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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NATURALLY OCCURRING ESTERS OF PHENOLIC ACIDS

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SUMMARY

High-performance liquid chromatographic (HPLC) data of cinnamic and benzoic acid derived phenols, esterified with quinic acid at the $C_{(5)}$ hydroxyl, and with glucose at the $C_{(1)}$ hydroxyl (β anomeric form), are presented for the first time. These naturally occurring compounds have been obtained synthetically. They are chromatographed on reversed-phase and on diol HPLC systems. The four chlorogenic acid isomers are also chromatographed on these columns.

INTRODUCTION

Recently, Wulf and Nagel[1], Court[2], Murphy and Stutte[3] and Price *et al.*[4] have published data on the separation of plant phenolic acids on reversed-phase high-performance liquid chromatographic (HPLC) columns. These acids are found in higher plants together with combined forms such as esters and glycosides. No HPLC data are given in the literature for the last two classes of compounds. In this paper, twelve esters of phenolic acids (esterification with quinic acid and glucose), which are very frequently reported in the literature as plant constituents, are synthesized and their behaviour on reversed-phase HPLC is studied. Few normal-phase HPLC systems are known for the analysis of plant polyphenols. Rapp and Ziegler[5], Morot-Gaudry *et al.*[6] and Nagels and Parmentier[7] use silica gel columns for their analysis. We now report the use of a diol type of column for the normal-phase separation of these compounds.

MATERIALS AND METHODS

Chromatographic equipment

All analytical separations are performed with a Hewlett-Packard 1080B liquid chromatograph equipped with an automatic injector. Injection volumes are 10 μl. Detection is with an UV monitor at 280 nm. The reversed-phase C_8 column and the diol column have inner diameters of 4.6 mm and lengths of 25 cm. The stationary phases are LiChrosorb RP-8 and LiChrosorb 10 diol (E. Merck, Darmstadt, G.F.R.) respectively. Both columns are packed by Chrompack. For preparative

scale separations, we used an air-driven pneumatic amplifier pump from Haskel and a six-way injection valve (Valco). The preparative column (25 × 2.24 cm I.D.) is filled with LiChrosorb 10 RP-8 (Merck).

Syntheses

Quinic acid esters. These compounds have the structures shown in Fig. 1 (*cf.* also Table I). The phenolic acid is esterified with the $C_{(5)}$ hydroxyl of quinic acid (new numbering is used throughout this text).

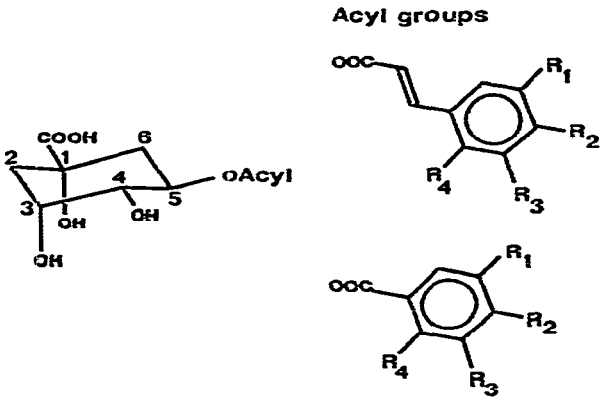

Fig. 1. Structure of quinic acid esters.

5-O-Cinnamoylquinic acid and 5-O-*p*-coumaroylquinic acid are synthesized as described by Nagels[8], 5-O-*o*-coumaroylquinic, 5-O-feruloylquinic and 5-O-sinapoylquinic acid by the methods of De Pooter and co-workers[9,10] and 5-O-*p*-hydroxybenzoylquinic acid by the methods of De Brucker[11].

3- and 4-O-caffeoylquinic acids are obtained in the following manner.

