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ANALYSIS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS IN PLANT MATERIAL

I. SAMPLE PREPARATION AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method is described for the extraction, hydrolysis of esters and glycosides and preliminary purification of phenolic acids derived from plant material. Extracts were analyzed by thin-layer chromatography on silica gel, examination of the chromatograms under ultraviolet light (254 nm, 366 nm), spraying with $TiCl_4$ or $FeCl_3$ solutions and overspraying with an alkaline diazobenzenesulphonate reagent. More than twenty phenolic acids could be differentiated by this procedure.

INTRODUCTION

In plants, phenolic acids occur in various forms[1]. Hydrolysis of derivatives was therefore considered a prerequisite to a qualitative and quantitative study of the overall distribution of these acids.

Although the phenolic acids have been the subject of many investigations (*cf.*, *e.g.*, references given by Maga[2]), there is unfortunately no simple yet precise method available for determining these compounds in biological samples. Hot water, methanol, ethanol, acetone and ethyl acetate are the main solvents recommended for extraction purposes, while columns of silica gel, cellulose, polyamide (polycaprolactam), polyclar (polyvinyl polypyrrolidone), lipophilic and hydrophilic dextran gels and various ion exchangers have been used to effect partial purification (*cf.*, references given by Drawert *et al.*[3]). Various systems employing paper chromatography (often mentioned in older publications) and thin-layer chromatography (silica gel, cellulose and polyamide layers)[4–12], with various solvent systems and spray reagents, have been recommended for qualitative analysis. For quantitative determination, spectrophotometry has been used[8,13]. Currently, increasing numbers of high-performance liquid chromatographic (HPLC) methods[12,14–18] are being reported, but gas–liquid chromatography (GLC) (after silylation or adequate derivatization) is still the most widely used technique (*e.g.*, refs. 19–23), combined in some cases with mass spectrometry.

Thin-layer chromatography (TLC) is a rapid, versatile and inexpensive method

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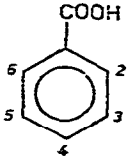
TABLE I

HYDROXYBENZOIC ACIDS



Substitution type	*Trivial name (Chemical Abstracts registry number)*		*Source of supply*
2-OH	Salicylic acid	(69-72-7)	Riedel de Haen (Seelze, G.F.R.)
3-OH	*m*-Hydroxybenzoic acid	(99-06-9)	Roth (Karlsruhe, G.F.R.)
4-OH	*p*-Hydroxybenzoic acid	(99-96-7)	Roth
2,3-di-OH	*o*-Pyrocatechuic acid	(303-38-8)	Fluka (Buchs, Switzerland)
2,4-di-OH	β-Resorcylic acid	(89-86-1)	Fluka
2,5-di-OH	Gentisic acid	(490-79-9)	Roth
2,6-di-OH	γ-Resorcylic acid	(303-07-1)	Fluka
3,4-di-OH	Protocatechuic acid	(99-50-3)	Roth
3,5-di-OH	α-Resorcylic acid	(99-10-5)	Fluka
3,4,5-tri-OH	Gallic acid	(149-91-7)	Riedel de Haen
2,3,4-tri-OH	Pyrogallolcarboxylic acid	(610-02-6)	Fluka
2,4,6-tri-OH	Phloroglucinolcarboxylic acid	(83-30-7)	E. Merck (Darmstadt, G.F.R.)
3-OCH_3, 4-OH	Vanillic acid	(121-34-6)	Fluka
3,5-di-OCH_3, 4-OH	Syringic acid	(530-57-4)	Fluka

for the determination of phenolic acids. It has the particular advantage of specificity and selectivity in detection. Its main disadvantages are insensitivity in trace analysis and limited possibilities for quantitative estimation. In the GLC determination described in Part II[29], supplementary TLC was found to be of great value for monitoring purposes.

EXPERIMENTAL

Evaporations were performed in a rotary vacuum evaporator at a temperature not higher than 40°C.

