# A NEW THIN-LAYER METHOD FOR PHENOLIC SUBSTANCES AND COUMARINS

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## INTRODUCTION

Since BATE-SMITH first developed the paper chromatography of phenols, several methods have been proposed for the separation of naturally occurring phenolic substances and coumarins[1–25].

Indeed, the discovery and identification of a wide variety of phenolics and related compounds in animal organisms and in the plant kingdom, and the realization that these substances can be metabolically active, have furthered the need for adequate and improved identification techniques. Although a suitable paper chromatographic method may still be of great value in the analysis of phenolics, the fairly recent development of chromatostrip[26–30] and chromatoplate techniques[31] and the subsequent standardization of the methods in what is now called thin-layer chromatography[32–35] have opened up new horizons in the field of micro-analytical separation and identification of phenolics, especially since these compounds are very often present in only small amounts in animal and plant material.

In this connection it may be mentioned that BERNARD[36] studied the separation of coumarins on chromatostrips; PASTUSKA[37], STAHL AND SCHORN[33], SUNDT AND SACCARDI[39], HALMEKOWSKI[40,41], COPENHAVER AND CARVER[42] and MINAMIKAWA *et al.*[43] presented thin-layer methods for the separation of certain phenolics and (or) coumarins on silica gel, while LYMAN *et al.*[44] reported the separation of a variety of polyhydroxy aromatic compounds that do not fluoresce but do absorb under ultraviolet light in the wavelength range 230–290 mμ.

However, the detection of phenolics and coumarins on silica gel layers is usually performed by examination of the plates under ultraviolet light for fluorescent and absorbing zones, both before and after spraying with a 2 *N* sodium hydroxide solution. Subsequently the plates are developed with a chromogenic spray, either diazotized benzidine, diazotized sulphanilic acid or stable diazonium salts such as diazotized 5-nitro-2-aminoanisole (Echtrotsalz B), diazotized 1-amino-4-benzoylamino-2,5-diethyloxybenzene (Echtblausalz BB) or tetra-azotized di-*o*-anisidine (Echtblausalz B)[37]. STAHL[35] also recommends Folin-Ciocalteu reagent as a spray for phenolics. Nevertheless, in none of the thin-layer methods mentioned above, is the detection of the phenolics completely satisfactory. This is mainly due to the low specificity and imperfect colour reactions obtained with diazotized reagents on silica gel layers, and the non-specificity of the inorganic fluorescing agent as used by LYMAN *et al.*[44] for the detection of the phenolics; it is also partly due to the relatively poor separatory power

of some of the systems used and their need for improvement. For all these reasons, and bearing in mind the advantages of thin-layer chromatography which offers a combination of high speed, sensitivity and powerful resolution, we have tried to improve the existing thin-layer techniques by preparing the plates with a mixture of 50 % silica gel and 50 % cellulose powder. In many ways the advantages of thin-layer and paper chromatography are combined by such plates. The stationary phase of silica gel plates and silica gel–cellulose plates can be further improved by steaming the plates just before irrigation with the chosen solvent. This treatment usually results in a much better separation of substances having a slightly different polarity (partition chromatography).

The method is described below and the results obtained from the investigation of the thin-layer chromatographic behaviour of 93 different compounds are given.

## METHODS

### *Preparation of chromatographic plates*

The thin layers, 250 $\mu$ thick, were prepared on 20 cm by 20 cm glass plates of uniform thickness with the Desaga applicator. For the preparation of silica gel plates, Silica gel G (Merck, Germany) was used as supplied, and was applied to the standardized glass plates as a slurry (30 g silica gel in 60 ml water)[35]. For the preparation of silica gel–cellulose plates, Silica gel G and MN Cellulose powder 300, average particle size *ca.* 10 $\mu$ (Macherey, Nagel and Co., Düren, Germany) were used. In order to prepare 5 plates of 20 × 20 cm, 10 g Silica gel G and 10 g cellulose powder were slurried in a beaker with 80 ml water. Before application of the mixture to the plates, it was thoroughly homogenized in a fast electric mixer. After waiting a few minutes (removal of air bubbles) the plates were prepared in the usual way. The thin layers were dried for about 6 h at room temperature before use.

### *Steaming of the chromatographic plates*

In certain experiments the internal water phase of the plates was increased by steaming the thin layers after spotting and before irrigation.

The increase of the stationary water phase was best obtained by keeping the plates in water vapour escaping from an ordinary tea kettle and continuing the steaming until the plates were uniformly wet. However, this treatment requires care, since over-steaming of the plates can destroy the thin layers. After the steaming, the plates were allowed to dry for about 5 min at room temperature, before irrigation was started; immediate use of the steamed thin layer results in the formation of a double front.

It is also clear that the $R_F$ values of the phenolics and coumarins must be very much dependent upon the water content of the plates. This means that in order to get reproducible $R_F$ values, steaming and drying of the plates must always be carried out under the same conditions and for the same length of time. More reproducible results would be expected by developing the thin layers in a tank with an above normal but constant atmospheric moisture content. Experiments in such tanks are under way. Nevertheless, steaming of the plates is very useful and very handy since the inconvenience of the poor reproducibility of the $R_F$ values can be overcome by spotting known test substances adjacent to the unknown mixtures.

*Developing solvents and chromatography*

The development of the chromatograms was carried out at room temperature in two different solvent systems:

System I: Toluol–ethyl formate–formic acid (5:4:1) (T.E.F.).

System II: Chloroform–acetic acid–water (4:1:1) (C.A.W.).

The system utilized was placed in the bottom of a rectangular tank, the inner walls of which were lined with a disposable paper wick saturated with the developing solvent in order to saturate the chromatography chamber uniformly with the solvent vapour. The plates were removed when the solvent had ascended to a distance 10 cm from the start. The time required for developing the different plates in either solvent is given in Table I.

TABLE I

TIME FOR DEVELOPMENT OF THE SILICA GEL AND SILICA GEL–CELLULOSE PLATES TO A HEIGHT OF 10 CM WITH T.E.F. AND C.A.W.

