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QUANTITATIVE ANALYSIS OF FLAVANONES AND 3-HYDROXYFLAVANONES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of flavanones and 3-hydroxyflavanones occurring in plant material, if necessary after suitable hydrolysis. The plant extract is first separated on a polyamide column, and then further separated on thin-layer plates of silica gel G. The individual phenolic compounds have been determined spectrophotometrically.

INTRODUCTION

It is well known that citrus fruits contain flavanone glycosides[1], which contribute to the bitter taste of grapefruit and bitter oranges. Although hesperetin was isolated by Hoffmann in 1876 and naringenin was isolated by Will in 1885, there is almost no reliable information about their occurrence in other food plants, except for naringenin in the tomatopeels[2,3]. On the other hand, 3-hydroxyflavanones seem to be widely distributed in hard woods[4].

Recently we published a method for the quantitative determination of phenolic acids[5], which consists of UV-spectroscopic measurement of the phenolic acids after their separation on a polyamide column and by thin-layer chromatography (TLC) on silica gel. By means of a modification of this method, flavanones and 3-hydroxyflavanones can also be determined in the ppm range.

EXPERIMENTAL

Reagents

The following reagents were used: polyamide SC 6 (0.05–0.16 mm) (Macherey, Nagèl & Co., Düren, G.F.R.); silica gel G Type 60 TLC plates (E. Merck, Darmstadt, G.F.R.), thickness of gel layer 0.3 mm, dried at 20°; zinc dust; 6 *N* hydrochloric acid; a 30% aqueous solution of sodium bisulphite; methanol; acetone; methanol–acetone (1:3); the lower phase of dichloromethane–acetic acid–water (2:1:1); 1% methanolic ferric chloride solution; a solution of 100 mg of diazobenzenesulphonic

TABLE I

DATA FOR SOME FLAVANONES AND 3-HYDROXYFLAVANONES

I = Dichloromethane–acetic acid–water (2:1:1, lower phase); II = benzene–acetic acid (45:4). Calibration factor = mg of substance in 100 ml of methanol corresponding to an absorbance of 0.500.

Compound	*Structure*	*hR_f Values in solvent*		*Main maximum (nm)*	*Calibration factor*	*Colour reactions with*		
		I	*II*			*$FeCl_3$*	*diazobenzene-sulphonic acid reagent*	*$NaBH_4/AlCl_3$*
Naringenin	4′,5,7-trihydroxyflavanone	59	12	288	0.71	red-violet	orange	red
Eriodictyol	3′,4′,5,7-tetrahydroxyflavanone	30	4	289	0.68	blue-violet	red-brown	blue-violet
Hesperetin	4′,5,7-trihydroxy-3′-methoxyflavanone	86	22	287	0.73	red-violet	orange	red
Dihydroquercetin	3,3′,4′,5,7-pentahydroxy flavanone	14	0	290	0.78	blue-violet	brown	colourless
Dihydrofisetin	3,3′,4′,7-tetrahydroxyflavanone	14	0	277	0.93	grey	light brown	red-violet
Dihydrorobinetin	3,3′,4′,5′,7-pentahydroxyflavanone	4	0	275	1.08	dark blue	grey-brown	blue-violet

acid in 50 ml of 0.5 *N* potassium hydroxide; a 2% methanolic solution of sodium tetrahydroborate; a 5% methanolic solution of aluminium chloride.

Extraction and polyamide column chromatography of the extracts

The chopped plant material (150–300 g) was covered with boiling water (*ca.* 300 ml), boiled for 10–15 min and finally homogenized and centrifuged. The residue was boiled and centrifuged twice with 200 ml of water. A small quantity of plant material that could not be separated by centrifugation was removed by pouring the extract through glass wool.

