# PAPER CHROMATOGRAPHY OF MIXTURES OF PHENOLIC COMPOUNDS

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## INTRODUCTION

Chromatography could greatly simplify the analysis of the degradation products of hardwood lignin, provided the use of a single eluent could give, on the sheets, a good separation of the main degradation products, *viz.*: vanillic, *p*-hydroxybenzoic and syringic aldehydes and acids, and ferulic and *p*-coumaric acids.

Many authors have published papers on the qualitative and quantitative analysis of phenolic compounds by paper chromatography[1,2]. In the generally employed technique[2,4-7] two or more eluents are used, and even recently, in a series of papers on the alkaline hydrolysis of hardwoods, PEARL and co-workers[8-11] were able to separate the above-mentioned compounds by using as many as four eluents.

If we consider REIO's[12] determination of the $R_F$ values of 450 compounds, we find that none of the six eluents used by him gives a complete separation of all the eight products; to obtain a separation good enough for quantitative purposes, the $R_F$ values must differ by at least 0.04. We have succeeded in developing a chromatographic technique with which a mixture of these compounds can be separated with a single eluent for qualitative and quantitative evaluation. At the end of this paper the $R_F$ values of 34 pure phenolic compounds obtained with our technique are given.

## EXPERIMENTAL

In our experiments, an hermetically sealed double-walled chromatographic tank was used, generally at room temperature (20 ± 2°); for experiments at other temperatures, the tank was placed in a thermostatically controlled oven. The sheets were cut from Whatman papers, across[1] the fibre direction.

Descending elution was employed; the distance of the starting line from the edge was 10 cm. Ethanolic solutions of the phenolic compounds with concentrations of 10 $\mu g/\mu l$ were applied on the starting line as spots of 2 $\mu l$ for each component, either pure or in a mixture.

The eluent was freshly prepared by shaking equal volumes of *n*-butanol and 2% aqueous ammonia for 10 min; the aqueous phase was separated and put at the bottom of the tank.

*Paper and eluent*

Of the four basic Whatman papers Nos. 1, 7, 20, 54, cut across the fibre direction, we found that Whatman No. 7 is the most suitable for the separation of single compounds; dense, uniform, fairly round spots without trails or beards were obtained, with an elution speed of 33 to 35 cm in 15 h. However, separation of the substances from a mixture was impossible; the three aldehydes were not resolved, nor were vanillic and *p*-hydroxybenzoic acid.

Various authors have obtained better results by using buffered paper. Other variables being kept constant, the $R_F$ values are more reproducible and the separation is somewhat better when buffered paper is used, due to complex formation or to the influence of the pH value at which the paper has been conditioned.

McFARREN[13] found that the molarity of the buffer is of no consequence, whereas the $R_F$ values are sensitive to the particular salt used in preparing the buffer solution. We found, for instance, that at the same pH value the results were different when phosphate-buffered or borate-buffered papers were used, probably because phenols can react with the boric ion.

GARDON AND LEOPOLD[14] applied McFARREN'S technique to the study of the products of lignin oxidation; the $R_F$'s were determined for each compound on a series of papers buffered at different pH values. Since the $R_F$'s of ionisable substances are sensitive to pH variations, a diagram of $R_F$ *versus* pH will show the values of the pH at which the separation of the different compounds is optimal.

We impregnated Whatman No. 7 sheets with mixtures of $H_3BO_3$ and 1 *N* NaOH, in a pH range from 3.5 to 9.2. It was found that care is needed to obtain a uniformly impregnated sheet, which is necessary to prevent the formation of irregular spots. A sufficiently uniform impregnation was never obtained by spraying, so we resorted to immersing the sheet in the appropriate bath, removing the excess of solution by gently pressing the sheet between two filter papers, and then allowing it to dry in air.

At this point we also changed the eluent, going over to *n*-butanol saturated with the same solutions that were used to impregnate the sheets, in order to prevent pH gradients along the sheets. Otherwise the mobility of the spots would be so high that separation and identification would be impaired.

In Fig. 1 the diagram of $R_F$ *versus* pH for the eight pure compounds studied is shown; the hydroxyaldehydes are best separated at pH 9.3 and the phenolic acids at pH 8.0. On repeating the experiment with a mixture of the various compounds, no complete separation could be obtained whatever pH value was used. This might be due to an interaction of the molecules of the different phenolic compounds, so that their mobility is different from that of the pure compounds.