Room temperature *ca.* 22°.

| *Carrier* | *Approximate time for solvent (min)* | |
|---|---|---|
| | *T.E.F.* | *C.A.W.* |
| Silica gel | 16 | 33 |
| Steamed silica gel | 15 | 26 |
| Silica gel–cellulose | 20 | 33 |
| Steamed silica gel–cellulose | 27 | 36 |

*Detection of the compounds*

The compounds were detected by examination of the plates under U.V. light (2537 Å) both before and after spraying the thin layers with 2 *N* sodium hydroxide. After this, the position of the phenolics and coumarins was revealed by spraying with diazotized *p*-nitroaniline[45]. This chromogenic spray was prepared by mixing 5 ml *p*-nitroaniline (0.5 % in 2 *N* HCl) and 0.5 ml $NaNO_2$ (5 %, w/v), and adding 15 ml sodium acetate (20 %, w/v) to this mixture[23]. Since the thin layer was frequently sprayed only lightly with NaOH and then rather heavily with the acetate buffer, a final spray with sodium carbonate (5 %, w/v) was normally used to return the plates to an alkaline condition.

Most of the compounds also react positively with a spray[35] consisting of 1 % potassium permanganate in 0.1 *N* $H_2SO_4$. Certain acids could only be detected with an indicator-spray made by mixing 0.04 g bromcresol green with 100 ml of 96 % ethanol. 0.1 *N* NaOH was added to the reagent until a green colour resulted[35].

MATERIALS

Most of the phenolic acids and related compounds and some coumarins were purchased from Fluka A.G., Buchs (Switzerland).

The phenolic acid β-glucosides were prepared according to KRATZL AND BILLEK[46]. The phenolic acid tetraacetylglucoside was deacetylated by dissolving it in 10 parts of methanol in which 0.3 % sodium metal had been previously dissolved. After

standing for 24 h at room temperature, a little water and a few drops of hydrochloric acid were added and the glucoside was filtered off and purified by recrystallization from ethanol[47]. The phenolic acid methyl esters were prepared according to VOGEL[48].

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## RESULTS AND DISCUSSION

Tables II and III show the results obtained. In Table II the $R_F$ values of 93 compounds on silica gel plates (A), silica gel–cellulose plates (C), steamed silica gel plates (B) and steamed silica gel–cellulose plates (D), developed with either toluol–ethyl formate–formic acid (T.E.F.) or chloroform–acetic acid–water (C.A.W.) can be found. Table III shows the fluorescence (2537 Å) of some of the compounds before and after NaOH treatment and also the colour reaction with diazotized *p*-nitroaniline. The colours found in the table were standardized and matched against the "Derwent" colour pencil* shades.

Table III also indicates the positive or negative reaction of the compounds with 1% potassium permanganate in 0.1 *N* sulphuric acid.

Thin layers made by mixing 50% silica gel and 50% cellulose powder show the following characteristics:

* "Derwent" colour pencils, series No. 19, The Cumberland Pencil Co. Ltd., Keswick, England.

TABLE II

$R_F$ VALUES OF THE COMPOUNDS STUDIED

On (A) silica gel, (B) steamed silica gel plates, (C) silica gel–cellulose plates, and (D) steamed silica–gel cellulose plates

