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Note

High-performance liquid chromatography of free and bound phenolic acids in the egg-plant (*Solanum melongena* L.)

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It is well known that polyphenols are important in the physiology of the growth and development of plants[1]. Since phenolic substituents usually improve the solubility characteristics of compounds and can interact with specific receptor groups by hydrogen bonding and/or by more stable covalent bonds, phenols are expected to influence a broad range of biological phenomena[1–8].

During a programme of plant breeding to obtain cultivars of the egg-plant for processing, the rôle of phenolic compounds on "browning" of vegetable tissues was considered. Most papers published on the high-performance liquid chromatography (HPLC) separations of polyphenolic compounds have dealt with a limited number of substances[9–13]. Vande Casteele *et al.*[14] studied the retention times of some 140 flavonoids and separated complex mixtures of about 40 substances.

The purpose of the present study was to develop a method for extending techniques previously applied to benzoic and cinnamic acid derivatives to more complex flavonoids extracted from plant tissues. The method, consisting of a combination of isocratic and linear gradient elution and a concave gradient elution, was applied to simple phenolic acids in the egg-plant.

EXPERIMENTAL

Chromatography

A Perkin-Elmer Series 2 liquid chromatograph, equipped with a spectrophotometric detector LC-55 and a Sigma 10B chromatography data station, was used. The column was a stainless-steel tube (30 cm × 4 mm I.D.) packed with μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.), having an average particle size of 10 μm. A short stainless-steel precolumn, packed with μBondapak C_{18}–Corasil (37–50 μm), was used. The UV detector was set at 280 nm and 325 nm.

Two solvents were used: A, methanol; B, acetic acid–water (5:95 v/v). The elution profile of the linear gradient (Programme I) was: 0–25 min, 15–40% A; 25–30 min, 40% A (isocratic); 30–45 min, 40–63% A; 45–47 min, 63% A (isocratic); 47–51 min, 63–99% A. The concave gradient (Programme II) was: initial conditions 10% A; programme time 44 min; final conditions 99% A. The flow-rate was 2 ml/min and the column pressure was 2000–2200 p.s.i.

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TABLE I

RETENTION TIMES (t_R) OF BENZOIC AND CINNAMIC ACID DERIVATIVES AND FLAVONOIDS

I = Linear gradient; II = concave gradient.

Compound	t_R^I *(min)*	t_R^{II} *(min)*	*Compound*	t_R^I *(min)*	t_R^{II} *(min)*
Phloroglucinol	2.18	2.21	Sinapic acid	15.60	21.85
Gallic acid	2.48	2.52	Luteolin-7-glucoside	21.87	28.48
Protocatechuic acid	3.71	3.82	Quercetin-3-glucoside + Rutin	23.35	29.20
Catechol	4.01	4.13			
(+)-Catechin	4.73	5.76	Cinnamic acid	25.08	29.65
p-Hydroxybenzoic acid	5.81	6.30	Myricetin	25.90	30.63
Chlorogenic acid	6.53	7.68	Quercitrin	26.96	31.27
Esculetin	6.94	7.80	Morin + Quercetin	28.27	32.02
Vanillic acid	7.29	8.76	Naringenin	30.85	33.45
Caffeic acid	7.79	9.36	Fisetin	33.68	35.00
Syringic acid	8.83	11.20	Hesperetin	34.49	35.40
Cynarin	9.00	13.34	Luteolin	37.16	37.26
p-Coumaric acid	12.24	15.46	Kaempferol	40,27	39.60
Dihydroquercetin	12.82	16.72	Apigenin	41.79	41.01
Ferulic acid	14.33	19.25	Galangin	43.00	42.50