We repeated the experiments with pure products on paper impregnated at four pH values, while 10 ml of a 10 % $NH_3$ solution was placed at the bottom of the chromatographic tank, together with the aqueous phase of the eluent (*n*-butanol saturated with the impregnating solution). Fig. 2 shows a diagram of the $R_F$ values *versus* pH; it can be seen that the separation of the phenolic compounds is markedly improved. We also found that the pH values of the sheets which varied from 3.5 to 8.5 at the

beginning of the experiment, were all 8.6 at the end of the experiment. In the case of papers impregnated at pH 9.3, however, not only the pH remained the same throughout the whole experiment, but also the relative positions of the spots were almost unaltered; at this value of the pH, the presence of $NH_3$ vapour in the vessel is of no consequence.

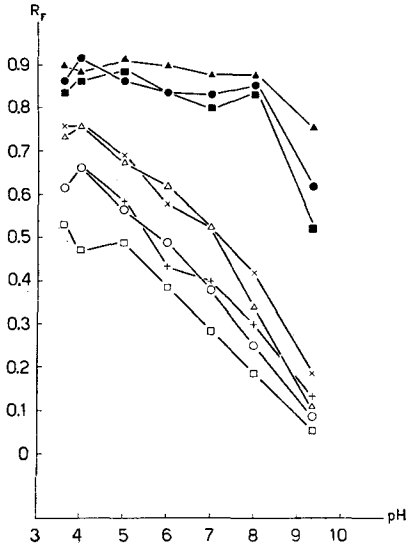


Fig. 1. $R_F$ *versus* pH values. ● Vanillin; ■ Syringaldehyde; ▲ *p*-Hydroxybenzaldehyde; ○ Vanillic acid; △ *p*-Hydroxybenzoic acid; □ Syringic acid; + Ferulic acid; × *p*-Coumaric acid.

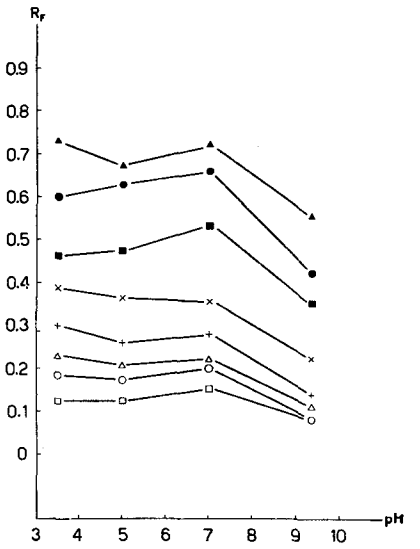


Fig. 2. $R_F$ *versus* pH values, in the presence of $NH_3$ vapours. ● Vanillin; ■ Syringaldehyde; ▲ *p*-Hydroxybenzaldehyde; ○ Vanillic acid; △ *p*-Hydroxybenzoic acid; □ Syringic acid; + Ferulic acid; × *p*-Coumaric acid.

On the other hand, the separation of the mixture was appreciably improved by the presence of ammonia vapours at all pH values. It is not necessary to buffer the paper, a simple impregnation with boric acid is sufficient; it is also not necessary to saturate the eluent with the impregnating solution.

Accordingly, in all successive experiments, the sheets were impregnated with a $H_3BO_3$ solution saturated at 20°, and the eluent was *n*-butanol saturated with a 2% $NH_3$ solution, the aqueous phase of which was placed at the bottom of the vessel.

At the end of the elution, the pH of the sheet was uniformly 8.6 from the starting line to the eluent front, and from there to the edge of the paper. This procedure has given the best separation of a mixture of ten phenolic compounds, whose $R_F$ values are given in Table I.

