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QUANTITATIVE ANALYSIS FOR PHENOLIC ACIDS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method is described by which phenolic acid compounds occurring in plant material can be separated on a polyamide column after suitable hydrolysis. The mixture of phenolic acids was also separated on silica gel G thin-layer plates, and the individual phenolic acids were determined spectrophotometrically. Phenolic acid compounds were hydrolysed by a technical enzyme or by treatment with 1% barium hydroxide solution, followed by 1% sulphuric acid. The method was applied successfully to fruit and vegetables.

INTRODUCTION

The presence of hydroxycinnamic acid compounds in many plants has been reported[1–4], and hydroxybenzoic acid compounds also occur frequently in nature[5]. Although there are no obvious difficulties in detecting phenolic acids by paper or thin-layer chromatography (TLC), there are almost no reports on quantitative analysis for these compounds, except some methods for particular acids, such as chlorogenic acid (3-caffeoylquinic acid), which occurs in higher concentrations in coffee or tobacco.

Quantitative analysis for phenolic acids in plant material involves considerable difficulties, because the acids are mostly present as, for example, esters or glycosides. Because of the relatively great number of possible combinations, the separation and determination of all of the original compounds seems to be almost impossible. Therefore, it is necessary to liberate the free phenolic acids, which poses the problem of devising optimum conditions of hydrolysis. For this purpose, we have evolved two different schemes. First, we found a technical enzyme (EL 45-68, Röhm, Darmstadt, G.F.R.) which was useful in hydrolysing natural phenolic acid compounds, and we have developed a method involving enzymic hydrolysis for determining hydroxycinnamic acids[6] and applied it to fruit[7,8] and vegetables[9]. However, it was apparent that some of the compounds present could not be completely hydrolysed by the enzyme, so we devised a second method of hydrolysis, involving a combination of alkali and acid treatment.

EXPERIMENTAL

Reagents

The following reagents were used: 1 *N* hydrochloric acid; 1 *N* potassium hydroxide; 30% aqueous solutions of potassium hydroxide and sodium bisulphite; 10% aqueous sulphuric acid; concentrated sulphuric acid; anhydrous sodium sulphate; barium hydroxide; sodium tetrahydroborate; methanol; acetone; ethyl acetate; polyamide SC 6 (0.05–0.16 mm) (Macherey, Nagel & Co., Düren, G.F.R.); silica gel G TLC plates (Merck nach Stahl, Type 60), thickness of gel layer, 0.25 mm, dried at 20°; technical enzyme with esterase and pectinase activity (EL 45-68, Röhm); methanol–25% aqueous ammonia (9:1); the lower phase of dichloromethane–acetic acid–water (2:1:1); benzene–acetic acid (45:4); 1% methanolic ferric chloride solution; solution of 100 mg of diazobenzenesulphonic acid in 50 ml of 0.5 *N* potassium hydroxide.

Preparation of polyamide columns

An aqueous suspension of polyamide, which had been mixed at least 3 h beforehand, was poured into a double-walled tube (length 30 cm; I.D. 5 cm) that could be heated. After the tube had been filled to a height of *ca.* 25 cm, the column was washed with 1 l of methanol–25% aqueous ammonia (9:1) and with another 1 l of methanol to remove the soluble portion of the polyamide; finally, it was washed with 1 l of water.

Extraction and enzymic hydrolysis of phenolic acid compounds

The plant material (150–300 g) was chopped with a rustproof knife, covered with 300 ml of boiling water, boiled for 15 min and finally homogenised. After the suspension had cooled to 45°, its pH was adjusted to 4.3 with 1 *N* potassium hydroxide or 1 *N* hydrochloric acid, with continuous stirring. Then a solution of 1.0 g of the technical enzyme in 10 ml of water was added, and the pH was again adjusted to 4.3 (the enzyme showed an alkaline reaction), and the mixture was allowed to stand at 45° for 20 h; it was then boiled for 5 min and immediately centrifuged for 10 min. The residue was boiled and centrifuged twice with 200 ml of water, and a small quantity of plant material that could not be separated by centrifugation was removed by pouring the extract through glass wool.

