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Constituents of Pine Heartwood

XX. * Separation of Phenolic Heartwood Constituents by Paper Partition Chromatography

GÖSTA LINDSTEDT

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In the previous papers of this series, the extraction of heartwood from different pine species and the isolation of crystalline phenolic compounds from the extracts have been described. The complete investigation of such extracts requires several kilograms of wood and is rather tedious and time-consuming. Also, one can never be sure that all crystalline substances occuring in the extracts have been detected. If there is a very small percentage of one substance along with an excess of syrupy resinous products, it can easily be overlooked. Therefore, it would be of great value to have access to a method which would enable a rapid and safe identification of all the different constituents of a heartwood extract from a small sample of wood. The method of paper partition chromatography, introduced by Consden, Gordon, and Martin [1], fulfils these requirements.

Some investigations concerning paper chromatography of flavones and their glycosides have been published recently. Bate-Smith separated antocyanin and flavone pigments from petal extracts [2], and later made a thorough study of the paper chromatography of antocyanins, flavones, and phenols [3–5]. Wender and co-workers also reported the successful separation of a few flavonoid pigments [6, 7].

The phenolic substances which have been isolated from pine heartwood are flavones (chrysin, tectochrysin), flavanones (pinocembrin, pinobanksin, pinostrobin, strobopinin, and cryptostrobin), and hydroxystilbenes (pinosylvin and its monomethyl ether). The paper-chromatographic separation of these substances in various solvents has been studied. The best results were obtained

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with a water-saturated mixture of equal volumes of benzene and ligroin, containing traces of methanol. This mixture will be referred to as the »standard solvent». Other solvents which gave rather good results were: chloroform-methanol-ligroin-water (2 : 1 : 7 : 5 vols., the supernatant layer used), ethyl ether-ligroin (1 : 5 vols., water-saturated), and carbon disulphide (water-saturated).

Since most of the substances mentioned above are colourless (chrysin and tectochrysin are yellow), they must be made visible by spraying the paper with some reagent after the chromatogram has been run. The best colouring reagent for these substances is a solution of bis-diazotised benzidine, as introduced by Koch and Krieg [8]. It has been used throughout this investigation for qualitative estimation of the phenol content of heartwood by direct staining. It forms red or yellow azo dyes with phenolic substances, and the spots on the filter paper are permanent and very intensely coloured (except for tectochrysin). The heartwood substances differ not only in R_F values and the colour of the spot, but also in the time required for the spot to become visible after spraying with benzidine reagent (See Table 1).

Table 1. Approximate R_F values at 20° in standard solvent and colour reactions with benzidine reagent. (Paper: Munktell OB.)

Substance	Structure	R_F	Colour of spot	The colour becomes visible
Pinosylvin	3,5-Dihydroxy-stilbene	0.05	Dark red	Immediately
Pinobanksin	3,5,7-Trihydroxyflavanone	0.14	Red	Within 0.5—1 min.
Chrysin	5,7-Dihydroxyflavone	0.17	Red	» 3—5 »
Pinocembrin	5,7-Dihydroxyflavanone	0.44	Red	» 1—3 »
Cryptostrobin [9]	Unknown, probably C-methyl dihydroxy-flavanones	0.48	Orange yellow	» 2—4 »
Strobopinin		0.65	yellow	» 1—3 »
Pinosylvin monomethyl ether	3-Hydroxy-5-methoxy-stilbene	0.71	Brick red	Immediately
Tectochrysin	5-Hydroxy-7-methoxy-flavone	0.91	Very pale yellow	Within 10 min.
Pinostrobin	5-Hydroxy-7-methoxy-flavanone	0.93	Orange red	» 7—10 »
Dihydropinosylvin	3,5-Dihydroxydibenzyl	0.11	Dark red	Immediately
3-Hydroxystilbene		0.78	Reddish violet	»
3-Hydroxydibenzyl		0.89	Bright yellow	»

The center of each spot is very difficult to define, the spots often being elliptical and their upper part more or less diffuse. Therefore, the distance from the starting point to the lower edge of the spot has been measured, and this distance divided by the distance travelled by the solvent front at the same time, is here defined as the R_F value.

