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Chromatographic Studies on Spruce Bark Tannin Extract

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Solid technical spruce bark tannin extract as well as extracts from fresh bark of Norway spruce, *Picea abies* (L) Karst., have been studied by two-dimensional chromatography. A large number of phenolic and fluorescent components were detected along with some aldehydes or ketones. A few components of the technical extract could not be found in fresh bark, and *vice versa*. The acetone extract of the technical extract was fractionated by Craig distribution between ethyl acetate and water (51 fractions). The intermediate fractions, which had the least complicated composition, were further investigated by partition chromatography. Some phenolic components having a strong bluish-violet fluorescence were greatly concentrated but could not be isolated in a pure state. One component of the technical extract, which is probably an artefact containing sulphur, showed a tendency to crystallise but was very unstable when isolated. The ultraviolet light absorption of three of the substances was investigated after elution from the chromatograms.

In spite of the great technical importance of the vegetable tannins, very little is at present known about their chemical structure. The main reason for this is the extremely complex composition of vegetable tannin extracts, as has been shown in recent papers by White and co-workers [1-6]. These authors, using paper chromatography, were able to demonstrate that all vegetable tannin extracts contain a very large number of components. Fractionation of these components by extraction and precipitation methods could be easily followed by paper chromatography.

In recent years, a few crystalline tannins of known composition have been isolated from divi-divi and from myrobalans by Schmidt and co-workers [7]. These substances belong to the so-called hydrolysable tannins. From the condensed tannins, no pure compound with tanning properties has been isolated so far, all publications about their chemical structure being entirely speculative. With the information now available concerning the complexity of these tannins, it must be regarded as an almost hopeless task to try to isolate any pure compounds from them by classical methods. The application of modern

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methods of fractionation, such as chromatography and counter-current extraction, might be expected to give better results.

The bark of Norway spruce, *Picea abies* (L) Karst., is the richest potential source of vegetable tannins in Scandinavia. At present it is utilised for tanning to a very small extent. Previous investigations on spruce bark extract have mainly dealt with its practical value as a tannin extract, and the changes in tanning properties on fractionation [8-14]. In lectures, Grassmann has twice reported the isolation of crystalline substances from spruce bark [15-16], but no further publications have appeared so far.

The present investigation was mainly carried out on solid technical spruce bark extract from AB Tannin, Västervik, Sweden. For comparison, a few extracts of fresh spruce bark were also made in the laboratory. The investigation of the technical spruce bark extract was limited to the acetone-soluble part of it, varying between 25 and 40 % of different samples of solid extract.

Küntzel and Melzer [11] and, later, Haglund [13] have carried out fractionations of aqueous spruce bark extract by precipitation with ethanol. According to Haglund [13], the fraction which is not precipitated in 80 % ethanolic solution contains the greater part of the substances which are valuable for tanning. The ethanol-insoluble fraction contains large quantities of salts and yields a poor leather on tanning. The properties of our acetone extract were found to correspond roughly to those of Haglund's soluble fraction. The acetone-insoluble residue, containing large amounts of salts (ashes 5.1 %), gives very diffuse spots on the paper chromatogram and must be regarded to be less suitable for attempts to isolate pure substances.

PAPER CHROMATOGRAPHY

For analysis of the different fractions of the spruce bark extract, we used two-dimensional paper chromatography. The solvents were those recommended by White and co-workers for other tannins [3], namely 0.1 % acetic acid in water, saturated with *tert.*-amyl alcohol in one direction and *sec.*-butanol (water-saturated) in the other. To make the spots visible, we employed the following methods: 1) Observation of the paper in ultraviolet light before and after treatment with ammonia vapour. 2) Spraying the paper with *bis*-diazotised benzidine solution [17]. This method has previously been used successfully by one of us for identifying phenolic pine heartwood constituents [18]. 3) Spraying with ammoniacal silver nitrate and 4) Spraying with a solution of 2,4-dinitrophenylhydrazine hydrochloride [19].