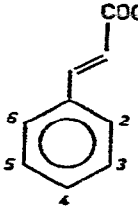
Sample preparation

The plant material (100 g, or corresponding amounts of dry samples), was covered with about 200 ml hot water and boiled for 10 min to denature enzymes and facilitate sample handling. The material was homogenized for about 10 min using an Ultra-Turrax (Janke & Kunkel, Staufen i.Br., G.F.R.). Extremely coarse material should be comminuted beforehand, *e.g.*, in a household blender. The suspension was cooled and adjusted to pH 4.6 with dilute HCl or KOH. After adding 1 g of the non-specific glycosidase EL 1-77 (Röhm, Darmstadt, G.F.R.) with stirring, the mixture was kept at 45°C for 15 h. This enzymatic treatment greatly facilitated the subsequent procedures.

The phenolics were extracted by boiling the suspension and centrifuging while

TABLE II

HYDROXYCINNAMIC [3-(HYDROXYPHENYL)-2-PROPENOIC] ACIDS



Substitution type	*Trivial name (Chemical Abstracts registry number)*	*Source of supply**
2-OH	*o*-Coumaric acid (583-17-5; *trans*-: 614-60-8)	Roth
3-OH	*m*-Coumaric acid (588-30-7)	Roth
4-OH	*p*-Coumaric acid (7400-08-0; *trans*-: 501-98-4; *cis*-: 4501-31-9)	Fluka
3,4-di-OH	Caffeic acid (331-39-5; *trans*-: 501-16-6; *cis*-: 4361-87-9)	Fluka
3-OCH_3, 4-OH	Ferulic acid (1135-24-6; *trans*-: 537-98-4; *cis*-: 1014-83-1)	Roth
4-OCH_3, 3-OH	Isoferulic acid (537-73-5; *trans*-: 25522-33-2)	Roth
3,5-di-OCH_3, 4-OH	Sinapic acid (530-59-6; *trans*-: 7362-37-0; *cis*-: 7361-90-2)	Riedel de Haen

* All as *trans*-isomers.

still hot. The sediment was re-extracted twice by homogenizing with about 150 ml hot water, boiling and centrifuging while hot. The combined extracts were filtered through glass wool into a suitable round-bottomed flask (*e.g.*, 1 l) to permit vigorous swirling (by hand) to reduce foaming during alkaline hydrolysis.

The filtrate was adjusted to pH 7.0–7.2 with KOH (1 *N*, containing *ca.* 10 mg of sodium borohydride per 50 ml). Sodium borohydride (2 g) and thereafter $Ba(OH)_2$ (1 g for each 100 ml solution) was added carefully with continuous stirring. When foaming was under control, the solution was boiled for 15 min to hydrolyze the extract. Where foaming was excessive, lower temperatures and longer time spans were employed to hydrolyze the phenolic acid esters. The maintenance of a reducing atmosphere (owing to slow decomposition of the added borohydride) throughout the period of hydrolysis was essential to protect the phenolic acids, especially caffeic acid, from oxidation[24].

An acid hydrolysis was then carried out to liberate phenolic acids bound glycosidically. Possible partial degradation of hydroxycinnamic acids (especially ferulic acid) should be taken considered, even when analyzing plant extracts (buffered complex systems). Van Brussel *et al.*[25] investigated the destruction of pure ferulic acid solutions treated with boiling mineral acids for more than 1 h.

The alkaline solution was adjusted carefully to pH 6.5 with 10% sulphuric acid with continuous stirring to avoid excessive foaming. Concentrated sulphuric acid was then added to a concentration of 1%, and the mixture was refluxed for 10 min. Phenolic acids which had possibly entered the condenser were recovered by washing it with a small quantity of hot water. The combined washings and hydrolysate were centrifuged and the sediment ($BaSO_4$) was suspended in hot water and recentrifuged. The combined extracts were adjusted to pH 2.5, and then transferred to a 1-l long-necked flask.