Extraction and alkaline hydrolysis of esters, and acid hydrolysis of glycosides

Plant material (150–300 g) was homogenised as described above, boiled for 15 min, stirred occasionally and immediately centrifuged for 15 min. The residue was boiled and centrifuged twice with 200 ml of water, and the extract was filtered through glass wool.

During sample preparation, certain materials (*e.g.*, potatoes, onions and peas) formed a gelatinous suspension that could not be centrifuged properly. We avoided this problem by hydrolysing the suspension with 0.5 g of technical enzyme at 45° during 20 h; after that treatment, the mixture could be centrifuged easily.

Having adjusted the pH of the combined extract to 7.0 with 1 *N* potassium hydroxide, the mixture was transferred to a 2-l beaker, and 2 g of sodium tetrahydroborate were added in small portions, with gentle stirring (rapid addition of the borate

causes excessive foaming). Then the pH was adjusted to 12–13 by adding 1% barium hydroxide solution, and the mixture was boiled for 15 min and cooled to 20°.

During neutralisation with 10% sulphuric acid, sodium tetrahydroborate in excess decomposed, causing heavy foaming. After adjusting the pH to 1.5 with concentrated sulphuric acid (giving a solution containing 1% of this acid), the mixture was boiled under reflux for 15 min, then immediately centrifuged for 15 min; the white residue (barium sulphate) was washed with 100 ml of boiling water.

Polyamide column chromatography of the extracts

The pH of the combined extracts was adjusted to 3.3 with 1 *N* hydrochloric acid (enzymic hydrolysis) or 30% potassium hydroxide (alkali–acid hydrolysis), then the solution was mixed with 20 ml of 30% aqueous sodium bisulphite solution and 20 g of polyamide suspended in water, and allowed to percolate through the polyamide column. To remove accompanying substances, the column was washed with 500–750 ml of water; the phenolic acids, hydroxycoumarins (*e.g.*, umbelliferone, scopoletin and aesculetin) and hydroxyflavanones (such as naringenin) were eluted at 40° with 1.5 l of methanol (eluate I). Under these conditions, salicylic and gentisic acids remained adsorbed on the column; they could be eluted with 1 l of methanol–25% aqueous ammonia (9:1) (eluate II).

Eluate I was evaporated to dryness *in vacuo*, the residue was dissolved in 20 ml of methanol, and this solution was mixed with 100 ml of acetone. The flocculent precipitate that formed was separated by filtration on a G4 fritted-glass filter and was washed with 40 ml of methanol–acetone (1:3). The combined filtrates were concentrated to a few millilitres *in vacuo*, transferred to a 10-ml volumetric flask and made up to volume with methanol (solution A).

Eluate II was concentrated to 50 ml *in vacuo*, its pH was adjusted to 3.0, and it was extracted three times with 100-ml portions of ethyl acetate. The combined extracts were dried with anhydrous sodium sulphate, concentrated to a few millilitres *in vacuo*, transferred to a 10-ml flask and made up to volume with methanol (solution B). Ellagic acid could not be extracted with ethyl acetate and remained in the aqueous solution[10].

Detection of phenolic acids

For detection, 0.25–0.5 ml of solutions A and B were applied (as bands 6 cm in length) with a Dibbern Microdoser (Desaga) and a 0.5-ml Hamilton syringe to a silica gel G plate. The plate was also spotted with methanolic solutions of authentic phenolic acids (20 mg per 100 ml of methanol). For the separation of phenolic acids having only hydroxyl groups (except salicylic acid), the chromatogram was developed with dichloromethane–acetic acid–water (2:1:1) (developing solvent 1); phenolic acids having both hydroxyl and methoxyl groups (and salicylic acid) were separated by developing the chromatogram with benzene–acetic acid (45:4) (developing solvent 2). After drying the chromatograms, the zones of single phenolic acids were detected by examination in UV radiation and by spraying with 1% methanolic ferric chloride solution or diazobenzenesulfonic acid reagent.

