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ANALYSIS OF HYDROXYCINNAMIC ACID ESTERS AND THEIR GLUCOSIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER POLYAMIDE SEPARATION

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SUMMARY

The derivatives of hydroxycinnamic acids are important phenolic compounds of plant extracts. A method for their analysis is presented which qualitatively and quantitatively separates the plant extract into two fractions (hydroxycinnamic acid derivatives of glucose and their esters with quinic acid) by means of column chromatography on polyamide, which simultaneously cleans up the extract. Subsequently the fractions are analysed by high-performance liquid chromatography on RP-18 columns.

INTRODUCTION

Hydroxycinnamic acid derivatives are widespread in nature. Therefore they are of great interest for plant physiology and food chemistry. A considerable number of these derivatives exist and are well known[1,2]. Analysis of these compounds has recently been carried out by means of high-performance liquid chromatography (HPLC) with great success (3–11).

Complex plant extracts are usually prepurified quantitatively by open column chromatography, especially when large amounts of different plant phenolics are expected. Möller and Herrmann[7] separated hydroxycinnamoyl-D-quinic acids from fruit extracts using polyamide columns. Ong and Nagel[3] eluted hydroxycinnamoyltartaric acids from *Vitis vinifera* grapes by the same method. Krause[4] carried out the preparative separation of glucose esters of hydroxycinnamic acids from their D-quinic esters from *Spirodella polyrrhiza*.

The behaviour of the glucosides of hydroxycinnamic acids on polyamide columns has not been systematically examined. Owing to the chemical similarity of the different hydroxycinnamic acid esters and glucosides and also their possible isomers, a quantitative preseparation on polyamide columns seems to be necessary to enable a complete separation of these compounds by HPLC.

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EXPERIMENTAL

Reference samples

Chlorogenic acid is the only commercially available substance of the hydroxycinnamic acid derivatives (Roth, Karlsruhe, F.R.G.). In our laboratory 3-O-feruloyl-D-quinic acid was isolated from green coffee beans and 5-*p*-coumaroyl-D-quinic acid was received from unripe morello cherries by preparative HPLC. Sinapoylglucose was also obtained by preparative HPLC from garden cress[11]. *p*-Coumaroylglucose was placed at our disposal by Birkofer. The *β*-glucosides of *p*-coumaric acid, ferulic acid and caffeic acid were synthesized[12–16].

The test solution was prepared with these compounds. For our colum tests, caffeoylglucose and feruloylglucose were not available in a sufficient amount. On polyamide columns, both derivatives showed behaviour analogous to that of the other tested glucose esters, which was ascertained with plant extracts. Therefore we dispensed with their time-consuming synthesis.

Sample preparation

About 100 g of plant material was cut into small pieces. After the addition of 500 ml of methanol and homogenization, the plant pulp was filtered. The residue was once more extracted with 500 ml of methanol in a water-bath at 40°C. After filtration, 50 ml of water was added to the combined extracts. This solution was evaporated *in vacuo* at 40°C. The residual aqueous solution was made up to a defined volume. This extract can be used directly for the preseparation on a polyamide column.

Preseparation and purification on polyamide column

About 50 g of polyamide powder MN-SC-6 (Macherey & Nagel, Düren, F.R.G.) was suspended in methanol. The suspension was poured into a glass column (35 × 4 cm I.D.) up to 25 cm. For preliminary treatment the column was flushed with 1.0 l of methanol–formic acid (995:5) followed by 1.0 l of water.

The plant extract sample or the test solution was then poured on to the column. The test solution containing the compounds listed as "reference samples" was collected in fractions of 100 ml. Elution was started with 1.2 l of water (fractions 1–12) and continued with 1.0 l of methanol (fractions 13–22). Finally the column was eluted with 1.0 l of methanol–formic acid (995:5) (Fractions 23–32). The flow-rate was kept between 8 and 10 ml/min.

