Journal of Chromatography, 441 (1988) 335–345
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 379

ANALYSIS OF NATURALLY OCCURRING HYDROXYBENZOIC ACID GLUCOSIDES AND HYDROXYBENZOYL GLUCOSES AND THEIR BEHAVIOUR ON A POLYAMIDE COLUMN

SILKE KLICK and KARL HERRMANN*
Institut für Lebensmittelchemie der Universität Hannover, Wunstorfer Strasse 14, D-3000 Hannover 91 (F.R.G.)
(First received October 30th, 1987; revised manuscript received January 29th, 1988)

SUMMARY

A procedure is described to determine 4-β-D-glucosides of vanillic, syringic, 4-hydroxybenzoic, protocatechuic and gallic acids, salicylic acid 2-β-D-glucoside and the 1-O-β-D-glucose esters of 4-hydroxybenzoic, vanillic and syringic acids in plant extracts. The method includes sample extraction, polyamide column chromatography, anion-exchange chromatography and quantitative analyses by reversed-phase high-performance liquid chromatography, capillary gas chromatography and silica gel high-performance liquid chromatography. The elution profiles of pure standard solutions and of standard solutions of glucosides and glucose esters in the presence of sample matrices on the polyamide column were investigated.

INTRODUCTION

While the wide distribution of the common hydroxybenzoic acids, salicylic (2-OH), 4-hydroxybenzoic, protocatechuic (3,4-OH), gallic (3,4,5-OH), vanillic (4-OH, 3-OCH_3) and syringic acid (4-OH, 3,5-OCH_3), in plants has been demonstrated many times by analysing the hydrolysates of plant extracts, little evidence has been forthcoming as to the forms, such as glucosides or glucose esters, in which these acids occur in intact plants[1]. So far, 4-β-D-glucosides of hydroxybenzoic, protocatechuic and, in some cases, gallic acids have been found in soft fruits[2,3]. Hydroxybenzoic acid 4-β-D-glucoside is present in a number of *Apiaceae*[4,5] and syringic acid 4-β-D-glucoside in two plant species[6,7].

The 1-O-β-D-glucose esters of vanillic and syringic acids have apparently not yet been isolated or proved to be present in plants. Birkofer *et al.*[8] isolated 1-O-(4-hydroxybenzoyl)-β-D-glucose from the flowers of *Catalpa bignonioides* and characterized it by spectroscopic methods[9]. No other hydroxybenzoyl glucoses are known.

In contrast to other hydroxybenzoic acids, gallic acid seems to occur preferentially as glucose esters and forms part of hydrolysable tannins[1,10].

Analysis of phenolic acid compounds, especially hydroxycinnamic acid derivatives, has recently been carried out by means of high-performance liquid chromato-

0021-9673/88/$03.50

graphy (HPLC) with great success[2,5,11–16], partly after pre-purification on polyamide columns[2,5,11,14,16] and with anion exchangers[2,5,12]. Glycosides of several plant phenolics have also been separated by capillary gas chromatography (GC) as trimethylsilyl derivatives[2,17]. Glucose compounds of hydroxybenzoic acids have not been determined by GC or HPLC with the exceptions mentioned above.

In order to acquire further information on the distribution of glucosides and glucose esters of hydroxybenzoic acids in plants, we have developed an effective method for sample clean-up, necessary for GC or HPLC analysis. We investigated the behaviour of 4-β-D-glucosides of 4-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids, salicylic acid 2-β-D-glucoside and of 1-O-β-D-glucose esters of 4-hydroxybenzoic, vanillic and syringic acids on a polyamide column, using standard solutions in water and in two sample matrices. The glucoside and glucose ester contents of each fraction were determined by reversed-phase HPLC. The sample extracts required further clean-up after pre-purification by polyamide, which was performed by anion exchange[2,18].

Quantitative analyses of glucosides were done by reversed-phase HPLC and capillary GC (TMS derivatives) and of glucose esters by reversed-phase HPLC and, after further purification on a C_{18} cartridge and subsequent benzoylation[19], by HPLC on silica gel.

EXPERIMENTAL

Reference substances

The 4-β-D-glucosides of hydroxybenzoic[4], protocatechuic and gallic acids[3] were available in our Institute. The glucosides of salicylic, vanillic and syringic acids were synthesized by the Koenigs–Knorr reaction of the methyl esters with 2,3,4,6-tetra-O-acetyl-D-glucose-α-bromide in the presence of quinoline and silver oxide[20,21] and subsequent saponification of the ester groups.

