	12	03	00	Methylmalonic acid	—	++
81	00	68	28	13	00	Dimethylmalonic acid	—	++
82	00	67	22	05	00	α-Methylsuccinic acid	—	+
86	01	79	41	13	00	α-Dimethylsuccinic acid	—	+
74	02	70	12	00	00	Tetrafluorosuccinic acid	—	+
90	00	80	41	11	00	α-Methylglutaric acid	—	+
85	00	76	38	09	00	β-Methylglutaric acid	—	+
93	06	85	83	77	00	*d*-Camphoric acid	—	+
92	06	62	93	94	92	Pinonic acid	—	+
94	00	84	74	46	07	Pinic acid	—	+

The compounds listed in this Table gave no reaction with the following reagents: U.V., D1, D2, D3, D4, DB, DN, Fe and Mo.

TABLE XX

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC HYDROXY ACIDS

R_F values × 100						Compounds	Detection			Remarks
F	E	A	B	C	D		Fe	Mn	Ind	
51	01	14	06	00	00	Glycolic acid	—	+	++	
68	00	35	15	04	00	Lactic acid	—	+	++	
13	00	00	00	00	00	Hydracrylic acid	—	+	++	
35	00	03	00	00	00	Glyceric acid	—	++	++	
52	02	10	01	00	00	Tartronic acid	+	++	++	
48	00	07	02	00	00	DL-Malic acid	03	+	++	D3 +
32	00	02	00	00	00	DL-Tartaric acid	44	+	++	
47	00	06	00	00	00	Dihydroxytartaric acid	58	+	++	
48	03	05	00	00	00	Citric acid	03	+	++	
40	00	03	00	00	00	Isocitric acid	—	—	+	
05	00	00	00	00	00	Mucic acid	—	+	+	
05	00	00	00	00	00	D-Galacturonic acid	—	+	+	
07	00	02	00	00	00	D-Glucuronic acid	—	+	+	
27	00	01	01	00	00	D-Glucuronic acid lactone	—	+	—	
05	00	00	00	00	00	D-Gluconic acid	03	+	++	D1-7, D2-17, Mo-30
28	00	01	00	00	00	D-Gluconic acid-δ-lactone	—	+	—	
71	03	32	19	01	00	2-Oxogulonic acid	—	+	+	

Most of the compounds listed in this Table gave no reaction with the following reagents: U.V., D1, D2, D3, D4, DB, DN and Mo; for exceptions see Remarks.

TABLE XXI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC KETO ACIDS AND OTHER KETO AND HYDROXY COMPOUNDS

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
92	35	87	93	77	46	Pyruvic acid	—	08	62	24 64	09	—	03	17	71	+	++
83	00	64	00	00	00	α-Ketobutyric acid	—	—	07	65 60	02	—	03	71	71	++	++
65	00	31	08	00	00	α-Ketoglutaric acid	—	—	07 62	65	01	—	03	60	—	+	++
79	01	44	04	00	00	β-Ketoglutaric acid	—	07	65	56	60	—	+	63	71	++	++
76	00	45	13	01	00	γ-Ketopimelic acid	—	—	—	—	—	—	+	—	—	—	++
78	04	62	71	29	09	Laevulinic acid	—	—	+	+	—	—	03	—	—	—	+
23	00	00	00	00	00	Mesoxalic acid	34	—	—	—	—	—	+	03	—	+	++
42	03	03	02	00	00	Oxalacetic acid	34	07	24 64	15 25	09	—	+	60	71	+	++
97	85	95	95	90	75	Ketipic acid monoethyl ester	34	—	—	62	62	—	+	13	71	—	+
94	88	93	94	92	90	Ketipic acid diethyl ester	33	17	68	55	08	—	+	64	71	++	++
92	93	96	92	93	95	Parasorbic acid	34	—	13	21	—	—	—	—	—	+	—
48	00	11	01	00	00	*trans*-Aconitic acid anhydride	47	—	52	20	07	—	—	—	—	++	++
Miscellaneous keto and hydroxy compounds																	
95	29	33	28	01	00	1,3-Dihydroxyacetone	—	—	—	—	—	—	05	—	71	+	—
85	82	82	93	80	66	Diacetyl	—	06	64	64	—	—	06	—	30	+	—
85	08	91	84	66	20	Acetoin	—	03	+	—	—	18	06	—	—	+	—
77	70	49	73	23	06	Acetaldol	—	—	66	65	—	—	06	—	—	+	—

to the great differences in R_F shown by compounds with nitro-, amino- and hydroxy-groups, in the case of solvents B, C and D. The differences in effect occurring in solvent E are not so marked except when an additional hydroxy-group is present.

