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Fractionation of Some Plant Estrogens and their Animal Excretion Metabolites on Dextran Gels

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Some experiments with Sephadex gel filtration in the separation of isoflavones and their animal excretion products are reported and discussed. The fractionation of the compounds is rather a result of different adsorption to the gel matrix than of a molecular sieving.

Gel-filtration has been used for separating substances of different molecular size [1]. Besides the molecular sieve action, the gel material also exerts an adsorptive effect on substances of aromatic and heterocyclic character [2]. This secondary effect was taken advantage of in the present separation of four plant estrogens in a mixture: biochanin A, genistein, formononetin and daidzein. The compounds are all isoflavones. Biochanin A, genistein and formononetin occur in the common forage plant, red clover *(Trifolium pratense)* [3–5]. The substances are also found in other species of the family *Leguminosae* [6]. Biochanin A is on account of its higher concentration responsible for the main estrogenic activity of the red clover [3]. The specific activity of both biochanin A and genistein is about 10^{-5} times that of diethylstilbestrol [7]. As to formononetin its estrogenic activity is negligible [8]. Daidzein never occurs free in nature but is found in soybeans *(Soja hispida)*, bound as a glucoside [9]. Some authors report that the compound is completely inactive as an estrogen by injection [6], others that its estrogenic activity when given orally is even greater than that of genistein [8].

Biochanin A, labelled with tritium, was used in metabolic studies with rats and sheep [10]. In the present study the gel filtration technique was used for separating conjugates and free metabolites of biochanin A from urine and feces extracts of rats, which had been given an intraperitoneal injection of the compound.

EXPERIMENTAL

Materials. Sephadex G-25, coarse, (manufactured by Pharmacia, Ltd, Uppsala), was used in this investigation throughout. This type of gel allows an entry of molecules with a maximum molecular weight of 1 000 – 4 000.

The columns were packed according to directions in the paper of Porath [9]. An account of the size of the columns and the elution performance is given in connection with each experiment described.

The four isoflavones were synthesized according to known methods [11,12].

The tritium labelling of biochanin A was performed by the Wilzbach method [13] at the Pharm. Dept., Kungl. Veterinärhögskolan, Stockholm. By chromatography and recrystallizations, the labelled compound was purified from tritiated by-products and labile tritium [10]. The labelled substance was mixed with carrier biochanin A to give a final specific activity of 0.2—0.5 μC/mg. Adult rats were given biochanin A by intraperitoneal injections. A very fine suspension of the compound in physiological saline was used as the injection medium. Urine and feces were collected from the period 0—48 h following injection.

The urine samples were put on the columns directly or else following a pretreatment described below. An extract of feces was prepared. The details of the extraction procedure are given along with the description of the gel filtration experiment.

Analytical methods. Ultraviolet absorbing zones in the eluates from the columns were recorded at 2537 Å with an Uvicord Ultraviolet Absorptiometer (LKB-products, Stockholm). The isoflavones were identified by paper chromatography in different solvent systems together with reference substances [14]. Glucuronides were determined by the naphthoresorcinol method according to Fischman-Green [15]. Sulphuric esters were determined by the turbidimetric method of Sperber [16]. The occurrence of urea in the eluates was measured by the urease method of Van Slyke and Cohen [17]. The quantity of sodium chloride, as a representative of the inorganic salts, was determined by Volhard-Arnhold titration [18].

Aliquots of the fractions were analyzed for radioactivity in an Ekco 612 liquid scintillation counter at a temperature of −20°C. The samples were counted both with and without an internal standard of a known amount of biochanin A of high activity. The radioactivity in each eluate could therefore be determined quantitatively.

RESULTS AND DISCUSSION

Fractionation of four isoflavones in a model experiment.

The molecular structures of the four compounds are given in Fig. 1. These isoflavones are soluble in alkaline solution but insoluble in diluted acid or water. They are, however, susceptible to even very mild alkaline hydrolysis, which opens the pyrone ring in the molecule. It was therefore convenient to choose the relatively weak base of 0.1 M ammonium hydroxide as the eluant. This eluant also has the advantage of being volatile. A mixture of 5 mg of each of the isoflavones, biochanin A, genistein, formononetin, and daidzein, was filtered through the first column (Fig. 2). It was difficult to dissolve the isoflavone crystals directly in the diluted ammonium hydroxide solution in a sufficiently small volume. They were therefore first dissolved in 5 ml 0.1 N NaOH. This solution was neutralized with diluted HCl. A very fine suspension of the com-

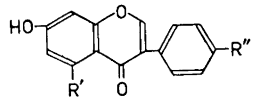


Fig. 1. Molecular structure of genistein, biochanin A, formononetin and daidzein.