For synthesis of 1-O-β-D-glucose esters of 4-hydroxybenzoic, vanillic and syringic acids, a method described by Zane and Wender[22] was modified. 4-Hydroxybenzoic acid chlorides react with 4,6-benzylideneglucose–sodium to yield esters; reductive elimination of the protective groups with hydrogen/palladium yields the corresponding glucose esters.

The crude products were purified by preparative HPLC. Structure elucidation with UV, IR, ^{1}H NMR, ^{13}C NMR and fast atom bombardment-mass spectroscopy (FAB-MS) confirmed the identity of the reference substances synthesized. Details of the synthesis parameters and spectroscopic data have been published elsewhere[23].

Sample extraction and polyamide chromatography

About 100 g of deep-frozen, fresh plant material (for elution profiles: dill and koriander leaves) were homogenized with 250–500 ml of 80% methanol for 10 min. After filtration, the residue was extracted further (twice) with 200 ml of 80% methanol. The pooled extracts were evaporated *in vacuo* at 40°C, the aqueous solution was made up to 100 ml and kept in a refrigerator overnight to precipitate chlorophyll and lipids. A 25-ml volume of the filtered aqueous solution were applied to the polyamide column and eluted with 600 ml of water and then 1 l of methanol. The eluates were pooled, evaporated *in vacuo* at $\leq$40°C and made up to 25 ml.

Anion exchange

An aliquot of the sample polyamide eluate (1–10 ml) was applied to an anion exchanger (4 g Dowex 1-X2, 100–200 mesh, counter ion $HCOO^-$) in a glass column (200 mm × 15 mm). The column was flushed with 100 ml of 50% methanol. This neutral eluate was evaporated *in vacuo* ($\leqslant 40°C$), made up to a defined volume and used for HPLC analysis or benzoylation of the glucose esters.

After flushing with 25 ml of methanol–acetone–water (1:1:1) and 10 ml of 90% acetone, the glucosides were eluted with 150 ml of acetone–water–formic acid (90:5:5). The acidic eluate was diluted in water and evaporated *in vacuo* at $\leqslant 40°C$ to an aqueous solution. Water and formic acid were removed by freeze-drying. The residue was made up to a defined volume and used for HPLC and GC analysis of the glucosides.

Reversed-phase HPLC of glucosides and glucose esters

The high-performance liquid chromatograph used for analyses of glucosides and esters was a Pye Unicam LC-XPD (Philips, Kassel, F.R.G.) consisting of a Pye Unicam LC-XPD pump, gradient programmer and LC-UV detector and an injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 20-μl sample loop. For qualitative analysis, a 1040 HP A (diode-array detector) with HP 85 and HP 82901 M flexible disc drive (Hewlett-Packard) was used. A Gynkotek C-R3A integrator (Gynkotek, München, F.R.G.) was employed to integrate peak areas. A 250 mm × 4.6 mm I.D. stainless-steel column (Gynkotek) packed with Shandon ODS-Hypersil, 5 μm, was employed and the mobile phases were 2% acetic acid (A) and methanol (B) with a gradient elution from 0 to 20% B in A over 25 min. Solvents were degassed with helium. The UV detector was set to 261 nm (syringic acid glucoside), 285 nm (salicylic acid glucoside), 262 nm (4-hydroxybenzoyl glucose and vanilloyl glucose), 284 nm (syringoyl glucose) and 250 nm (remaining glucosides). Quantification was performed using external standards.

Capillary GC of glucosides

An aliquot of the acidic anion exchanger eluate was evaporated to dryness and silylated at 70°C for 1 h with bis(trimethylsilyl)acetamide–trimethylchlorosilane (BSA–TMCS) (20:1). The alkanes C_{18} and C_{20} were used as internal standards.

GC separation of glucosides was accomplished using a Fractovap 2105 gas chromatograph (Carlo Erba, Hofheim, F.R.G.) equipped with a flame ionization detector. The columns were a 35 m × 0.3 mm I.D. wall-coated open-tribular glass capillary coated with SE-30 (0.2% static) or a 32 m × 0.3 mm I.D. OV-1701 glass capillary (0.1% static). Conditions: carrier gas, 1 ml/min (N_2); injector and detector, 300°C; temperature programme, 230–250°C at 4°C/min. 250–270°C at 6°C/min, then isothermal (SE-30); 220–270°C at 4°C/min, then isothermal (OV-1701); splitting ratio 1:10–1:20.

