Large-Scale Processing of Alfalfa Meal for Coumestrol

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Dehydrated alfalfa meal, custom-produced to contain several times the normal amount of coumestrol, was processed to yield an acetone extract having a coumestrol content of 0.5% and containing 3% of the solids of the original meal. The concentrate was further processed to yield 2500 grams of crystalline coumestrol.

Increased growth rates have been obtained with sheep (*4*, *9*) and cattle (*8*) fed coumestrol-rich alfalfa meal. This growth promotion may be attributable either to the coumestrol or to unknown factors. A shortage of plant estrogen has prevented large-animal feeding tests to establish its effectiveness as a growth promoter. The work described in the present paper was undertaken to obtain enough coumestrol in concentrate and crystalline form to permit evaluation of its potential as a growth promoter.

Coumestrol in commercially produced alfalfa meal varies widely, ranging from less than 5 to about 100 p.p.m. (*3*). However, work undertaken by this laboratory in cooperation with the Crops Research Division of the Agricultural Research Service of the U. S. Department of Agriculture recently demonstrated that alfalfa containing over 600 p.p.m. can be produced (*5*).

In this study it was found that differences in coumestrol content between varieties were very small. At each cutting and location, coumestrol content usually increased with successive stages of growth and reached a maximum 25 days after full bloom.

Through cooperative arrangements with a number of commercial dehydrators, the authors collected 75 tons of dehydrated alfalfa meal which contained 340 to 560 p.p.m. of coumestrol and which was allowed to mature to seed pod stage before harvest (*6*). Some meal was employed directly in animal-feeding studies (*4*), and about 45 tons were processed to prepare a high-coumestrol concentrate. A portion of this concentrate was used directly for growth studies with large animals, and the remainder was used as a source of crystalline coumestrol. The procedure was similar to the original isolation of coumestrol in 1957 (*2*). However, the large amount of meal being handled required a number of modifications.

Coumestrol Recovery

Since no equipment for handling large quantities of meal was available in this laboratory, Nutrilite, Inc., San Jacinto, Calif., carried on the preliminary coumestrol recovery.

They treated 45 tons (37,530 kg. on a dry-weight basis) in U-shaped extractors, about 14 feet long by 6 feet wide by 8 feet deep. Each contained a multiple-ribbon flight agitator about 5 feet in diameter that turned at 33 r.p.m. Each extractor could handle 1 ton of alfalfa plus the requisite volume of solvent. The meal was first treated with hexane for 16 hours at a temperature between 38° and 55° C. The temperature of the mass was at first adjusted so that it was just below the boiling point of the solvent. The slurry was then filtered through a rotary vacuum filter, where most of the solvent was removed under vacuum, and the meal was washed with fresh solvent. The meal from this operation was passed through a vertical bank of four horizontally disposed, steam-jacketed screw conveyors for removal of solvent. The dried meal from this operation was recharged to the extractors and treated with acetone. Acetone was employed for extraction of coumestrol in an analytical method for coumestrol (*1*). The solvent extract from this operation was then pumped to a holding tank, from which it was fed to a large Rodney-Hunt Turba-Film evaporator operating under vacuum (25 to 26 inches of Hg). The solvent mixture was concentrated in this apparatus until the solids approximated 15%. The material from the evaporator was collected in surge tanks and pumped to a pot still for final concentration. The final result was a dark-green, extremely viscous sludge. It was frozen in nine 55-gallon steel drums to await further processing.

The source of the meal and data pertinent to the individual drums are presented in Table I. The total coumestrol extracted was 6215 grams or about 37% of that present, together with only 2.8% of the solids initially present in the meal. About 425 kg. of the concentrated acetone extract was employed in cooperative sheep- and steer-feeding experiments.

Sludge (1102 kg.), equivalent to 904-kg. dry weight and containing about 5000 grams of coumestrol, was processed in a batch operation by shaking 1500 ml. of sludge, 700 ml. of chloroform, and 350 ml. of 2.5*N* sodium hydroxide together in a 1-gallon container for 1 minute on a mechanical shaker. Longer shaking tended to form fairly stable emulsions. The entire contents of the container were centrifuged at 5° to 10° C. for 10 minutes. At the end of this time, the upper and lower phases were completely separated by a layer of partially solidified interfacial material. The aqueous upper phases from each of the tubes were then siphoned off, combined, and acidified to pH 3.5 with 6*N* hydrochloric acid. Solids began to precipitate immediately upon neutralization. After 30 minutes, they had coagulated into a gummy mass adhering to the bottom of the container.