The collected fractions of the test solution and the plant extract sample were evaporated at 40°C *in vacuo*. After making up to a defined volume, these solutions can be used for HPLC analysis.

Analytical HPLC

The HPLC analysis was carried out with a Pye Unicam gradient chromatograph (Philips, Kassel, F.R.G.) equipped with a LC-XPD pump, a LC-XP gradient programmer and a LC-UV detector. The Rheodyne 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) was supplied with a 10-*μ*l sample loop. The results were registrated by an HP-3390 A reporting integrator (Hewlett-Packard, Frankfurt, F.R.G.).

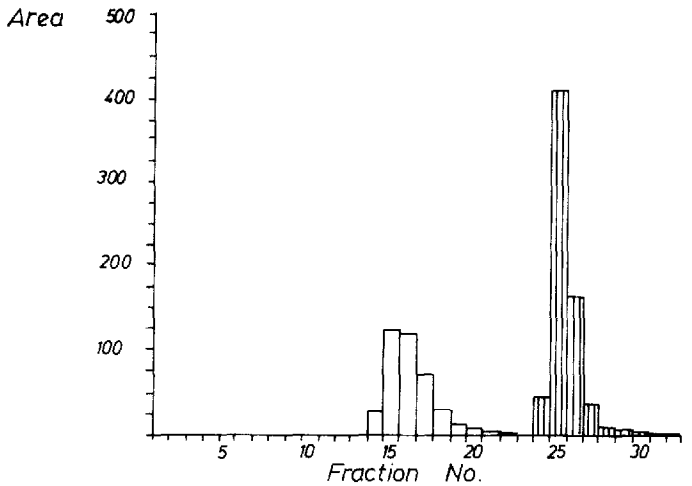

Fig. 1. Elution profile of caffeic acid derivatives. ◫ = Caffeoyl-D-quinic acids; □ = caffeic acid-4-β-D-glucoside.

Two different systems of columns with suitable gradient programs were used for reversed-phase analysis.

System A was a Hibar stainless-steel column (250 × 4 mm I.D.) filled with LiChrosorb RP-18, 5 μm (E. Merck, Darmstadt, F.R.G.). Gradient elution was carried out using solvent A (10 m*M* H_3PO_4) and solvent B (methanol) from 10% to 26% B in A in 35 min (linear), continuing from 26% to 30% B in A in 1 min, and finally holding at 30% B in A for 10 min.

System B was a stainless-steel column (250 × 4.6 mm I.D.) containing Shandon ODS Hypersil, 5 μm (Gynkotek, München, F.R.G.). Gradient elution was performed with solvent A (2% acetic acid) and solvent B (methanol) from 10% to 30% B in A (linear) in 45 min.

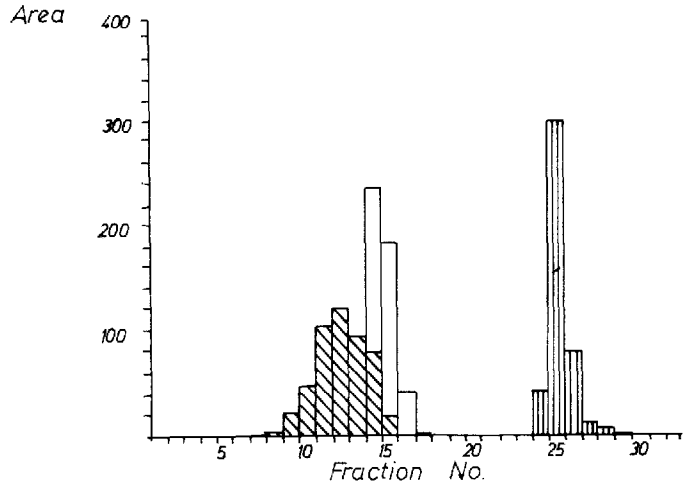

Fig. 2. Elution profile of *p*-coumaric acid derivatives. ◫ = *p*-Coumaroyl-D-quinic acids; □ = *p*-coumaric acid-β-D-glucoside; ◰ = *p*-coumaroylglucose.