The nine first substances in Table 1 have been isolated from pine heartwood extracts. For a comparison of R_F values, the three last substances, which are all structurally related to pinosylvin, have also been investigated. Dihydropinosylvin was not isolated in a crystalline state in the original communication by Erdtman. (See Part II [10]). It has recently been obtained in a crystalline state in this laboratory (m. p. 80—82°, private communication from Dr. B. Lindberg). 3-Hydroxydibenzyl has recently been isolated from subfossil Norwegian pine heartwood [11].

Removal or methylation of a hydroxyl group naturally lowers the water-solubility of the substance and thus increases the R_F value. This relation between structure and chromatographic behaviour was first observed by Bate-Smith [3,4]. (Compare also pinosylvin with its monomethyl ether and with 3-hydroxystilbene, chrysin with tectochrysin, and pinocembrin with pinostrobin, Table 1.) Another such relation is evident from the data given in Table 1. Hydrogenation of a double bond causes an increase in R_F, which is, however, smaller than that caused by removal or methylation of a hydroxyl group. (Compare pinosylvin with dihydropinosylvin, chrysin with pinocembrin, tectochrysin with pinostrobin, and 3-hydroxystilbene with 3-hydroxydibenzyl.)

If a mixture of all nine heartwood substances listed in Table 1 is chromatographed, some of the spots will appear too close to each other to make definite identification of each substance possible. Pinobanksin-chrysin, cryptostrobin-pinocembrin, strobopinin-pinosylvin monomethyl ether, and tectochrysin-pinostrobin are substance pairs which do not form two individual spots on the paper, since the differences in R are too small and the upper part of the spots are more or less trailing. Cryoptostrobin and strobopinin can sometimes be distinguished from their partners by the difference in colour reaction, but this is not possible when they occur in small quantities compared to the partners. A second difficulty lies in the identification of tectochrysin, which gives such a weak colour with benzidine reagent that the spot often escapes detection.

The heartwood extracts from pines belonging to the section *Diploxylon* (Hard Pines) do not seem to contain more than four of the phenolic heartwood constituents, namely pinosylvin and its monomethyl ether, pinocembrin, and pinobanksin. A one-dimensional chromatogram gives an excellent separation of this mixture. As follows from the R_F values, the pinosylvin spot comes out

just below the starting point, followed by the spots of pinobanksin, pinocembrin, and pinosylvin monomethyl ether at sufficient intervals. As the R_F values may vary within limits of a few percent, two chromatograms are always run on the same paper side by side, one containing the heartwood extract and the other a mixture of known substances.

Much greater difficulties arise if a mixture of all nine heartwood phenols is to be separated. As mentioned above, the tectochrysin spot is often difficult to observe. The safest identification of this flavone consists of observing the fluorescence of the paper in ultraviolet light. The flavones (chrysin and tectochrysin) give a yellowish-brown fluorescence, which is intensified by spraying with sodium carbonate solution, as indicated by Wender and Gage [6]. Pinosylvin and its monomethyl ether give a bluish-violet fluorescence in ultraviolet light which is independent of the treatment of the paper. Pinostrobin shows a very strong green fluorescence after treatment with sodium carbonate, which facilitates its identification. The colours of the other heartwood substances in ultraviolet light are of little value for their identification.

To achieve a better separation of the phenolic mixture, two-dimensional chromatograms were tried. By running the chromatogram with standard solvent in one direction and with chloroform-methanol-ligroin (2 : 1 : 7 + 5 water) in the other, chrysin can be distinguished from pinobanksin, cryptostrobin from pinocembrin, and strobopinin from pinosylvin monomethyl ether. Since the order of the R_F values is the same in all solvents hitherto tried, the spots do not move quite apart from each other, but the two substances either give different colour reactions (the two last-mentioned pairs), or require different time to become visible (pinobanksin-chrysin). Chrysin, being very sparingly soluble in both water and the organic solvent, causes trailing of the upper part of the spot and is thus distributed over a large area on a two-dimensional chromatogram. When the paper is sprayed with benzidine reagent, the pinosylvin and pinobanksin spots come out first, and after a few minutes they are surrounded by the pale red chrysin spot. Pinocembrin and pinosylvin monomethyl ether also give rather large and diffuse spots on the two-dimensional chromatogram. Fig. 1 shows a chromatogram containing all substances except tectochrysin.