If known glycosides of flavanones and 3-hydroxyflavanones are present in the homogenate, they must be hydrolyzed in a suitable manner, *e.g.*, by use of specific enzymes or acids (sulphuric or hydrochloric acid). In the case of unknown glycosides, we preferred a technical enzyme having esterase and pectinase activity (EL 45-68; Röhm, Darmstadt, G.F.R.) which also completely hydrolyses most of the phenolic acid compounds[5]. The homogenate was cooled to 45°, and adjusted to pH 4.3 with 1 *N* potassium hydroxide or 1 *N* hydrochloric acid, with continuous stirring. A solution of 1.0 g of the technical enzyme in 10 ml of water was then added, the pH was again adjusted to 4.3 and the mixture was allowed to stand at 45° for 20 h; it was then boiled for 5 min and centrifuged immediately.

After adjusting the combined extracts to pH 3.3, the solution was mixed with 20 ml of a 30% aqueous solution of sodium bisulphite and 20 g of polyamide suspended in water, and allowed to percolate through a polyamide column (25 × 5 cm I.D.) in a double-walled tube that could be heated. In order to remove substances such as sugars and salts, the column was washed with 500–750 ml of water; flavanones, 3-hydroxyflavanones and phenolic acids were eluted at 40° with 1.5 l of methanol. The eluate was evaporated to dryness *in vacuo*, the residue was dissolved in 20 ml of methanol and the resulting solution was mixed with 100 ml of acetone. The flocculent precipitate was separated by filtration on a G4 fritted-glass filter and 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 I).

Detection, purification and determination of flavanones and 3-hydroxyflavanones

For detection, 0.25–0.5 ml of solution I were applied (as a band 6 cm in length) to a plate of silica gel G by means a Dibbern Microdoser (Desaga) and a 0.5-ml Hamilton syringe. The plate was also spotted with methanolic solutions of authentic substances (20 mg per 100 ml of methanol). The chromatograms were developed with dichloromethane–acetic acid–water (2:1:1). After drying the chromatograms, the zones of the individual substances were detected by spraying with a 1% methanolic solution of ferric chloride or with diazobenzenesulphonic acid reagent. First, the chromatograms were sprayed with reagents which react specifically with our groups of compounds. We used two colour reactions: (a) zinc dust suspended in acetone followed by 6 *N* hydrochloric acid (3-hydroxyflavanones are reduced to blue-violet compounds); and (b) methanolic solutions of sodium tetrahydroborate and aluminium chloride followed by heating of the chromatograms to 120° for 1.5 min (for the colour reactions see Table I).

For purification, a portion (0.1–1.0 ml) of solution I was applied as a band

(12 cm in length) to a plate of silica gel G; beside this main band and 2 cm from it, we also applied an identification band (4 cm in length) containing the authentic substances. After development of the chromatogram with dichloromethane–acetic acid–water (2:1:1) and drying, the main chromatogram was covered with a glass plate and the identification band was sprayed with the ferric chloride solution. The zones corresponding to an individual flavanone or 3-hydroxyflavanone in the main chromatogram could 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 substance in the zone) and made up to volume with methanol p.a. (solution II).

The UV spectra of these solutions were recorded with an Unicam SP 800 spectrophotometer in 1-cm quartz cells, using methanol p.a. as a blank, and the absorbances at the main maxima in the spectra were determined. The content (in mg/kg) of flavanones and 3-hydroxyflavanones in the original plant material was calculated from the expression $100 \cdot AB/EC$, where A is the concentration (mg per 100 ml of methanol) of phenolics corresponding to the measured absorbance, B is the volume of solution II, C is the volume of solution I applied to the plate and E is the original weight (in g) of plant material.

RESULTS AND DISCUSSION

It would be advantageous to be able to apply specific enzymes to the hydrolysis of flavanone and 3-hydroxyflavanone glycosides, but for this purpose a greater knowledge is required of the compounds occurring in nature. Therefore, we used a technical enzyme, which also completely hydrolyzes most of the phenolic acid compounds. On the other hand, extracts should not be hydrolyzed with alkaline solutions[5] because flavanones and 3-hydroxyflavanones are very sensitive to alkali. Under these conditions, the dihydro-γ-pyrone ring breaks up, forming chalcones which decompose to phenols and cinnamic acid derivatives[6].