| | *Compounds* | *T.E.F.* | | | | *C.A.W.* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Silica gel* | | *Cellulose–silica gel* | | *Silica gel* | | *Cellulose–silica gel* | |
| | | *A* | *B** | *C* | *D** | *A* | *B** | *C* | *D** |
| 1 | Benzoic acid | 0.67 | 0.80 | 0.83 | 0.77 | 0.90 | 0.99 | 0.99 | 0.99 |
| 2 | *o*-Hydroxybenzoic acid | 0.59 | 0.90 | 0.73 | 0.81 | 0.78 | 0.75 | 0.90 | 0.88 |
| 3 | *m*-Hydroxybenzoic acid | 0.44 | 0.37 | 0.51 | 0.34 | 0.30 | 0.24 | 0.42 | 0.33 |
| 4 | *p*-Hydroxybenzoic acid | 0.46 | 0.47 | 0.50 | 0.24 | 0.29 | 0.34 | 0.47 | 0.50 |
| 5 | *p*-Hydroxybenzoic acid methyl ester | 0.58 | 0.54 | 0.66 | 0.73 | 0.55 | 0.42 | 0.82 | 0.76 |
| 6 | 2,4-Dihydroxybenzoic acid (β-resorcylic acid) | 0.50 | 0.34 | 0.49 | 0.44 | 0.31 | 0.34 | 0.44 | 0.22 |
| 7 | 2,5-Dihydroxybenzoic acid (gentisic acid) | 0.47 | 0.40 | 0.50 | 0.26 | 0.28 | 0.13 | 0.30 | 0.13 |
| 8 | 2,6-Dihydroxybenzoic acid (γ-resorcylic acid) | 0.45 | 0.30 | 0.44 | 0.33 | 0.03 | 0.07 | 0.03 | 0.05 |
| 9 | 3,4-Dihydroxybenzoic acid (protocatechuic acid) | 0.40 | 0.28 | 0.36 | 0.24 | 0.09 | 0.00 | 0.08 | 0.05 |
| 10 | 3,5-Dihydroxybenzoic acid (α-resorcylic acid) | 0.39 | 0.20 | 0.26 | 0.24 | 0.02 | 0.00 | 0.03 | 0.00 |
| 11 | 3-Methoxy-4-hydroxybenzoic acid (vanillic acid) | 0.51 | 0.52 | 0.55 | 0.35 | 0.70 | 0.54 | 0.90 | 0.84 |
| 12 | 3,4,5-Trihydroxybenzoic acid (gallic acid) | 0.32 | 0.18 | 0.12 | 0.08 | 0.04 | 0.00 | 0.02 | 0.00 |
| 13 | *o*-Hydroxybenzaldehyde | 0.69 | — | 0.82 | 0.95 | 0.73 | — | 0.85 | — |
| 14 | 2,4-Dihydroxybenzaldehyde (β-resorcylaldehyde) | 0.55 | 0.85 | 0.61 | 0.73 | 0.55 | 0.81 | 0.68 | 0.84 |
| 15 | 3,4-Dihydroxybenzaldehyde (protocatechuic aldehyde) | 0.39 | 0.67 | 0.33 | 0.33 | 0.14 | 0.22 | 0.22 | 0.16 |
| 16 | 3,4-Dihydroxy-5-methoxybenzaldehyde | 0.44 | 0.40 | 0.46 | 0.24 | 0.38 | 0.55 | 0.50 | 0.48 |
| 17 | 2,3-Dimethoxybenzaldehyde | 0.65 | — | 0.75 | — | 0.88 | — | 0.98 | — |
| 18 | 2,5-Dimethoxybenzaldehyde | 0.66 | 0.98 | 0.74 | 0.91 | 0.98 | 0.98 | 0.98 | 0.99 |
| 19 | 3,4-Dimethoxybenzaldehyde (veratraldehyde) | 0.50 | 0.70 | 0.63 | 0.85 | 0.98 | 0.92 | 0.80 | 0.90 |
| 20 | 3-Hydroxy-2-aminobenzoic acid (3-hydroxyanthranilic acid) | 0.20 | 0.24 | 0.13 | 0.21 | 0.19 | 0.20 | 0.33 | 0.54 |
| 21 | Phenylacetic acid | — | — | 0.89 | 0.85 | — | — | — | — |
| 22 | *o*-Hydroxyphenylacetic acid | 0.48 | 0.33 | 0.47 | 0.41 | 0.37 | 0.41 | 0.52 | 0.64 |
| 23 | *p*-Hydroxyphenylacetic acid | 0.44 | 0.29 | 0.43 | 0.36 | 0.27 | 0.29 | 0.44 | 0.30 |
| 24 | 3,6-Dihydroxyphenylacetic acid | 0.30 | 0.30 | 0.23 | 0.41 | 0.03 | 0.02 | 0.04 | 0.00 |
| 25 | Mandelic acid | 0.26 | 0.24 | 0.56 | 0.28 | 0.27 | 0.42 | 0.49 | 0.51 |
| 26 | 3,4-Dihydroxymandelic acid | 0.23 | 0.25 | 0.20 | 0.20 | 0.00 | 0.02 | 0.00 | 0.00 |
| 27 | 3-Methoxy-4-hydroxymandelic acid | 0.22 | 0.24 | 0.24 | 0.21 | 0.03 | 0.05 | 0.10 | 0.05 |
| 28 | Phenylpropionic acid | 0.49 | 0.25 | 0.57 | 0.40 | 0.45 | 0.62 | 0.57 | 0.68 |
| 29 | *m*-Hydroxyphenylpropionic acid | 0.38 | 0.18 | 0.31 | 0.26 | 0.32 | 0.65 | 0.73 | 0.70 |
| 30 | *p*-Hydroxyphenylpropionic acid | 0.47 | 0.40 | 0.50 | 0.41 | 0.50 | 0.50 | 0.58 | 0.60 |
| 31 | 2,3-Dihydroxyphenylpropionic acid | 0.46 | 0.33 | 0.46 | 0.22 | 0.30 | 0.23 | 0.40 | 0.27 |
| 32 | Phenylpyruvic acid | — | — | — | — | — | — | — | — |
| 33 | *p*-Hydroxyphenylpyruvic acid | 0.43 | 0.39 | 0.45 | 0.35 | 0.17 | 0.29 | 0.41 | 0.41 |
| 34 | Phenyllactic acid | 0.26 | 0.32 | 0.62 | — | 0.18 | 0.43 | 0.76 | 0.65 |
| 35 | *o*-Hydroxyphenyllactic acid | 0.24 | 0.05 | 0.18 | 0.12 | 0.06 | — | 0.21 | 0.21 |
| 36 | *p*-Hydroxyphenyllactic acid | 0.23 | 0.20 | 0.25 | 0.22 | 0.00 | 0.03 | 0.05 | 0.02 |
| 37 | Cinnamic acid | 0.67 | 0.87 | 0.84 | 0.83 | 0.95 | 0.99 | 0.99 | 0.99 |
| 38 | *o*-Hydroxycinnamic acid (*o*-coumaric acid) | 0.49 | 0.65 | 0.56 | 0.47 | 0.35 | 0.65 | 0.62 | 0.65 |
| 39 | *p*-Hydroxycinnamic acid (*p*-coumaric acid) | 0.48 | 0.57 | 0.50 | 0.35 | 0.34 | 0.53 | 0.55 | 0.62 |
| 40 | *m*-Hydroxycinnamic acid (*m*-coumaric acid) | 0.53 | 0.50 | 0.58 | 0.49 | 0.45 | 0.71 | 0.55 | 0.73 |
| 41 | *o*-Hydroxyhydrocinnamic acid | 0.50 | 0.59 | 0.53 | 0.59 | 0.50 | 0.61 | 0.69 | 0.80 |
| 42 | *o*-Hydroxycinnamic acid methyl ester | 0.54 | 0.58 | 0.65 | 0.76 | 0.88 | 0.68 | 0.97 | 0.94 |
| 43 | *p*-Hydroxycinnamic acid methyl ester | 0.50 | 0.55 | 0.65 | 0.75 | 0.61 | 0.47 | 0.87 | 0.89 |
| 44 | *o*-Coumaroylglycine | 0.58 | 0.28 | 0.30 | — | 0.66 | — | — | — |
| 45 | *o*-Hydroxycinnamic acid β-glucoside | 0.65 | — | 0.48 | — | 0.52 | — | — | — |
| 46 | *p*-Hydroxycinnamic acid β-glucoside | 0.50 | — | 0.41 | — | 0.70 | — | — | — |

(*continued on p. 53*)

TABLE II (*continued*)