TABLE I

TRIVIAL NAMES OF QUINIC ACID ESTERS

Ester	R_1	R_2	R_3	R_4
Phenylpropanoic				
5-O-Cinnamoylquinic acid	H	H	H	H
5-O-*p*-Coumaroylquinic acid	H	OH	H	H
5-O-*o*-Coumaroylquinic acid	H	H	H	OH
1-O-Caffeoylquinic acid	OH	OH	H	H
3-O-Caffeoylquinic acid	OH	OH	H	H
4-O-Caffeoylquinic acid	OH	OH	H	H
5-O-Caffeoylquinic acid (chlorogenic acid)	OH	OH	H	H
5-O-Feruloylquinic acid	OCH_3	OH	H	H
5-O-Sinapoylquinic acid	OCH_3	OH	OCH_3	H
Benzoic				
5-O-*p*-Hydroxybenzoylquinic acid	H	OH	H	H

Chlorogenic acid (Fluka, Buchs, Switzerland) is dissolved in a saturated solution of $NaHCO_3$. The temperature is raised to 90°. Heating is stopped after 30 min and H_2SO_4 added to pH 3. The obtained mixture of isomeric caffeoylquinic acids is extracted with ethyl acetate. The ethyl acetate phase is concentrated to dryness, the residue dissolved in water and chromatographed on preparative-scale reversed-phase HPLC (*cf.*, *Chromatographic equipment*). Eluent: 10 m*M* H_3PO_4–methanol (80:20). Three isomers are obtained in this way, namely 3-, 4- and 5-O-caffeoylquinic acid, the 1-isomer being present in negligible amounts only. The 3- and 4-isomers are collected. Fractions are concentrated in vacuum to remove methanol and the residual aqueous phase is extracted with ethyl acetate. This ethyl acetate phase is concentrated to dryness. 1-O-Caffeoylquinic acid is synthesized from 1-(3′, 4′-dicarboethoxycaffeoyl)acetonequinide by refluxing with water for 6 h. The 1-isomer is isolated from the reaction mixture by preparative scale RP HPLC (see other chlorogenic acid isomers).

Glucose esters. Structural formulae of these compounds are shown in Fig. 2 (*cf.*, Table II for substitution patterns).

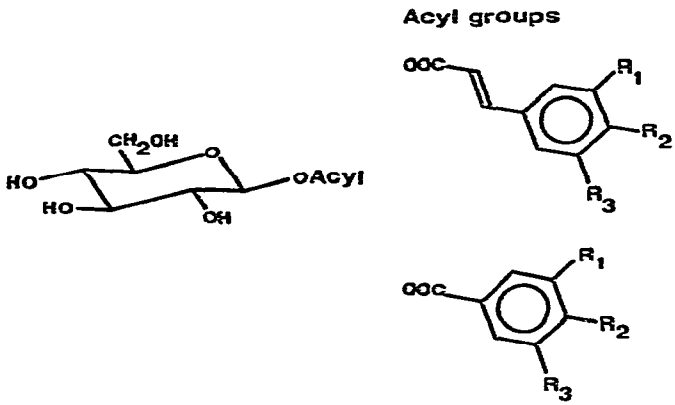

Fig. 2. Structure of glucose esters.

TABLE II

TRIVIAL NAMES OF GLUCOSE ESTERS

Esters	R_1	R_2	R_3
Phenylpropanoic			
1-O-*p*-Coumaroylglucose	H	OH	H
1-O-Caffeoylglucose	OH	OH	H
1-O-Feruloylglucose	OCH_3	OH	H
Benzoic esters			
1-O-Benzoylglucose	H	H	H
1-O-Galloylglucose (glucogallin)	OH	OH	OH

Benzoylglucose is synthesized by reaction of ethyl 1-thio-β-D-glucopyranoside with silver benzoate in acetonitrile. The compound is also purified with preparative reversed-phase HPLC. *p*-Coumaroyl-, caffeoyl-, feruloyl- and galloylglucose are

obtained from their acetyl derivatives by deacetylation with sodium methanolate in methanol[12,13]. After removal of the acetyl protecting groups, acetic acid is added to the reaction mixture to slightly acidic pH. Water is added and methanol removed by vacuum evaporation. Sodium ions are eliminated by eluting the resultant aqueous phase over a Servacel cellulose ion exchanger (H^+) (Serva, Heidelberg, G.F.R.). The crude reaction mixture is extracted with ethyl acetate. The water phase contains a mixture of isomeric glucose esters. These are separated by preparative reversed-phase column chromatography. The structures of all the described compounds are verified with nuclear magnetic resonance spectroscopy.