In both systems the UV detector was set at 320 nm (band width 8 nm) and the flow-rate at 1.0 ml/min. The solvents were degassed by helium.

RESULTS AND DISCUSSION

The fractionated separation of the test solution on the polyamide column showed that the glucose esters were found in fraction 7 (water). The glucosides and the rest of the glucose esters eluted completely with methanol. Finally the quinic acid esters were washed from the column by methanol–formic acid (Figs. 1–4).

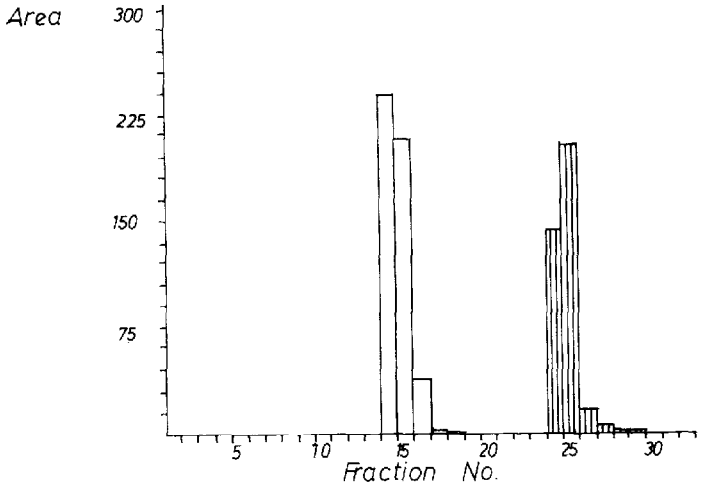

Fig. 3. Elution profile of ferulic acid derivatives. ▥ = Feruloyl-D-quinic acids; □ = ferulic acid-β-D-glucoside.

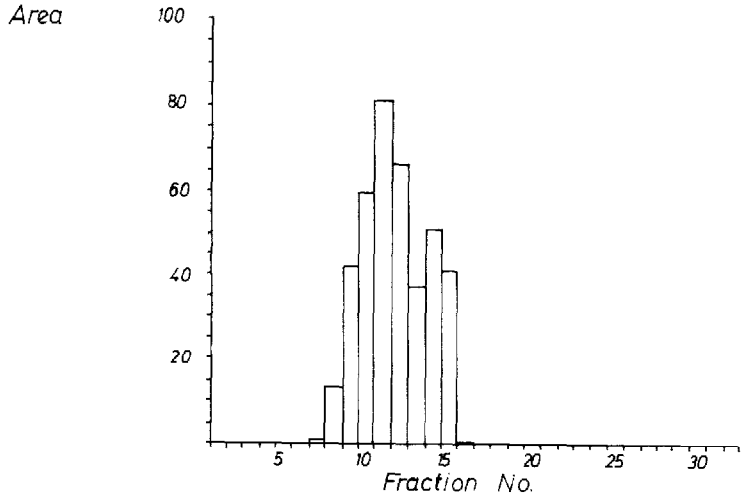

Fig. 4. Elution profile of sinapic acid derivatives. □ = Sinapoylglucose.