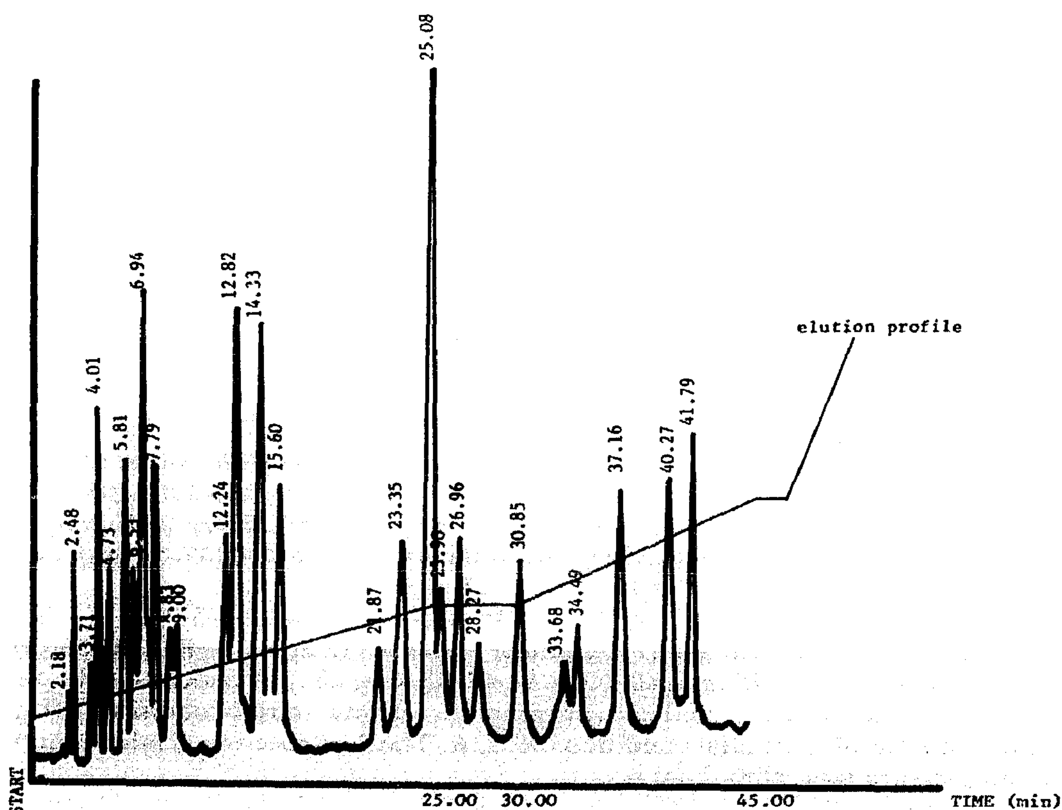

Fig 1. The retention times of phenolic compounds eluted using the linear gradient on a μBondapak C_{18} column (30 cm × 4 mm I.D.). A list of the compounds separated is given in Table I. For the elution system see Experimental.

Samples

Standards of benzoic and cinnamic acid derivatives and flavonoids were dissolved in methanol. Peeled fruits of the egg-plant were extracted with methanol–ethanol (1:1) by the procedure developed by the Laboratory of Plant Biochemistry of Ghent[16,17]. The alcoholic extract, after concentration, was partitioned between 1-butanol and 6% Na_2CO_3. The aqueous layer was acidified to pH 3.5 and re-extracted with diethyl ether giving fraction A. The acidic aqueous layer was made alkaline with concentrated NaOH until 2 *M*, then refluxed, acidified and extracted with diethyl ether to give fraction B. The 1-butanol layer was refluxed in 2 *M* NaOH and the aqueous layer then acidified to pH 3.5 and re-extracted with diethyl ether to give fraction C. The residue insoluble in methanol–ethanol, after alkaline hydrolysis, acidification and ether extraction, gave fraction D. The four fractions obtained were: A, free phenolics; B, carbonate-soluble, alkali-labile bound phenolics; C, carbonate-insoluble, alkali-labile bound phenolics; D, alcohol-insoluble, alkali-labile bound phenolics.

The ether fractions of free and bound phenolics were dried *in vacı o* at 30°C and the residue was dissolved in methanol. The injected volume of standard solutions and vegetable extracts was 10 μl.