A better separation of strobopinin from pinosylvin monomethyl ether is obtained by running the chromatogram with standard solvent in one direction and with carbon disulphide in the other. The two substances then form separate spots. In every other respect, however, carbon disulphide is inferior to the chloroform-ligroin-methanol mixture.

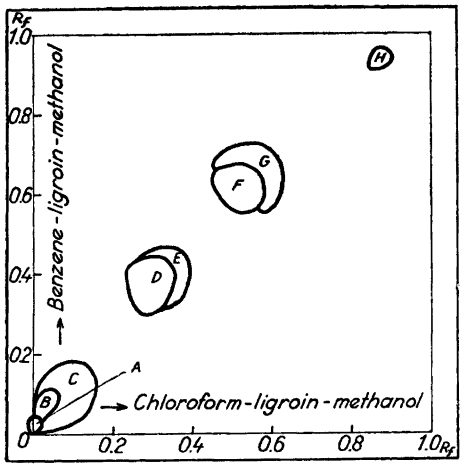

Fig. 1. Two-dimensional chromatogram of eight phenolic heartwood substances after spraying with benzidine reagent. A: Pinosylvin (dark red). B: Pinobanksin (red). C: Chrysin (pale red). D: Pinocembrin (red). E: Cryptostrobin (orange yellow). F: Strobopinin (yellow). G: Pinosylvin monomethyl ether (brick red). H: Pinostrobin (orange red).

Thus, the separation of all nine substances involves the following three operations:

1. A one-dimensional chromatogram with standard solvent, which gives good information as to the main constituents of the mixture. Some of them can be identified accurately (pinosylvin and its monomethyl ether, pinocembrin, and very often pinostrobin).

2. A one-dimensional chromatogram for identification of tectochrysin and pinostrobin. The best solvent for this separation is ethyl ether-ligroin (1 : 5), in which the R_F values are about 0.95 for pinostrobin and 0.8 for tectochrysin. The paper is observed in ultraviolet light before and after spraying with sodium carbonate solution. Chrysin, pinosylvin, and its monomethyl ether may also be identified by this method.

3. A two-dimensional chromatogram using as solvents the standard mixture and chloroform-methanol-ligroin-water (2 : 1 : 7 : 5) respectively, and bis-diazotised benzidine as developing reagent.

The chromatographic methods described above have been applied to heartwood extracts from a great number of pines. Most of the pines from the section *Diploxylon* contain the four substances pinosylvin, pinobanksin, pinocembrin, and pinosylvin monomethyl ether. Owing to the great sensitivity of the chromatographic method, the presence of all four substances can often be demonstrated even when only one or two of them have been isolated by classical methods. Pinosylvin seems to be the substance which is most easily overlooked in preparative work.

The chromatograms of heartwood extracts from pines belonging to the section *Haploxylon* are of far more complicated nature. They generally indicate the presence of all or most of the nine substances hitherto isolated, and, in addition, there are some spots which must be due to unknown substances.

Thus, the paper chromatography method will make possible a rapid and sensitive qualitative analysis of pine heartwood extracts without tedious fractionation by classical methods. It is also a valuable tool for discovering new components. A detailed report of the results obtained with heartwood extracts from numerous pines will be published later.

EXPERIMENTAL

One-dimensional chromatograms

The apparatus used for one-dimensional chromatograms was similar to that described by Wender and Gage [6]. A broad glass cylinder with a glass lid served as vapour chamber. An evaporating dish, standing on a framework of glass rods and metal wire, served as through. The paper strips, 3.5 × 42 cm (Munktell O or OB) were kept in place by a second evaporating dish of smaller dimensions, standing inside the first one. Two chromatograms were run on each paper, one of the unknown mixture and one of the reference mixture. Approximately 0.01—0.02 ml of an alcoholic solution of each mixture were applied as circular spots on the filter paper by means of a glass capillary. The spots were applied on a horisontal line 14 cm from one end of the paper strip at an interval of 1.8 cm. The most suitable concentration for the reference solution is 0.1 % with respect to each component. Too high concentrations cause too high R_F values and vigorous trailing of the spots. For a solution of unknown strength, several chromatograms with different dilutions must sometimes be run.