| | *Compounds* | *T.E.F.* | | | | *C.A.W.* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Silica gel* | | *Cellulose–silica gel* | | *Silica gel* | | *Cellulose–silica gel* | |
| | | *A* | *B** | *C* | *D** | *A* | *B** | *C* | *D** |
| 47 | 3,4-Dihydroxycinnamic acid (caffeic acid) | 0.40 | 0.34 | 0.35 | 0.25 | 0.14 | 0.09 | 0.13 | 0.12 |
| 48 | 3,4-Dihydroxyhydrocinnamic acid (hydrocaffeic acid) | 0.40 | 0.23 | 0.39 | 0.14 | 0.17 | 0.10 | 0.21 | 0.08 |
| 49 | 3-Methoxy-4-hydroxycinnamic acid (ferulic acid) | 0.50 | 0.74 | 0.56 | 0.44 | 0.63 | 0.98 | 0.75 | 0.99 |
| 50 | 3-Methoxy-4-hydroxycinnamic acid β-glucoside | 0.49 | — | — | — | 0.70 | — | — | — |
| 51 | 3,4-Dimethoxycinnamic acid methyl ester | 0.50 | 0.59 | 0.75 | 0.81 | 0.90 | 0.99 | 0.99 | 0.99 |
| 52 | 3,4,5-Trihydroxycinnamic acid (syringic acid) | 0.43 | 0.48 | 0.54 | 0.65 | 0.79 | 0.55 | 0.90 | 0.90 |
| 53 | 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid) | 0.45 | 0.37 | 0.46 | 0.40 | 0.76 | 0.97 | 0.92 | 0.94 |
| 54 | Cinnamaldehyde | 0.67 | — | 0.80 | — | 0.98 | — | — | — |
| 55 | *p*-Methoxycinnamaldehyde | 0.63 | 0.99 | 0.69 | 0.93 | 0.98 | 0.99 | 0.98 | 0.99 |
| 56 | 3,4-Dimethoxycinnamaldehyde | 0.54 | 0.98 | 0.64 | 0.85 | 0.98 | 0.99 | 0.97 | 0.99 |
| 57 | 3-Methoxy-4-hydroxycinnamaldehyde (coniferylaldehyde) | 0.51 | 0.92 | 0.59 | 0.79 | 0.79 | 0.98 | 0.90 | 0.94 |
| 58 | Coumarin | 0.55 | 0.96 | 0.68 | 0.93 | 0.75 | 0.96 | 0.98 | 0.99 |
| 59 | 3-Hydroxycoumarin | 0.55 | 0.93 | 0.65 | 0.88 | 0.70 | 0.98 | 0.96 | 0.92 |
| 60 | 3-Carboxycoumarin | 0.47 | 0.80 | 0.59 | 0.66 | 0.60 | 0.98 | 0.88 | 0.99 |
| 61 | 4-Hydroxycoumarin | 0.75 | 0.65 | 0.54 | 0.51 | 0.27 | 0.63 | 0.59 | 0.93 |
| 62 | 5-Hydroxycoumarin | 0.45 | 0.79 | 0.52 | 0.52 | 0.30 | 0.92 | 0.72 | 0.76 |
| 63 | 6-Hydroxycoumarin | 0.43 | 0.63 | 0.44 | 0.68 | 0.25 | 0.66 | 0.49 | 0.65 |
| 64 | 7-Hydroxycoumarin (umbelliferone) | 0.45 | 0.43 | 0.44 | 0.61 | 0.44 | 0.27 | 0.61 | 0.56 |
| 65 | 7-Hydroxycoumarin-3-carboxylic acid | 0.46 | 0.30 | 0.49 | 0.39 | 0.20 | 0.41 | 0.26 | 0.38 |
| 66 | 7-Hydroxycoumarin-3-carboxylic acid ethyl ester | 0.44 | 0.61 | 0.53 | 0.59 | 0.37 | 0.86 | 0.45 | 0.99 |
| 67 | 7-Methoxycoumarin-3-carboxylic acid ethyl ester | 0.50 | 0.93 | 0.63 | 0.92 | 0.86 | 0.98 | 0.89 | 0.99 |
| 68 | 7-Propoxycoumarin | 0.65 | 0.95 | 0.74 | 0.95 | 0.92 | 0.99 | 0.97 | 0.98 |
| 69 | 7-Propoxycoumarin-3-carboxylic acid | 0.60 | 0.94 | 0.67 | 0.92 | 0.83 | 0.97 | 0.90 | 0.99 |
| 70 | 8-Methoxycoumarin (herniarin) | 0.55 | 0.97 | 0.94 | 0.95 | 0.80 | 0.86 | 0.98 | 0.99 |
| 71 | 6,7-Dihydroxycoumarin (esculetin) | 0.28 | 0.28 | 0.40 | 0.43 | 0.08 | 0.04 | 0.15 | 0.15 |
| 72 | 6,7-Dihydroxycoumarin-6-β-glucoside (esculin) | 0.04 | 0.13 | 0.05 | 0.10 | 0.00 | 0.00 | 0.00 | 0.00 |
| 73 | 7,8-Dihydroxycoumarin (daphnetin) | 0.42 | 0.36 | 0.44 | 0.34 | 0.20 | 0.28 | 0.35 | 0.36 |
| 74 | 6-Methoxy-7-hydroxycoumarin (scopoletin) | 0.42 | 0.67 | 0.46 | 0.54 | 0.66 | 0.84 | 0.83 | 0.96 |
| 75 | 6,7-Dimethoxycoumarin-3-carboxylic acid ethyl ester | 0.46 | 0.77 | 0.55 | 0.88 | 0.85 | 0.98 | 0.91 | 0.99 |
| 76 | 4-Methyl-5-acetoxy-7-methoxycoumarin | 0.48 | 0.59 | 0.60 | 0.86 | 0.82 | 0.83 | 0.90 | 0.94 |
| 77 | 4-Methyl-5,7-dimethoxycoumarin | 0.53 | 0.92 | 0.65 | 0.99 | 0.81 | 0.98 | 0.98 | 0.99 |
| 78 | 4-Methyl-5-methoxy-7-hydroxycoumarin | 0.47 | 0.57 | 0.41 | 0.79 | 0.35 | 0.72 | 0.80 | 0.94 |
| 79 | Peucedanin | 0.56 | 0.88 | 0.63 | 0.97 | 0.91 | 0.98 | 0.98 | 0.99 |
| 80 | Oreoselon | 0.62 | 0.84 | 0.73 | 0.95 | 0.93 | 0.99 | 0.98 | 0.95 |
| 81 | Marmelosin | 0.61 | 0.67 | 0.72 | 0.98 | 0.89 | 0.99 | 0.98 | 0.99 |
| 82 | Quinic acid | 0.02 | 0.20 | 0.02 | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 |
| 83 | 3-O-*p*-Coumaroyl-quinic acid | 0.04 | 0.17 | 0.09 | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 |
| 84 | 1-O-Cinnamoyl-quinic acid | 0.15 | 0.30 | 0.19 | 0.15 | 0.04 | 0.17 | 0.04 | 0.19 |
| 85 | 3-O-Cinnamoyl-quinic acid | 0.16 | — | 0.24 | 0.20 | 0.02 | 0.43 | 0.05 | 0.68 |
| 86 | Chlorogenic acid | 0.02 | 0.17 | 0.05 | 0.14 | 0.00 | 0.00 | 0.00 | 0.00 |
| 87 | Isochlorogenic acid | 0.45 | 0.25 | 0.42 | — | 0.52 | — | 0.84 | — |
| 88 | 3-O-Feruoyl-quinic acid | 0.07 | 0.18 | 0.10 | 0.12 | 0.00 | 0.05 | 0.02 | 0.04 |
| 89 | Phloridzin | 0.09 | 0.27 | 0.07 | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 |
| 90 | Quercetin | 0.43 | 0.28 | 0.30 | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 |
| 91 | Quercitrin | 0.10 | 0.27 | 0.07 | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 |
| 92 | Rutin | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 93 | Vitexin | 0.04 | 0.00 | 0.05 | 0.18 | 0.00 | 0.00 | 0.00 | 0.00 |

* Colums B and D refer to the steamed plates.