Methods 1) and 2) are the most important ones, method 2) being specific to phenols (especially polyphenols) and aromatic amines, but method 1) generally being more sensitive. Both methods gives spots of varying colours, which facilitates the identification. The fluorescent spots are generally bluish violet, but in some cases yellow or green. The colours produced by diazotised benzidine are red, yellow or brown. Method 3) is applicable to reducing substances in general, many of which also react with reagent 2). The reducing sugars, which do not react with any of the other reagents, are also detected by method 3). Reagent 4) was used only in special cases for the detection of aldehydes and ketones.

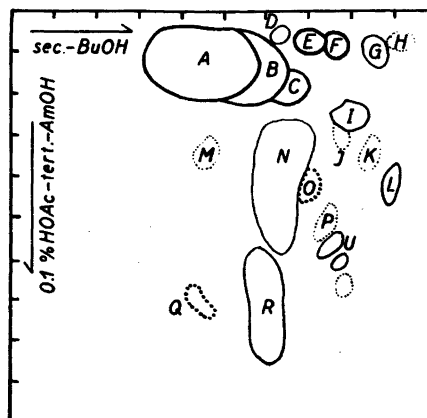


Fig. 1. Two-dimensional paper chromatogram of the acetone extract of solid technical spruce bark extract before fractionation. The starting point is at the upper left corner. Paper: Whatman No 2. Full contours: fluorescent areas. Dotted contours: non-fluorescent areas, detectable by colour reaction with diazotised benzidine. The letters refer to Table 1.

In Fig. 1, a two-dimensional chromatogram of the entire acetone-soluble fraction, before further fractionation, is demonstrated. The picture has been slightly simplified by omitting some very weak and diffuse spots. The chromatogram is entirely dominated by the three spots A, B, and C, giving a strong bluish-violet fluorescence and strong red or brown colours with diazotised benzidine. Although there are many other fluorescent spots on the chromatogram, these three substances must be mainly responsible for the strong bluish-

Table 1. Colour reactions of the strongest spots on the chromatograms of spruce bark extract. This table refers to the spots visible on Fig. 1 and later figures.

Spot	Fluorescence	Fluorescence in NH_3 vapour	Colour reaction with diazotised benzidine
A	bluish-violet	bluish-green	brown
B	» »	bluish-white	red
C	» »	bluish-violet	red
D	yellow	yellow	red
E	blue	green	red
F	blue	blue	red
G	—	green	red
H	—	—	red
I	—	blue	red
J	—	—	red
K	—	—	red
L	—	blue	—
M	—	—	brownish-yellow
N	—	blue	—
O	—	—	brownish-yellow
P	—	—	red
Q	—	—	red
R	—	blue	—
S	—	—	red
T	—	—	brownish-yellow
U	blue	green	red
V	—	—	brownish-yellow
W	—	—	brownish-yellow

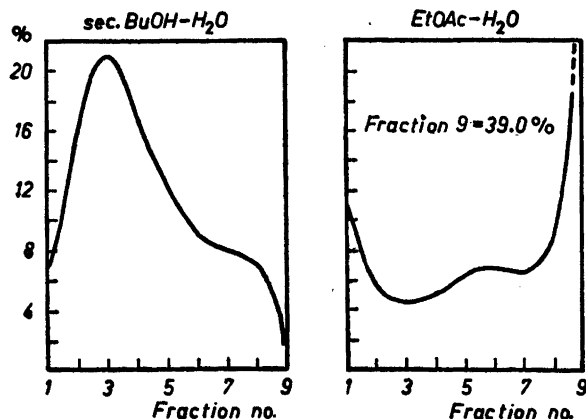


Fig. 2. Craig distribution diagrams (nine fractions) for the acetone extract of solid technical spruce bark extract between sec.*-butanol and water (left) and between ethyl acetate and water (right).*

violet fluorescence of spruce bark. Many years ago, Gerngross made an attempt to isolate the fluorescent component from an ether extract of spruce bark [20–21]. He called it "violettin", believing it to be a single substance, but it is evident that his preparation must have been a very complex mixture.