RESULTS AND DISCUSSION

Chromatograms of the twelve synthesized esters and of the 4 chlorogenic acid isomers on a reversed-phase column are shown in Figs. 3 and 4, respectively. These components are not completely separated by the system. It is clear that complex mixtures of phenolic compounds (as found in plant extracts) will seldom be resolved by one technique, such as reversed phase HPLC, alone. A group separation of the phenolic compounds will usually be necessary. In an earlier publication[14]

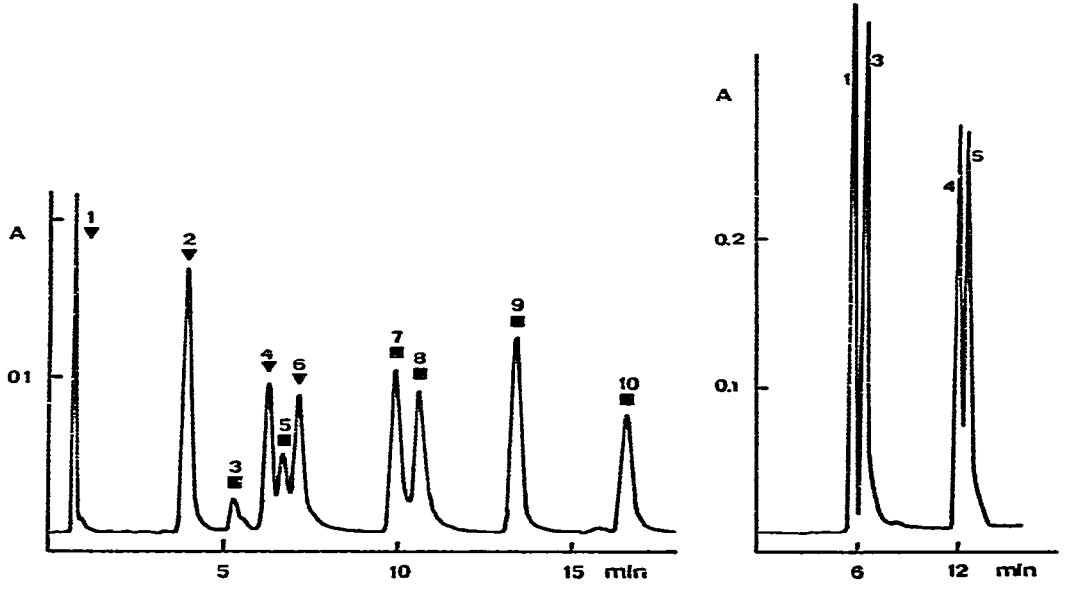

Fig. 3. Separation of quinic acid and glucose esters of phenolic acids on reversed-phase HPLC. Separated compounds: 1 = galloylglucose; 2 = caffeoylglucose; 3 = *p*-hydroxybenzoylquinic acid; 4 = *p*-coumaroylglucose and benzoylglucose; 5 = caffeoylquinic acid; 6 = feruloylglucose; 7 = *p*-coumaroylquinic acid; 8 = feruloylquinic acid and sinapoylquinic acid; 9 = *o*-coumaroylquinic acid; and 10 = cinnamoylquinic acid. Flow-rate, 4.0 ml/min. Gradient elution: solvent A = 10 m*M* H_3PO_4, solvent B = methanol; from 10% B to 80% B in 40 min.