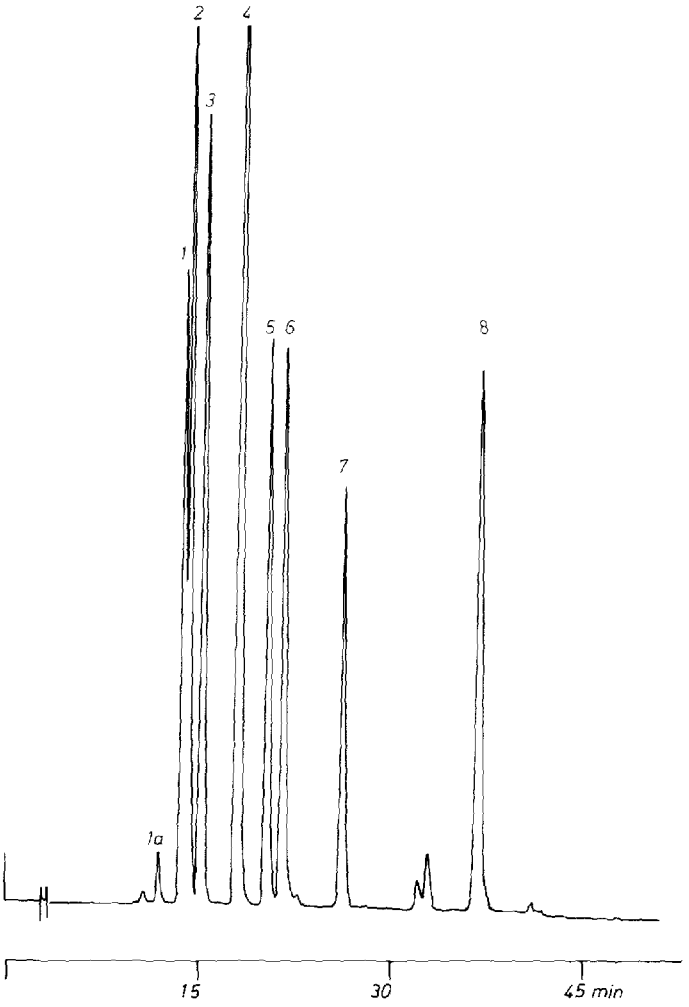

Fig. 5. HPLC chromatogram of the reference solution (System B). Peaks: 1 = 5-*p*-coumaroyl-D-quinic acid; 2 = *p*-coumaric acid-*β*-D-glucoside; 3 = caffeic acid-4-*β*-D-glucoside; 4 = *p*-coumaroylglucose; 5 = ferulic acid-*β*-D-glucoside; 6 = chlorogenic acid; 7 = sinapoylglucose; 8 = 3-feruloyl-D-quinic acid. "a" indicates the *cis*-isomer.

With regard to the behaviour of the test solution, the volume of water was reduced to 500 ml and the volumes for methanol and methanol–formic acid were not changed. After elution with the reduced volume of water no interesting compounds could be found in this fraction. Therefore only the other two fractions needed to be analysed.

The behaviour of the glucosides on the polyamide column is interesting. As they have a free carboxylic group and a very polar glucose moiety, elution could have occurred in both fractions. The tests indicate that the influence of the glucose part is predominant.

According to the elution sequence, the hydroxycinnamic acid compounds were distributed into glucose derivatives and quinic acid esters (Fig. 5–10).