RESULTS AND DISCUSSION

Table I shows the retention times of the phenolic compounds eluted according to two elution profiles. The values are averages from six runs, the error being about

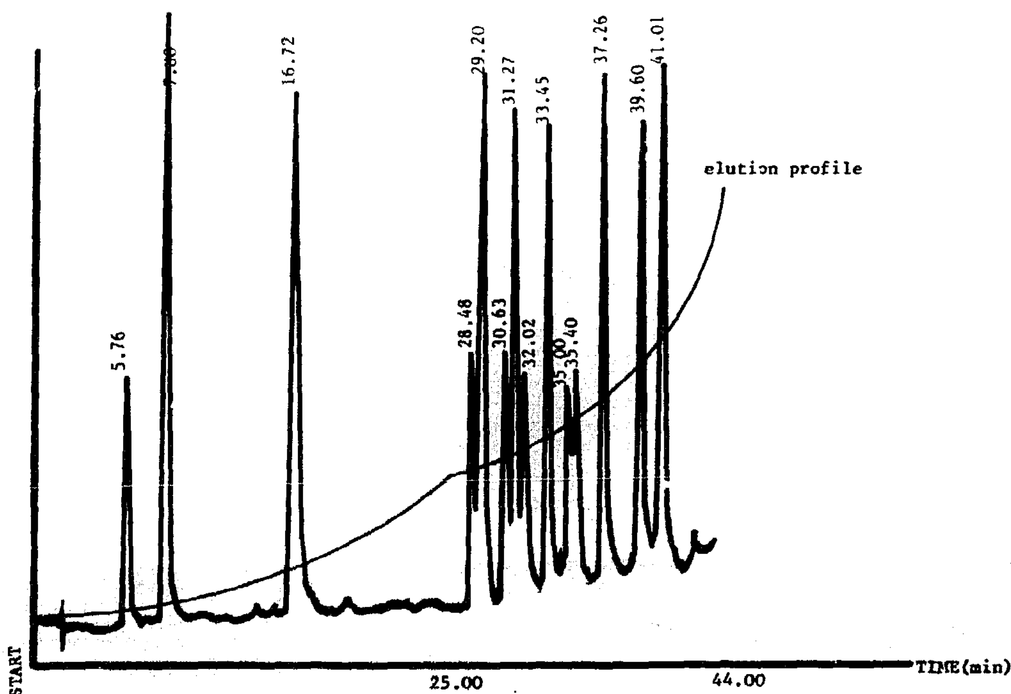

Fig. 2. The retention times of flavonoids eluted using the concave gradient on a μBondapak C_{18} column (30 cm × 4 mm I.D.). Other details as in Fig. 1.