Benzoylation and silica gel HPLC of glucose esters

For determination of glucose esters, the neutral anion exchanger eluate was purified on a C_{18} cartridge (500 mg, cartridge volume 3 ml; J. T. Baker, Phillipsburgh, NJ, U.S.A.). The cartridge was flushed with methanol (2 ml), then water (4 × 2 ml). A sample aliquot (0.5–2 ml) was applied and the cartridge flushed with water (3 ×

1 ml) to remove carbohydrates and other polar substances. The esters were eluted with methanol (4 × 2 ml) and the solution was evaporated to dryness. The residue was benzoylated with benzoyl chloride (0.5 ml) in the presence of pyridine (4 ml) by a procedure described[19]. The benzoates obtained were made up to a defined volume with isooctane–diethyl ether–acetonitrile (150:100:40). For HPLC apparatus, see above. A 125 mm × 4.6 mm I.D. stainless-steel column was packed with Shandon Hypersil, 3 μm (Gynkotek). The eluent was isooctane–diethyl ether–acetonitrile (100:30:9), and UV detector wavelength was 231 nm. Quantification was carried out with external standards, which had been benzoylated in the same way as the samples.

Elution profiles on polyamide

A suspension of polyamide MN-SC-6 (Macherey & Nagel, Düren, F.R.G.) in methanol was poured into a glass column (250 mm × 35 mm I.D.) to an height of 20 cm and the column was then flushed with 600 ml of methanol followed by 1 l of water.

For elution profiles, 25 ml of an aqueous standard solution containing 0.2 mg of each reference substance or a 25-ml sample of a raw extract containing the same amounts of glucoside and ester references were applied to the polyamide column. Elution was started with 600 ml of water and continued with 1.6 l of methanol. For a pure standard solution, a further 800 ml methanol–4.5 ml formic acid mixture was used. Fractions were collected in 100–300 ml aliquots (see Figs. 7 and 8), evaporated *in vacuo* at $\leqslant 40°C$ and made up to a defined volume. These solutions were used for reversed-phase HPLC analysis.

RESULTS AND DISCUSSION

Determination of hydroxybenzoic acid glucosides

The determination of hydroxybenzoic acid glucosides is possible with GC and HPLC (standard chromatograms and applications in Figs. 1–5). The advantages of capillary GC are the better separation and the fact that all glucosides can be determined in a single experiment. Disadvantages are the lower selectivity of the flame ionization detector and the high temperatures required for substances with higher molecular weights, such as glucosides. The glucosides were not affected by high temperatures, but in some cases decomposition of other substances caused interference with sample peaks.

HPLC analysis with UV detection is more selective than GC with a flame ionization detector and can give better results for fruit and vegetable extracts. For more complex sample matrices, such as spices, containing a great number of substances with UV activity, the separation efficiency is a limiting factor. In these cases, GC is more suitable. The HPLC determination of low amounts of salicylic acid glucoside (< 5 ppm) was limited by a low extinction coefficient of this substance compared with the other glucosides. In GC determinations, the detection level was 1 ppm for all glucosides. HPLC separation of the plant extracts made it possible to confirm the identity of the glucosides (and glucose esters) by additional use of a diode-array detector.

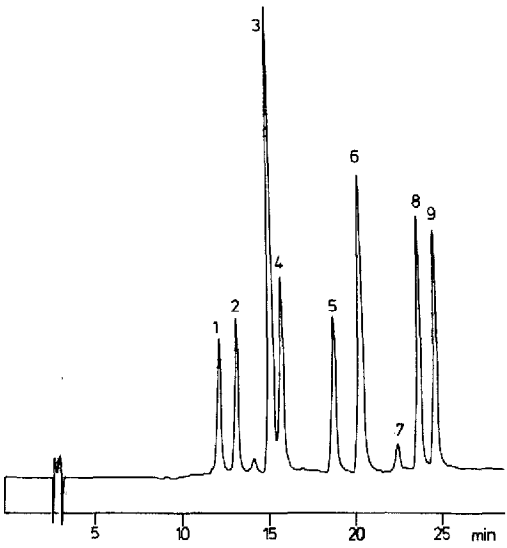

Fig. 1. Reversed-phase HPLC of glucosides and glucose esters of hydroxybenzoic acids detected at 270 nm. Peaks: 1 = hydroxybenzoic acid 4-β-D-glucoside; 2 = protocatechuic acid 4-β-D-glucoside; 3 = 1-O-(4-hydroxybenzoyl)-β-D-glucose; 4 = gallic acid 4-β-D-glucoside; 5 = vanillic acid 4-β-D-glucoside; 6 = 1-O-vanilloyl-β-D-glucose; 7 = salicylic acid 2-β-D-glucoside; 8 = syringic acid 4-β-D-glucoside; 9 = 1-O-syringoyl-β-D-glucose.