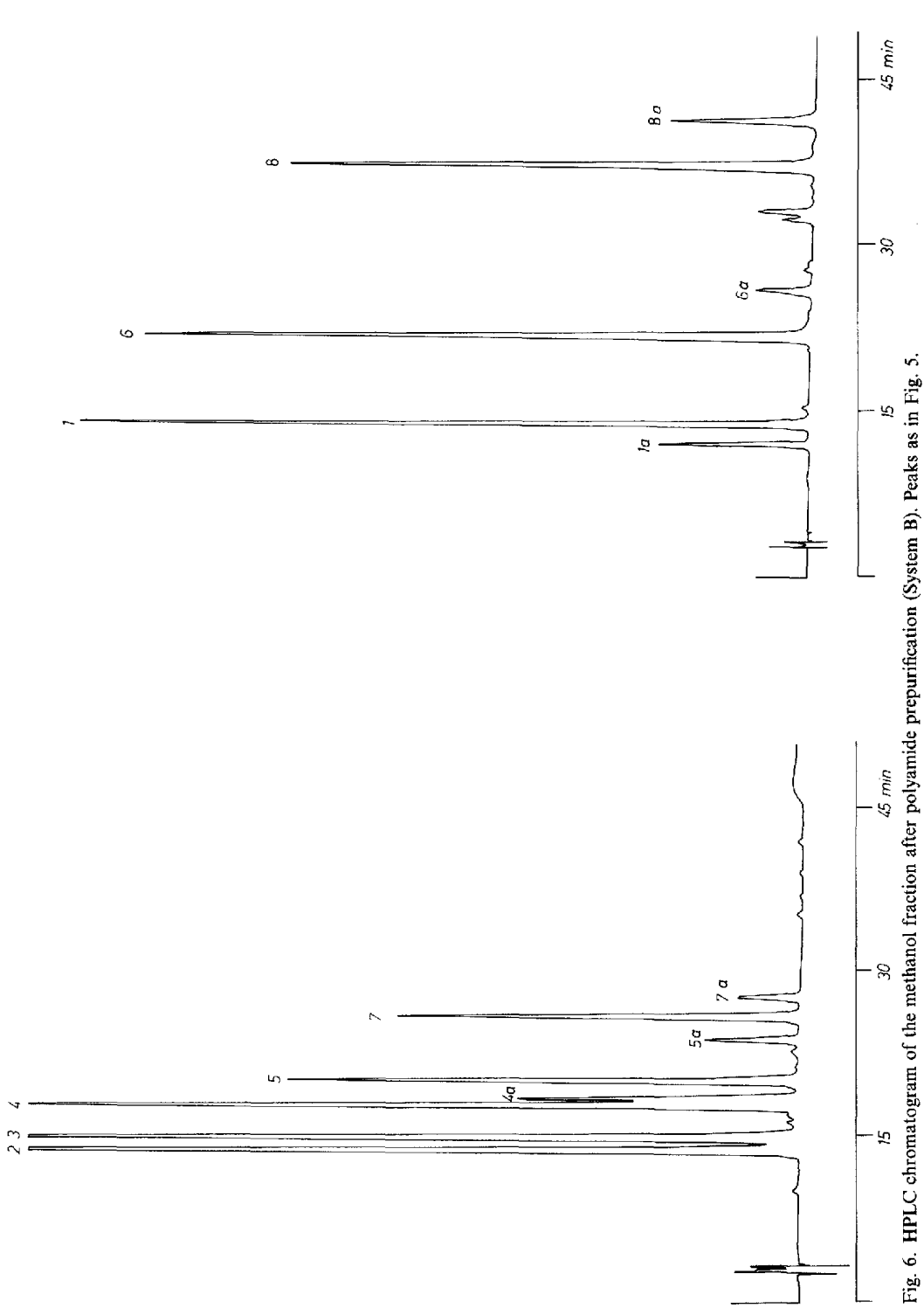

Fig. 6. HPLC chromatogram of the methanol fraction after polyamide prepurification (System B). Peaks as in Fig. 5.

Fig. 7. HPLC chromatogram of the methanol-formic acid fraction after polyamide prepurification (System B). Peaks as in Fig. 5.