At this stage the extract could be left overnight, if desired, providing that it was kept in the dark and contained some polyamide from the column to be used in the next step.

Preparation of polyamide columns

About 150 g polycaprolactam powder (MN-Polyamid-SC 6, 0.05–0.16 mm; Macherey, Nagel & Co., Düren, G.F.R.) were suspended in *ca.* 1 l of water–methanol (1:1). After about 3 h the suspension was poured into a double-walled heatable tube (25 × 5 cm I.D., stoppered with glass wool). For complete removal of soluble polyamide components the column was washed with 1 l methanol–25% aqueous ammonia (9:1) followed by 1 l methanol and at least 2 l water–acetic acid (999:1). The polyamide was then free of methanol. Such a column could generally be used only once.

The aqueous extract (pH 2.5) was applied to the polyamide column from the long-necked flask, and the flask and column were washed with water (2.0 l) to remove carbohydrates, salts and other undesired compounds. The column was thermostatted to 40°C, and three eluates, A, B and C, were collected (separately) as follows.

(A) Elution with about 0.5 l methanol. The point at which methanol began to exit from the column could be recognized by a visible "streakiness" due to density changes within the eluate droplets and by a sudden acceleration in the drop rate. This was the moment from which eluate A was collected.

(B) Elution with a mixture of 1 l methanol and three drops 25% aqueous ammonia.

(C) Final elution with about 0.5 l methanol–25% aqueous ammonia (99:1).

Most of the phenolic acids were found in fractions A and B. Fraction C served only to ensure complete elution of the strongly adsorbed salicylic and gentisic acids. Many interfering compounds were also eluted in fraction C. (Possibilities of removing these undesired substances exist, *e.g.*, by ethyl acetate extraction[8] or by precipitation with acetone[8,26].)

The ammonia was removed from eluates B and C as rapidly as possible by concentrating the solutions in a rotary vacuum evaporator at 40°C. Eluates A, B and C were then combined and concentrated further, any remaining water being removed azeotropically by addition of methanol. Precipitation of ammonium sulphate, occurring sometimes during concentration, was filtered off. The resultant concentrate, diluted to 25 ml, could be stored in a refrigerator for several weeks This is the parent solution for analysis by TLC or GLC (Part II).

Thin-layer chromatography

The parent solution (*ca.* 5 ml) was concentrated to about 0.5 ml and spotted

on to two silica gel plates (Silica-Rapid-Platten Woelm F 254; Woelm, Eschwege, G.F.R.). It is noteworthy that not every type of commercial silica gel TLC plate was found to be suitable. Some types yielded a pink background which caused interference when the plates were sprayed with $FeCl_3$. One plate was developed in freshly prepared solvent I, dichloromethane–toluene–formic acid (50:40:10, v/v/v), and the other in solvent II, dichloromethane–water–acetic acid (100:50:50, v/v/v, lower phase). The separation in solvent I can be improved by double development.

Evaluation of the chromatograms was done by examination first in UV light, 366 nm and 254 nm, followed by visualization after gentle spraying with 1% methanolic $FeCl_3$ solution (the background of the plates should be pale yellow). The spots were then fanned with hot air at a distance of less than 1 cm above the sorbent layer (this causes the background to become white again). Finally, the chromatograms were oversprayed lightly with a solution of 0.5 g diazobenzenesulphonic acid in 100 ml of 1 *N* NaOH and heated at 80°C for 15 min. A second respraying and heating are recommended to obtain the best results.

A solution of 20% $TiCl_4$ in concentrated HCl may be used instead of the $FeCl_3$ reagent without impairing the subsequent reaction with diazobenzenesulphonate. The phenol-specific titanium reagent[9] is especially suitable for quantitative estimation by TLC scanning because of the uniformity of the yellow light brown colour development, but the lack of colour differentiation with different compounds is a disadvantage for identification purposes.