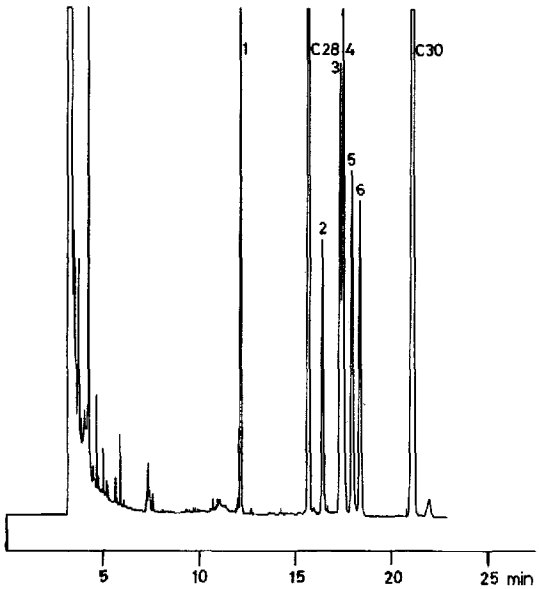

Fig. 2. Capillary GC of hydroxybenzoic acid glucosides (trimethylsilyl derivatives) on SE-30. Peaks: 1 = salicylic acid 2-β-D-glucoside; 2 = hydroxybenzoic acid 4-β-D-glucoside; 3 = protocatechuic acid 4-β-D-glucoside; 4 = gallic acid 4-β-D-glucoside; 5 = vanillic acid 4-β-D-glucoside; 6 = syringic acid 4-β-D-glucoside.

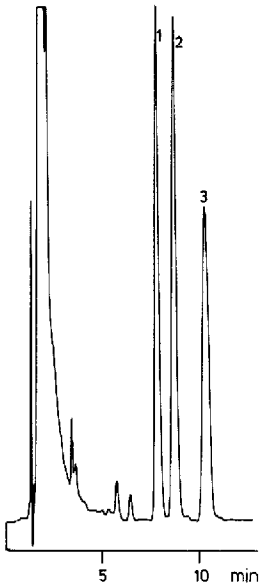

Fig. 3. Silica gel HPLC of hydroxybenzoyl glucoses after benzoylation. Detection at 231 nm. Peaks: 1 = 1-O-(4-hydroxybenzoyl)-β-D-glucose; 2 = 1-O-vanilloyl-β-D-glucose; 3 = 1-O-syringoyl-β-D-glucose.

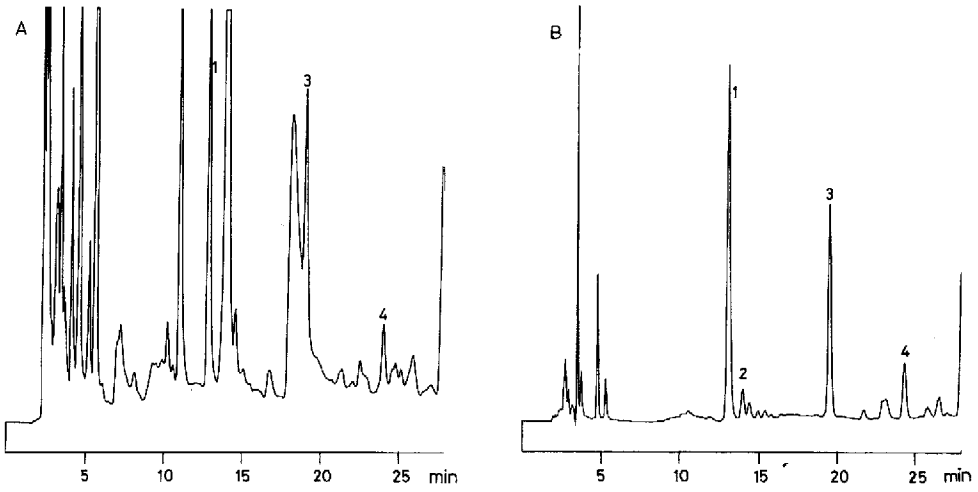

Fig. 4. Reversed-phase HPLC of hydroxybenzoic acid glucosides of a dill leaf extract detected at 250 nm. (A) after polyamide column chromatography and (B) after polyamide column chromatography and anion-exchange chromatography. Peaks: 1 = hydroxybenzoic acid 4-β-D-glucoside; 2 = protocatechuic acid 4-β-D-glucoside; 3 = vanillic acid 4-β-D-glucoside; 4 = salicylic acid 2-β-D-glucoside.

