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PREPARATIVE AND ANALYTICAL LIQUID CHROMATOGRAPHY OF COMPLEX CAFFEOYL ESTERS

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SUMMARY

Two liquid chromatography techniques for the analysis of caffeoylated phenylethanol glycosides in plant extracts are described. Preparative separations can be achieved by polyamide-6 column chromatography employing gradient ethanol elution. An isocratic reversed-phase high-performance liquid chromatographic separation of these compounds is also presented.

INTRODUCTION

During the past fifteen years, there have been numerous reports of the isolation and identification of caffeoylated phenylethanol glycosides (CPEGs) from plant tissues[1–12]. Their detection, however, has been limited by the lack of specific analytical techniques and thus their occurrence in plants may be more widespread than previously suspected[13]. Many CPEGs are structural isomers or possess only minor substitution differences which leads to difficulties in their separation. In this report we present new preparative and analytical methods for their analysis and identification.

EXPERIMENTAL

Plant material

Syringa vulgaris and *Forsythia* × *intermedia* were obtained from the campus of the University of Guelph. Cultures were established and maintained as described previously[14].

Standard compounds

Verbascoside was isolated previously from *S. vulgaris* culture[14]. Isoverbascoside was synthesized from verbascoside according to the method of Schilling *et al.*[10]. Forsythoside, echinacoside and orobanchoside were the generous gifts of Dr. H. Hikino, Tohuko University, Dr. H. Becker, University of Heidelberg, and Dr. C. Andary, University of Montpellier, respectively.

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Apparatus

The high-performance liquid chromatographic (HPLC) apparatus consisted of an Eldex dual piston model AA pump, Valco injection valve model CV-6-UHPa-N60 (15-μl injection loop), and a Beckman Model 153 UV detector (280 nm) equipped with a 10-mm path length, 8-μl analytical cell. The column used was a 150 × 4.1 mm I.D. Hamilton PRP-1 reversed-phase analytical column (column packing, 10-μm beads of styrene–divinylbenzene copolymer). For preparative separations, the UV detector was fitted with a 0.5-mm path length, 2-μl preparative cell. The particle size of the polyamide-6 used was 100–300 μm (Woelm).

Solvents

The HPLC solvent consisted of acetonitrile–9.3 m*M* sodium dihydrogen phosphate (20:80). The acetonitrile used (Caledon Laboratories, Georgetown, Ontario, Canada) was HPLC grade. Thin-layer chromatography (TLC) was carried out with cellulose TLC plates (Polygram CEL 400, 0.1 mm microcrystalline cellulose) using *n*-butanol–ethanol–water (4:1:1) and 10% acetic acid as solvents.

Preparative extraction

S. vulgaris L. suspension culture (2 kg) was extracted in 5 l of 70% ethanol under reflux for 1 h. The extract was filtered and then concentrated *in vacuo*. The residue was redissolved in 250 ml of water and applied to a 40 × 5.5 cm I.D. column of polyamide-6 powder. Elution with 1.0 l of glass-distilled water was followed by 2.0 l of 50% ethanol. The ethanolic eluate was concentrated *in vacuo* and lyophilized to give 7.5 g of amorphous product.

Preparative fractionation

Lyophilized product (1.0 g) was applied to a 21 × 2.5 cm I.D. column of polyamide-6 powder and eluted (100 ml/h) with a linear aqueous ethanol gradient from 0 to 50% ethanol at 4%/h. The column eluate was monitored for UV absorbance at 280 nm and 15-ml fractions were collected. Fractions representing peaks were pooled, dried *in vacuo*, and examined by TLC and HPLC.

Analytical extraction

Plant material (1.0 g) was extracted in 50 ml of 70% ethanol as described under Preparative extraction, except the polyamide-6 column was 12 × 1 cm I.D. and the 50% ethanol eluate (50 ml) was concentrated and taken up in water for analysis by HPLC.

RESULTS

Preparative fractionation

Gradient elution of the polyamide column gave a UV absorption profile with four major peaks (Fig. 1). Fractions 1 (14 mg), 3 (475 mg), and 4 (120 mg) each gave single, unique peaks when analyzed by HPLC. Fraction 2 (59 mg) yielded two unique peaks. The HPLC profile of the combined peaks 1–4 is shown in Fig. 1 inset. The order of elution from the analytical PRP-1 column corresponds to the order of elution from the polyamide column. Fraction 3 has been identified as verbascoside by com-