In Table XXIII, five examples are also given of salicylic acid derivatives with different substituents at position 5. The variations in R_F values followed the general rule as described previously for dihydric phenols. Colour reactions in U.V. and with ferric chloride were observed throughout, but the shades were slightly different. Of these compounds, 5-aminosalicylic acid was the most reactive, whereas 5-hydroxy-salicylic acid showed a typical hydroquinone colour pattern.

In Table XXIV (p. 371), three typical colour-reaction sequences are presented, which may serve as a guide when determining, with colour reactions only, whether a substance is a pyrocatechol, resorcinol or hydroquinone derivative. As far as the examples from the reference system are concerned, these colour patterns were fairly constant and were not influenced by additional substituents. An additional hydroxy group forms an exception, since it gives colour patterns identical to those of trihydric phenols.

Naphthols show intense colour patterns of the type indicated. Their reactions with DB and Fe are also strong, although the colour varies; this has, however, not been indicated in the Table. It was striking how easily the aliphatic keto acids reacted with diazonium reagents. However, the colours were more or less equal in shade, and it was difficult to discern any distinct differences, except with ferric chloride.

Four stabilized diazonium reagents were chosen from commercial sources. During the testing of several diazonium reagents (some of which are described in the section *Additional reagents*) it was observed that many of them failed to react under standard conditions, the principle of which is that diazo coupling occurs when the *para* or *ortho* position in a phenolic structure is free. Other reagents gave fairly specific colours with certain types of compounds. As can be seen from the Tables, the choice of at least 4 different diazonium reagents is justified, since in many cases only one reagent gives a positive reaction. Phenols substituted in the *meta* position are in all cases favoured. Coupling occurred even if the hydroxyl group was etherified. Pyrones, pyridoxine, barbituric acid and aliphatic keto acids are some of the non-phenolic compounds that undergo diazo-coupling.

2,6-Dibromoquinone-4-chloroimide is known to be a typical phenolic reagent, coupling predominantly with phenols with a free *para* position. With a few exceptions, reaction also occurred when the group in the *para* position could be decarboxylated or oxidized into a volatile aldehyde[18]. Some examples of other types of coupling compounds are *o*- and *m*-aminobenzoic and -toluic acids, indole, pyridoxine, and kojic acid. Very rarely a red colour was produced, for instance with γ-methyltetronic acid, thiosalicylic acid and uric acid.

2,4-Dinitrophenylhydrazine was used for detecting aldehyde and keto compounds. Some examples of other compounds giving positive reactions are haematoxylin, α-thujaplicin, indole, pyrogallol and pyrocatechol.

The colour reactions of ferric chloride with phenolic compounds are usually

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described as rather unspecific. However, most pyrocatechol derivatives gave green colours. Many *meta*-substituted phenols of resorcinol type failed to give colours. This was even the case with simple monohydric phenols, probably due to the low concentration level used. Some examples of non-aromatic compounds with a positive reaction are tetronic acids and some aliphatic acids, which gave yellow colours.

TABLE XXII

A COMPREHENSIVE LIST OF TYPES OF COMPOUNDS INVESTIGATED, WITH THEIR APPROXIMATE R_F VALUES (± 10) IN SIX DIFFERENT SOLVENT SYSTEMS