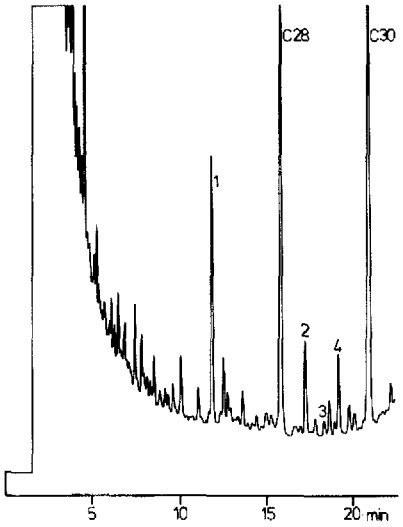

Fig. 5. Capillary GC of hydroxybenzoic acid glucosides (trimethylsilyl derivatives) of a dill leaf extract on SE-30. Peaks: 1 = salicylic acid 2-β-D-glucoside; 2 = hydroxybenzoic acid 4-β-D-glucoside; 3 = protocatechuic acid 4-β-D-glucoside; 4 = vanillic acid 4-β-D-glucoside.

Determination of hydroxybenzoyl glucoses

The general problem in the determination of glucose esters, occurring in lower amounts than the corresponding glucosides in plant matrices, is the separation from interfering substances. Direct reversed-phase HPLC of the neutral anion exchanger eluate was sufficient in all cases for quantification of these substances. As a second method, benzoylation and subsequent separation of the benzoates obtained by silica gel HPLC was employed. This method is effective in the analysis of many substances containing hydroxyl groups and has been used successfully in food analysis[19]. For the investigation of the neutral anion exchanger eluate, further purification on a C_{18} cartridge was necessary before benzoylation to remove polar substances, such as carbohydrates, and to produce evaluable chromatograms.

Silica gel HPLC of the benzoylated samples was successfully used as a second method to confirm quantitative results obtained by reversed-phase HPLC of the underivatized samples. It is less suitable for samples with a more complicated matrix. The analysis of hydroxybenzoyl glucoses with both methods is illustrated in Fig. 6.

GC separation of hydroxybenzoyl glucoses as trimethylsilyl derivatives was also tried, but was not successful as the substances decomposed during analysis. Related hydroxycinnamoyl glucoses showed the same behaviour and could not be determined by GC (unpublished results).

Elution behaviour on polyamide

The elution profiles of the standard solutions (Fig. 7) demonstrate that the aqueous fractions were free of glucosides, while the three glucose esters occurred almost quantitatively in these fractions. When elution with methanol was first started, only glucosides without free phenolic hydroxyl groups were found in the eluate. Protocatechuic acid glucoside and gallic acid glucoside containing free phenolic hy-