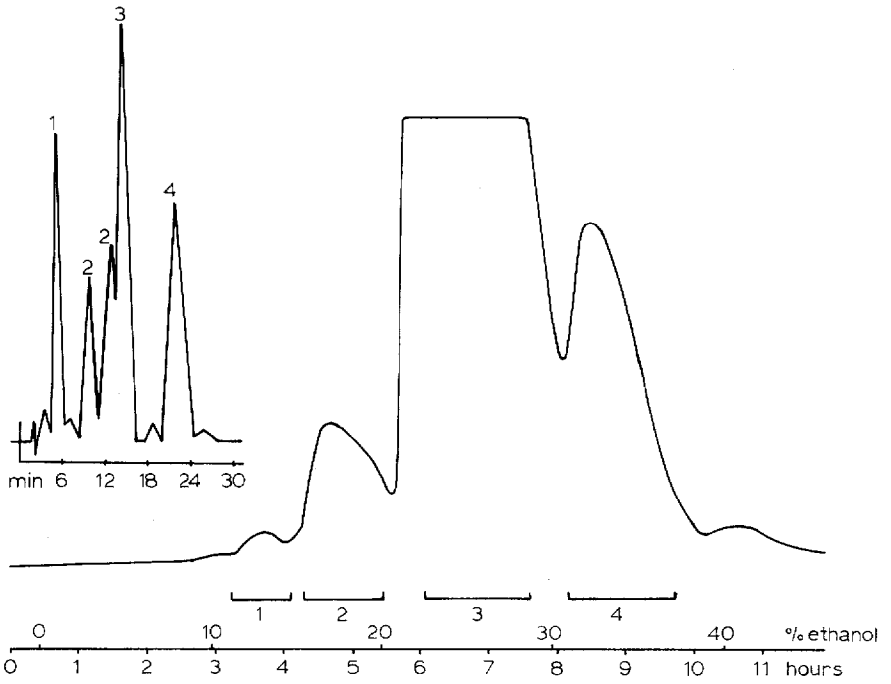


Fig. 1. Fractionation of *Syringa vulgaris* extract on polyamide-6 by gradient ethanol elution. Bars 1–4 represent peak fractions. Detection, 280 nm; full scale, 2.56 a.u. Inset: HPLC profile of combined samples of fractions 1–4 on PRP-1 column. Detection, 280 nm; full scale, 0.04 a.u.; flow-rate, 1.0 ml/min; mobile phase, acetonitrile–9.3 m*M* sodium dihydrogen phosphate (20:80).

parison with standard verbascoside based on TLC, HPLC, and ^{13}C nuclear magnetic resonance spectroscopy (NMR). The identity of fraction 4 as isoverbascoside was confirmed by TLC, ^{13}C NMR and by HPLC comparison with the authentic compound.

HPLC

HPLC analysis of a mixture of the standard compounds available is shown in Fig. 2. Echinacoside eluted first possibly due to the increased hydrophilicity induced by the presence of a third sugar residue. Forsythoside and verbascoside are cleanly separated. Isoverbascoside and orobanchoside are not totally separated, but have sufficiently different retention times to allow their identification. The capacity factor (k') value for each compound is given in Table I. The HPLC profiles of various plant extracts are shown in Fig. 3. Both *S. vulgaris* samples are seen to contain verbascoside and isoverbascoside as well as several other unidentified components (Fig. 3a and b). *Forsythia* × *intermedia* blossom extract contains predominantly forsythoside while *Forsythia* tissue cultured *in vitro* produces mainly verbascoside (Fig. 3c and d).

Verbascoside isomerization

Injection of the verbascoside isomerization mixture provided peaks identified as isoverbascoside and residual verbascoside, and two peaks which were likely hy-

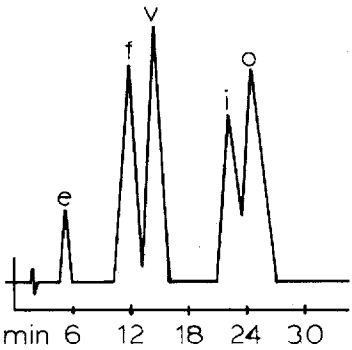


Fig. 2. Separation of standard CPEGs by HPLC using PRP-1 column. Detection, 280 nm; full scale, 0.04 a.u.; flow-rate, 1.0 ml/min; mobile phase, acetonitrile–9.3 m*M* sodium dihydrogen phosphate (20:80). Abbreviations: e = echinacoside; f = forsythoside; v = verbascoside; i = isoverbascosde; o = orobanchoside.

drolysis products (Fig. 4). The identities of the latter were confirmed by comparison with standard caffeic acid and the corresponding decaffeoylated glycoside. The HPLC profile of the reaction products after 5 min of incubation indicates a high degree of hydrolysis. Samples taken at 30 min showed that the verbascoside and isoverbascoside were almost totally hydrolyzed.

DISCUSSION

The structures of the five standard compounds available for this study are shown in Table II. Three are structural isomers; only in echinacoside and orobanchoside are unique substitutions present.

CPEGs are subject to oxidation, hydrolysis and isomerization[10], therefore mild chromatographic methods are required. Preparative techniques previously applied to the study of CPEGs include paper chromatography (PC)[3], liquid chromatography[11], and droplet counter-current chromatography (DCCC)[2,4]. Each suffers one or more of the following difficulties: (i) limited capacity; (ii) poor recovery; (iii) specialized equipment requirements. Polyamide chromatography is known to provide mild conditions although its full potential has seldom been exploited. The use of polyamide chromatography with gradient ethanol elution proves to be a gentle, efficient and high capacity method for the fractionation of CPEG-containing extracts.