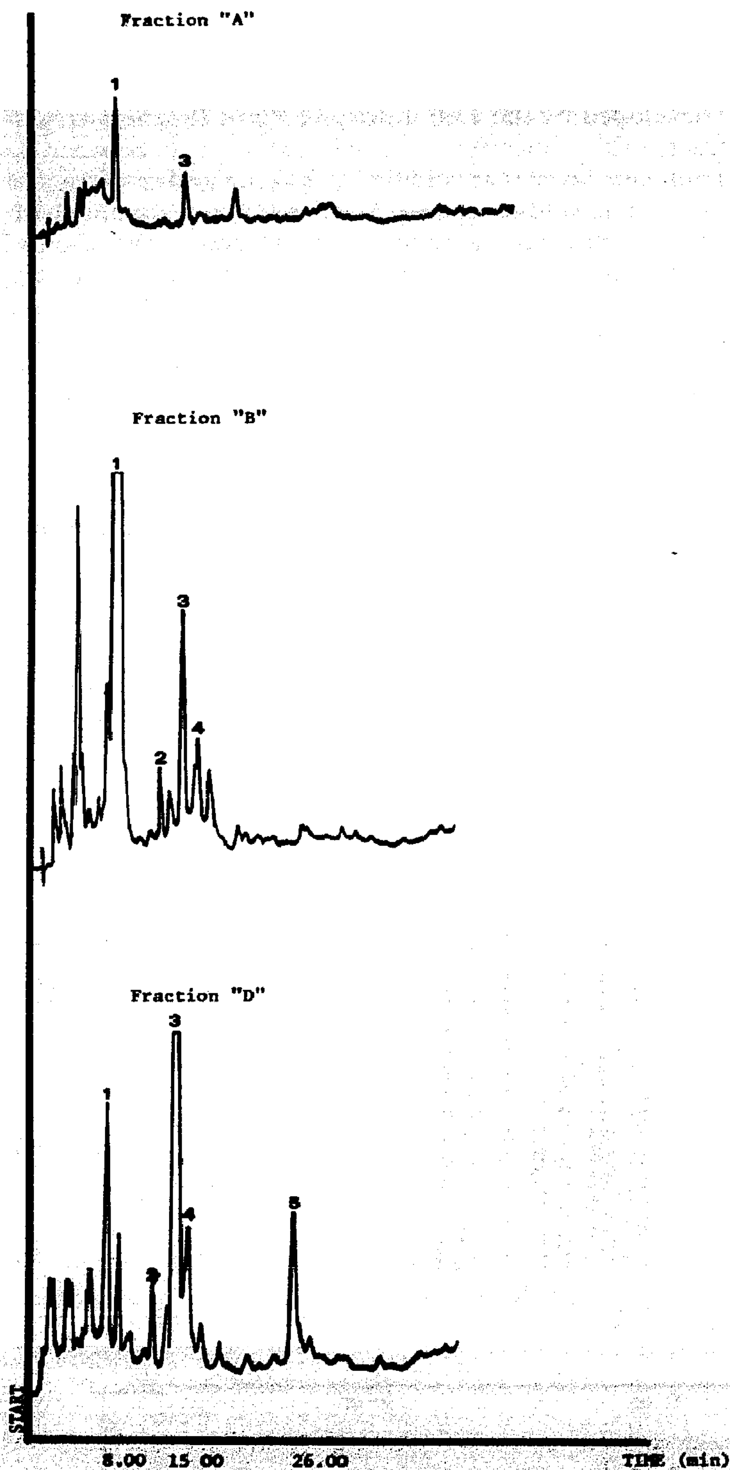

Fig. 3. The separation of simple phenols in vegetable extracts of the egg-plant using the linear gradient. Peaks: 1 = Caffeic acid; 2 = *p*-coumaric acid; 3 = ferulic acid; 4 = sinapic acid; 5 = cinnamic acid. For the elution system see Experimental.

TABLE II

SIMPLE PHENOLS IN VEGETABLE EXTRACTS OF THE EGG-PLANT

Cultivar	*Caffeic acid*	*p-Coumaric acid*	*Ferulic acid*	*Sinapic acid*	*Cinnamic acid*
Fraction A					
L39/78	+		+		
L17/79	+		+		
L.V.					
Fraction B					
L39/78	+	+	+	+	+
L17/79	+	+	+	+	
L.V.	+	+	+	+	
Fraction D					
L39/78	+	+	+	+	
L17/79	+	+	+	+	+
L.V.	+	+	+	+	

2%. Fig. 1 shows the separation, using the linear gradient, in a single run of 30 substances: only quercetin-3-glucoside and rutin at 23.35 min and morin and quercetin at 28.27 min are not separated. Fig. 2 shows the elution of a set of flavonoids using the concave gradient. The two elution programmes are nearly equivalent, but permit more information to be obtained from the two different t_R values. Fig. 3 shows the separation of simple phenols from vegetable extracts of egg-plant fruits. A list of the compounds identified, according to their two different t_R values, is given in Table II. The UV spectra of the peaks identified corresponded to those of standard compounds. In addition to the components reported, a number of minor constituents in fractions B and D have not as yet been identified.

The highest content of simple phenols was found in the fractions B and D, while in fraction C they were absent. The content of free phenolics (fraction A) was very low and among them were identified caffeic and ferulic acids, the only ones existing in all fractions. Analysis carried out on cultivars of egg-plant with different "browning" characteristics showed differences mainly in the content of caffeic and ferulic acids, the most abundant components.

These results are in agreement with the theory that cinnamic acid and its derivatives, widely distributed in vascular plants, generally are found as esters rather than as free acids[2-5,8,16,18]. In addition, large amounts of cinnamic acid derivatives, particularly *p*-coumaric acid and ferulic acid, are found after alkaline hydrolysis of the insoluble residue remaining after alcoholic extraction.

In the fraction D the content of ferulic acid was very high. El-Basyouni *et al.*[3] suggested an alcohol insoluble enzyme ester of hydroxycinnamic acids as the active intermediate of lignin biosynthesis. Other possible interactions between alcohol insoluble phenolic acids and proteins are hydrogen bonding and irreversible oxidation followed by covalent condensation. Ferulic acid may also be bound by means of an amide linkage[8]. Fraction B contains phenolic acids esterified with the hydroxyls of compounds such as glucose or quinic acid. Among these phenolics, caffeic acid was particularly abundant and their rôle in plants is related to the biosynthesis of flavonoids and coumarins.

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