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QUANTITATIVE DETERMINATION OF CAFFEIC ACID ESTERS AND CATECHINS BY DIRECT MEASUREMENT ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

A method is described by which the main caffeic acid esters (chlorogenic acid, neochlorogenic acid, rosmarinic acid, dicaffeoyltartaric acid) as well as the catechins can be analysed quantitatively by direct measurement of spots on thin-layer plates. Accurate determination is possible to lower concentrations of 1 mg per kg for chlorogenic acid and 10 mg per kg for catechins. The method was applied successfully to various fruits and vegetables cultivated in Germany.

INTRODUCTION

Several quantitative procedures have been described for the determination of chlorogenic acid (3-caffeoylquinic acid) or total chlorogenic acids[1–7]. In general, however, their application is restricted to such plant materials as coffee or tobacco in which these acids occur in concentrations of at least 1%, and the methods are either too expensive or too inaccurate for application to products in which the chlorogenic acid content is below this level. In those methods based on absorption measurements at 324 nm, substantial losses can occur during purification of the extracts, and inadequate purification can lead to results that are too high.

Initially, we tried to develop a quantitative method based on the reaction of *o*-diphenols with sodium nitrite–acetic acid to produce a red colour; the method included chromatography of the plant extracts on a polyamide column, elution of the caffeic acid derivatives at 40° with 0.5% methanolic ammonia, clean-up by thin-layer chromatography (TLC) on cellulose, with 3% aqueous sodium chloride–methanol (10:3) as developing solvent and spectrophotometric measurement of the resulting colour at 500 nm. However, the results were unsatisfactory owing to the low recoveries, which varied between 45 and 62%.

We have subsequently described a spectrophotometric method for determining catechins in the p.p.m. range following purification by column chromatography and TLC[8], and no reliable method other than this is known to us. However, in order to

reduce the time of analysis, we have now developed methods for determining these plant phenolic compounds by direct measurement of their concentrations on TLC plates with use of a chromatogram spectrophotometer.

EXPERIMENTAL

Reagents and materials

The reagents and materials used were: methanol; 80% and 50% aqueous methanol; concentrated hydrochloric acid; light petroleum (boiling range 40–60°); methanol–25% aqueous ammonia (200:1); 1% methanolic vanillin; polyamide SC 6, particle size 0.05–0.16 mm (Macherey, Nagel & Co., Düren, G.F.R.).

The TLC was performed on 0.3-mm layers of cellulose (Avicel; Merck, Darmstadt, G.F.R.), the solvents listed in Table I being used.

A Zeiss Chromatogram Spectrophotometer equipped with a compensator for simultaneous reflectance and transmittance measurements was used for determining spot intensities.

Preparation of polyamide columns

A suspension of polyamide (free from traces of iron) in 50% methanol, premixed for at least 3 h, was poured into a water-jacketed glass tube (30 × 3 cm I.D.), which was filled to give a column height of about 25 cm. The column was washed with methanol (500 ml) and then with water (500 ml) at 40° to remove soluble impurities.

Extraction and polyamide column chromatography of the extracts

The plant material (50–500 g, weighed to the nearest 0.1 g) was homogenised with 200–500 ml of 80% methanol by means of a mixer and then an Ultra-Turrax homogeniser. After centrifuging for 10 min at 4000 rpm, the solution was filtered through glass wool into a 2-l round-bottomed flask. The residue was re-extracted twice as described above, and the combined extracts were concentrated in a rotary vacuum evaporator until free from methanol. Extracts containing chlorophyll were shaken three or four times with 150-ml portions of light petroleum, and the aqueous phase was freed from residual organic solvent by vacuum evaporation. The aqueous extract was divided into two equal parts, one being used for the determination of the catechins, and the other for the caffeic acid derivatives.