By this procedure, 90 kg. of precipitate containing about 2500 grams of coumestrol were recovered. The coumestrol represented about 3% of the weight of the precipitate. The remaining coumestrol was distributed among several fractions: chloroform phase,

Table I. Preparation of Acetone-Extracted Concentrate from Alfalfa Meal

(Step 1 of Processing Operation)

Meal Source	Meal Extracted, Kg.	Concentrate Recovered, Kg.	Solids in Concentrate, %	Coumestrol Concn. Extract, Grams/Kg. (M.F.B.)	Total Coumestrol in Extract, Grams	Coumestrol Concn. in Original Meal, Gram/Kg.	Total Coumestrol in Meal, Grams	Proportion Extracted, %
Keystone	22,602	1155	53.2	5.63	3436	0.380	8,587	40.0
Sioux	9,238	458	57.1	4.33	1133	0.330	3,048	37.2
A. D. M.	9,101	462	56.0	6.36	1646	0.560	5,096	32.3
Total	40,941	2075			6215		16,731	
Mean			54.7	5.65		0.409		37.3

Table II. Data from a Typical Solvent Distribution Run (Step 3)

Fraction	Solids Distribution,[a] %	Coumestrol Distribution,[b] %
Precipitate from Step 2	...	...
First extract	4.8	35.6
Second extract	5.6	37.8
Third extract	2.5	12.2
Fourth extract	2.0	2.7
Fifth extract	1.5	2.5
Extracted residue	82.5	5.3

[a] 158 grams of starting material. [b] 4.49 grams of coumestrol in starting material.

915 grams; interfacial residue, 576 grams; aqueous filtrate, 592 grams. Most of this coumestrol was recovered by processing these fractions through the regular extraction sequence again.

The acid-precipitated material was dissolved in hot methanol by agitation in a 50-gallon, steam-jacketed, stainless steel kettle. This methanol extract, which contained 35% solids, was poured into 2-liter separatory funnels in 450-ml. portions with 150 ml. of water, 750 ml. of ether, and 300 ml. of hexane, in that order. The separatory funnels had to be shaken vigorously after the addition of each solvent to prevent a gummy mass from precipitating. A battery of twenty 2-liter separatory funnels was employed. The battery sequence allowed sufficient time for the emulsions to break in any one separatory funnel and the two phases to separate completely before starting the next operation. After the two phases had separated completely, the lower phase was drawn off and re-extracted four times with 750 ml. of ether and 300 ml. of hexane. The upper phases were all retained and combined. The combined upper phases now contained 9.75 kg. of solids, including about 1700 grams of coumestrol or about 17% of the dried weight of this fraction. One such typical distribution is given in Table II.

The combined upper phases from this operation were transferred to a battery of large evaporating dishes and the solvent was evaporated in a fume hood under a slow stream of air. As the solvent evaporated, the coumestrol and a number of other phenolic compounds crystallized. This technique was employed because concentration of this solution in vacuo or on a steam bath did not give crystals. The crystals were filtered off and washed with a minimum of methanol. The crude crystals at this point weighed 1880 grams and contained 1380 grams of coumestrol by analysis (7).

The crude greenish product was recrystallized several times from 2-propanol and dimethylformamide. Recrystallization removed most of the green contaminant, leaving a light yellow crystalline product which was 85 to 90% coumestrol. Two-dimensional paper chromatography [solvent systems: in the first direction, 2-propanol–concentrated ammonium hydroxide (2 to 1); in the second direction, 50% aqueous acetic acid] showed about 15 other blue- or yellow-fluorescing contaminants which could not be removed by repeated recrystallizations.

The entire crystalline mass was acetylated by heating with acetic anhydride and anhydrous sodium acetate for 30 minutes. Repeated recrystallization from chloroform yielded crystalline coumestrol acetate. The coumestrol acetate was deacetylated in 80-gram batches by addition to 4 liters of ice-cold 0.5% potassium hydroxide in methanol. The reaction mixture was stirred for $^1/_2$ hour in an ice bath. Then the ice bath was removed and the stirring continued until complete solution occurred (3 to 4 hours). The light yellow reaction mixture was filtered into 8 to 10 liters of ice-cold water and the pH slowly adjusted to 5 to 6 with dilute hydrochloric acid. The resultant white slurry was kept at 4° to 5° C. for several hours. The pH was then adjusted to 2 to 3 and the mixture filtered. The solid product was recrystallized from a mixture of dimethylformamide and methanol, giving 60 grams of an off-white solid from each starting batch of 80 grams. Analysis showed the material to be 100% coumestrol. Working up the various fractions obtained in the course of the processing produced a total of 2500 grams of coumestrol.

Portions of this coumestrol have been supplied to several cooperating agencies to evaluate its growth-promoting properties with sheep and cattle.

The filtrate from the final recrystallization contained several hundred grams of crystalline material, which included a number of compounds closely related to coumestrol. The isolation of a number of these compounds is now in progress.

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