The colour reactions of the strongest spots on the chromatograms are listed in Table 1. The R_F values are not mentioned in the table but can be approximately estimated from Fig. 1. It is evident that most of the spots give a fluorescence in ultraviolet light. With few exceptions, these spots also give a colour reaction with diazotised benzidine. In some cases, the failure of a colour reaction may be due to a too low concentration of the substance, since the fluorescence is more sensitive than the colour reaction. Finally, there are some non-fluorescent spots giving a colour reaction. A few of these spots give a strong brownish-yellow colour (M, O), while others give a red colour with diazotised benzidine (H, J *etc.*).

COUNTER-CURRENT DISTRIBUTION

For further fractionation of the extract, we used the Craig counter-current distribution technique, which has been previously employed by White and coworkers for fractionation of other tannin extracts [3,5]. Fig. 2 shows the weight distribution curves from two pilot experiments with ethyl acetate-water and *sec.*-butanol-water as the solvent pairs. The number of fractions was nine in both cases. Fig. 2 clearly shows that ethyl acetate-water sharply divides spruce bark extract into an ethyl acetate-soluble and a water-soluble fraction with relatively small intermediate fractions. On distribution between *sec.*-butanol and water, on the contrary, the yield is mainly concentrated to the intermediate fractions. Reducing sugars (detectable with aniline hydrogen phtalate) were found only in fractions 7—9 from the ethyl acetate-water distribution, but in

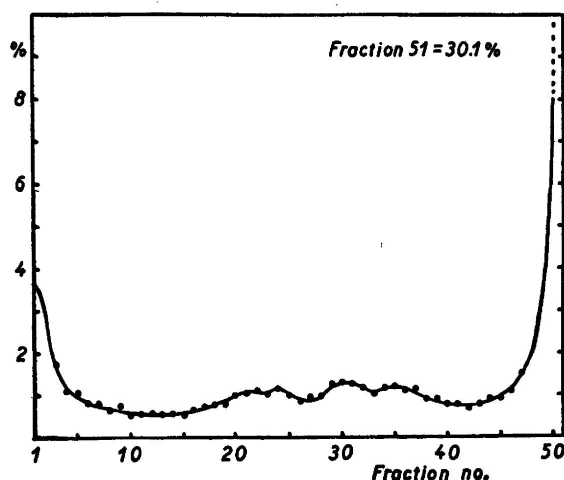


Fig. 3. Craig distribution diagram for the same material as in Fig. 2 between ethyl acetate and water (51 fractions).

the *sec.*-butanol experiment, fractions 3—9 gave a positive sugar reaction. A paper chromatographic analysis of the fractions also showed that the ethyl acetate fractionation is far more promising with regard to the separation of the phenolic spruce bark constituents.

A larger quantity (75 g) of the acetone extract was distributed between ethyl acetate and water in 51 fractions. Fig. 3 shows the weight distribution curve from this experiment. As can be seen from the curve, the fraction most soluble in water (No. 51) contains 30 % of the starting material and fractions 47—51 together more than 45 %. Another maximum is represented by the fractions most soluble in ethyl acetate. (Fractions 1—5 contain more than 10 % together.) In the intermediate fractions, the material is more evenly distributed, but some less pronounced maxima and minima can be observed. None of these fractions contains more than 1.5 % of the starting material.

The chromatograms of the fractions from the counter-current distributions indicate that a rather good fractionation of the material has been achieved. The total number of spots visible on the different chromatograms exceeds that which can be observed on Fig. 1. In Fig. 4, a number of typical chromatograms from some of the fractions are represented. As regards the minor components, one must not neglect the possibility of new substances being formed by oxidation or other reactions during the distribution, which lasted for more than one month. Although a fairly good enrichment of the components has been achieved in certain fractions, we are, however, at this stage still a long way from the isolation of any pure compounds. Each one of the main components occurs in at least ten different fractions, and each fraction still contains several components visible on the chromatograms.