TABLE III

APPEARANCE OF THE COMPOUNDS STUDIED IN U.V. LIGHT AND AFTER SPRAYING WITH DIAZOTIZED *p*-NITROANILINE

| *Compound* | *Fluorescence in U.V. light (2537 Å)* | | *Colour reaction with diazotized p-nitroaniline (Cellulose/silica gel plates)* | *Reaction with 1% $KMnO_4$ in 0.1 N $H_2SO_4$ (Silica gel plates)* |
|---|---|---|---|---|
| | *Before NaOH* | *After NaOH* | | |
| 1 Benzoic acid | — | — | — | + |
| 2 *o*-Hydroxybenzoic acid | — | — | middle chrome | + |
| 3 *m*-Hydroxybenzoic acid | — | blue | crimson lake | + |
| 4 *p*-Hydroxybenzoic acid | — | beige | crimson lake | + |
| 5 *p*-Hydroxybenzoic acid methyl ester | — | absorbs | pale vermilion | + |
| 6 2,4-Hydroxybenzoic acid (β-resorcylic acid) | absorbs | absorbs | deep chrome | + |
| 7 2,5-Dihydroxybenzoic acid (gentisic acid) | blue | blue | raw umber | + |
| 8 2,6-Dihydroxybenzoic acid (γ-resorcylic acid) | absorbs | absorbs | deep chrome | + |
| 9 3,4-Dihydroxybenzoic acid (protocatechuic acid) | absorbs | absorbs | raw umber | + |
| 10 3,5-Dihydroxybenzoic acid (α-resorcylic acid) | — | purple | deep chrome | + |
| 11 3-Methoxy-4-hydroxybenzoic acid (vanillic acid) | — | — | imperial purple | + |
| 12 3,4,5-Trihydroxybenzoic acid (gallic acid) | — | absorbs | brown ochre | + |
| 13 *o*-Hydroxybenzaldehyde | grey | grey | middle chrome | + |
| 14 2,4-Dihydroxybenzaldehyde (β-resorcylaldehyde) | blue | grey | Van Dyke brown | + |
| 15 3,4-Dihydroxybenzaldehyde (protocatechuic aldehyde) | absorbs | absorbs | raw umber | + |
| 16 3,4-Dihydroxy-5-methoxybenzaldehyde | absorbs | absorbs | brown ochre | + |
| 17 2,3-Dimethoxybenzaldehyde | — | — | — | + |
| 18 2,5-Dimethoxybenzaldehyde | blue | blue | — | + |
| 19 3,4-Dimethoxybenzaldehyde (veratraldehyde) | yellow | grey | — | + |
| 20 3-Hydroxy-2-aminobenzoic acid (3-hydroxyanthranilic acid) | grey | grey | raw umber | — |
| 21 Phenylacetic acid | — | — | — | + |
| 22 *o*-Hydroxyphenylacetic acid | — | — | dark violet | + |
| 23 *p*-Hydroxyphenylacetic acid | absorbs | absorbs | red-violet lake | + |
| 24 3,6-Dihydroxyphenylacetic acid | — | — | — | + |
| 25 Mandelic acid | — | — | — | + |
| 26 3,4-Dihydroxymandelic acid | absorbs | absorbs | crimson lake | + |
| 27 3-Methoxy-4-hydroxymandelic acid | absorbs | absorbs | dark violet | + |
| 28 Phenylpropionic acid | absorbs | absorbs | red-violet lake | + |
| 29 *m*-Hydroxyphenylpropionic acid | absorbs | absorbs | crimson lake | + |
| 30 *p*-Hydroxyphenylpropionic acid | — | — | red-violet lake | + |
| 31 2,3-Dihydroxyphenylpropionic acid | — | absorbs | raw umber | + |
| 32 Phenylpyruvic acid | — | — | — | — |
| 33 *p*-Hydroxyphenylpyruvic acid | blue | grey | red-violet lake | + |
| 34 Phenyllactic acid | — | — | — | — |
| 35 *o*-Hydroxyphenyllactic acid | — | — | red-violet lake | + |
| 36 *p*-Hydroxyphenyllactic acid | absorbs | — | imperial purple | + |
| 37 Cinnamic acid | — | — | — | — |
| 38 *o*-Hydroxycinnamic acid (*o*-coumaric acid) | blue | green | imperial purple | + |
| 39 *m*-Hydroxycinnamic acid (*m*-coumaric acid) | blue | yellow | crimson lake | + |
| 40 *p*-Hydroxycinnamic acid (*p*-coumaric acid) | dark violet | blue | blue | + |
| 41 *o*-Hydroxyhydrocinnamic acid | — | — | raw umber | + |
| 42 *o*-Hydroxycinnamic acid methyl ester | grey | yellow | red-violet lake | + |
| 43 *p*-Hydroxycinnamic acid methyl ester | absorbs | blue | red-violet lake | + |
| 44 *o*-Coumaroylglycine | yellow | yellow | deep vermilion | + |
| 45 *o*-Hydroxycinnamic acid β-glucoside | — | — | — | + |
| 46 *p*-Hydroxycinnamic acid β-glucoside | — | — | — | + |
| 47 3,4-Dihydroxycinnamic acid (caffeic acid) | blue-green | absorbs | raw umber | + |

(*continued on p. 55*)

