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FORAGE ESTROGENS

Detection of Daidzein, Formononetin, Genistein, and Biochanin A in Forages

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Daidzein, not previously reported in forages, was found in alfalfa, and ladino, red, and subterranean clovers. Formononetin and genistein, isolated earlier from red and subterranean clovers, have now also been found in alfalfa and ladino clover. Biochanin A, previously reported in red and subterranean clovers, was found in alfalfa but not in ladino clover. A combination of three sequential purifications on paper chromatograms followed by one on silicic acid chromatostrips was used to isolate the isoflavones. The identity of the compounds was confirmed by absorption spectra as well as by comparison of paper and silicic acid chromatograms of the isolated compounds with those of authentic samples of the isoflavones.

Interest in naturally occurring forage estrogens has increased in recent years, especially in view of the restrictions on the use of synthetic estrogens in animal feeds. Although coumestrol has been found in a number of more important forages (*2, 4*), the estrogenic isoflavones, formononetin, genistein, and biochanin A, known to be present in red and subterranean clovers (*7*), have not as yet been reported in alfalfa nor in any varieties of white clover. The closely related isoflavone, daidzein, has been reported only in *Pueraria sp.* root (*9*). Since genistein (4′,5,7-trihydroxyisoflavone) has been found together with its 4′-methyl ether, biochanin A, in some forages, it also seems logical to expect the presence of daidzein (4′,7-dihydroxyisoflavone) in those forages which contain its 4′-methyl ether, formononetin. The present experiments were undertaken to determine the presence or absence of daidzein, formononetin, genistein, and biochanin A in several important forages.

Experimental

For this study four representative forage meals were employed: alfalfa (*Medicago sativa*), ladino clover (*Trifolium repens*, var. *ladino*), red clover (*Trifolium pratense*), and subterranean clover (*Trifolium subterraneum*). Each meal was shown to be estrogenically potent by a mouse assay (*1*).

Forage extracts were made in Soxhlet apparatus of 300 grams of each of the dried meals. Preliminary extraction of the meals with Skellysolve B for 24 hours removed the fats and lipochromes, which were discarded. A subsequent extraction of the meal with acetone for 24 hours removed the estrogens (*1*). The resulting acetone extracts were concentrated under reduced pressure to 300 ml. each and kept as stock solutions for all further work. The same chromatographic procedures were used to examine and to isolate the isoflavones, if present, from each of the four forages. To serve as guide spots, samples of the authentic isoflavones were applied in solution to each chromatogram in the purification procedures.

Detection of Daidzein (Table I). Step 1. One hundred milliliters of the acetone extract was applied in 10-ml. portions to 10 sheets of Eaton and Dikeman No. 301 paper. The extract was applied as a narrow band 5 cm. from the long edge of an $18^1/_4 \times 22^1/_2$ inch sheet and a 5-cm. zone was left at one end, so that a 25-μg. reference spot of daidzein could be chromatographed at the same time. The sheets were developed simultaneously by ascending chromatography with the upper phase of a benzene–acetic acid–water (2:2:1) solvent mixture for 4 hours at room temperature. Daidzein and formononetin were located easily on the paper by visual inspection under an ultraviolet source with a peak at 3600 A. The exposure of the paper to ammonia vapors greatly increased the intensity of fluorescence of both daidzein and formononetin.

Under our laboratory conditions, the reference spots of daidzein had an average R_f = 0.22, measured to the leading edge of the spot. However, the daidzein from the extract appeared to have been carried somewhat further, probably because of the presence of other materials. After the developed chromatograms had been dried at room temperature, the paper in an area between the origin and R_f = 0.40 was removed, cut into small pieces, and treated with four 800-ml. portions of warm methanol.

Step 2. The combined methanol solution was concentrated under reduced pressure to 100 ml. and applied to 33 sheets of Whatman 3-mm. paper (each $18^1/_4 \times 22^1/_2$ inches) in the manner described previously. These sheets were developed in acetone-water (3:7) for 16 hours. Daidzein reference spots advanced to R_f = 0.85. Corresponding fluorescent bands on the sheets were cut out, the absorbed material was extracted

with four 200-ml. portions of warm methanol, and the solution was concentrated and rechromatographed with the same developing system.