The aqueous solution was allowed to percolate slowly through the polyamide column prepared as described, and the column was washed with 500 ml of water. The catechins were then eluted with 500 ml of methanol, the column temperature being kept at 40°. The caffeic acid derivatives were recovered from a second similarly prepared column by elution at 40° with 500 ml of methanol containing 0.5% of ammonia. The solutions were evaporated to dryness *in vacuo*, and the residues were transferred to volumetric flasks (10 or 50 ml) and made up to volume with methanol. Turbid solutions were clarified by centrifugation.

Concentrations in the range 0.1–0.8 mg/ml for catechins and 0.1–2.0 mg/ml for caffeic acid esters were the most convenient for subsequent analysis.

Chromatography and measurement

To maintain a baseline as constant as possible over the entire area of the

chromatographic scan, reflectance and transmittance values were measured simultaneously (see p. 394). Further, it was necessary to wash the TLC plates once with the developing solvent before chromatography in order to remove soluble impurities.

Chromatography was performed in a direction parallel to that in which the layer was applied, with a layer-free margin about 0.5 cm wide at either side of the plate to eliminate, as far as possible, irregularities at the edges due to variations in layer thickness. It was also essential to ensure that the rear of the plate was completely clean by scraping off any adhering cellulose after drying, and then wiping the (rear) surface with a damp cloth.

Each plate must include spots representing the sample and four or five standard concentrations in order to permit a calibration curve to be established for each individual chromatogram. Poor reproducibility of chromatographic conditions precludes comparison of results from different plates. It is also necessary to apply the same volume of solution (1 μl) in respect of the sample and each standard to ensure as far as possible that all spots are of the same size.

Five standard spots and three sample spots were applied at 2-cm intervals to each cellulose plate by means of calibrated capillary tubes, and chromatograms were developed (to a distance of 17 cm from the start) with the solvents listed in Table I.

TABLE I

DEVELOPING SOLVENTS USED FOR TLC OF CAFFEIC ACID DERIVATIVES AND CATECHINS

Solvent	*Compounds separated**
3% Aqueous sodium chloride–methanol (10:3)	ChA, RA, DCTA, CA
Propan-2-ol–water (5:1)	ChA, NeoChA, RA, CA
5% Acetic acid	ChA, NeoChA, CA
Propan-2-ol–methanol–acetic acid–water (120:40:3:57)	(+)-Catechin, (−)-epicatechin, (−)-epigallocatechin
Propan-2-ol–methanol–acetic acid–water (20:30:2:38)	Catechins from certain plant pigments

* ChA = Chlorogenic acid; NeoChA = neochlorogenic (5-caffeoylquinic) acid; DCTA = dicaffeoyltartaric acid; RA = rosmarinic acid [2-caffeoyl-3-(3,4-dihydroxyphenyl)-D-lactic acid; the depside of caffeic acid and α-hydroxyhydrocaffeic acid]; CA = caffeic acid.

Standard solutions of chlorogenic acid are stable for several days, slight decomposition being detectable only after 2 weeks or longer. However, standard solutions of catechins show high losses (about 20%) after only 2 days, and should therefore be freshly prepared immediately before use.

For the quantitative analysis of caffeic acid esters, measurements were made at their absorption maxima between 355–365 nm (see p. 394). Because *cis–trans* isomerization occurs in solution, the contribution of each isomer must be taken into account in calculating the total ester concentration (dicaffeoyltartaric acid gives three peaks resulting from the two caffeic acid components).

For measuring the catechins, the developed chromatogram was dried and sprayed evenly with methanolic vanillin solution, care being taken not to wet the plate sufficiently for the spray reagent to run; after a short drying period, a second light spraying was given. After re-drying, the chromatogram was exposed to hydrogen chloride by placing it in a large desiccator over concentrated hydrochloric acid for at

least 1 min. The excess gas was blown away with a fan, and the intensities of the developed spots were immediately measured at 500 nm.

Various methods exist for calculating the results from the Gaussian graphs of the recorder trace. Peak areas were determined from the product of height and width at half height or by means of an integrator, and concentrations were found to be directly proportional to the squares of the corresponding peak areas.

