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Note

Chromatographic separation of phenolic compounds on Amberlite IR-45

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Strongly and weakly basic anion-exchange resins have been utilized for the chromatographic separation of phenols[1–5] and for their isolation from waste water[6,7] and vegetable extracts[8,9]. Chromatographic methods for the analysis of phenols have often been studied in aqueous solution containing an organic solvent[4,5,10].

The separation of several groups of phenolic compounds from vegetable extracts on weakly basic anion-exchange resins has not so far been achieved. This paper reports on the chromatographic separation of a number of phenolic compounds on the weakly basic anion exchanger Amberlite IR-45. A stepwise elution both with aqueous solutions containing an increasing concentration of methanol and with alkaline solutions was carried out.

MATERIALS AND METHODS

Resin and chemicals

Amberlite IR-45, analytical grade, was used (Rohm and Haas, Philadelphia, Pa., U.S.A.).

The compounds used were as follows:

(I) Coumarins: coumarin (J. T. Baker, Phillipsburgh, N.J., U.S.A.); esculetin (Fluka, Buchs, Switzerland); 4-methylesculetin (Aldrich, Milwaukee, Wisc., U.S.A.); scopoletin, daphnetin (NIPA).

(II) Phenols: hydroquinone, pyrogallol, 3-methoxycatechol, 4-chlorocatechol, 4-nitrocatechol, DL-DOPA (Fluka); catechol, resorcin, phloroglucin, *p*-methoxyphenol, *o*-cresol, *p*-cresol (Carlo Erba, Milan, Italy); 3-methylcatechol (EGA Chemie); 2,6-dimethylphenol, 2,5-dimethoxyphenol (Eastman-Kodak, Rochester, N.Y., U.S.A.); thymol, phenol (Merck); 4-methylcatechol (Aldrich); saligenin (K & K, Plainview, N. J., U.S.A.).

(III) Phenolic acids: salicylic acid (Carlo Erba); *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (Aldrich); 2,3-dihydroxybenzoic acid, 2,3-dihydroxy-4-methoxybenzoic acid, *o*-coumaric acid, chlorogenic acid, 3,4-dihydroxy-β-phenylpropionic acid (NIPA); gallic acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, siringic acid, vanillic acid (Fluka).

(IV) Non-phenolic acids: benzoic acid, cinnamic acid (Carlo Erba); shikimic acid (Fluka).

(V) Catechins and tannins: D(+)-catechin (Schuchardt, Munich, G.F.R.); L(−)-epicatechin, tannic acid (Fluka).

(VI) Flavonols: fisetin, myricetin (J. T. Baker); quercetin, kaempferol (Fluka); morin, robinetin (NIPA).

(VII) Flavanones: hesperetin, dihydrofisetin, taxifolin (J. T. Baker); naringenin (NIPA).

(VIII) Flavanols ond flavanone glycosides: myricitrin (J. T. Baker); rutin, quercitrin, naringin (Fluka).

Samples

Samples containing five or six phenols of the same group were prepared in methanol–water (2:3) and acidified to pH 5.0 with dilute hydrochloric acid. The concentration of each compound was 0.05 mg/ml. The analyzed substances were also individually chromatographed.

The same conditions were used in preparing a mixture of four phenolic compounds of different classes (D(+)-catechin, catechol, quercetin and 2,3-dihydroxybenzoic acid).

Preparation of the resin

The resin was equilibrated in boiled distilled water, then in 0.1 *N* HCl, 0.1 *N* NaOH and methanol, which were removed, after each step, by repeated washing with boiled distilled water. The column (20 × 10 cm) was water-jacketed; the bed dimensions were 1 × 5 cm.

Chromatographic procedure

Volumes of 2 ml of the samples were diluted with an equal volume of boiled distilled water and the solutions passed through the resin. Elution was carried out as follows: water; methanol–water (2:3); methanol–water (3:1); absolute methanol; 0.1 *N* NaOH in water; and finally 0.2 *N* NaOH in methanol. The flow-rate was 30 ml/h at a temperature of 20°. Fractions of 5 ml were collected and immediately acidified to pH 5 with dilute hydrochloric acid in order to prevent the auto-oxidation of the solutes.