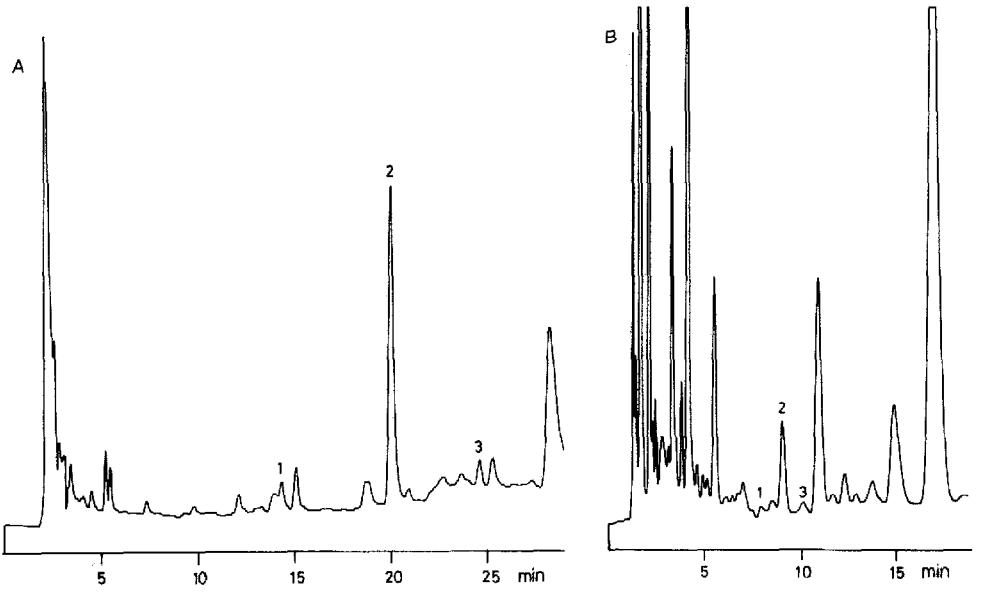

Fig. 6. HPLC of hydroxybenzoyl glucoses of a cress extract. (A) Reversed-phase HPLC of the underivatized sample detected at 262 nm. (B) Silica gel HPLC after benzoylation, detection at 231 nm. Peaks: 1 = 1-O-(4-hydroxybenzoyl)-β-D-glucose; 2 = 1-O-vanilloyl-β-D-glucose; 3 = 1-O-syringoyl-β-D-glucose.

droxyl groups were eluted in later methanol fractions. The latter could not be desorbed from the stationary phase quantitatively by using 1.6 l of pure methanol and a methanol–formic acid mixture was required[2].

These results indicate that the affinity of the phenolic acid glucosides to polyamide is greater for substances with free phenolic hydroxyl groups than for those with only one hydroxy group, involved in the glucosidic binding. On the other hand, the different behaviour of glucosides and isomeric glucose esters shows a stronger polyamide affinity for the aromatic carboxyl group than for the phenolic hydroxyl group.

Comparing elution profiles of pure standard solutions with those of standard addition experiments (Fig. 8), the presence of the sample matrix caused a "break-through" of the glucosides into the aqueous fraction. Moreover, only 800 ml of methanol were necessary in this case to obtain quantitative elution, even of gallic acid glucoside. Dill and koriander as sample matrices had the same effect on the elution behaviour, and even 5–10 ml extracts (corresponding to 5–10 g fresh material) were enough to cause the "break-through". The fact that the sample elution profiles have two maxima leads to the suggestion of an overload of the stationary phase. The active sites of the polyamide are obviously occupied by other phenolic substances, and the affinity of glucosides and glucose esters is not strong enough to complete with them. This also applies to protocatechuic and gallic acid glucosides, which were not eluted in the aqueous fraction in soft fruit matrices[2].

Hydroxycinnamic acid glucosides and glucose esters behaved similarly to hydroxybenzoic acid derivatives on polyamide[16]. In pure standard solutions, esters were eluted earlier than corresponding glucosides and occurred partly in the aqueous frac-