TABLE III (*continued*)

| | *Compound* | *Fluorescence in U.V. light (2537 Å)* | | *Colour reaction with diazotized p-nitroaniline (Cellulose/silica gel plates)* | *Reaction with 1% $KMnO_4$ in 0.1 N $H_2SO_4$ (Silica gel plates)* |
|---|---|---|---|---|---|
| | | *Before NaOH* | *After NaOH* | | |
| 48 | 3,4-Dihydroxyhydrocinnamic acid (hydrocaffeic acid) | blue-green | absorbs | raw umber | + |
| 49 | 3-Methoxy-4-hydroxycinnamic acid (ferulic acid | blue | white-blue | cobalt blue | + |
| 50 | 3-Methoxy-4-hydroxycinnamic acid β-glucoside | — | — | — | + |
| 51 | 3,4-Dimethoxycinnamic acid methyl ester | weak blue | blue | — | + |
| 52 | 3,4,5-Trihydroxycinnamic acid (syringic acid) | absorbs | absorbs | spectrum blue | + |
| 53 | 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid) | grey | blue | gun metal | + |
| 54 | Cinnamaldehyde | — | — | — | + |
| 55 | *p*-Methoxycinnamaldehyde | — | — | — | + |
| 56 | 3,4-Dimethoxycinnamaldehyde | grey | grey | — | + |
| 57 | 3-Methoxy-4-hydroxycinnamaldehyde (coniferylaldehyde) | absorbs | yellow-green | olive green | + |
| 58 | Coumarin | — | green | imperial purple | + |
| 59 | 3-Hydroxycoumarin | — | blue | venetian red | + |
| 60 | 3-Carboxycoumarin | grey | blue | imperial purple | + |
| 61 | 4-Hydroxycoumarin | grey | brown | brown ochre | + |
| 62 | 5-Hydroxycoumarin | grey | beige | Van Dyke brown | — |
| 63 | 6-Hydroxycoumarin | white-blue | yell | Van Dyke brown | + |
| 64 | 7-Hydroxycoumarin (umbelliferone) | blue | bl | Van Dy rown | + |
| 65 | 7-Hydroxycoumarin-3-carboxylic acid | purple | bl | venetian | + |
| 66 | 7-Hydroxycoumarin-3-carboxylic acid ethyl ester | blue | gr en | lemon cadmium | + |
| 67 | 7-Methoxycoumarin-3-carboxylic acid ethyl ester | purple | yellow | dark violet | + |
| 68 | 7-Propoxycoumarin | purple | blue | imperial purple | + |
| 69 | 7-Propoxycoumarin-3-carboxylic acid | purple | blue | imperial purple | + |
| 70 | 8-Methoxycoumarin (herniarin) | purple | blue | dark violet | + |
| 71 | 6,7-Dihydroxycoumarin (esculetin) | beige | blue | raw umber | + |
| 72 | 6,7-Dihydroxycoumarin-6-β-glucoside (esculin) | grey | blue | raw umber | + |
| 73 | 7,8-Dihydroxycoumarin (daphnetin) | absorbs | absorbs | raw umber | + |
| 74 | 6-Methoxy-7-hydroxycoumarin (scopoletin) | blue | blue | gun metal | + |
| 75 | 6,7-Dimethoxycoumarin-3-carboxylic acid | blue | yellow | — | + |
| 76 | 4-Methyl-5-acetoxy-7-methoxycoumarin | blue | green | terra cotta | + |
| 77 | 4-Methyl-5,7-dimethoxycoumarin | blue | purple | raw sienna | + |
| 78 | 4-Methyl-5-methoxy-7-hydroxycoumarin | blue | blue | spectrum orange | + |
| 79 | Peucedanin | blue | blue | raw umber | + |
| 80 | Oreoselon | — | grey | imperial purple | + |
| 81 | Marmelosin | yellow | grey | gun metal | + |
| 82 | Quinic acid | — | — | — | + |
| 83 | 3-O-*p*-Coumaroyl-quinic acid | — | blue | raw umber | + |
| 84 | 1-O-Cinnamoyl-quinic acid | — | — | — | + |
| 85 | 3-O-Cinnamoyl-quinic acid | — | — | light violet | + |
| 86 | Chlorogenic acid | yellow-green | absorbs | raw sienna | + |
| 87 | Isochlorogenic acid | grey | grey | raw sienna | + |
| 88 | 3-O-Feruoyl-quinic acid | blue | blue | delft blue | + |
| 89 | Phloridzin | grey | absorbs | orange chrome | + |
| 90 | Quercetin | yellow-green | brown | middle chrome | — |
| 91 | Quercitrin | yellow-green | yellow-brown | middle chrome | + |
| 92 | Rutin | yellow-green | brown | middle chrome | + |
| 93 | Vitexin | — | yellow-brown | middle chrome | + |

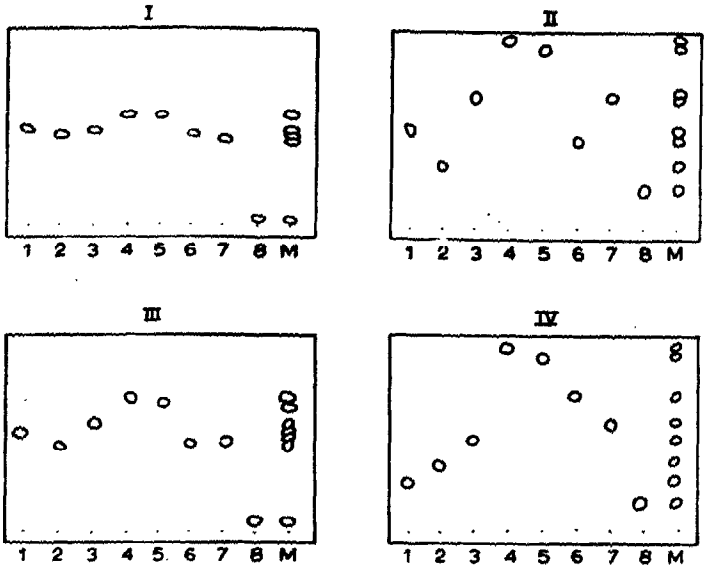


Fig. 1. Separation of phenolic acids and coumarins by thin-layer chromatography on: (I) silica gel plates dried at room temperature for 6 h; (II) steamed silica gel plates; (III) silica gel–cellulose plates dried at room temperature for 6 h; (IV) steamed silica gel–cellulose plates. Solvent: T.E.F. 1 = *p*-Hydroxybenzoic acid; 2 = *γ*-resorcylic acid; 3 = *o*-hydroxycinnamic acid; 4 = coumarin; 5 = 3-hydroxycoumarin; 6 = umbelliferone; 7 = scopoletin; 8 = chlorogenic acid; M = mixture.