Analysis of the column effluent

The eluate from the column was treated with diazotized benzidine[12] and after 1 min the absorbance at 440 nm was measured with a Beckman colorimeter *versus* a blank. Also, thin-layer chromatography of the eluted products was carried out on silica gel 1B Baker-flex sheets. The following eluents were used: (1) benzene–methanol–acetic acid (45:8:4) and benzene–dioxan–acetic acid (90:25:4) for phenols and phenolic acids[13]; (2) dioxan–*n*-heptane–acetone–formic acid (20:5:1:1) for flavonoids[14] (aglycones and glycosides).

After drying, the chromatograms were examined in UV light and sprayed with diazotized benzidine solution. In order to reveal the phenolic acids, the plates were sprayed with a 0.04% solution of bromocresol purple indicator in ethanol–water (1:1) at pH 10.0 (ref. 15), before spraying with diazotized benzidine.

RESULTS AND DISCUSSION

The phenolic compounds considered and the eluents used are listed in Table I. The methanol–water mixtures and absolute methanol revealed a low tendency to detach the phenolic compounds from the binding resin, but solutions containing hydroxyl ions showed a greater eluting power.

TABLE I

ELUTION OF PHENOLIC COMPOUNDS ON AMBERLITE IR-45 (OH^-)

Compound	K_{diss}*	*Eluent*				
		Methanol–H_2O (2:3)	*Methanol–H_2O (3:1)*	*Methanol*	*0.1 N NaOH*	*Methanolic 0.2 N NaOH*
Coumarins						
Coumarin		+				
Esculetin			+			
Daphnetin			+			
4-Methylesculetin		+				
Scopoletin		+				
Phenols						
Phenol			+			
o-Cresol			+			
p-Cresol	$6.7 \cdot 10^{-11}$		+			
p-Methoxyphenol	$1.53 \cdot 10^{-11}$		+			
Thymol			+			
2,6-Dimethylphenol	$2.57 \cdot 10^{-11}$		+			
2,6-Dimethoxyphenol			+			
Catechol	$7.5 \cdot 10^{-10}$		+			
Resorcinol	$1.55 \cdot 10^{-10}$		+			
Hydroquinone	$1.41 \cdot 10^{-11}$		+			
Pyrogallol	$9.67 \cdot 10^{-10}$					
Phloroglucinol	$3.56 \cdot 10^{-9}$		+			
3-Methylcatechol				+		
4-Methylcatechol			+			
3-Methoxycatechol				+		
4-Chlorocatechol				+		
4-Nitrocatechol					+	
Saligenin	$1.02 \cdot 10^{-10}$	+				
DL-DOPA		+				
Phenolic acids						
Salicylic	$1.07 \cdot 10^{-3}$					
p-Hydroxybenzoic	$2.6 \cdot 10^{-5}$				+	
2,3-Dihydroxybenzoic					+	
3,4-Dihydroxybenzoic	$3.3 \cdot 10^{-5}$				+	
2,3-Dihydroxy-4-methoxybenzoic				+		
Gallic	$3.9 \cdot 10^{-5}$				+	
Vanillic					+	
Siringic				+		
o-Coumaric	$2.43 \cdot 10^{-5}$				+	

* Dissociation constants in aqueous solutions at 25°. Values are taken from ref. 16.