TABLE I

CPEG CAPACITY FACTORS

PRP-1 analytical column; solvent, acetonitrile–9.3 m*M* sodium dihydrogen phosphate (20:80).

Compound	*k'*
Echinacoside	1.0
Forsythoside	4.7
Verbascoside	6.1
Isoverbascoside	9.6
Orobanchoside	10.7

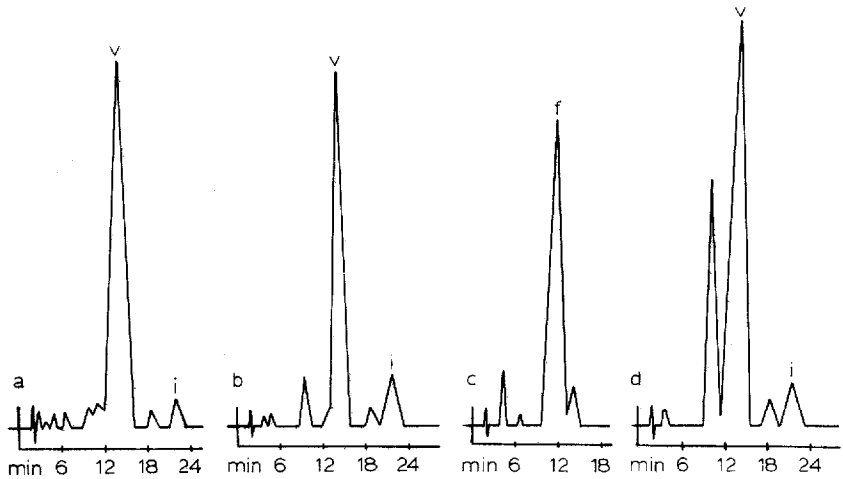


Fig. 3. HPLC analysis of plant extract. (a) *Syringa vulgaris* blossom. (b) *Syringa vulgaris* culture. (c) *Forsythia* × *intermedia* blossom. (d) *Forsythia* × *intermedia* culture. Chromatographic conditions and abbreviations as in Fig. 2.

Complex mixtures of CPEGs typical of plant extracts are incompletely resolved by analytical TLC and PC while the only application of HPLC to CPEGs has been the separation of verbascoside and myricoside peracetates[4]. The reversed-phase HPLC system reported here provides an efficient, isocratic separation of the underivatized glycosides. It is capable of very discrete separations such as that of verbascoside and its isomer forsythoside. The system also readily discriminates between disaccharide CPEGs and the widespread, simpler caffeoyl esters chlorogenic acid and

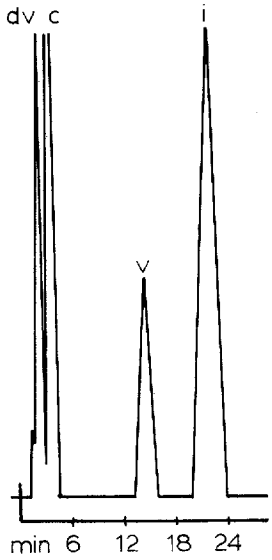
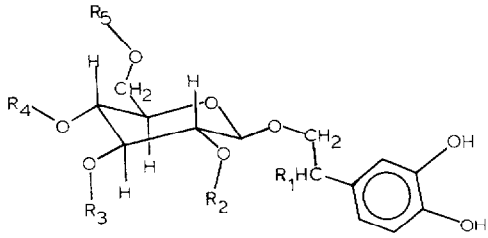


Fig. 4. HPLC separation of verbascoside isomerization products after 5-min incubation. Chromatographic conditions and abbreviations as in Fig. 2. Additional abbreviations: dv = decaffeoylated verbascoside; c = caffeic acid.

TABLE II

STRUCTURE OF CPEGs



Compound	R_1	R_2	R_3	R_4	R_5
Echinacoside	H	H	Rhamnose	Caffeoyl	Glucose
Forsythoside	H	H	H	Caffeoyl	Rhamnose
Verbascoside	H	H	Rhamnose	Caffeoyl	H
Isoverbascoside	H	H	Rhamnose	H	Caffeoyl
Orobanchoside	OH	Rhamnose	H	Caffeoyl	H

rosmarinic acid which elute at or near the solvent peak. All CPEGs investigated are sufficiently separated to be tentatively identified in extracts by their k' values. It has previously been assumed that polyamide binds polyphenolics through hydrogen-bonding of its amide residues to catechol hydroxyls[15]. It is interesting to note the striking similarity between the elution patterns of the polyamide gradient system and that produced by reversed-phase HPLC.

Fig. 3 illustrates how the HPLC technique can be applied to various plant extracts for the characterization of their production patterns. For example, the secondary peak seen in *Forsythia* cultures is most likely identical to the unidentified compound in *Syringa* cultures which eluted in fraction 2 of the polyamide gradient elution (Fig. 1).

HPLC analysis of plant extracts would aid in the detection of novel CPEGs and would be of value in chemotaxonomic work. While the ability of the PRP-1 column to separate isomeric CPEGs containing three sugar moieties has yet to be tested, preliminary results suggest that they may be separated on columns known to separate oligosaccharides.

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