Fig. 4. Reversed-phase chromatography of chlorogenic acid isomers (numbers refer to position of esterification, *cf.*, Fig. 1). Linear gradient elution at a flow-rate of 4 ml/min: solvents as in Fig. 3; from 5% B to 20% B in 15 min.

we described such a separation (based on the charge differences of phenolic compounds at pH 7) using ECTEOLA-cellulose. When the above fifteen phenolic compounds are subjected to this separation, we obtain two groups: glucose esters (marked ▲ in Fig. 3) and quinic acid esters (marked ■). Poorly resolved components such as *p*-coumaroylglucose, chlorogenic acids and feruloylglocuse (see Fig. 3) are completely separated by the use of this technique. (Since chlorogenic acid is a very abundant phenolic compound in higher plant tissues, the lower amounts of these two glucose esters will be completely masked by the former compound if no group separation is used.) Benzoylglucose and *p*-coumaroylglucose are unresolved by this reversed-phase chromatography. *p*-Coumaroylglucose has one phenolic hydroxyl function, benzoylglucose has none. The increase in polarity caused by this grouping is matched by the increase in hydrophobic character of the molecule caused by the conjugated double bond.

As will be shown below, a diol column differentiates these two compounds (hydrogen bonding is more important here). Another material (low pressure) that can separate these two components is ecteola cellulose[14]. This material separates neutral phenolics on the basis of their hydrogen-bonding capacities. When it is used as a complementary purification step before HPLC analysis on RP columns, the two glucose esters are determined without interference. Feruloyl- and sinapoylquinic acid also are unresolved on RP columns. They are partly separated by chromatography on a diol column ($R_s = 0.6$). According to Corse and Patterson[15], 3-O-sinapoylquinic acid and chlorogenic acid are unresolved on paper chromatography. They are separated on RP columns (Fig. 3), while on diol the resolution between these components is much higher (Fig. 7).

The chlorogenic acid isomers are resolved on RP columns, (Fig. 4). The isomers with the caffeoyl moiety axially disposed (1- and 3-O-caffeoylquinic acid) elute first; 4- and 5-O-caffeoylquinic acid, where the caffeoyl group is in an equatorial position, have higher retention volumes. The reverse elution order has been given by Hanson[16] for the four cinnamoylquinic acid isomers on a low-pressure silica gel column. For the chlorogenic acid isomers, a diol type HPLC column and a low-pressure silica gel column have the same selectivity. This can be concluded when comparing Fig. 5 with chromatographic data from the literature[16]. The 1- and 3-isomers are only partly resolved by the diol column. The selectivity of this material and of silica gel columns are also comparable for other phenolic compounds (see refs. 6 and 17). Silica gel columns have the disadvantage that their water content is very critical, and varies during a gradient run. This is not the case with a diol column, which also has the advantage that low viscosity organic solvents can be used, resulting in low column pressures.

A complete separation of the five synthesized glucose esters, together with some commercially available phenolic compounds, is shown in Fig. 6.

The quinic acid esters are also chromatographed on the diol column (Fig. 7). When the retention orders of the phenolic components on RP and on diol columns are compared, it is interesting to note that they are not merely inverted. This means that both columns have different selectivities, and may be used to complement each other.

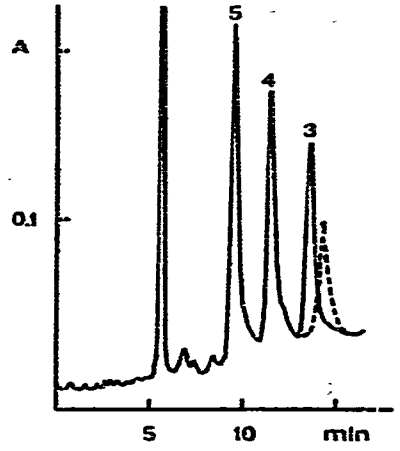

Fig. 5. Diol column chromatography of chlorogenic acid isomers (numbering refers to position of esterification, *cf.*, Fig. 1). The first eluted peak is caffeic acid (not numbered). The 1-isomer is injected separately (dotted line). Flow-rate, 4 ml/min. Linear gradient elution: from 3% B to 20% B in 20 min; solvent A = CH_2Cl_2–acetic acid (99:1), solvent B = 1-propanol.