Genistein	R′ = OH	R″ = OH	Formononetin	R′ = H	R″ = OCH_3
Biochanin A	R′ = OH	R″ = OCH_3	Daidzein	R′ = H	R″ = OH

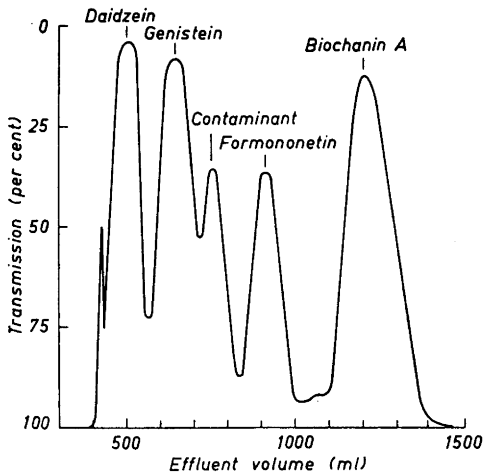


Fig. 2. Filtration diagram of a mixture of genistein, biochanin A, formononetin and daidzein on a gel column of the size 4.5 × 37 cm. Eluant: ammonium hydroxide.

pounds in saline was then obtained. The suspended particles were now readily dissolved when the solution was adjusted to be 0.1 M with regard to NH_4OH in a final volume of 10 ml. The sodium chloride in the solution left the column far ahead of the isoflavones.

The column was of the dimensions 4.5 × 37 cm and had a volume of 500 ml. The rate of the elution was 1 ml per minute. The outer or void volume was experimentally determined as the elution volume for serum albumin from horse, with 0.1 M NH_4OH as the eluant. It was found to be 165 ml. It is not possible to explain the position of the different isoflavones in the Sephadex chromatogram from the differences in their chemical configurations.

The elution volumes are the same if the compounds are put on the column alone or together in a mixture.

Fractionation of urine metabolites

Pretreated urine samples. Each of 35 rats were injected intraperitoneally with 30 mg of biochanin A (specific activity 0.23 μC/mg). The urine, 330 ml, contained about 30 % of the injected radioactivity. By four extractions with 300 ml butanol, 80 % of the radioactivity in the urine was transferred into the butanol phase. The solvent was evaporated under vacuum and the dry residue, 12 g, was dissolved in hot methanol and cooled. To its volume was added three times this volume of ether. The methanol-ether soluble part of the urine contained 56 % of the radioactivity of the untreated urine. The precipitate which was formed when the ether was added to the methanolic solution contained the rest of the butanol extractable substances, 7.5 g, and 24 % of the radioactivity. It contained no free isoflavones.

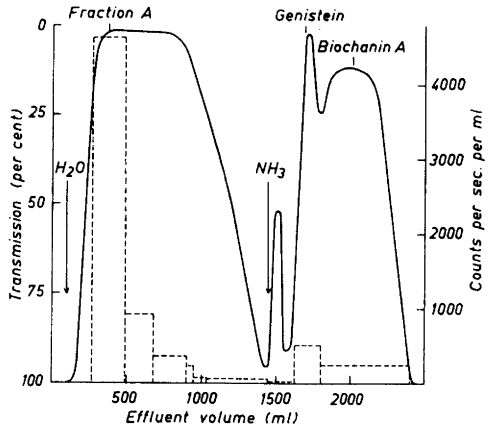


Fig. 3. Diagram showing the fractionation of a methanol-ether extract of 330 ml of urine on a column of the size 4.5 × 26 cm. Eluant: I water and II ammonium hydroxide. Ultraviolet absorption curve ————. Radioactivity of pooled eluates --------. Fraction A contains unknown metabolites.

In Fig. 3 is illustrated the separation of the biochanin A metabolites in the methanol-ether soluble part of the urine. The solvent was evaporated and the dry residue, 4.5 g, was dissolved in 18 ml H_2O. The separation was performed on a Sephadex column, 4.5 × 26 cm, and with a gel volume of 360 ml. 1400 ml distilled water was filtered through the column. When the ultraviolet absorption curves, recorded during the elution, returned to zero, the eluant was changed to 0.1 M NH_4OH. The rate of the elution was 3 ml/min.