In Fig. 4, the chromatograms from six Craig fractions of different types are represented. The most ethyl acetate-soluble fraction (No. 1) contains a large

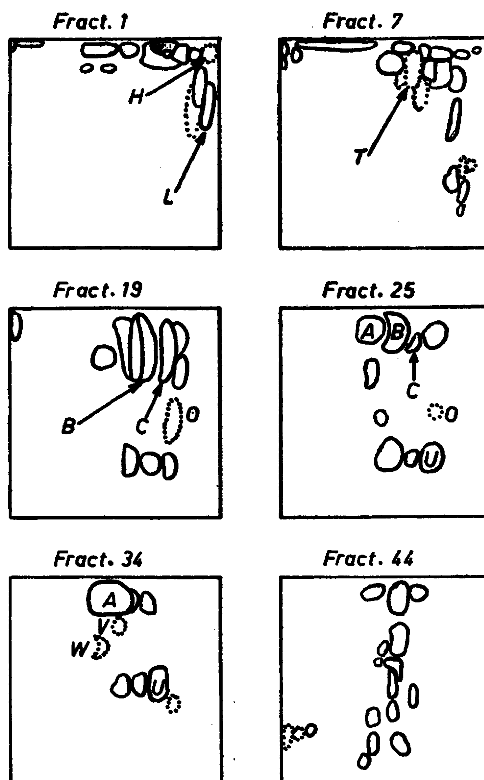


Fig. 4. Two-dimensional chromatograms of six fractions from the Craig distribution. The letters refer to Table 1.

number of substances. Most spots on the chromatogram are crowded along its upper edge. Some of the spots have a yellow or brownish fluorescence, in contrast to the dominating blue or violet colour. One of the strongest spots on the chromatogram is L, which always has an oblong shape and is detected by its strong blue fluorescence in ammonia vapour. This spot has been found in fractions 1—6.

The chromatogram of fraction 7 is rich in spots, many of which are weak and diffuse. The dominating spot is T, which is non-fluorescent. On the chromatogram of the whole acetone extract, it is very likely covered by the strong spots A and B.

The intermediate fractions (about Nos. 13—40) seem to be of less complicated composition than the first and last ones. All the strongest spots on the chromatogram reach their maximum intensity within this interval, and the number of minor components is relatively small. These fractions must be judged to be the most suitable starting material for attempts to isolate any pure substances. In Fig. 4, three fractions from this group are represented.

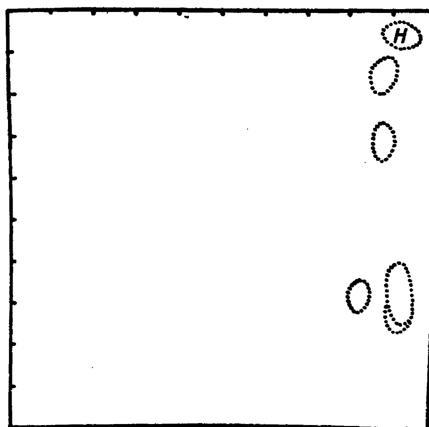


Fig. 5. Position of spots giving a colour reaction with 2,4-dinitrophenylhydrazine, which are found on the chromatograms of the Craig fractions.

About fraction 19, the spots C and O (which is not very strong on Fig. 1) reach their maxima. Fraction 25 represents the maximum of B, while C and O have almost vanished and A is still weak. Two rather strong spots have developed below O. In fraction 34, A dominates together with U, a spot giving a green fluorescence in ammonia vapour. Two small non-fluorescent spots (V, W), giving a similar colour reaction as O, have developed below A.

Fraction 44 is an example of the last fractions, giving a chromatogram containing a large number of relatively weak spots, most of which are gathered along a straight line across the middle of the chromatogram. These fractions must be less suited for isolation experiments.