The chromatographic separation of a mixture of phenolic compounds by this technique is very sensitive to the concentration of ammonia in the aqueous solution used to prepare the eluent. Increasing the concentration of ammonia produces a slowing down of the phenolic acids and hydroxyaldehydes: at an ammonia concentration higher than 5% the separation is seriously impaired. On the other hand, if the am-

monia concentration is raised to 10%, the separation of those phenolic compounds that run with the eluent at lower ammonia concentrations is improved. An $R_F$ of 0.88 is obtained for 2,6-dimethoxyphenol and one of 0.76 for resorcinol, instead of the values 0.86 and 0.81 respectively, as given in Table I, and hence the separation is much better.

TABLE I

$R_F$ VALUES OBTAINED ON CHROMATOGRAPHY OF A MIXTURE OF TEN PHENOLIC COMPOUNDS, AND THE EFFECT OF TEMPERATURE

| *Phenolic compounds* | *$R_F$ values* | |
|---|---|---|
| | *Tank at 20°* | *Tank at 35°* |
| Syringic acid | 0.16 | 0.15 |
| Vanillic acid | 0.20 | 0.19 |
| *p*-Hydroxybenzoic acid | 0.24 | 0.23 |
| Ferulic acid | 0.32 | 0.33 |
| *p*-Coumaric acid | 0.39 | 0.40 |
| Syringaldehyde | 0.52 | 0.52 |
| Vanillin | 0.62 | 0.64 |
| Hydroxybenzaldehyde | 0.72 | 0.71 |
| Resorcinol | 0.81 | — |
| 2,6-Dimethoxyphenol | 0.86 | — |

*Ambient factors*

The temperature variations in the laboratory were ± 2°; with our final technique, the $R_F$ values of the various compounds were always constant. The relative positions of the spots of the compounds are the same, although their absolute positions may change, but the variations in the $R_F$ values are never higher than 0.02. Some experiments were made at 35°, at which the solvent speed was somewhat increased and consequently also the mobilities of the spots, but the $R_F$'s were unchanged, as is shown in the third column of Table I.

The saturation of the atmosphere of the chromatographic tank and the equilibration of the sheet are much more important. Only the aqueous phase should be put at the bottom of the tank; an excess of ammonia or of the phase rich in organic solvent gives elongated spots.

If the sheets are left for a long time in the tank before starting the elution, very diffuse spots with overlapping trails are produced. We found that good spots are obtained if the paper is allowed to equilibrate for one hour in the vessel.

*Spraying solutions*

All the classical developers for phenolic compounds[1,2], as well as some new ones, were tested and finally the following techniques were adopted:

(1) *Mäule's test.* The chromatogram is first dried, then exposed for ten minutes to chlorine vapours, and finally sprayed with a 10% $Na_2SO_3$ solution. Spots of syringic acid or aldehyde assume a cherry-red colour. It was found that on examination with U.V. light some compounds show characteristic yellow or blue spots.

(2) *Other tests.* The chromatogram is subjected to the following treatment:

(a) U.V. examination before and after exposure to ammonia vapours.

(b) Development of the phenolic acids: the sheet is first sprayed with a solution of diazotized sulphanilic acid prepared according to BLOCK[15] and dried in air. Subsequently, it is exposed to ammonia vapours. At this moment, coloured spots appear, but the colours are not brilliant and not fast enough; they fade in a short time.

(c) We then introduced a second spray, this time with a solution of diazotized *p*-nitroaniline prepared according to BRAY[16]; this changes the colours, enhances their brilliance and makes them completely fast even after weeks of exposure to air.

The sheet is dried at 50°. The hues of the spots are characteristic for the various acids. Care has to be exercised when carrying out this last spray, in order to prevent soaking of the paper, otherwise a yellowish background appears and the sheets become brittle when dried, due to the action of the hydrochloric acid present in the solution. Of paramount importance in this technique is the exposure of the sheet to ammonia vapour between the two sprayings with the diazotized solutions; if this stage is omitted, the benefits obtained by spraying with the second solution are lost.