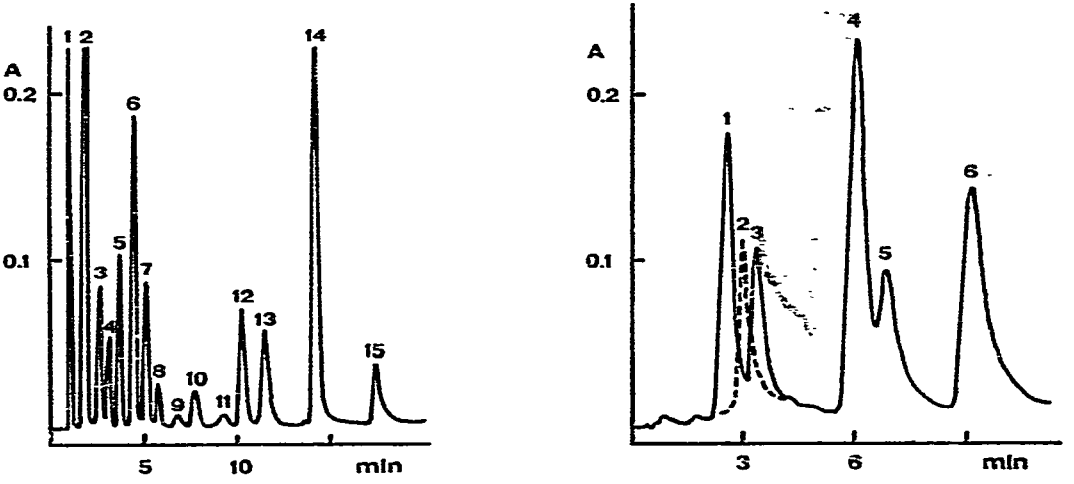

Fig. 6. Separation of phenolic glucose esters together with commercial phenolics on a diol column. Peaks: 1 = cinnamic acid; 2 = catechol; 3 = *p*-coumaric acid; 4 = *p*-hydroxy benzoic acid; 5 = resorcinol; 6 = methyl gallate; 7 = caffeic acid; 8 = 3,4-dihydroxybenzoic acid; 9 = benzoylglucose; 10 = feruloylglucose; 11 = chlorogenic acid; 12 = gallic acid; 13 = *p*-coumaroylglucose; 14 = caffeoylglucose; 15 = galloylglucose. Flow-rate, 4 ml/min. Gradient elution: from 3% B to 12% B in 10 min and then to 40% B in 10 min; solvent A = CH_2Cl_2–acetic acid (99.5:0.5), solvent B = 1-propanol.

Fig. 7. Behaviour of phenolic quinic acid esters on a diol column. Peaks: 1 = 5-O-cinnamoylquinic acid; 2 = 5-O-sinapoylquinic acid (separate injection); 3 = feruloylquinic acid; 4 = *p*-coumaroylquinic acid; 5 = *p*-hydroxybenzoylquinic acid; and 6 = chlorogenic acid. Linear gradient elution at a flow-rate of 4 ml/min: from 3% B to 90% B in 40 min; solvent A = CH_2Cl_2–acetic acid (9:1), solvent B = 1-propanol.

CONCLUSION

HPLC offers a convenient method for the analysis of plant phenolic esters. Reversed-phase chromatography and normal-phase chromatography on the weakly polar diol column can be used to complement each other. For complex mixtures, prior to injection on one of these columns, are recommended, group separations on low-pressure column chromatography on carbohydrate-based anion exchangers.

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