Approx. range of R_F values × 100						*Types of compounds*
F	*E*	*A*	*B*	*C*	*D*	
						Monohydric phenols
90	90	90	90	90	90	+ alkyl or OCH_3 or Cl
50	80	50	30	10	00	+ NH_2
90	70	90	90	90	70	+ 1-2 NO_2
90	70	90	30	50	70	+ 3 NO_2
90	90	90	70	70	40	+ CH_2OH
90	80	90	90	80	60	+ $COCH_3$
90	90	90	90	90	60	+ CHO
90	10	90	40	30	00	+ COOH
90	90	90	90	90	70	+ COOR
40	20	10	00	00	00	+ COOH + NH_2
80	10	60	20	30	00	+ COOH + NO_2
90	60	90	90	90	70	+ COOH + CH_3
						Dihydric phenols
90	90	90	60	60	10	1,2
90	90	90	30	30	00	1,3
90	90	90	20	20	00	1,4
80	10–30	80	10–30	10–30	00	1,2 + COOH
80	10–70	70	10	10–20	00	1,3 + COOH
80	40	80	20	20	00	1,4 + COOH
80	00	60	00	00	00	1,2 + 2 COOH
80	00	50	00	00	00	1,3 + 2 COOH
60	00	20	00	00	00	1,4 + 2 COOH
80	00–60	60	00	00	00	*Trihydric phenols*
70	00	40–80	00	00	00	+ COOH
90	90	90	50	50–70	10	*Dihydric naphthols*
						Benzoic acid derivs.
90	00	80	50–70	20–60	10–30	+ NH_2
90	25	80	90	90	80	+ CH_3
90	20–40	90	90	90	40–80	+ NO_2
80	00	30–80	10–40	00	00	+ 2–3 COOH
20–60	10	10–50	00–50	10	00	*Aromatic and heterocyclic amino acids*
20–50	10–80	10	00	00	00	*Biogenic amines*
90	20–80	80	80	70	30	*Coumarins*
80	10–50	10–90	10–90	10–80	10–80	*Pyrones*
90	90	90	90	90	90	*Tropolones*
40–80	10–40	10–40	10	00	00	+ COOH
20–90	10–40	10–70	10–70	10–40	10–40	*Tetronic acids*
						Aliphatic carboxylic acids
90	10	90	80	80	40–90	Monocarboxylic acids, unsaturated
80	00	60	20	10	00	Dicarboxylic acids, unsaturated
70–80	10	40–80	10–70	10–60	00	Dicarboxylic acids, saturated
10–60	00	10–30	10	00	00	Hydroxy acids
30–80	10–30	10–90	10–80	10–70	00–40	Keto acids

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TABLE XXIII

THE INFLUENCE OF SUBSTITUENTS ON R_F VALUES AND COLOUR REACTIONS

1. A few examples are given of compounds 5-X-salicylic acid, where X designates the chloro-, methyl-, droxy-, carboxyl- and amino-group.
2. A few examples are given of compounds *ortho*-Y-benzoic acid, where Y designates the chloro-, methyl-, droxy-, amino-, thio-, nitro- and carboxyl-group.
3. A few examples are given of compounds *para*-Z-benzoic acid, where Z designates the chloro-, nitro-, thyl-, amino-, hydroxy- and carboxyl-group.

The influence of these substituents on the R_F values as well as on the characteristics of the colour reactions clearly demonstrated.

R_F values × 100					Compounds	Detection									
E	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
67	86	87	85	70	5-Chlorosalicylic acid	33	—	—	—	08	—	—	25	—	+	+
46	89	90	89	65	5-Methylsalicylic acid	38	—	—	—	—	—	—	25	—	+	+
36	82	19	25	00	5-Hydroxysalicylic acid	38	72	—	—	58	70	—	28	68	+	+
02	92	24	13	00	5-Carboxysalicylic acid	30	—	—	—	—	—	—	65	—	—	+
21	09	00	00	00	5-Aminosalicylic acid	48	—	62	64	08	53	—	65	68	+ +	+
28	85	88	86	80	*o*-Chlorobenzoic acid	—	—	—	—	—	—	—	—	—	—	+ +
18	89	93	83	82	*o*-Methylbenzoic acid	—	—	—	—	—	—	—	—	—	—	+
51	92	87	86	52	*o*-Hydroxybenzoic acid	33	06	—	—	07	—	—	24	—	+	+
10	84	81	72	40	*o*-Aminobenzoic acid	33	—	—	08	09	41	—	57	—	+ +	+
11	92	78	88	33	*o*-Thiobenzoic acid	33	01	07	07	08	21	—	58	30	+ +	+
16	88	90	80	29	*o*-Nitrobenzoic acid	70	—	—	—	—	—	—	—	—	—	+
00	62	38	08	00	*o*-Carboxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+
37	91	92	89	92	*p*-Chlorobenzoic acid	—	—	—	—	—	—	—	—	—	—	+
22	92	88	88	88	*p*-Nitrobenzoic acid	70	—	—	—	—	—	—	—	—	—	+ +
34	78	89	83	80	*p*-Methylbenzoic acid	—	—	—	—	—	—	—	—	—	—	+
02	77	42	27	05	*p*-Aminobenzoic acid	—	—	—	—	08	—	—	58	—	+ +	+
08	91	37	28	01	*p*-Hydroxybenzoic acid	—	06	—	58	07	—	—	03	—	+	+
00	89	03	02	00	*p*-Carboxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+

TABLE XXIV

A LIST OF SOME TYPICAL COLOUR-REACTION SEQUENCES THAT ARE INDEPENDENT OF FURTHER SUBSTITUENTS

Few exceptions have so far been observed. (The code numbers have been given with a good approximation.)