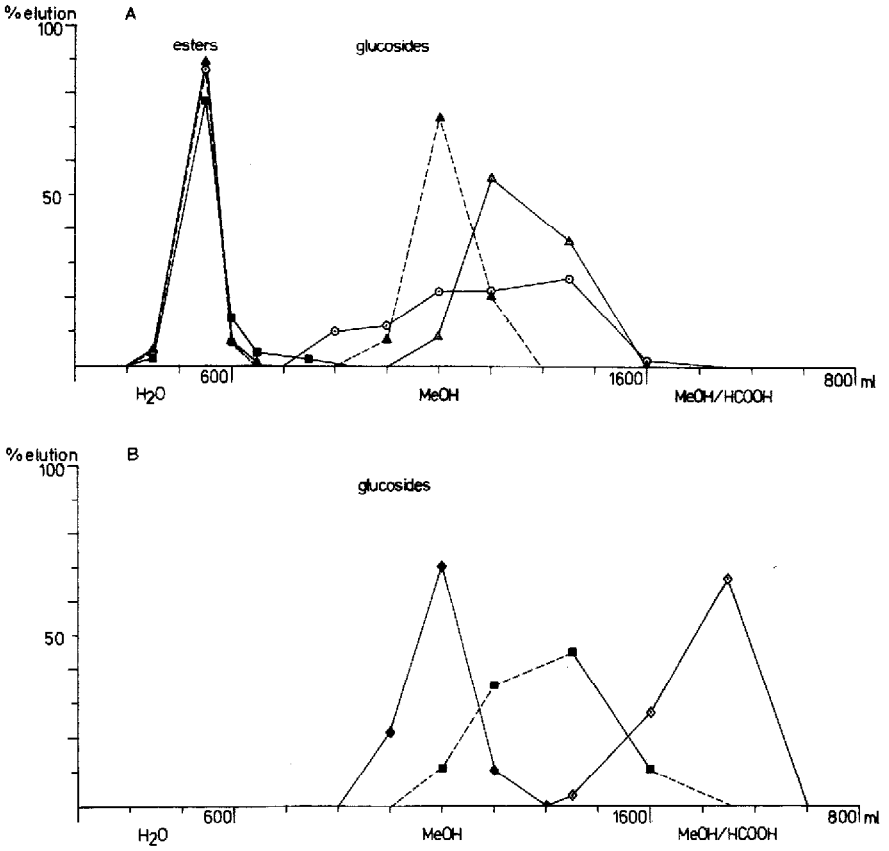

Fig. 7. Elution profiles of a standard solution of hydroxybenzoic acid glucosides and glucose esters on polyamide. (A) (Glucosides) (○) salicylic acid 2-β-D-glucoside; (▲) vanillic acid 4-β-D-glucoside; (△) syringic acid 4-β-D-glucoside; (esters) (■) 1-O-(4-hydroxybenzoyl)-β-D-glucose; (▲), 1-O-vanilloyl-β-D-glucose; (○), 1-O-syringoyl-β-D-glucose. (B) (◆) Hydroxybenzoic acid 4-β-D-glucoside; (■), protocatechuic acid 4-β-D-glucoside; (◇), gallic acid 4-β-D-glucoside. MeOH = methanol.

tions, whereas glucosides could be detected only in the methanol fractions. In contrast to hydroxybenzoic acid compounds, hydroxycinnamic acid glucosides did not break through into the aqueous fraction when sample matrix was present.

With regard to elution profiles, for sample clean-up, the volume of methanol was reduced to 1.0 l. Each sample was eluted with a further 200 ml of methanol to make sure that elution was quantitative. Aqueous and methanolic fractions were pooled.

Anion exchange

The break-through of hydroxybenzoic acid compounds into the aqueous fraction made it impossible to separate them from interfering substances, such as carbohydrates, organic acids or mineral salts. Therefore, in most cases, purification on polyamide was not sufficient to give satisfactory results in GC and HPLC analysis