STEP 3. The methanol solution obtained from Step 2 was concentrated under reduced pressure to 10 ml. and streaked on 10 sheets of Whatman 3-mm. paper for development with acetic acid–water–hydrochloric acid (3:6:1) for 16 hours. The well defined bands corresponding to the daidzein reference spots (R_f = 0.64) were cut out, the material was extracted with four 50-ml. portions of warm methanol, and its solution was concentrated to 2.0 ml.

STEP 4. A total of 0.4 ml. of this concentrate was applied to twenty $^1/_2$-inch-wide silicic acid chromatostrips (*5*, *6*) with a 5-μl. capillary pipet. The strips were developed in test tubes with ethyl acetate–Skellysolve B (3:1); daidzein appearing as an absorbing spot at R_f = 0.76 when viewed under ultraviolet light from a 2537-A. source. Reference daidzein was spotted and developed concomitantly on separate strips.

The silicic acid containing the dark spots was carefully removed from the glass strips and eluted with methanol, and the solution was filtered and evaporated to dryness. The solids were washed twice with 1- to 2-ml. portions of water to remove additional impurities. The washed residue was dried and taken up in 5.0 ml. of methanol. The methanol solution of the purified daidzein was used to obtain ultraviolet absorption spectra and for further chromatographic comparisons with an authentic sample of daidzein. Ultraviolet spectra were determined on a recording spectrophotometer, normally in a 1-cm. fused silica cell. The addition of aqueous sodium hydroxide solution to bring the solution to 0.001*N* produced an auxochromic effect for comparison with that produced by alkali on authentic daidzein.

Detection of Formononetin, Biochanin A, and Genistein. Formononetin, biochanin A, and genistein were isolated and identified by the general procedure employed for the daidzein, with only minor changes. Since neither genistein nor biochanin A fluoresces under ultraviolet light (3600 or 2537 A.), it was necessary to spray the section of chromatogram containing the guide spots with diazotized sulfanillic acid–sodium carbonate in order to determine the location of these compounds on the chromatographic papers. R_f values of the reference spots of each of the isoflavones are summarized in Table I.

An ethyl acetate–Skellysolve B (1:1) system (Step 5, Table I) proved to be better for development of formononetin on silicic acid chromatostrips than the developer used for the other isoflavones. The chromatographic technique used for the purification of each of the isoflavones is summarized in Table I.

Table I. Sequence of Chromatographic Solvent Systems for Isolation of Daidzein, Formononetin, Genistein, and Biochanin A

Step	Type of Chromatography	Solvent System	R_f Values: Daidzein	Formononetin	Genistein	Biochanin A
1	Heavy paper (E & D No. 301)	Benzene–acetic acid–water (2:2:1, upper phase	0.22	0.70	0.15	0.84
2	Medium paper (Whatman 3 mm.)	Acetone-water (3:7)	0.85	0.70	0.76	0.78
3	Medium paper (Whatman 3 mm.)	Acetic acid–water–hydrochloric acid (3:6:1)	0.64	0.72	0.60	0.66
4	Silicic acid chromatostrip	Ethyl acetate–Skellysolve B (3:1)	0.76	...	0.50	0.61
5	Silicic acid chromatostrip	Ethyl acetate–Skellysolve B (1:1)	...	0.56	...	...

Table II. R_f Values of Daidzein, Formononetin, Genistein, and Biochanin A on Two Types of Chromatograms

Solvent System	R_f Values: Daidzein	Formononetin	Genistein	Biochanin A
Whatman No. 1 Chromatographic Paper				
Acetone-water (3:7)	0.66	0.70	0.68	0.68
1-Butanol–acetic acid–water (4:1:5)	0.94	0.95	0.95	0.97
Potassium carbonate–water (5% by weight)	0.20	0.12	0.14	0.15
Acetic acid–water (1:1)	0.81	0.83	0.79	0.84
Isopropyl alcohol–water (6:4)	0.91	0.96	...	...
Benzene–acetic acid–water (4:4:2) upper phase	0.08	0.85	0.10	0.90
Ethyl acetate–water to saturate	1.00	0.98	0.93	0.97
Acetic acid–water–hydrochloric acid (3:6:1)	0.80	0.72	0.67	0.71
Silicic Acid Chromatostrips				
Ethyl acetate–Skellysolve B (3:1)	0.76	0.88	0.78	0.82
Ethyl acetate–Skellysolve B (1:1)	0.38	0.56	0.50	0.61
Acetone–ethyl acetate–Skellysolve B (4:3:3)	0.81	0.95	0.80	0.90
Ethyl alcohol–chloroform (1:3)	0.62	0.81	...	0.71
Ethyl alcohol–chloroform (1:1)	0.84	0.95	0.68	0.82
Ethyl ether–Skellysolve B (7:3)	...	...	0.36	0.60