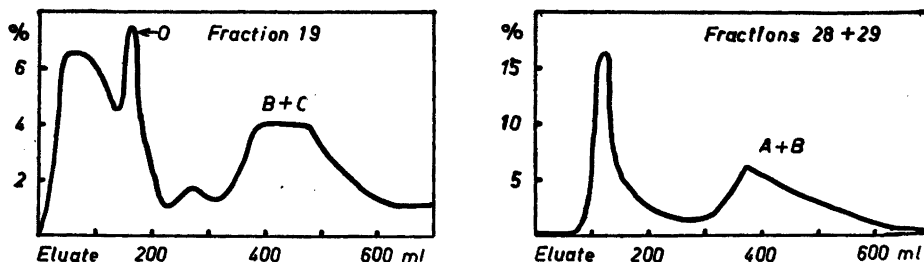
The very last fractions (48—51) give hardly any spots which are visible by fluorescence or benzidine colour reaction. Instead, a very strong reaction of reducing sugars is obtained on spraying with ammoniacal silver nitrate or aniline hydrogen phtalate. At present, nothing can be said about the composition of these fractions, except that they contain carbohydrates. In fraction 51, the content of "tannins", determined by the shake method, was found to be 11.6 %. The corresponding figure for the whole acetone extract was 48 %. Thus, fraction 51 contains only very little of the material which is determined as "tannins" by the official analysis. Of course, the substances in fraction 51 may still play an important part in tanning, although they do not act as tannins when separated from the other constituents of the extract.

At present, it is impossible to distinguish between "tannin" and "non-tannin" spots on the chromatograms.

As mentioned above, 2,4-dinitrophenylhydrazine was also used as a spraying reagent for the detection of aldehydes and ketones. Chromatograms of fractions 1—14 were treated with this reagent, and Fig. 5 shows all the "carbonyl" spots found on these chromatograms. The spots which are situated high up on the paper are found in the first fractions, the lower spots coming out in the higher fractions. All these spots are non-fluorescent. One of them, developing in fractions 1—5, seems to be identical with spot H (see Fig. 1).

PARTITION CHROMATOGRAPHY

As mentioned above, the Craig distribution did not lead to isolation of any pure substances. In order to separate the components of fractions 14—40, partition chromatography on cellulose columns was therefore tried. Water-acetone (4:1), containing 0.1 % acetic acid, was used as a solvent. The fractions of the eluate were evaporated to dryness, weighed and analysed by paper chromatography. Fig. 6 shows a weight distribution curve of the eluate from fraction 19. (*Cf.* Fig. 4). There are three major weight maxima, the second of which corresponds to substance O and the third to B+C and some minor components. The first one contains the fluorescent compounds coming out below O on the chromatogram, but since the intensity of these spots is much lower than that of O, B or C, one must assume that the bulk of the first weight maximum must be due to some substance which cannot be detected on the chromatogram by the methods used here.



Figs. 6 and 7. Weight distribution in the eluate from the cellulose column separations of Craig fraction 19 (Fig. 6) and fractions 28 + 29 (Fig. 7). Each fraction of eluate = 25 ml.

Fig. 7 represents the weight distribution curve from the separation of fractions 28+29 on a cellulose column. The first very sharp weight maximum did not give any strong spots on the chromatogram. The second one is mainly due to compounds A and B but also to some minor components.

The other column separations gave results similar to those demonstrated in Figs. 6 and 7. In no case did we obtain a fraction containing only one of the fluorescent compounds, although they had been greatly concentrated compared with the starting material, and none of these fractions showed a tendency to crystallise. Some fractions, however, seemed to consist mainly of the non-fluorescent compound O (see Table 1 and Fig. 6), and a light brownish crystalline material was obtained on evaporation to dryness. When attempting to recrystallise it, however, brown amorphous products were formed. A qualitative analysis of the crude product gave a positive reaction for sulphur. Substance O may be assumed to be a phenolic sulphonic acid, which has been formed during the weak sulphitation of spruce bark which is carried out when preparing the technical extract. There are some other non-fluorescent spots found on the chromatograms giving the same brownish-yellow colour with diazotised benzidine. None of these spots were found on the chromatogram of fresh spruce bark extract (see below).