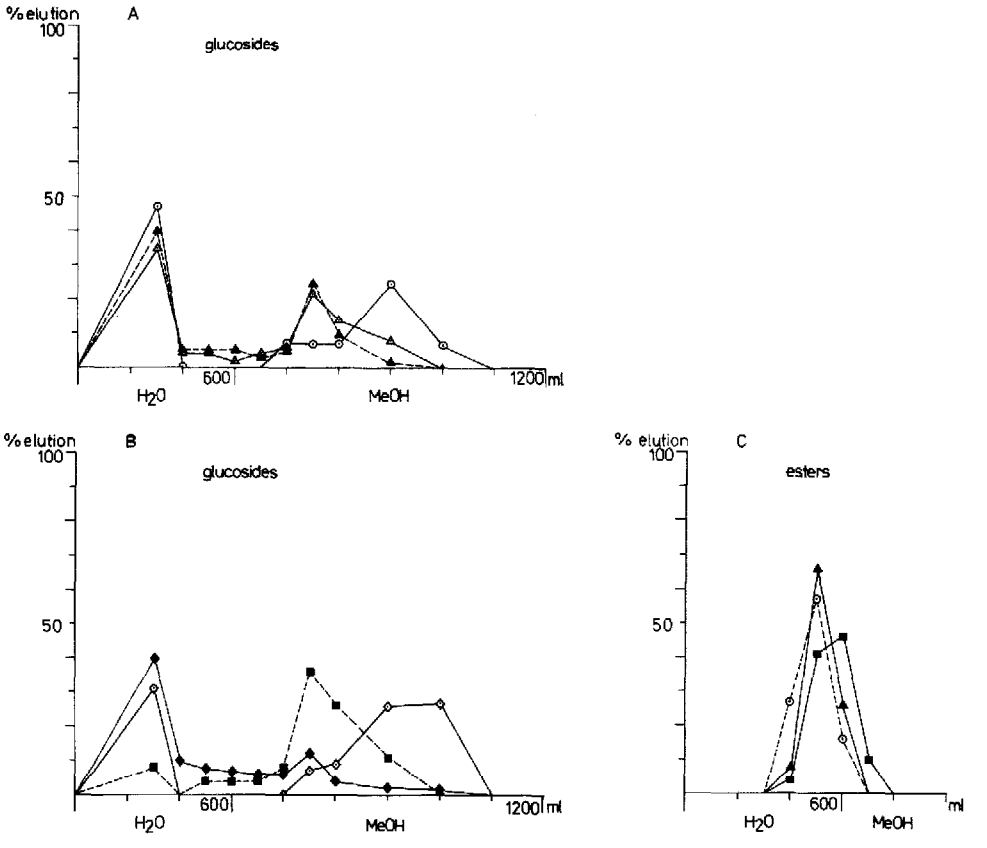

Fig. 8. Elution profiles of a standard solution of hydroxybenzoic acid glucosides and glucose esters on polyamide after addition to a koriander leaf extract. (A) (○), Salicylic acid 2-β-D-glucoside; (▲), vanillic acid 4-β-D-glucoside; (△), syringic acid 4-β-D-glucoside. (B) (◆) Hydroxybenzoic acid 4-β-D-glucoside; (■), protocatechuic acid 4-β-D-glucoside; (◇), gallic acid 4-β-D-glucoside. (C) (■), 1-O-(4-Hydroxybenzoyl)-β-D-glucose; (▲), 1-O-vanilloyl-β-D-glucose; (○), 1-O-syringoyl-β-D-glucose. MeOH = methanol.

(*cf.*, Fig. 4A and B). For further clean-up, anion exchange was necessary to give a separation into neutral (esters) and acidic compounds (glucosides).

Evaporation of the anion exchanger eluate containing formic acid caused a partial hydrolysis of glucosides and reduced the recovery of protocatechuic and gallic acid glucosides to 55–60%, and to 69% for 4-hydroxybenzoic acid glucoside. Other glucosides were less sensitive to hydrolysis (recovery 83–87%). Glucose esters were not affected by the ion-exchange procedure and had recoveries of 93–97% for direct reversed-phase HPLC analysis and for determination after benzoylation.

Application

The method described and the subsequent GC and HPLC analysis were used successfully for the qualitative and quantitative detection of hydroxybenzoic acid

glucosides and glucose esters in different plant materials. The results obtained from 24 species of 10 plant families have been published elsewhere[23].

ACKNOWLEDGEMENT

This work was supported by a grant from Deutsche Forschungsgemeinschaft, which is gratefully acknowledged.

REFERENCES

1 K. Herrmann, *Fortschr. Chem. Org. Naturst.*, 35 (1978) 73.
2 B. Schuster and K. Herrmann, *Phytochemistry*, 24 (1985) 2761.
3 B. Schuster, M. Winter and K. Herrmann, *Z. Naturforsch., Teil C*, 41 (1986) 511.
4 U. Dirks and K. Herrmann, *Phytochemistry*, 23 (1984) 1811.
5 U. Dirks and K. Herrmann, *Z. Lebensm.-Unters.-Forsch.*, 179 (1984) 12.
6 K. Shima, S. Hisada and I. Inagaki, *Phytochemistry*, 10 (1971) 894.
7 M. Ogawa and Y. Ogihara, *Chem. Pharm. Bull.*, 24 (1976) 2102.
8 L. Birkofer, C. Kaiser, W. Nouvertné and U. Thomas, *Z. Naturforsch., Teil B*, 16 (1961) 249.
9 L. Birkofer, C. Kaiser, B. Hillges and F. Becker, *Liebigs Ann. Chem.*, 725 (1969) 196.
10 E. Haslam, in E. E. Conn (Editor), *Biochemistry of Plants*, Vol. 7, Academic Press, London, 1981, p. 527.
11 B. Y. Ong and C. W. Nagel, *J. Chromatogr.*, 157 (1978) 345.
12 L. Nagels, W. Van Dongen, J. De Brucker and H. De Pooter, *J. Chromatogr.*, 187 (1980) 181.
13 D. A. Roston and P. T. Kissinger, *J. Liq. Chromatogr.*, 5 (Suppl. 1) (1982) 75.
14 A. G. Marwan and C. W. Nagel, *J. Food Sci.*, 47 (1982) 585.
15 K. Vande Casteele, H. Geiger and C. F. Van Sumere, *J. Chromatogr.*, 258 (1983) 111.
16 M. Winter and K. Herrmann, *J. Chromatogr.*, 315 (1984) 243.
17 R. Julkunen-Tiitto, *J. Chromatogr.*, 324 (1985) 129.
18 D. W. Baker, *J. Assoc. Off. Anal. Chem.*, 56 (1973) 383.
19 R. Galensa, *Z. Lebensm.-Unters.-Forsch.*, 178 (1984) 199.
20 G. Wagner, *Arch. Pharm.*, 291 (1958) 278.
21 A. B. Durkee and I. R. Siddiqui, *Carbohydr. Res.*, 77 (1979) 252.
22 A. Zane and S. H. Wender, *J. Org. Chem.*, 29 (1964) 2078.
23 S. Klick and K. Herrmann, *Phytochemistry*, 27 (1988) in press.