The paper strips were left hanging down from the dish (five to six strips at one time) in the closed cylinder for one to two hours to become saturated with the vapours of water and solvent, which were present in a liquid state on the bottom of the cylinder. The dish was then filled with solvent from a pipette by means of a small hole in the lid, which was normally covered by a piece of glass. The chromatogram was allowed to run till the solvent front had reached to 2—3 cm above the lower end of the strips. The strips were then taken out of the cylinder, the solvent front marked with a pencil line, and the solvent allowed to evaporate in the air. The time required for the standard solvent to travel the suitable distance (~ 35 cm) was 80—85 min. It travels 3—4 times faster than the butanol-acetic acid mixture used for chromatography of amino acids or sugars [1], when compared on the same type of paper.

The experiments were carried out in a room with the temperature thermostatically maintained at 20° ± 1°. Chromatograms run in an ordinary laboratory, without taking special care to maintain constant temperature, did not differ very much from those run in the thermostat room.

After drying in the air, the paper strips were dipped into bis-diazotised benzidine solution (composition, see below) and allowed to hang moistened with the solution for about two minutes. They were then carefully rinsed with cold water and hung up for drying at room temperature. The coloured spots continue to develop even during and

after rinsing. The rinsing is necessary to remove excess benzidine reagent, which would otherwise give the paper a brownish colour after some time.

The spots are best observed if the paper is made more transparent by painting with cellulose lacquer or better by soaking in molten paraffin.

For the identification of tectochrysin and pinostrobin, the ascending technique [12] was often preferred. The apparatus was similar to that described by Ma and Fontaine [13]. The paper sheet, 24 × 24 cm, allowed ten chromatograms to be run simultaneously. After drying, the paper was investigated under the quartz lamp and the visible spots marked with a pencil. It was then sprayed with 5 % sodium carbonate solution and again observed under the quartz lamp when still wet. The tectochrysin was now clearly visible by means of its yellowish-brown fluorescence, and the pinostrobin spot had a still stronger greenish colour.

Two-dimensional chromatograms

The two-dimensional chromatograms were run in the same apparatus as used for ascending one-dimensional chromatograms. A spot of the solution to be tested was applied near one corner of the paper. (Munktell OB, 24 × 24 cm). A steel rod through the central axis of the paper holder [13] prevented the paper from reaching the bottom of the glass cylinder. The cylinder contained enough solvent (both phases) to reach to a few cm below the lower edge of the paper. It was closed by a cork, and the paper left in the cylinder for one to two hours to attain equilibrium with solvent vapours. The chromatogram was started by siphoning an additional quantity of solvent (organic phase) into the cylinder, so that the lower edge of the paper reached into the solvent. For this purpose, a narrow glass tube was led through the cork to the bottom of the cylinder close to the wall. When the solvent front had mounted to a few cm from the upper edge of the paper, the paper holder was taken out of the cylinder, and the paper allowed to dry in the air. It was then inserted into the paper holder again at right angles to the direction previously used, and the chromatogram run with the second solvent. Finally, the paper was sprayed with the benzidine reagent and rinsed with water as described above.

Reagents

Standard solvent: Benzene (50 vols.), ligroin (b. p. 85—105°, 50 vols.), methanol (1 vol.) and water (50 vols.) were shaken in a separatory funnel, and the organic phase used as the solvent. The funnel was left standing in the thermostat room overnight before the solvent was used. New solvent must be prepared once a week.

Other solvents: A: Methanol (1 vol.), chloroform (2 vols.), ligroin (7 vols.), water (5 vols.).

B: Ethyl ether (1 vol.), ligroin (5 vols.), water (5 vols.).

Benzidine spraying reagent:[8] Benzidine (5 g) was stirred with conc. hydrochloric acid (14 ml) and the suspension dissolved in water (980 ml). A suitable amount of this solution was mixed with an equal volume of a 10 % sodium nitrite solution and the mixture stirred till it was clear and pale yellow. It must be used for spraying within 10 minutes after the mixing.

SUMMARY

It has been shown that all nine phenolic substances hitherto isolated from pine heartwood extracts can be separated and identified by paper partition chromatography.

With the aid of this method, a rapid and extremely sensitive qualitative analysis of pine heartwood extracts can be carried out with very small samples of wood.

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