It is remarkable that chebulinic acid, chebulagic acid and corilagin (kindly put at our disposal by Prof. O. Th. Schmidt, Heidelberg) give a colour reaction which is similar to that given by the substances just mentioned. These three substances have been isolated from divi-divi and myrobalans [7].

From the fractionation experiments just described, one can draw the conclusion that the compounds corresponding to the strongest spots on the chromatogram (A, B, C, and O) constitute a considerable part of the intermediate fractions, and thus their isolation should not be impossible. On the other hand, these same fractions contain a large percentage of substances which cannot be made visible on the chromatogram by the methods used here. At present, nothing can be said about the chemical nature of these substances, except that some of them may be glycosides. On boiling with dilute hydrochloric acid, reducing sugars could be detected in the solution. These substances always had higher R_F values on the cellulose column than the phenolic substances.

EXTRACTS OF FRESH SPRUCE BARK

The experiments hitherto described were all carried out with commercial spruce bark extract, which is available on the market in large quantities. This extract is prepared by treating the bark with hot water containing a little sodium sulphite or bisulphite, which renders part of the so-called phlobaphenes soluble, thus increasing the yield of extract (see below). From the tanner's point of view, the value of the sulphitation is dubious [11]. Thus, the technical extract may contain sulphur compounds which are not present in the bark, and, in addition, the components of the bark may have been changed by oxidation before the extraction. Extracts of bark, which had just been removed from a living spruce, were therefore prepared. The greater part of the outermost layer of the bark was removed before extraction. The yields are listed in Table 2.

Table 2. Yields of extract of fresh spruce bark with different solvents.

Solvent	Yield, %
Acetone	11
Water	17
Water with Na_2SO_3 + $NaHSO_3$ (0.5 % of the bark of each)	33

A chromatogram of the acetone extract is shown in Fig. 8. The chromatogram of the water extract is very similar, and the higher yield of the latter extract is probably due to an increased extraction of carbohydrates. The sulphite extract, on the other hand, contains some phenolic components which are found neither in the other extracts nor in the technical extract.

If Fig. 1 and Fig. 8 are compared, it is evident that the three strongest fluorescent spots, A, B, and C, are present on both chromatograms. Thus, to study these substances, it is possible to start from the technical extract as well as from fresh spruce bark. The most important difference between the two

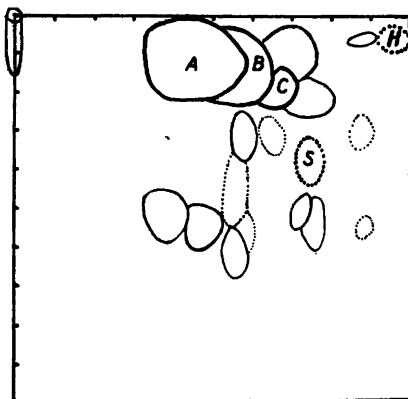


Fig. 8. Two-dimensional chromatogram of the acetone extract of fresh spruce bark. The letters refer to Table 1.

extracts is that compound O and the other non-fluorescent substances with a brownish-yellow colour reaction are totally absent in fresh spruce bark. On the contrary, fresh spruce bark contains, as a main constituent, a non-fluorescent substance giving a strong reddish colour with the benzidine reagent (S), which is absent in the technical extract. On the whole, the total number of minor constituents seems to be larger in the technical extract.

All attempts to obtain compound O in the laboratory by extracting spruce bark with water or sulphite solutions of varying strength failed. This compound must have been formed under the special conditions prevailing during the technical extraction.