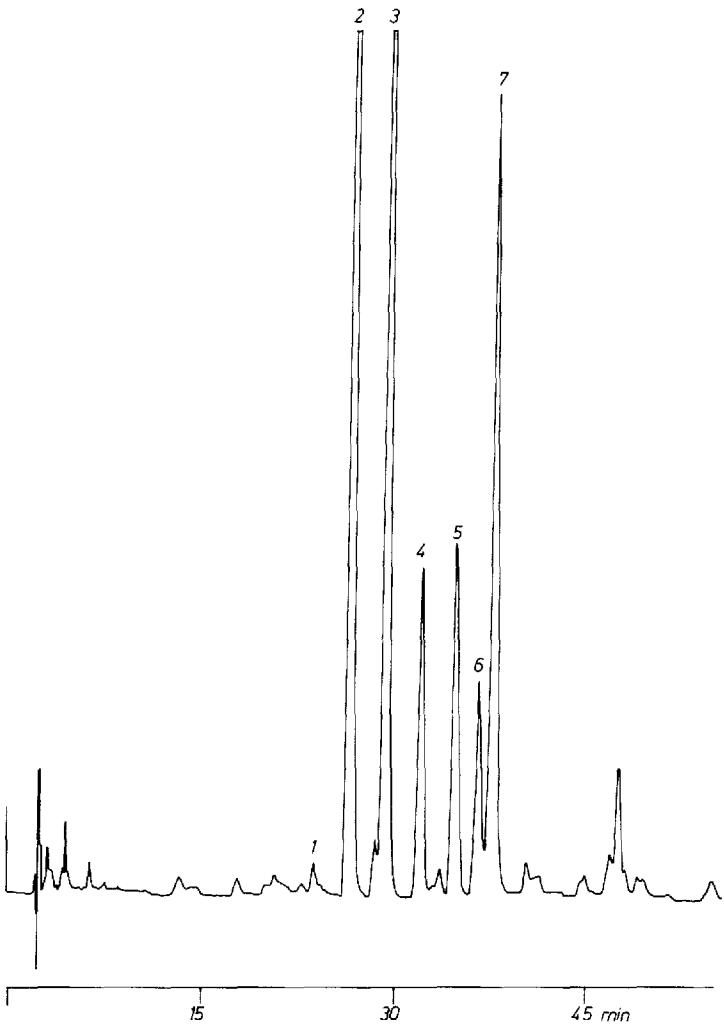

Fig. 8. HPLC chromatogram of a tomato extract without polyamide prepurification (System A). Peaks: 1 = caffeoylglucose; 2 = *p*-coumaric acid-β-D-glucoside; 3 = caffeic acid-4-β-D-glucoside; 4 = *p*-coumaroylglucose; 5 = ferulic acid-β-D-glucoside; 6 = 4-caffeoyl-D-quinic acid; 7 = 3-caffeoyl-D-quinic acid.

Quantitative analysis with a standard solution consisting of chlorogenic acid, 5-*p*-coumaroyl-D-quinic acid, ferulic acid-β-D-glucoside and *p*-coumaroylglucose was satisfactory; the recoveries were chlorogenic acid 97%, 5-*p*-coumaroyl-D-quinic acid 99%, ferulic acid-β-D-glucoside 96% and *p*-coumaroylglucose 91%. These results were coroborated by the analysis of a tomato extract which contained a standard solution of chlorogenic acid and ferulic acid-β-D-glucoside. The capacity of the column amounted to 200 ppm for these substances. During the elution a proportion of *cis*-isomers was formed from the *trans*-isomers (Figs. 5 and 7).

Short glass columns (20 × 2.8 cm I.D.) produced the same separation effect. The column was filled up to 15 cm with polyamide. The elution sequence was water