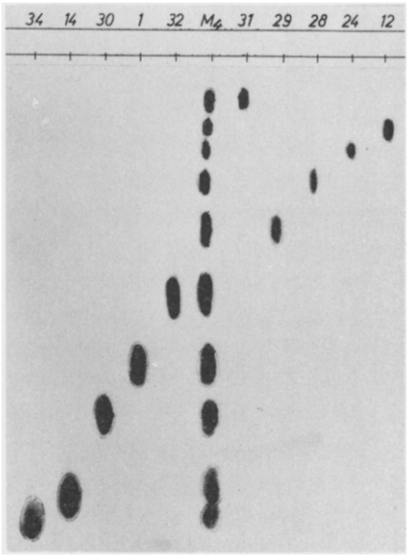


Fig. 3. Chromatographic separation of a mixture of ten phenolic compounds. (12) Vanillic acid; (24) *p*-Hydroxybenzoic acid; (28) Ferulic acid; (29) *p*-Coumaric acid; (31) Syringic acid; (32) Syringaldehyde; (1) Vanillin; (30) *p*-Hydroxybenzaldehyde; (14) Resorcinol; (34) 2,6-Dimethoxyphenol.

(d) Development of the hydroxyaldehydes: the dried sheet is sprayed with a solution obtained by mixing equal volumes of a 0.1 *M* benzidine solution in ethanol and 1 *N* hydrochloric acid. The sheet is then dried in an oven at 50°. The colours obtained with this technique are more brilliant than those obtained by spraying with 2,4-dinitrophenylhydrazine. Furthermore, vanillin and syringaldehyde give different colours.