ULTRAVIOLET LIGHT ABSORPTION OF SOME OF THE COMPOUNDS

The ultraviolet light absorption of three of the substances (A, O, S) in ethanol solution was determined by elution of the spots from a number of paper chromatograms. All absorption curves had one maximum and one minimum, the wavelengths of which are listed in Table 3.

Table 3. Maxima and minima of ultraviolet light absorption.

Substance	Maximum	Minimum
A	251 mμ	240 mμ
O	262 mμ	239 mμ
S	272 mμ	256 mμ

EXPERIMENTAL

The acetone extractions of solid technical extract were carried out in a large percolator for 24 h. The extract was concentrated in a nitrogen atmosphere and finally dried in a vacuum drying oven at 40°. The extractions of fresh bark were carried out in a Sox hlet apparatus.

The Craig distribution was carried out as follows: Ordinary bottles, provided with sealed-in outlet tubes, were used as separating vessels. Each bottle, in the beginning,

contained 500 ml of water-saturated ethyl acetate. In the first bottle, 500 ml of water, saturated with ethyl acetate, and 75 g of the acetone extract (dry weight 70 g) were also introduced. The bottle was shaken, and the two phases allowed to settle. The aqueous phase was then drawn off through the bottom tube into bottle No. 2, and fresh aqueous phase (500 ml) was introduced into bottle No. 1. Both bottles were then shaken, whereupon the bottom phase of bottle No. 2 was transferred to bottle No. 3, that of bottle No. 1 to bottle No. 2, and fresh water added to bottle No. 1. The fractionation was carried on in this way until the material was divided into 51 fractions. Sometimes, the liquid phases formed emulsions which had to be broken up by centrifugation. Both phases of each fraction were concentrated together by distillation in a nitrogen atmosphere and finally dried in a vacuum. The residues were weighed and the residual moisture content determined by drying a small sample at 105°.

Glass tubes (size 60 × 3.5 cm), packed with cellulose powder (200 mesh), which had previously been boiled with 5 % nitric acid and thoroughly washed with water and acetone, were used for the chromatographic separations. The solvent consisted of 0.1 % acetic acid in water (4 vols.) and acetone (1 vol.). About 0.5 g of material, dissolved in a small volume of solvent, was added to the top of the column by a pipette. The top was covered by a little more cellulose and the solvent allowed to flow through the column. The movement of the fluorescent zones could be observed by illuminating with a quartz lamp. The eluate was divided into 10 to 25 ml fractions, which were evaporated to dryness, weighed and analysed by paper chromatography.

For two-dimensional chromatography, Whatman No. 2 papers (size 23 × 23 cm) were used. The solvents were those described by White and co-workers [3].

Solvent 1: 0.1 % acetic acid in water, saturated with *tert.*-amyl alcohol.

Solvent 2: *Sec.*-butanol, saturated with water.

The chromatograms were run with Solvent 1 for 8 h, dried and then run at right angles with Solvent 2 for 2 h.

Spraying reagents

Diazotised benzidine [17, 18]. Benzidine (5 g) was stirred with conc. hydrochloric acid (14 ml), and the suspension dissolved in water (980 ml). Just before spraying, a 10 % sodium nitrite solution was added to this solution dropwise under stirring until the dark colour first formed turned to light yellow. The mixed solution is not stable. After spraying with this reagent, the papers must be immediately washed with water to avoid a dark background coloration.

Ammoniacal silver nitrate [3]. 5 % aqueous silver nitrate solution (20 ml) was mixed with sufficient 10 % ammonia solution to obtain a clear solution, and the volume then made up to 100 ml. After spraying, the paper was dried at 105° for 10 min. The excess reagent may be removed by washing with sodium thiosulphate solution.

2,4-dinitrophenylhydrazine hydrochloride [19]. Dinitrophenylhydrazine (1 g) was dissolved in conc. hydrochloric acid (300 ml), and the solution diluted to 1 l with water. This reagent forms yellow spots with aldehydes and ketones. The spots must be marked with a pencil immediately after spraying, since they soon become less distinct owing to background coloration.

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