Purification and determination of phenolic acids

A portion (0.1–1.0 ml) of solution A or B was applied as a band 12 cm in

length to a silica gel G plate; beside this main band and 2 cm from it, we applied an identification band (4 cm in length) containing the authentic phenolic acids. After developing the chromatogram in the solvents mentioned above and drying it, the main chromatogram was covered with a glass plate, and the identification band was sprayed with the ferric chloride solution; the zones corresponding to the single phenolic acids in the main chromatogram could then be easily located and were marked, scraped off and transferred to a G4 fritted-glass filter. After extraction of these zones with methanol p.a., the extracts were concentrated *in vacuo*, transferred to volumetric flasks (10–100 ml, depending on the amount of each phenolic acid in the zone) and made up to volume with methanol p.a. (solution C).

The UV spectra of these solutions were recorded with a Unicam SP800 spectrophotometer in 1-cm quartz cells, using methanol p.a. as blank, and the absorbances at the main maxima in the spectra were determined.

Calibration factors

Purified phenolic acids were obtained by re-crystallisation of commercial products with active charcoal in water and drying with phosphorus pentoxide *in vacuo* at 110°. To prepare calibration graphs, 100 mg of each phenolic acid were dissolved in methanol p.a., the solution was diluted to 100 ml with methanol p.a., and 0.1, 0.3, 0.5-ml portions (etc.) of these solutions were transferred to 100-ml flasks and made up to volume. With methanol p.a. as blank, we recorded the UV spectra and determined the absorbances at the main maxima. The calibration graphs were all rectilinear. Calibration factors, UV maxima and R_F values are shown in Table I for some phenolic acids.

The content of phenolic acid in the original plant material (in mg/kg) was calculated from the expression 100 AB/EC, where A is the concentration of phenolic acid (mg per 100 ml of methanol) corresponding to the measured absorbance, B is the volume of solution C (in ml), C is the volume of solution A or B applied to the plate, and E is the original weight of plant material (in g).

RESULTS AND DISCUSSION

We intentionally limited our quantitative work to free phenolic acids. For reliable determinations we could use the absorbance at the maximum of the UV spectrum, because the colour reactions are not highly specific. Without doubt, adequate purification of the extracts is a most important step in obtaining reliable quantitative results. The main difficulties in the method are in developing suitable conditions for hydrolysis. We found that, for the species of fruits examined, and for many species of vegetables, it was sufficient to hydrolyse with the technical enzyme EL 45-68. However, the enzymic method did not work well with Brassica species and other Cruciferae, which contained sinapic and ferulic acid compounds that could only be partly hydrolysed. The application of specific enzymes would be advantageous, but for this purpose greater knowledge of the compounds occurring in nature is required. Often, combined alkali and acid hydrolysis yields better results. If only esters are present, an additional acid hydrolysis can be omitted. On the other hand, it is well known that alkaline treatment of sensitive esters like caffeic acid compounds can cause high losses, as with acid hydrolysis of hydroxyflavone glycosides[11]. Thus, the conditions of

TABLE I

CALIBRATION FACTORS, MAIN MAXIMA IN THE UV SPECTRA AND hR_F VALUES OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS

Phenolic acid	*Substituent*	*Calibration factor**	*Main max. (nm)*	hR_F	
				Solvent 1	*Solvent 2*
Hydroxybenzoic acids					
Salicylic acid	2-OH	1.74	305	100	67
3-Hydroxybenzoic acid	3-OH	2.60	298	79	33
4-Hydroxybenzoic acid	4-OH	0.49	253	86	33
2,3-Dihydroxybenzoic aicd	2,3-OH	2.43	318	89	34
2,4-Dihydroxybenzoic acid	2,4-OH	1.34	295	78	30
Gentisic acid	2,5-OH	1.78	330	63	25
2,6-Dihydroxybenzoic acid	2,6-OH	2.47	317	33	14
Protocatechuic acid	3,4-OH	0.82	258	44	16
3,5-Dihydroxybenzoic acid	3,5-OH	2.88	310	20	8
Gallic acid	3,4,5-OH	0.89	271	11	6
Phloroglucine acid	2,4,6-OH	0.61	261	10	5
Vanillic acid	3-OCH_3, 4-OH	0.81	258	100	58
Syringic acid	3,5-OCH_3, 4-OH	0.88	272	100	44
Hydroxycinnamic acids					
o-Coumaric acid	2-OH	0.98	325	95	34
m-Coumaric acid	3-OH	0.44	276	89	33
p-Coumaric acid	4-OH	0.40	310	90	35
Caffeic acid	3,4-OH	0.56	325	51	14
Ferulic acid	3-OCH_3, 4-OH	0.58	320	100	54
Isoferulic acid	3-OH, 4-OCH_3	0.64	322	100	49
Sinapic acid	3,5-OCH_3, 4-OH	0.61	322	100	39

* Calibration factor: Concentration (mg per 100 ml of methanol) corresponding to an absorbance of 0.500.

hydrolysis should be as mild as possible, but strong enough to hydrolyse compounds occurring in plant material. For alkaline hydrolysis of phenolic acid esters, we found it best to make the extract alkaline by adding 1 % barium hydroxide solution and boiling for 15 min.

For the acid hydrolysis of phenolic acid glycosides, we recommend the same time for boiling after the addition of 1 % sulphuric acid to the neutralised extract. Addition of sodium tetrahydroborate prevents oxidation of cinnamic and benzoic acid derivatives having two hydroxyl groups in positions *ortho* or *para* to each other[12], *e.g.*, caffeic, protocatechuic, gentisic and gallic acids.

For quantitative analysis of phenolic acids, we recommend the use of both methods of hydrolysis in separate operations, provided that the reaction products do not make the alkali–acid hydrolysis difficult, as, for instance, with fruits containing catechins.

Quantitative analysis yields reliable results if the concentrations of phenolic acids are higher than 5 mg/kg of plant material; for less than 1 mg/kg the error is so large that only the order of magnitude can be estimated.

In recovery tests, we subjected aqueous solutions of chlorogenic acid and free phenolic acids to both methods of hydrolysis, purified the products by polyamide column chromatography and determined the individual phenolic acids spectropho-

tometrically after TLC on silica gel G plates. The results in Table II show that those phenolic acids that do not have two hydroxyl groups *ortho* or *para* to each other can be recovered almost completely; protocatechuic acid is an exception. We conclude that mainly oxidation during adsorption on the silica gel layer is responsible for the losses of chlorogenic, caffeic, gentisic and gallic acids.

TABLE II

RECOVERY DATA FROM BOTH METHODS OF HYDROLYSIS

Phenolic acid	*Recovery (%)*	
	Alkali–acid hydrolysis	*Enzymic hydrolysis*
Chlorogenic acid	81	85
Caffeic acid	85	90
p-Coumaric acid	90	92
Ferulic acid	92	93
Sinapic acid	95	95
Salicylic acid	99	100
4-Hydroxybenzoic acid	98	99
Gentisic acid	85	88
Protocatechuic acid	95	99
Gallic acid	83	89
Vanillic acid	99	98
Syringic acid	97	99

CONCLUSION

By using the methods described here, all species of fruits and vegetables grown in Germany and fruits from tropical countries were examined by Schmidtlein and Stöhr. The concentrations of the single phenolic acids were generally low (up to 50 mg/kg) and often only traces (0.5 mg/kg) were found. Detailed results will be reported elsewhere[10,13].

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