TABLE II

$R_F$ VALUES AND IDENTIFICATION OF 34 PURE PHENOLIC COMPOUNDS*

| *Compounds* | $R_F$ | *Mäule test* | *Mäule test Ultraviolet* | *U.V.* | *U.V. after exposure to $NH_3$ vapours* | *Diazotized sulphanilic acid spray* | *Diazotized sulphanilic acid and p-nitroaniline sprays* | *Benzidine spray* |
|---|---|---|---|---|---|---|---|---|
| Gallic acid | 0.01 | Magenta 27142 | — | — | — | Straw-yellow 33793 | Straw-yellow 33793 | — |
| Syringic acid | 0.16 | Cherry-red 22356 | Water-green 24554 | — | — | Cherry-red 22356 | Crimson lake 21158 | — |
| Vanillic acid | 0.20 | — | Lemon-cadmium 33814 | — | — | Middle chrome 33538 | Rose-pink 32356 | — |
| *p*-Hydroxybenzoic acid | 0.24 | — | Sky-blue 35231 | — | — | Deep cadmium 13432 | Gold 33793 | — |
| Catechol | 0.29 | Zinc yellow 33434 | Lemon-cadmium 33814 | — | — | Lemon-cadmium 33814 | Naples yellow 10371 | — |
| Ferulic acid | 0.32 | — | Sky-blue 35231 | Light blue 15123 | Light blue 15123 | Pink madder lake 31433 | Light violet 37144 | — |
| Sinapic acid | 0.35 | Cherry-red 22356 | Turquoise-green 15123 | Sky-blue 35231 | Sky-blue 35231 | Cherry-red 22356 | Blue-grey 36173 | — |
| Vanillil | 0.35 | — | Yellow-green 34552 | — | Gold 33793 | Gold 33793 | — | — |
| *p*-Coumaric acid | 0.39 | — | Sky-blue 35231 | — | Sky-blue 35231 | Pale vermilion 22276 | Magenta 27142 | — |
| 3,4-Dimethoxycinnamic acid | 0.42 | — | Sky-blue 35231 | Sky-blue 35231 | Sky-blue 35231 | — | — | — |
| *o*-Coumaric acid | 0.45 | — | Light blue 15123 | Sky-blue 35231 | Sky-blue 35231 | Middle chrome 33538 | Hazel 12648 | — |
| Veratric acid | 0.46 | — | Yellow-green 34552 | — | — | — | — | — |
| Syringaldehyde | 0.50 | Cherry-red 22356 | Water-green 24554 | — | Light blue 15123 | — | — | Orange chrome 32246 |
| *p*-Anisic acid | 0.55 | — | Sky-blue 35231 | — | — | — | — | — |
| Vanillin | 0.60 | — | Lemon-cadmium 33814 | — | Light blue 15123 | — | — | Middle chrome 33538 |
| Trimethylgallic acid | 0.61 | Magenta 27142 | Water-green 34554 | Ultramarine 25414 | Ultramarine 25414 | — | — | — |
| Salicylic acid | 0.69 | — | Sky-blue 35231 | Sky-blue 35231 | Sky-blue 35231 | Deep cadmium 13432 | Gold 33793 | — |
| 2,4-Dimethoxybenzaldehyde | 0.70 | — | — | Ultramarine 25414 | Ultramarine 25414 | — | — | Middle chrome 33538 |
| Acetovanillone | 0.70 | Pale yellow 23695 | Sky-blue 35231 | Sky-blue 35231 | Dark blue 15102 | Flesh-pink 21575 | Gold 33793 | — |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *p*-Hydroxybenz-aldehyde | 0.71 | — | Sky-blue 35231 | — | Dark brown 10080 | — | — | Primrose-yellow 23655 |
| Resacetophenone | 0.71 | — | Yellow-green 24552 | Gold 33793 | Rose-pink 32356 | Gold 33793 | Gold 33793 | — |
| *p*-Methoxycin-namic acid | 0.74 | — | Sky-blue 35231 | Sky-blue 35231 | Sky-blue 35231 | — | — | — |
| Vanillyl alcohol | 0.80 | — | Lemon-cadmium 33814 | — | — | Middle chrome 33538 | Rose-pink 32356 | — |
| 2,4-Hydroxyace-tophenone | 0.81 | — | Sky-blue 35231 | Water-green 24554 | Water-green 24554 | Pale yellow 33481 | Gold 33793 | — |
| Resorcinol | 0.81 | Zinc yellow 33434 | Sky-blue 35231 | Sky-blue 35231 | Sky-blue 35231 | Straw-yellow 33793 | Deep cadium 13432 | — |
| Veratraldehyde | 0.85 | — | Ultramarine 25414 | Water-green 24554 | Water-green 24554 | — | — | Middle chrome 33538 |
| 2,6-Dimethoxy-phenol | 0.86 | Cherry-red 31136 | Yellow-green 34552 | — | — | Orange chrome 32246 | Geranium lake 12197 | — |
| Hydroquinone | 0.89 | — | — | — | — | — | Flesh-pink 21575 | — |
| Isoeugenol | 0.89 | Pale yellow 23695 | Lemon-cadmium 33814 | — | — | Flesh-pink 21575 | Flesh-pink 21575 | Primrose-yellow 23655 |
| Eugenol | 0.92 | Magenta 27142 | Sky-blue 35231 | — | — | Flesh-pink 21575 | Flesh-pink 21575 | — |
| 4,4′-Dihydroxy-biphenyl | 0.92 | Pale yellow | Sky-blue 35231 | Sky-blue 35231 | Sky-blue 35231 | — | — | — |
| Dihydroisoeuge-nol | 0.95 | — | Sky-blue 35231 | — | — | Flesh-pink 21575 | Flesh-pink 21575 | — |
| Guaiacol | 0.95 | — | Water-green 34554 | — | — | Flesh-pink 21575 | Flesh-pink 21575 | — |
| Coumarin | 0.95 | — | Sky-blue 35231 | — | — | Pale yellow 23695 | — | — |
| Phloroglucinol | streak | — | Sky-blue 35231 | — | — | Lemon-cadmium 33814 | Zinc yellow 33434 | — |
| Pyrogallol | streak | Cherry-red 22356 | Yellow-green 34552 | — | — | Raw sienna 33481 | Hazel 12648 | — |

* Colour recording of the spots of the chromatograms was performed according to Federal Standard No. 595, March 1, 1956 (U.S.A.).

*$R_F$ values of some phenolic compounds*

Fig. 3 shows a chromatogram of the separation of a mixture of ten compounds, and Table II gives the $R_F$ values for 34 pure phenolic compounds.

SUMMARY

A qualitative method of paper chromatography for phenolic compounds is discussed. Whatman No. 7 paper was impregnated with a saturated solution of boric acid. The solvent used was the organic phase of *n*-butanol saturated with 2% $NH_4OH$. The time of development was 15 hours.

Beside the classical methods of spot detection, two new processes were employed: (a) ultra-violet examination of the chromatogram after Mäule's test; (b) spraying with a solution of diazotized sulphanilic acid, exposure to ammonia vapours followed by spraying with a solution of diazotized *p*-nitroaniline.

A table of $R_F$ values of 34 pure phenolic compounds is given, together with methods for their identification.

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