While flavones and flavonols tend to give tailing on silica gel plates and are very easily oxidized, flavanones and 3-hydroxyflavanones form sharply bordered bands and can be eluted easily with methanol. Flavanones and 3-hydroxyflavanones can be detected in the presence of phenolic acids[5] on chromatograms by means of their hR_F values and by their usual colour reactions. The chromatograms are first sprayed with reagents[7] which react specifically with flavanones and 3-hydroxyflavanones.

Colour reactions which allow of the determination of the type of flavonoid have been tabulated by Venkataraman[7]. The pairs flavones/flavonols and flavanones/3-hydroxyflavanones can be distinguished since flavanones and 3-hydroxyflavanones may be reduced to flavylium ions[8]. Thus the Shinoda test (reduction by magnesium and HCl in methanol solution), the specificity of which was tested by Sauer[9] for 49 natural or synthetic flavanones and 3-hydroxyflavanones, yields intensively orange, red and violet colours. Flavones and flavonols show almost no reaction. Substitution of magnesium by zinc in this test allows[10] 3-hydroxyflavanones and flavanones to be distinguished since only 3-hydroxyflavanones are reduced to blue-violet compounds. This test can be applied[11] to thin-layer plates by spraying first with zinc dust suspended

in acetone and then with 6 *N* HCl. The Horowitz reduction test, modified by Koeppen[12], can also be applied to thin-layer plates. In this test, the chromatograms are first sprayed with methanolic solutions of $NaBH_4$ and $AlCl_3$, and then heated to 120° for 1.5 min. However, 3-hydroxyflavanones derived from phloroglucinol, such as dihydroquercetin, do not show a positive reaction.

In contrast to flavones and flavonols, flavanones and 3-hydroxyflavanones only absorb in the UV-region, due to the lack of conjugation between A and B rings of the molecules. The main maxima occur at wavelengths between 275 and 290 nm. Moreover, there are always shoulders at 310–325 nm. Valuable clues for structural analysis can be obtained by a determination of the shifts in the UV spectra produced by addition of sodium methoxide, aluminium chloride and sodium acetate[13]. Table I shows hR_F values, spectral data, calibration factors and colour reactions of three flavanones and three 3-hydroxyflavanones.

Quantitative analysis yields reliable results if the concentrations of the phenolic compounds are higher than 5 mg per kg of plant material. The recovery of the aglycones (Table I) is *ca.* 85–90%. We conclude that the losses in these compounds are due mainly to oxidation during adsorption on the silica gel layer. The advantage of our method is that it can be used together with the analysis for naturally occurring phenolic acids[5]. With this method, it is usually possible to determine whether flavanones or 3-hydroxyflavanones occur in the plant together with other phenolics. Naringenin was determined quantitatively in tomatoes in the presence of phenolic acids[3].

CONCLUSIONS

By means of this method, we have been able to confirm the occurrence of naringenin in the tomatopeels[3]. Naringenin could not be detected in green immature tomatoes; it is formed during the ripening of the fruit, and its concentration reaches a maximum in a relatively short time. We found 12 mg of naringenin per kg fresh weight in green, slightly red, fruits of "Haubners Vollendung", 40 mg in bright red fruits, 40 mg in ripe red fruits and 23 mg in overripe fruits (195, 690, 700 and 415 mg of naringenin per kg dry weight, respectively).

This method can also be used for the determination of flavanone glycosides and aglycones in citrus fruits. However, our studies of phenolic acids in many native species and varieties of vegetables[14,15] and fruits[16,17] did not show any indications of the occurrence of flavanones and 3-hydroxyflavanones.

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