The bulk of the labelled compounds left the column without being strongly retarded. The radioactive eluates contained glucuronic acid and organic sulphates. A limited fractionation was achieved but these eluates have to be further purified before identification of labelled metabolites is possible.

A small amount of biochanin A passed the body unchanged. Another part of the compound was demethylated in the liver to genistein [19]. The free isoflavones could not be eluted with pure water, but were easily displaced from the column by the ammonium hydroxide solution and were recovered essentially free from contaminants. The two fractions containing the isoflavones overlap each other, but could be well separated by repeated gel filtration, Figs. 4 and 5. This second fractionation was performed at a later occasion. Samples had been taken for different analysis, and account for the deficit in material used for the second filtration. A Sephadex column was used with the dimensions 4.5 × 37 cm and with a gel volume of 500 ml. The dried eluates from the first separation were dissolved in 15 ml 0.1 M NH_4OH and the column was eluted with the same solvent, at the rate of 3 ml/min. An estimation of the recovered radioactivity gave 2.2 % in the genistein and 5.6 % in the biochanin A fraction of the total radioactivity in the urine sample. There is thus a very small amount of unconjugated isoflavones in the urine.

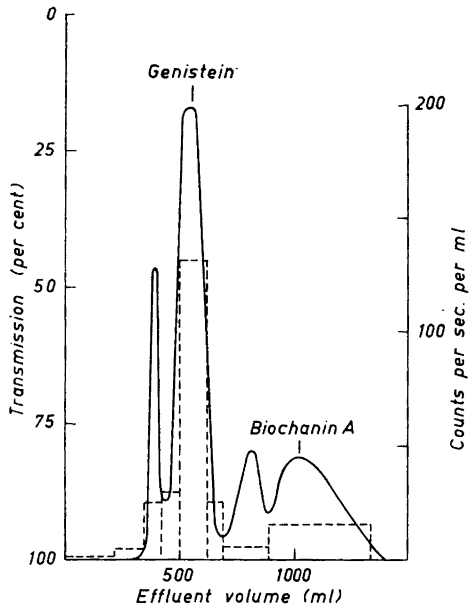


Fig. 4. Second filtration of the fraction "genistein" (Fig. 3) on a column of the size 4.5 × 37 cm. Eluant: ammonium hydroxide. Ultraviolet absorption curve ———. Radioactivity of pooled eluates ------.

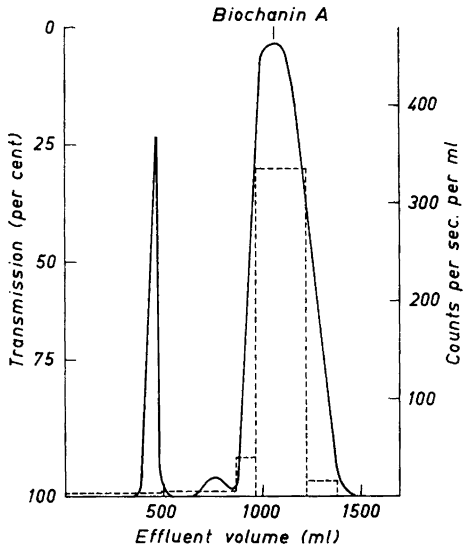


Fig. 5. Second filtration of the fraction "biochanin A" (Fig. 3) on the same column and under the same conditions as in Fig. 4.

Untreated urine. During the extraction procedures described above a large part of the metabolites was lost judging from the recovery of radioactivity in the extracts. It was therefore attempted to put the urine sample directly on the column without any preliminary purification. A total of 70 ml of urine was collected from five rats, each of which had been injected with 25 mg of labelled biochanin A (specific activity 0.5 μC/mg). The urine contained 28 % of the injected radioactivity.

The ultraviolet absorption of the isoflavone fractions from the crude urine would have been rather weak due to the low concentration of these compounds in the sample. Carrier isoflavones were therefore added to mark the ultraviolet absorbing zones of genistein and biochanin A. 10 mg each of unlabelled genistein and biochanin were suspended in 20 ml saline and added to the urine samples. There was no visible precipitate in the diluted urine.