Type of compounds	Detection										
	U.V.	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
Pyrocatechol derivatives		07	10	21	10	35		42		+	
		07	—	—	10	35		42		+	
Resorcinol derivatives		06	15	21	10	65		—		+	
		06	15	21	10	25		25		+	
Hydroquinone derivatives		72	—	—	72	53		—		+	
		72	72	72	72	69		28		+	
Naphthol derivatives		12	25	25	12	+		+		+	
Coumarin derivatives	33	+	+	+	+	51				+	
	44	+	+	+	+	71				+	
Benzoic acid, chloro- and methyl-derivatives only	—	—	—	—	—	—	—	—	—	—	+
Tetronic acids		—	—	—	—			07		+	+
Aliphatic keto acids		07	62	64	09	—	03	60	71	+	+

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Phosphomolybdic acid has been shown to give many colour shades, which were difficult to interpret.

Most compounds gave positive reactions with permanganate. Fully methylated phenols and methyl- and chloro-derivatives of benzoic acid, however, gave a negative reaction. The indicator reagent was specific for detecting carboxyl groups. The colour in U.V. of a non-sprayed compound was recorded. Since it was nonspecific, it was hard to interpret. Examination of the sprayed areas in U.V. yielded further data, but these have not been recorded in this paper.

On comparing the data for an unknown compound with the data of the R_F values presented as a diagram in the reference system in the order F, E, A, B, C and D, it was possible visually to establish its probable type and to exclude several other types as not probable. The identity of six R_F values with those of an unknown compound was not regarded as significant, unless several colour reactions were found to agree. This provided strong evidence for the identity of a certain type of compound, even before it had been isolated. This information facilitates, in some cases, the chemical isolation procedure or the choice of the preparative chromatographic method.

In view of these possibilities, the reference system was provided with several cross-indexing systems.

The inclusion of many natural products of vegetable origin indicates further possibilities of investigation in this field.

ACKNOWLEDGEMENTS

The author is greatly indebted to Prof. G. EHRENSVÄRD, head of the Department, who suggested this work, for his encouragement and financial support and for valuable suggestions and discussions.

His thanks are due to Dr. G. HÖGSTRÖM for permission to use the solvent systems that he has devised, and to Dr. S. LYBING for valuable collaboration in classifying the compounds studied.

For numerous samples of compounds belonging to mould species, the author owes much gratitude to Prof. H. RAISTRICK of the London School of Tropical Medicine, London, to Dr. J. GIERER of the Swedish Wood Research Laboratory, Stockholm, and to Dr. M. NILSSON of the Royal Institute of Technology, Stockholm, who generously put samples of the vanilline–lignin series and pinosylvin–thujaplicin series at his disposal. Drs. G. WIDMARK and T. HULTIN of the University of Stockholm, and E. BLADH, of the University of Lund, have also kindly contributed to the collection of compounds.

SUMMARY

A paper-chromatographic procedure is described for the identification of phenol derivatives, metabolic products from moulds, especially from *Penicillia* species, and aliphatic compounds of biochemical interest. Information is given concerning the application of the method to the characterization of unknown substances encountered in various biological investigations, mg quantities of material being sufficient. The R_F values in six different solvent systems together with the corresponding colour reactions produced by ten standard reagents have been collected and presented in 21 Tables. In all, approx. 450 compounds were investigated.

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Original recordings were made on standard cards, which made it possible to present the R_F values as diagrams and to give the code of colour reactions for each compound separately. A standard numerical colour index was used for these colour recordings.

The possible application of this procedure, within the biochemical, microbiological, medical and organic-chemical fields, to preliminary investigations of unknown mixtures is indicated.

A special set-up was constructed for rapid analysis by means of which spraying as well as evaluation of six chromatograms could be carried out simultaneously.

Two new stable diazonium reagents, *o*-dianisidine diazotate and 4-benzoylamino-2,5-dimethoxy-aniline, were found to be useful for detecting phenolic compounds and aliphatic keto acids. Several other reagents were tested with regard to their usefulness for spraying purposes in paper-chromatographic analysis.

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Received January 13th, 1958