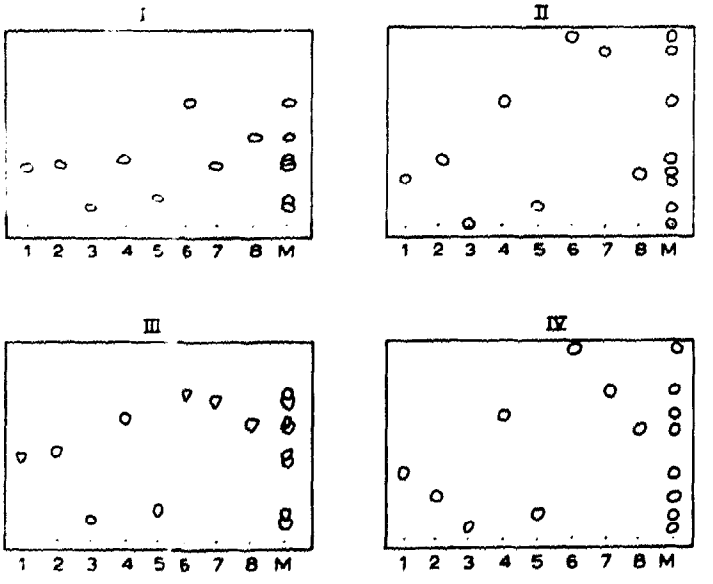


Fig. 2. Separation of phenolic acids and coumarins by thin-layer chromatography on: (I) silica gel plates dried at room temperature for 6 h; (II) steamed silica gel plates; (III) silica gel–cellulose plates, dried at room temperature for 6 h; (IV) steamed silica gel–cellulose plates. Solvent: C.A.W. 1 = *m*-Hydroxybenzoic acid; 2 = *β*-resorcylic acid; 3 = protocatechuic acid; 4 = *o*-hydroxycinnamic acid; 5 = caffeic acid; 6 = ferulic acid; 7 = 5-hydroxycoumarin; 8 = umbelliferone; M = mixture

(1) They possess a greater stability than the plates made from silica gel only. The silica gel–cellulose plates do not disintegrate as easily as the silica gel thin layers (addition of 1% starch even increases the stability). This is a very important factor when autoradiograms have to be made from the chromatograms or when a quantitative estimation has to be performed from the compounds on the thin layer.

(2) The development of the plates with diazotized *p*-nitroaniline gives rise to the same pronounced colour reactions with the phenolics and coumarins as on paper chromatograms. This result is of great significance in the identification of unknown substances, especially since the colours obtained with the same spray reagent on silica gel plates are not very much differentiated, all the phenolics and related substances producing a similar, more or less brown tint.

In many cases, there is the advantage that a somewhat smaller amount of the compound can be applied on the silica gel–cellulose plates than on the silica gel plates,

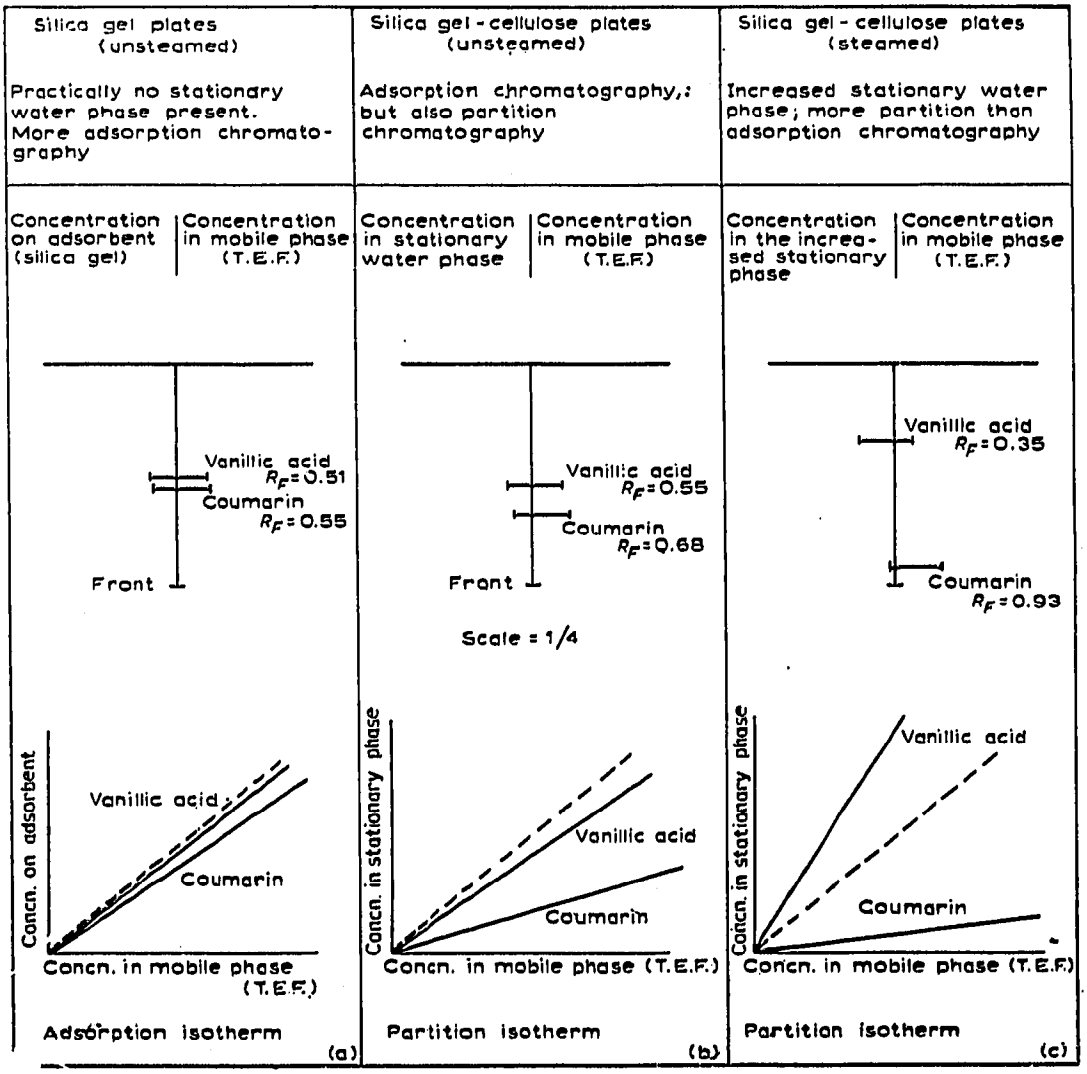


Fig. 3. Simplified scheme, explaining the better thin-layer separation of equal amounts of vanillic acid and coumarin on steamed silica gel–cellulose plates, with T.E.F. (5:4:1) as solvent. The better separation of both compounds on steamed silica gel–cellulose thin layers is compared to the separation obtained on silica gel and silica–cellulose plates. The scheme refers to the ideal situation (linear adsorption and partition isotherms).