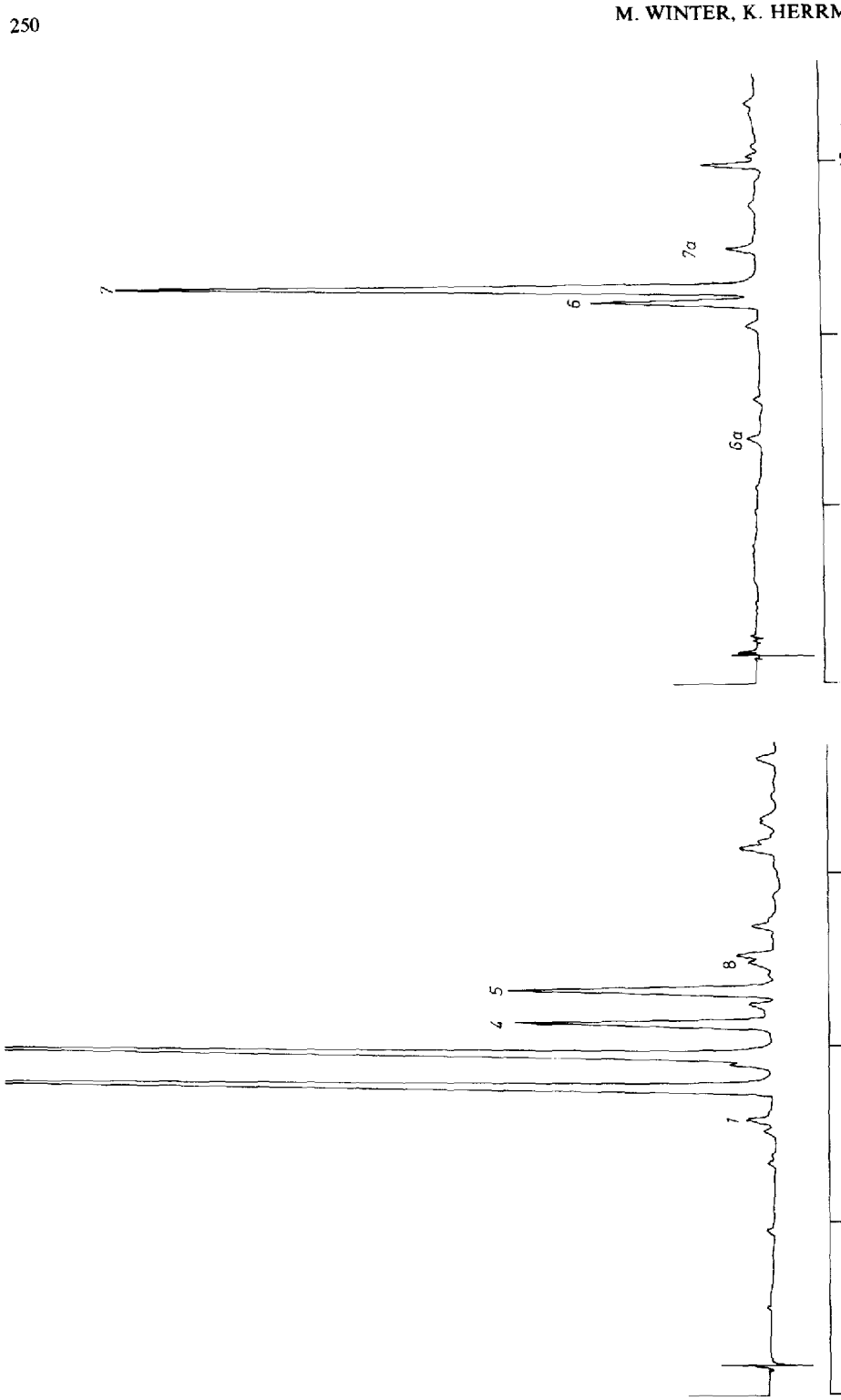

Fig. 9. HPLC chromatogram of the methanol fraction of the tomato extract after polyamide prepurification (System A). Peaks 1–5 as in Fig. 8; 8 = feruloylglucose.

Fig. 10. HPLC chromatogram of the methanol–formic acid fraction of the tomato extract after polyamide prepurification (System A). Peaks 6 and 7 as in Fig. 8; "a" indicates the *cis*-isomer.

(130 ml), methanol (250 ml), methanol–formic acid (250 ml). The capacity was 50 ppm for these substances.

This modified method means not only that a prepurification of a complex plant extract can be obtained, but also that hydroxycinnamic acid compounds can be separated into glucose derivatives and quinic acid esters. Therefore these compounds will not overlap in HPLC chromatograms and quantitative and qualitative misinterpretations will also be avoided.

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