RESULTS

The retention sequence of phenolic acids (including some coumarins) under the conditions employed is shown in Figs. 1 and 2. 2,3,4-Trihydroxybenzoic acid, 2,3-dihydroxybenzoic acid and particularly 2,6-dihydroxybenzoic acid show tailing and poor retention reproducibility. This is obviously caused by their very small pK_a values. The use of trichloroacetic acid in the developing solvent eliminates this undesirable phenomenon. Separation of the solvent and impairment of the reaction with $FeCl_3$ make a general application of this acid impracticable.

Under the conditions described, the *cis*-hydroxycinnamic acids are not separated from the *trans*-isomers, but this can be achieved using cellulose TLC plates and 1% aqueous acetic acid as solvent[27].

The colours of phenolic acids (including some coumarins) with the chromogenic spray reagents employed are summarized in Table III. The more concentrated the spots, the more intense and better differentiated are the colours, especially after spraying with the $FeCl_3$ reagent. For good distinction of the different fluorescence colours a powerful UV light with a high degree of monochromaticity (366 nm) is necessary.

It is difficult to specify the lower limit of detection of phenolic acids by TLC, because accompanying substances in plant extracts can impair the determination. In general, however, phenolic acids can be detected unequivocally at levels of 10 ppm, often at levels of only 1 ppm, in plant material. Because their colour reactions are less intense, *m*- and *p*-hydroxybenzoic acids are somewhat more difficult to detect in low concentrations. Gallic acid is eluted incompletely from the polyamide column during sample preparation with the result that it cannot be detected in concentrations below

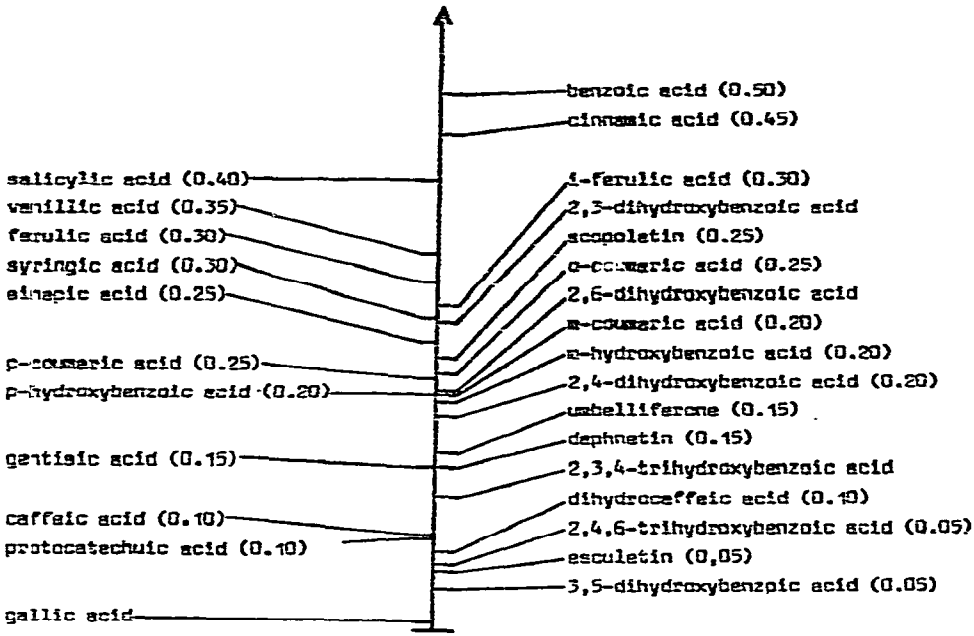


Fig. 1. Retention sequence of phenolic acids and coumarins (R_F values in parentheses) by TLC on silica gel with dichloromethane–toluene–formic acid (50:40:10, v/v/v) as solvent.