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TABLE I *(continued)*

Compound	K_{diss}*	*Eluent*				
		Methanol–H_2O (2:3)	*Methanol–H_2O (3:1)*	*Methanol*	*0.1 N NaOH*	*Methanolic 0.2 N NaOH*
m-Coumaric	$4.02 \cdot 10^{-5}$				+	
p-Coumaric					+	
Caffeic	$2.43 \cdot 10^{-5}$				+	
Ferulic					+	
Sinapic				+		
Chlorogenic	$2.2 \cdot 10^{-3}$					+
3,4-Dihydroxy-β-phenylpropionic						+
Non-phenolic acids						
Benzoic	$6.25 \cdot 10^{-5}$			+		
Cinnamic	$3.65 \cdot 10^{-5}$			+		
Shikimic	$7.1 \cdot 10^{-5}$			+		
Catechins and tannins						
D(+)-Catechin		+				
L(−)-Epicatechin		+				
Tannic acid				+		
Flavonols						
Fisetin						+
Myricetin						+
Quercetin						+
Kaemperol						+
Morin						+
Robinetin						+
Flavanones						
Hesperetin		+				
Naringenin			+			
Dihydrofisetin				+		
Taxifolin						+
Flavonol and flavanone glycosides						
Rutin						+
Quercitrin			+			
Myricitrin			+			
Naringin		+				

* Dissociation constants in aqueous solutions at 25°. Values are taken from ref. 16.

Phenols, with a K_a value of about 10^{-10} or 10^{-11} (as catechol, resorcinol, etc.) and hydroxycoumarins are virtually undissociated at the pH of the experiments and are very weakly bound to the chromatographic bed, so that they could very easily be eluted with aqueous or absolute methanol. However, 4-nitrocatechol and phenolic acids could be eluted only by means of the displacement effect of hydroxyl ions.

Flavonols were eluted only with 0.2 *N* NaOH in methanol. Flavans, flavanones and dihydroflavonols (except taxifolin) were easily eluted with neutral solvents, possibly because the B-ring is not conjugated with the A-ring in a

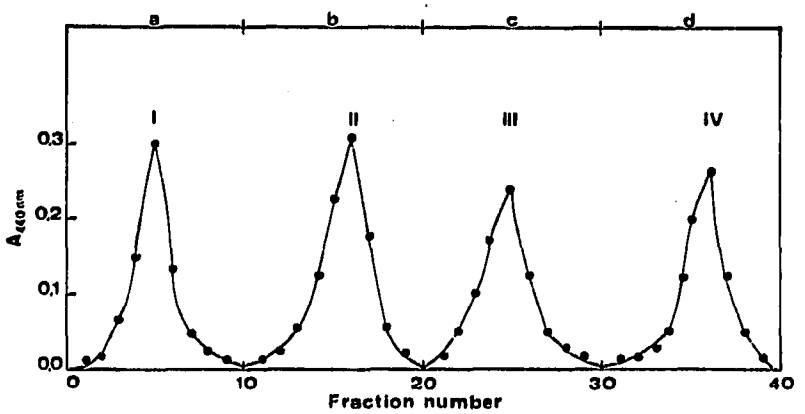

Fig. 1. Separation of D(+)-catechin (I), catechol (II), 2,3-dihydroxybenzoic acid (III), quercetin (IV), on Amberlite IR-45 with methanol–water (2:3) (a), methanol–water (3:1) (b), aqueous 0.1 *N* NaOH (c) and methanolic 0.2 *N* NaOH (d).

resonating system. A similar suggestion may be advanced in order to explain the different behaviour of shikimic acid and the phenolic acids.

The comparison between catechin and taxifolin confirms the importance of the role played by the carbonyl group. Moreover, the behaviour of taxifolin and dihydrofisetin show that the presence of a hydroxyl group in the 5-position is the major reason for the attachment of such substrates to the resin. Glycosylation of the 3- and 7-positions has a partial blocking effect, except for rutin, which can be eluted only with alkaline methanol, probably because of the different solubility compared with that of the other glycosides.

The adsorbtion by the resin of the phenolic compounds could be ascribed to the formation of a hydrogen bond between the electron pair on the amine nitrogen of the resin and the hydroxyl hydrogen of the phenols. Moreover, it is possible to suggest the formation of a bond between the hydrogen atoms of the amine group and any phenolate ion present[7].

The chromatography of a mixture of four phenols belonging to different classes gave predictable results. The four phenols were eluted in the following order: D(+)-catechin (methanol–water, 2:3); catechol (methanol–water, 3:1); 2,3-dihydroxybenzoic acid (aqueous 0.1 *N* NaOH); quercetin (methanolic 0.2 *N* NaOH).

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