COMMUNICATIONS

Analysis of Coumestrol, A Plant Estrogen, in Animal Feeds by High-Performance Liquid Chromatography

Coumestrol, a phytoestrogen, was determined in animal feeds by high-performance liquid chromatography. The coumestrol concentration was determined in feeds being fed to animals showing physiological effects of high estrogen levels and to animals where no physiological effect was noticed. The data implies that animals fed haylage containing 37 ppm coumestrol or more as their major feed will show physiological estrogenic effects.

Coumestrol is a plant estrogen found in many forage crops (Bickhoff et al., 1957; Guggolz et al., 1961; Wada and Yuhara, 1964; Knuckles et al., 1975; Knuckles et al., 1976). In a test of 16 vegetables, 13 contained coumestrol, with the highest levels in sprouted alfalfa and soybean seeds (Knuckels et al., 1976). High levels of estrogenic substances in forage crops fed to cattle, sheep, hogs, and mice result in severe biological effects, including increased teat length, gestation time, and uterine weight, as well as prolapsed vagina, cervix, and rectum (Bradbury and White, 1954; Bickoff et al., 1960, 1962; Magee, 1963; Braden et al., 1964; Cox and Braden, 1974; Trenkle and Burroughs, 1978). Beneficial effects of such substances have also been reported and include increased rate of growth and milk production (Cheng et al., 1953; Bradbury and White, 1954; Livingston et al., 1961; Bickoff et al., 1962; Oldfield et al., 1966; Trenkle and Burroughs, 1978). Recently, cattle implanted with Synovex estrogens and fed alfalfa haylage (stored in Harvestores) showed typical adverse effects of high estrogen levels (Turner, 1978; Jones, 1978). A method was needed to quickly identify and quantitate possible estrogens in the feed. Samples of these feeds and others from similar sources were examined by solvent extraction techniques previously used on soybeans (Lookhart, 1979) and red clover (Linder, 1967), followed by analyses with a high-performance liquid chromatography (LC) technique previously used to quantitate coumestrol in soybeans (Lookhart, 1979).

CHEMICALS AND REAGENTS.

Water was distilled and deionized; petroleum ether was analytical reagent grade; all other solvents were high-purity "HPLC" grade purchased from Fisher Scientific Co. Coumestrol (Eastman Organic Chemicals) was used without further purification since TLC showed only one spot.

Samples. Triplicate samples of alfalfa haylage from five sources (samples 1–5); a high-energy supplement containing oats, bran, grasses, and alfalfa; and a ground corn supplement were examined by extraction and LC analysis. Samples 1 and 2 were harvested in 1977 and 1978 respectively, from the same farm in southwestern Ohio, but were stored separately; sample 3 was harvested in 1978 from a farm 20 miles from the first farm; sample 4 was harvested in 1978 from a farm adjacent to the first; and sample 5 was harvested from a farm in a different region, Iowa. All the haylages were stored in Harvestore facilities. The feed supplements were commercial preparations. All feed samples were provided by J. L. Turner, DVM, Liberty, IN, and Rex Jones, DVM, Syntex Labs, Des Moines, IA.

Haylage, from which samples 1 and 2 were taken, supplemented with the corn and high-energy mixtures was fed to 170 estrogen implanted cattle (Synovex H and S for heifers and steers, respectively) in a total confinement feedlot. Haylage corresponding to sample 3 was fed to 100 nonimplanted cattle not under confinement. Sample 4 haylage was fed to 70 nonimplanted animals as 30% of their daily ration. Haylage corresponding to sample 5 was fed to 100 nonimplanted animals as 30% of their daily ration. Haylage corresponding to sample 5 was fed to 100 nonimplanted cattle not under confinement.

Extraction and Analysis. The air-dried feed samples were extracted following the method previously described for soybeans (Lookhart, 1979), omitting the Wiley mill grinding step. All LC analyses were performed with a Tracor Model 6970 LC pump utilizing a Waters 10-μm particle μBondapak C_{18} column, 30 × 0.40 cm i.d. The samples were injected via a Waters Associates WISP 710 autosampler. The detection system included a Tracor variable wavelength detector set at 343 nm and a Turner Designs Model 110 filter fluorometer with excitation filter peaking at 360 nm and emission filter passing wavelengths above 415 nm. The solvent system, methanol/water (65:35, v/v) at a flow rate of 1.0 mL/min, gave a retention time for coumestrol of about 9 min. The UV trace monitored on a recorder, and peak areas were determined by a Varian CDS-111C integrator. The fluoresecence trace was monitored and peaks integrated by a Hewlett-Packard 3385A printer-plotter integrator. Standard curves were determined and concentrations of extracts determined as previously described (Lookhart et al., 1978).