The dimensions of the column were 4.5 × 37 cm with a gel volume of 500 ml. Distilled water was used as the first eluant. The effluent was collected in fractions of 23—25 ml every 20 min. When 1400 ml water had passed through the column the eluting agent was changed to 0.1 M NH_4OH. The distribution of the radioactive metabolites is shown in Fig. 6. Two highly radioactive fractions (Fig. 6 A) eluted with water, contained both glucuronic acid and organic sulfate conjugates (Fig. 7). The labelled compounds amounted to only a minor

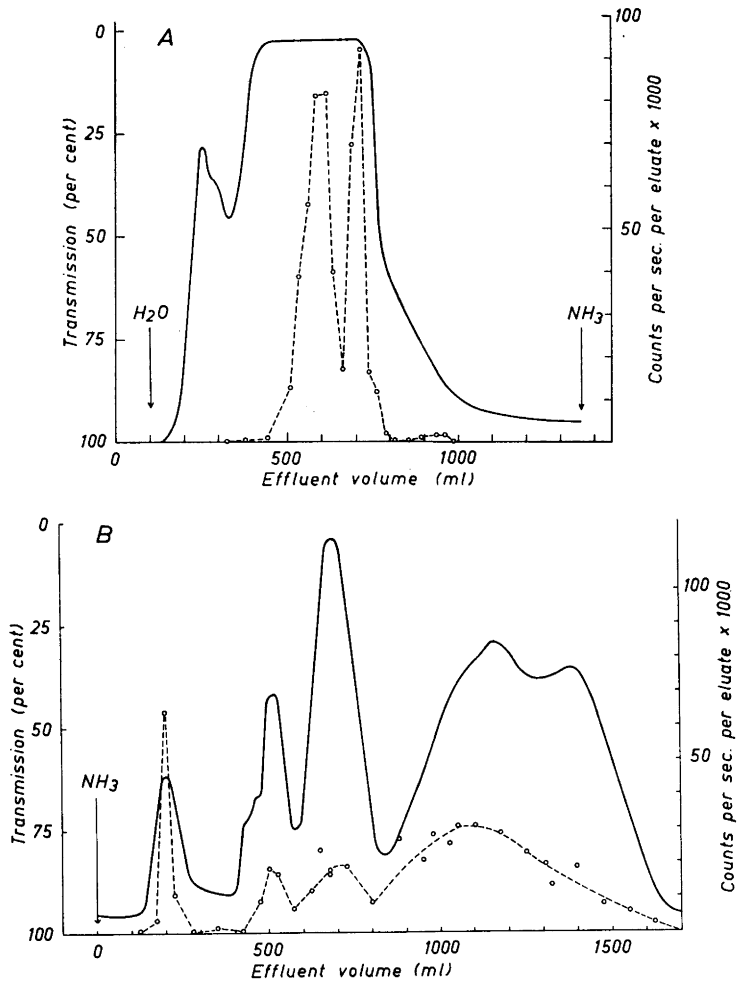


Fig. 6. Gel filtration of an untreated urine sample (70 ml). Column: 4,5 × 37 cm. Eluant: I water (Diagram A) and II ammonium hydroxide (Diagram B). Ultraviolet absorption curve ————. Radioactivity of individual eluates O-----O. Eluate volume 23—25 ml.

part of the substances in these eluates, which contained all the urine conjugates of glucuronic acid and of sulfate. Urea and sodium chloride move a little ahead of the conjugates (Fig. 8). An attempt was also made to separate the crude urine sample with 0.1 M NH_4OH, as the only eluant. However, a better separation of the conjugates was not obtained. The eluates with pure water were used directly for enzymatic hydrolysis with glucuronidase and sulfatase enzymes, while the ammonium hydroxide eluates had to be freed from ammonia before the enzymatic hydrolysis. It is therefore preferable to make the