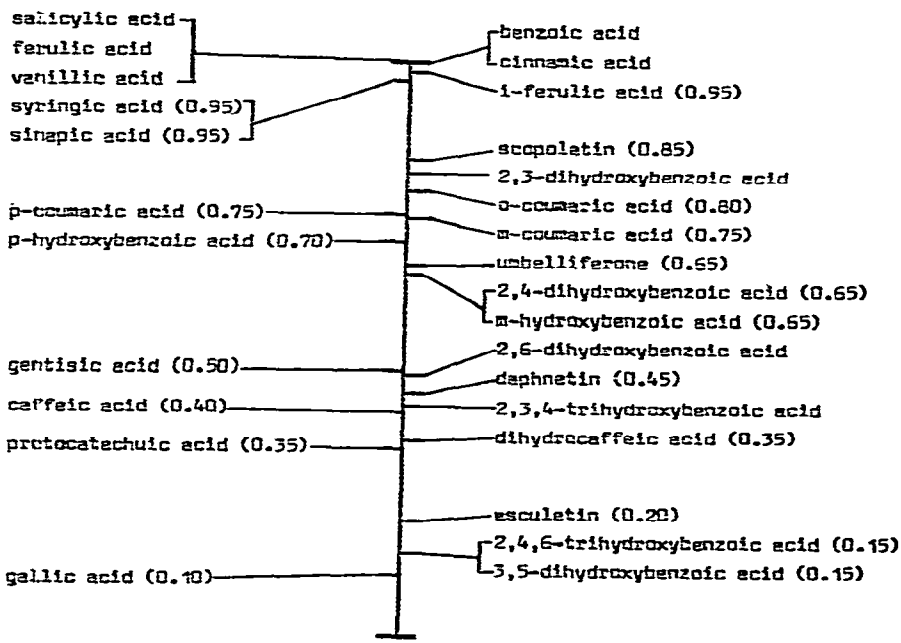


Fig. 2. Retention sequence of phenolic acids and coumarins (R_F values in parentheses) by TLC on silica gel with dichloromethane–water–acetic acid (100:50:50, v/v/v, lower phase) as solvent.

about 30 ppm in plant material. The preliminary purification (polyamide column) was necessary for successful chromatogram evaluation. Gallic acid showed insufficient recovery from this analytical step. Therefore the described method is unsuitable for its quantitation.

Hydroxybenzoic acid and hydroxycinnamic acid derivatives[1] are generally soluble in hot water. So we used the described aqueous extraction after an enzymatic pretreatment.

The conditions for a combined hydrolysis had been developed previously[8,28]. It was shown[28] that a combined acidic–alkaline treatment gives a maximum yield of individual acids, liberated from their derivatives in plant extracts. In Table IV the recovery of phenolic acids using the described analytical method is summarized.

TABLE III

COLOUR REACTIONS OF PHENOLIC ACIDS (INCLUDING SOME COUMARINS)

Compound	*Colour on examination under UV-light at*		*Colour on spraying with*		
	366 nm	*254 nm*	*1% methanolic $FeCl_3$*	*20% $TiCl_4$ in conc. HCl*	*Alkaline diazobenzene sulphonate after treatment with $FeCl_3$*
Acids					
Salicylic	bright blue	bright blue-grey	violet-brown	yellow	pale yellow
Vanillic	—	blue	bright brown	yellow-ochre	yellow
Ferulic	blue	grey-blue	dark red-brown	dirty-brightbrown	pink-grey
Syringic	—	grey-blue	dark brown	yellowochre	orange-bright red
Sinapic	blue	grey-blue	dark grey-violet	dirty bright brown	reddish grey
p-Coumaric	profound dark blue	blue	orange	yellow-ochre	pale yellow
p-Hydroxybenzoic	—	dark blue	pale yellow	pale yellow	pale yellow
Gentisic	white	bright grey (fluorescent)	brownish grey	orange brown	brown
Caffeic	blue	dark grey	grey to greenish black	dirty brown	grey-brown-black
Protocatechuic	—	blue	black to brown-black	dark red-brown	brown
Gallic	—	dark grey-blue	dark blue	brown	grey-brown-black
Benzoic	—	grey	—	—	—
m-Hydroxybenzoic	—	grey-blue	pale yellow	yellow	yellow
2,3-Dihydroxybenzoic	brownish yellow	grey-blue	blue-grey	brown	brown
2,4-Dihydroxybenzoic	—	blue	bright blue-grey	ochre	grey-yellow

