Separation of flavonoid compounds on Sephadex LH-20

In the study of plant phenolics, the isolation of flavonoids by adsorption chromatography is difficult because these compounds bind to many chromatographic media[1]. Countercurrent distribution and polyamide columns have been used successfully for some separations, but the number of techniques available for such investigations is otherwise very limited.

Several reports[2–4] have described the successful analytical separations of flavonoid compounds on Sephadex G-25, which has a well-known affinity for aromatic, heterocyclic and basic compounds[5–8]. SJOVALL AND VIHKO[9] and WILK and his collaborators[10] observed the analogous adsorptive properties of the polyalkylated dextran gel, Sephadex LH-20, so it was of interest to study this gel as a potentially valuable new tool for flavonoid separations.

Materials and methods

Sephadex solvent-resistant columns (2.5 × 45 cm), LH-20 gel and Blue Dextran (Pharmacia, Inc.)* were used throughout this investigation. All chemicals were commercially available or were synthesized. Preliminary results showed methanol to be a desirable eluant, so it was used exclusively.

After the swollen gel was poured and allowed to settle in the column, it was washed with a 5 ml/min downward flow of solvent, which compressed the gel slightly, before the upper adapter was adjusted flush to the top of the gel. Samples were injected directly into the solvent line, and were eluted immediately at a flow rate of 3–5 ml/min. Effluent was monitored by an ultraviolet spectrophotometer fitted with a flow cell. Void volume, V_0, was determined with blue dextran, which had been dissolved in water and then diluted with three parts methanol. Polyvinylpyrrolidine (mol.wt. ~40,000) in absolute methanol was eluted in the same volume. Data are reported in V_e/V_0, the ratio of elution volume to void volume.

Results and discussion

All compounds tested came off the column in reproducible elution volumes. Loss of samples onto the gel was negligible, although after several runs the gel did become colored, but without appreciable change in properties. This method is nondestructive and the purified compounds can easily be recovered from methanol. Band spreading is greater than on polyamide columns, which were tested under the same conditions, but the properties of the gel are considerably different[11].

The data in Table I indicate a definite correlation between elution volume and structure. These compounds are apparently adsorbed to the gel. Electrostatic interaction between these acidic compounds and charged groups on the gel would probably result in ionic exclusion and reduced elution volumes, rather than strong retention by the column. The degree of adsorption of the aglycones depends generally on the number of hydroxyl groups, but not on their acidity. It may be possible to explain the very strong adsorption of the flavonols as an effect of the intramolecular chelation of the hydroxyl groups at the 3 and 5 positions with the carbonyl at the 4 position[12]. This association stabilizes two "crossed" systems of conjugation, with a total of five

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

TABLE I

V_e/V_0 OF FLAVONOIDS ON SEPHADEX LH-20 IN METHANOL

Compound[a]	*Substituents*	V_e/V_0[b]
Flavone		
Apigenin	5,7,4′-OH	5.3
Luteolin	5,7,3′,4′-OH	6.3
Resokempferol	3,7,4′-OH	5.9
Fisetin	3,7,3′,4′-OH	6.6
Robinetin	3,7,3′,4′,5′-OH	7.4
Kempferol	3,5,7,4′-OH	7.5
Quercitin	3,5,7,3′,4′-OH	8.3
Morin	3,5,7,2′,4′,-OH	4.4
Myricitin	3,5,7,3′,4′,5′,-OH	9.2
3-O-Methylquercitin	5,7,3′,4′-OH; 3-OMe	5.6
Azaleatin	3,7,3′,4′-OH; 5-OMe	5.9
Isorhamnetin	3,5,7,4′-OH; 3′-OMe	7.0[c]
Tamarixetin	3,5,7,3′-OH; 4′-OMe	7.3
Rhamnetin	3,5,3′,4′-OH; 7-OMe	7.7
Penta-O-methylquercitin	3,5,7,3′,4′-OMe	3.4
Hexa-O-methylmyricitin	3,5,7,3′,4′,5′-OMe	3.1
Quercitin penta-acetate	3,5,7,3′,4′-OAc	2.7
Quercitrin	5,7,3′,4′-OH; 3-rhamnoside	4.9
Rutin	5,7,3′,4′-OH; 3-rhamno-glucoside	4.0
Robinin	3′,4′,5′-OH; 3-galactorhamno-side; 7-rhamnoside	3.3
Flavanone		
Naringenin	5,7,4′-OH	5.4
Eriodictyol	5,7,3′,4′-OH	5.8
Taxifolin	3,5,7,3′,4′-OH	5.5
Hesperetin	5,7,3′-OH; 4′-OMe	5.2[c]
Naringin	5,4′-OH; 7-rhamnoglucoside	3.6
Astilbin	5,7,3′,4′-OH; 3-rhamnoside	4.2
Hesperidin	5,3′-OH; 4′-OMe; 7-rhamno-glucoside	3.4[c]
Catechin		
d-Catechin	3,5,7,3′,4′-OH	5.2

[a] Samples: 2.5 mg/0.5 ml; flow rate, 3–5 ml/min.
[b] Under these conditions, $V_e/V_0 = 2.2\ K_d + 1$.
[c] Sample dissolved in dioxane–methanol (1:1).

rings in a planar conformation, decreasing the solubility of the molecule in the polar solvent phase. Thus, the flavones are eluted faster than their flavonol analogs, and 3-O-methylquercitin and azaleatin, faster than quercitin itself. Flavanones and catechins also lack this conjugation and come off the column at about the same time. Morin, although it is a flavone, is eluted even more easily due to out-of-plane association of the 2′-hydroxyl group with the pyranyl oxygen. The glycosides, with much larger molecular weights, undergo gel sieving as well as adsorption.

Several runs were made to determine whether the capacity of LH-20 was adequate for preparative-scale separations (see Fig. 1). Mixtures of impure quercitin and rutin, 250 mg/25 ml, came down the column in two pale yellow bands, leaving a brown deposit at the top. At slightly higher concentrations, these colored impurities remained in the phenolic fractions.

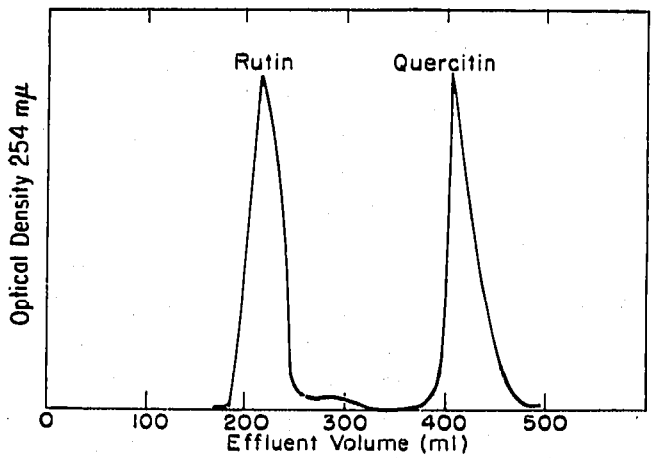

Fig. 1. Separation of rutin (166 mg) and quercitin (75 mg). Column: 40 g Sephadex LH-20, 2.5 × 33 cm. Sample size: 22 ml. Solvent: methanol; flow rate, 4 ml/min. Fractions of 10 ml collected.

Sephadex LH-20 combines adsorption with molecular sieving in the separation of flavonoid compounds. In non-aqueous solvents the gel is an efficient, high-capacity medium for analytical and preparative work.

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