RESULTS AND DISCUSSIONS

The 65% extraction efficiency was in agreement with the efficiency reported for the same method in soybeans (Lookhart, 1979) and red clover (Linder, 1967). Analysis of the haylages was hampered by the large discrepancies between UV and fluoresence detectors. The UV detector showed a very large peak eluting from the column at a retention time of 0.5 min less than the standard. The fluorometer output described a reasonably large peak at a similar retention time as the standard, but about $^1/_{50}$ the amount shown by UV. Therefore, it was concluded that some other compound elutes from the column slightly before coumestrol and adsorbs at 343 nm but does not fluoresce at the same excitation and emission wavelengths

Table I. Coumestrol Content of Alfalfa Haylages as Analyzed by LC

sample no.	coumestrol, ppm
1	37.7
2	20.9
3	10.1
4	53.3
5	184.0

as coumestrol. The samples were therefore analyzed by their fluorescence output and quantitated by the ratio of standards vs. samples and then the extraction efficiency, their respective moistures, and volume changes taken into account (Lookhart et al., 1978).

The haylage samples varied in coumestrol concentration from 10.1 to 184 ppm (Table I). The haylage (sample 1) fed to cattle implanted with Synovex estrogens that had shown physical effects of high estrogen intake, i.e., bulling of steers and udder development and prolapsed vagina, cervix, and rectum of heifers contained 37 ppm coumestrol or more. Of the 170 animals fed sample 1, 150 were heifers and 20 were steers. All of the heifers showed udder development and over 100 animals showed prolapse. Six of these animals had to be marketed early after being sewn up a second time and their flesh was noted as being very soft.

When animals fed sample 1 (37.7 ppm) plus the corn (no coumestrol) and high-energy mixture (5.8 ppm coumestrol) showing effects of high estrogen intake were switched to sample 2 (20.9 ppm) plus the corn and high-energy mixture, the incidence of the deleterious effects gradually subsided (Turner, 1978). Animals not implanted and fed haylage from which sample 3 was taken showed no estrogen effects. Nonimplanted animals fed up to 30% of their intake as haylage from which sample 4 was taken also showed no ill effects. Animals not implanted and fed haylage from which sample 5 was taken showed the effects of high estrogen levels (Jones, 1978). Our results indicate that cattle fed haylage containing 37 ppm coumestrol or more as their major feed will show deleterious estrogenic effects.

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Effect of Sterilization Methods on 3-Chloroaniline Behavior in Soil

The persistence and degradation of 3-[U-^{14}C]chloroaniline (3-CA) was examined in nonsterile, autoclaved and potassium azide, mercuric chloride, and ethylene oxide treated, Hagerstown silty clay loam. Treatments were monitored for ^{14}C volatilization, the formation of organosoluble products, and soil-bound residues. Highest levels of ^{14}C volatilization occurred from autoclaved and potassium azide treated soils, whereas lower ^{14}C volatilization occurred from nonsterile and mercuric chloride and ethylene oxide treated soils. ^{14}C volatilization from autoclaved and potassium azide, mercuric chloride, and ethylene oxide treated soils occurred primarily as 3-[^{14}C]CA, whereas both $^{14}CO_2$ and 3-[^{14}C]CA were evolved from nonsterile soil. Organosoluble products accounted for 22–31% of the ^{14}C recovered, whereas soil binding accounted for 34–80% of the total ^{14}C applied. The distribution of ^{14}C in organosoluble degradation products varied with each treatment. Alkaline extraction of the soil-bound residues released 5–7% of the total ^{14}C applied. Distribution of the residual ^{14}C in fulvic and humic acids and humin soil organic matter fractions varied with the soil sterilization method.

Pesticides may be degraded in soil by a variety of processes. One of the methods frequently used to distinguish biological reactions in soil is by comparison of nonsterile and sterilized soils. Soil sterilization procedures most commonly used, however, generally destroy the soils integrity to the extent that not only are biological reactions

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