(Continued on p. 92)

TABLE III *(continued)*

Compound	*Colour on examination under UV-light at*		*Colour on spraying with*		
	366 nm	*254 nm*	*1% methanolic $FeCl_3$*	*20% $TiCl_4$ in conc. HCl*	*Alkaline diazobenzene sulphonate after treatment with $FeCl_3$*
2,6-Dihydroxybenzoic	pale brownish	dark grey	grey	grey-ochre	dirty yellow
3,5-Dihydroxybenzoic	—	blue	pale yellow	ochre-yellow	yellow (pink leaking out)
2,3,4-Trihydroxybenzoic	—	black-blue	grey-blue	brown	grey-brown
2,4,6-Trihydroxybenzoic	—	grey-blue	dark grey-ochre	dark ochre	bright brown
Cinnamic	bright violet-white	dark blue	yellow	pale yellow	orange (very pale)
o-Coumaric	bright violet-white	grey-blue	ochre	yellow	yellow
m-Coumaric	bright grey-blue	blue	yellow	yellow	pink-yellow
Isoferulic	bright blue-violet	blue-grey	brown-red	ochre	pink-orange
Dihydrocaffeic	—	blue-grey	grey-black	orange-brown	violet-brown
Coumarins					
Scopoletin	bright blue	bright blue (fluorescent)	pale grey-yellow	yellow	pink-yellow
Umbelliferone	bright blue-white	bright blue (fluorescent)	—	pale yellow	pale brownish
Daphnetin	pale brownish	dark grey	greenish grey	ochre	grey-brown
Esculetin	bright blue-white	grey-blue (fluorescent)	grey	ochre-orange	brown

Gentisic acid showed unsatisfactory recovery. Obviously the losses are caused by the hydrolysis step.

Pure aqueous solutions of phenolic acids were less stable in the hydrolysis step than phenolic acids occurring in plant extracts. From 100 ppm phenolic acid admixed with coriander seed and bay leaves we recovered *p*-coumaric acid (91%), caffeic acid (88%), ferulic acid (92%) and sinapic acid (87%).

In the range below 10 ppm (phenolic acid in plant extract) the losses of phenolic acids can increase noticeably, so that in this range the method is only semi-quantitative.

When recovery tests were performed only partial losses of phenolic acids were noticed. No new compounds (decomposition products) were found by TLC or GLC.

TABLE IV

RECOVERY DATA

Aqueous solutions of 5 and 10 mg phenolic acid were submitted three times to the purification step and eight times to the whole method. Data were obtained by comparison with gas chromatograms of directly silylated standard solutions.

Acids	*Recovery (%) from purification (polyamide column)*	*Overall recovery (%) ± S.D. (%) from extraction + hydrolysis + purification*
Hydroxybenzoic acids		
Salicylic	96	89 ± 3.2
p-Hydroxybenzoic	98	94 ± 2.1
Syringic	97	91 ± 2.9
Vanillic	99	96 ± 1.8
Protocatechuic	96	92 ± 3.0
Gentisic	90	58 ± —*
*Hydroxycinnamic acids***		
p-Coumaric	94	87 ± 4.6
Ferulic	94	82 ± 4.3
Caffeic	92	78 ± 5.7
Sinapic	93	79 ± 6.4

* Not measured.

** *cis*- and *trans*-isomers are added.

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