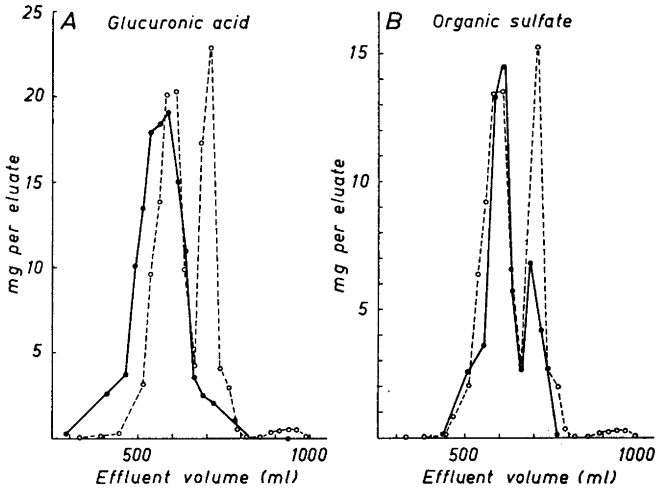


Fig. 7. Diagram showing the distribution of glucuronic acid ●———● (Diagram A) and organic sulfate ●———● (Diagram B) in the eluates of the urine sample from Fig. 6 A. Radioactivity curve O-----O.

elution in two steps: first with water and then with 0.1 M NH_4OH. The results from the analysis of the water soluble metabolites will be published later.

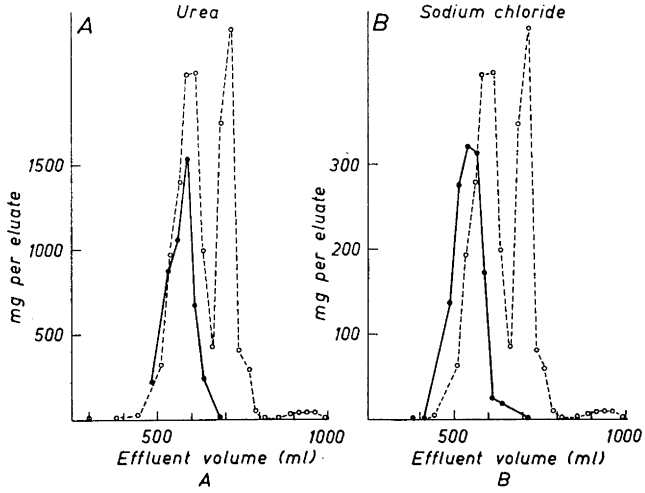


Fig. 8. Diagram showing the distribution of urea ●———● (Diagram A) and sodium chloride ●———● (Diagram B) in the eluates of the urine sample from Fig. 6 A. Radioactivity curve O-----O.

The radioactivity peaks in Fig. 6 B coincide with the peaks of the ultraviolet absorption curves for genistein and biochanin A. Two unknown metabolites of low concentration move ahead of the genistein and biochanin A fractions.

The recovery of the radioactivity was quantitative to the extent that it could be estimated from a summation of the analyzed individual eluates. About 90 % of the labelled compounds were obtained in the purely water soluble fractions. The genistein fraction contained about 1 % and the biochanin A about 8 % of the radioactivity in the urine. This corresponded to 0.34 mg genistein and 2,7 mg biochanin A from the labelled injected compound.

Fractionation of metabolites in feces

Feces samples were collected from 15 rats, each of which had been injected with 25 mg of tritiated biochanin A (specific activity 0.18 μC/mg). The feces sample was repeatedly extracted with hot 95 % alcohol. The alcohol extract contained 60 % of the injected radioactivity. No more radioactivity could be extracted from the feces by additional extractions with other solvents. The alcoholic extract was further purified as illustrated in the following scheme. The radioactivity of the crude alcoholic extract is indicated as 100 %. The recovered radioactivity during the purification steps are given within the parenthesis.

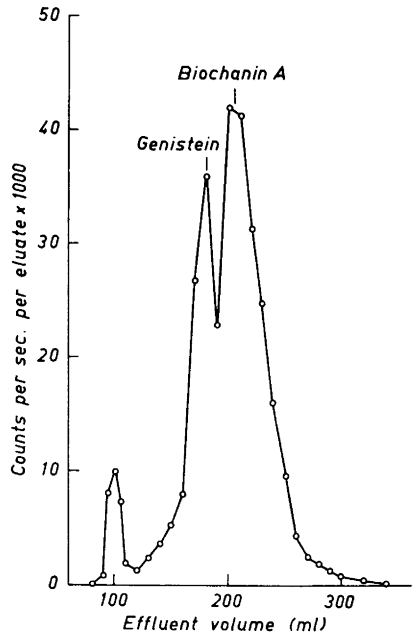
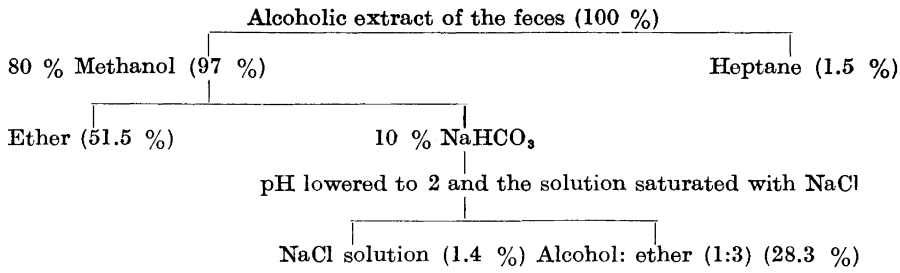