RESULTS AND DISCUSSION

The instrument used allows simultaneous reflectance and transmittance measurements on any particular spot at the same wavelength. A compensator accepts both signals and feeds them, as a single value, to the recorder. In determining reflectance only, the baseline value due to the layer alone is adjusted to 100%, absorption of the selected wavelength by the spots being measured resulting in a decrease in the amount of reflected light. Changes in layer properties (such as thickness, packing density, particle diameter or impurities) will cause changes in the 100% setting. In measuring transmittance, the same factors affect the baseline value, which now represents 100% transmittance. A compensated signal derived from simultaneous reflectance and transmittance measurements offers the advantages of (a) enhanced sensitivity to the sample because the individual measurements reinforce each other, and (b) greater baseline stability because the factors causing variation result in signal values that largely cancel each other.

Caffeic acid esters

To elute the caffeic acid derivatives completely from the polyamide column, ammonia must be added to the methanol eluent. The resulting error due to hydrolysis of the esters is smaller than that caused by non-quantitative elution with methanol only, and the reproducibility of the results is much improved.

Caffeic acid derivatives exhibit bathochromic shifts in absorption maxima on cellulose as opposed to in methanolic solution, and maxima at the following wavelengths were observed: chlorogenic acid, 360 nm; neochlorogeric and rosmarinic acids, 365 nm; dicaffeoyltartaric acid, 355 nm; and caffeic acid, 375 nm.

If caffeic acid esters are not available in sufficient quantity or purity to serve as standards, samples can be related to standards of chlorogenic acid. By using isolated dicaffeoyltartaric acid[9], the following relationship was established: $C_{ChA} \cdot 0.88 = C_{DCTA}$ (C = concentration).

For chlorogenic acid, the standard deviation (s) was $\pm$ 3.1% when purified apple extracts were analyzed. Based on a minimum signal-to-noise ratio of 3:1, the limit of detection was found to be 25 ng for chlorogenic acid and 10 ng for caffeic acid. At maximum sensitivity of the spectrophotometer and 3 mV for full-scale deflection of the recorder, accurate analysis of plant material was possible to lower limits of 1 mg/kg for chlorogenic acid and 10 mg/kg for catechins.

Catechins

For analysis of the catechins, there are three major possibilities:

(1) direct measurement of the absorption at 280 nm, with use of a deuterium lamp,

(2) measurement of the loss of fluorescence on indicator plates at 254 nm,

(3) measurement after colour reactions with vanillin at 500 nm, or with 4-dimethylaminocinnamaldehyde at 650 nm (refs. 10 and 11).

The reaction with vanillin is considerably more sensitive and gives better reproducibility than the fluorescence method ($s = \pm 3.9\%$ as against $s = \pm 8.7\%$). The reaction with 1% methanolic 4-dimethylaminocinnamaldehyde solution is of value when red plant-pigments interfere with measurement of the colour obtained with vanillin. We found a limit of detection of 50 ng of catechin for the vanillin method.

Recovery

Recovery values were determined by quintuplicate analysis of apple samples with and without the addition of the test substance at a level of 10 mg/100 g. The average recovery was 81% (range 79–82%) for chlorogenic acid, and 86% (84–89%) for the catechins.

CONCLUSION

All the important species of fruits and vegetables grown in Germany, as well as coffee, were analyzed by the method described; results will be reported in detail elsewhere.

The caffeic acid ester predominating in most fruits (sweet and sour cherries, plums, raspberries, blackberries, black- and red-currants and gooseberries) is neochlorogenic acid. Chlorogenic acid predominates in apples, pears and strawberries, and is also the main caffeic acid ester in vegetables. Dicaffeoyltartaric acid occurs in *Compositae*, such as lettuce, endive and chicory; neochlorogenic acid is found only in dill, and rosmarinic acid in dill, sweet fennel and spices of *Labiatae*. Catechins occur in all species of fruits of the *Rosaceae*, *Saxifragaceae* and *Ericaceae*, but of the vegetables examined catechins were found only in rhubarb.

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