Semiquantitative Estimations. To estimate the importance of each of the estrogenic compounds known to be present in forages, the size and intensity of color or fluorescence of spots developed by two-dimensional paper chromatography were compared with a graded series of spots from developed paper chromatograms containing known quantities of isoflavones. Acetic acid–water (60:40) followed by formic acid–water (40:60) were the developing systems found satisfactory for two-dimensional chromatography of the isoflavones. Daidzein and formononetin were observed as blue-violet fluorescent spots under 3600-A. ultraviolet light. Genistein and biochanin A spots were stained by spraying the developed chromatograms with bis-diazotized benzidine (*8*) and washed with water to eliminate the background color.

Coumestrol was determined by measurement of the intensity of fluorescence of the coumestrol spots on developed paper chromatograms (*3*). Although this method was developed specifically for alfalfa, the results obtained with the other forages are sufficiently accurate for the purposes of this comparison.

Results and Discussion

Although the paper and developing system used for the primary separation of the compounds only separated the components into rather broad bands, it was satisfactory. It permitted the loading and separation of large amounts of material, moving most of the remaining green pigment to the solvent front and widely separating daidzein and genistein from formononetin and biochanin A.

Table III. Approximate Amounts of Estrogenic Compounds Present in Each Forage

(P.p.m. in dry forage)

	Alfalfa	Ladino Clover	Red Clover	Subterranean Clover
Daidzein	<1	3	29	18
Formononetin	14	38	1700	900
Genistein	<1	1	40	850
Biochanin A	1–5	0	1000	500
Coumestrol	57	49	18	26

Closely following impurities made the four additional steps necessary for the complete purification of the isoflavones. A violet fluorescing zone which streaked up into the genistein and daidzein zone from below on the first chromatograms was eliminated by the 30% acetone development. Formononetin, with nearly identical fluorescing characteristics, tailed down into the daidzein zone on the first chromatograms and was not completely eliminated by the second system consisting of 30% acetone. The third system, consisting of acetic acid–water–hydrochloric acid, produced sharp separations of the reduced loadings and clearly separated the four isoflavones. The small amount of impurities still remaining were then readily eliminated by application of the silicic acid chromatostrip technique.

The identity of the isoflavones was confirmed by comparison of the ultraviolet absorption spectra of the isolated and authentic compounds, and by comparative paper and silicic acid chromatograms (Table II).

When the developed strips were viewed under ultraviolet light (2537 A.), absorbing spots of all four isoflavones were visible on the strips containing extracts of alfalfa and red and subterranean clovers. However, only genistein, daidzein, and formononetin were visible on the strips containing the ladino clover extract. Considering the sensitivity of the detection method, the sample of ladino clover examined must have contained less than 1 p.p.m. of biochanin A.

Table III shows the approximate quantities of the four isoflavones and of coumestrol found in the four forages studied.

Since coumestrol is more than 30 times as estrogenic as any of the isoflavones (*2*), the contribution of the isoflavones to the estrogenicity of alfalfa and ladino clover is insignificant. However, they are present in red and subterranean clovers in such large quantities that they account for most of the estrogenic activity of these forages.

Summary

The finding of genistein, daidzein, formononetin, and biochanin A brings the number of estrogenic compounds in alfalfa and red and subterranean clovers to five. Ladino clover extracts contain all these compounds except biochanin A, which was not detected by the methods used.

From visual inspection of the paper chromatograms of ladino clover and alfalfa extracts, the isoflavones appear to be present in much smaller quantity than coumestrol.

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