because the substances tend to spread a little more (diffusion) on the former than on the latter thin layers. However, this fact is not of much significance because 0.25 $\mu$g spots still show a positive colour reaction on silica gel–cellulose plates with diazotized *p*-nitroaniline.

Several of the fluorescent compounds can even be detected in as low a concentration as 0.02 $\mu$g on silica gel–cellulose plates, while the concentration on silica gel plates must be of the order of 0.10 $\mu$g. Spraying of the chromatograms with 2 *N* sodium hydroxide promotes the detection of fluorescent compounds.

(3) The $R_F$ values of the compounds on the unsteamed silica gel–cellulose plates are very reproducible and this again is an important benefit in comparison with the silica gel plates.

(4) The water content of the stationary phase of the silica gel and, *a fortiori*, of the silica gel–cellulose plates can be increased by steaming the plates just before irrigation[20]. This in general results, as mentioned above, in a much better separation of certain more polar, less polar and non-polar substance (see Figs. 1 and 2). For example, whereas thin-layer chromatography on activated silica gel layers is essentially an adsorption process, on silica gel–cellulose layers, as well as adsorption, there is also to some extent a partition process taking place, the cellulose powder serving as a carrier for a small, though important, stationary phase. Even the prescribed activation of the silica gel plates[32,35] by drying of the thin layers before use seems in many cases superfluous, since a higher water content of the thin layer enhances partition chromatography. Fig. 3 shows and explains the better separation of a relatively "more" polar phenolic acid, *e.g.* vanillic acid, and the less polar coumarin, on steamed silica gel–cellulose plates by comparing it with the separation obtained on silica gel and silica gel–cellulose layers.

However, we must bear in mind that the real situation is probably a more com-

TABLE IV

EFFECT OF THE LENGTH OF RUN ON THE $R_F$ VALUES OF A FEW PHENOLICS AND COUMARINS

| *Solvent* | *Compounds* | *Unsteamed plates* | | | | *Steamed plates* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Silica gel* | | *Silica gel–cellulose* | | *Silica gel* | | *Silica gel–cellulose* | |
| | | *A* | *B* | *A* | *B* | *A* | *B* | *A* | *B* |
| T.E.F. | 1 Coumarin | 0.55 | 0.45 | 0.68 | 0.62 | 0.96 | 0.71 | 0.93 | 0.8 |
| | 2 Ferulic acid | 0.50 | 0.36 | 0.56 | 0.51 | 0.74 | 0.39 | 0.44 | 0.6 |
| | 3 Vanillic acid | 0.51 | 0.37 | 0.55 | 0.50 | 0.52 | 0.33 | 0.35 | 0.6 |
| | 4 *p*-Hydroxybenzoic acid | 0.46 | 0.34 | 0.50 | 0.41 | 0.47 | — | 0.24 | 0.6 |
| | 5 *p*-Hydroxycinnamic acid | 0.48 | 0.33 | 0.50 | 0.43 | 0.57 | 0.28 | 0.35 | 0.6 |
| | 6 *o*-Hydroxycinnamic acid | 0.49 | 0.37 | 0.56 | 0.48 | 0.65 | 0.30 | 0.47 | 0.5 |
| C.A.W. | 1 Coumarin | 0.75 | — | 0.98 | 0.93 | 0.96 | 0.98 | 0.99 | 0.9 |
| | 2 Ferulic acid | 0.63 | — | 0.75 | 0.88 | 0.98 | 0.95 | 0.89 | 0.9 |
| | 3 Vanillic acid | 0.70 | 0.91 | 0.90 | 0.84 | 0.54 | 0.35 | 0.84 | 0.9 |
| | 4 *p*-Hydroxybenzoic acid | 0.29 | 0.64 | 0.47 | 0.62 | 0.34 | 0.51 | 0.50 | 0.3 |
| | 5 *p*-Hydroxycinnamic acid | 0.34 | 0.72 | 0.55 | 0.71 | 0.53 | 0.66 | 0.62 | 0.6 |
| | 6 *o*-Hydroxycinnamic acid | 0.35 | 0.77 | 0.62 | 0.77 | 0.65 | 0.71 | 0.65 | 0.7 |

A = Start at 1 cm from the edge of the glass plate.
B = Start at 6 cm from the edge of the glass plate.

plex one, since this simple picture is on one hand certainly obscured by frontal analysis[32,40–51], due to the fact that ternary solvents were used and the components of these solvents can be selectively retarded; consequently, the $R_F$ values of the coumarins and phenolic acids may be dependent on the length of the run. This was actually proved to be the case[52] and this effect can be seen for certain compounds in Table IV.

On the other hand, water deactivation of the silica gel (this can certainly be expected when thin layers containing silica gel are steamed) can affect the adsorption of phenolics and coumarins differently according to their structure, and the type, number and position of the functional groups in the aromatic ring system[8,53–55].

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SUMMARY

A new thin-layer method for the separation of naturally occurring and synthetic phenolics and coumarins was developed. In this method, thin layers prepared from equal amounts of Silica gel G and cellulose powder were used and the advantages of both thin-layer and paper chromatography were combined. The separation of 93 compounds was studied. Two solvent systems, toluol–ethyl formate–formic acid (5:4:1) and chloroform–acetic acid–water (4:1:1) were employed. Steaming of the plates just before irrigation resulted, in general, in a better separation of compounds of slightly different polarity. The advantages of the method are discussed.

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