Fig. 9. Gel filtration of an ether extract of feces. Column size: 1.8 × 42 cm. Eluant: ammonium hydroxide. Radioactivity curve O———O.



The ether extract was dried and the residue dissolved in 0.1 M NH_4OH and filtered through a Sephadex column 1.8 × 42 cm, with a gel volume of 110 ml. Eluates of 5 ml were collected every 30 min. The distribution of the radioactivity is shown in Fig. 9. The bulk of the radioactive compounds in the ether extract consisted of genistein and biochanin A. As the load of the column, 100 mg, was very large in proportion to its size (100 ml gel volume) it was not possible to get a complete separation of the two isoflavones. A second gel filtration with the genistein and biochanin A fractions on a column of the size 4.5 × 26 cm gave a better separation of the zones (Figs. 10 and 11). It was estimated that the feces contained about 40 % biochanin A and 14 % genistein, which were not conjugated. The alcohol-ether soluble part of the feces was also filtered through a gel column, but no fractionation was obtained in this experiment. The occurrence of such a large amount of free isoflavones in the feces may naturally be the result of hydrolysis of conjugates in the in-

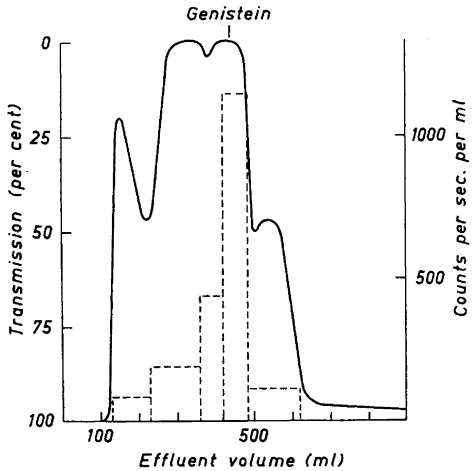


Fig. 10. Second filtration of the fraction "genistein" (Fig. 9) on a column of the size 4.5 × 26 cm. Eluant: ammonium hydroxide. Ultraviolet absorption curve ————. Radioactivity of pooled eluates --------.

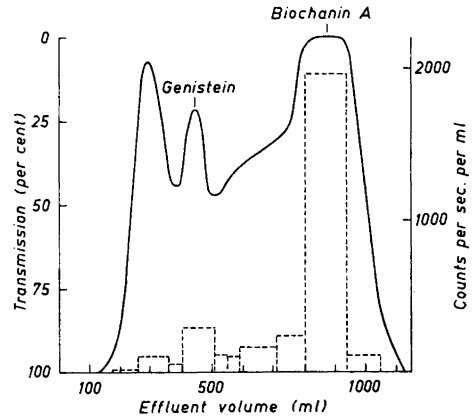


Fig. 11. Second filtration of the fraction "biochanin A" (Fig. 9) on the same column and under the same conditions as in Fig. 10.

testines by the fecal flora. The demethylation to genistein does however not take place in the intestines, but rather in the liver [19]. Incubation of biochanin A with intestine contents gave no genistein.

DISCUSSION

The four isoflavones in the model experiment are of very equal molecular size. The good separation obtained is therefore probably the result of a different adsorption to the gel matrix. The separation procedures were also applicable to biological fluids and extracts of biological material. When water was used as the eluant, the isoflavones were retained in the upper part of the column. They could be eluted with fairly high purity by ammonium hydroxide, when all water soluble components had left the column. The amount of substances and the load volumes, put on the columns, have been very large in these experiments. Nevertheless a good fractionation of the metabolites has been obtained. Work is in progress to study if the new finely ground Sephadex will give an improved separation.

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