which differed substantially in the relative amounts of their provitamin A components (Table IV). Two additional inbreds (Corns 4 and 5) included in the table show that wide differences in carotenoid distribution also occur in yellow corn grain samples of different genetic origin.

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ALFALFA ESTROGENS

Quantitative Determination of Coumestrol in Fresh and Dried Alfalfa

A. L. LIVINGSTON, E. M. BICKOFF, JACK GUGGOLZ, and C. R. THOMPSON[1]

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.

A simplified procedure has been developed for the paper chromatographic fluorometric determination of the plant estrogen, coumestrol, in fresh and dried alfalfa. The method is sensitive to about 2 p.p.m. of coumestrol, with a maximum error of about ±5%. The method makes feasible the assay of large numbers of samples required in studies correlating estrogenic activity and stage of maturity, heredity, environment, and other factors.

THE PRESENCE (*3*) and subsequent qualitative determination (*8*) of the plant estrogen coumestrol in alfalfa and other forages were given in previous reports. Estrogenic substances present in forages have been implicated as the cause of reproductive disturbances in sheep (*4*) and have also been suggested as having beneficial effects such as increased rate of growth and milk production (*5*). The bioassay procedures (*2*) used for determining the estrogenic activity of these plants are laborious and not adapted to assaying large numbers of samples required in studies correlating estrogenic activity with stage of maturity, heredity, environment, and other factors. However, since coumestrol appears to be the predominant plant estrogen in alfalfa, its quantitative determination should correlate closely with the total estrogenic activity of alfalfa.

A recent report from this laboratory (*7*) presented factors which are involved in the paper chromatographic, fluorometric measurement of coumestrol. Based on these observations a rapid quantitative paper chromatographic procedure has been developed for fresh and dried alfalfa. In addition to the proposed procedure two alternative methods have been devised to confirm the results obtained by the fluorometric procedure. In the first paper chromatography is used to purify the extract, and the developed chromatogram is read directly in an ultraviolet spectrophotometer. The second utilizes silicic acid chromatostrips (*6*) to purify the extract, followed by ultraviolet spectrophotometry to measure the solution of coumestrol eluted from the chromatostrips.

[1] Present address, University of California, Riverside, Calif.

Experimental Procedure

Extraction. FRESH ALFALFA. The fresh plant material is cut into 1-inch lengths and uniformly mixed, and a 10-gram sample is taken for moisture determination. For analysis, 400 grams of the material is blended with 1 liter of 95% alcohol at high speed for 1 minute in an electric blender. The mixture is filtered on a Büchner funnel through Whatman No. 1 paper, under very light suction to minimize evaporation losses. The filtrate thus obtained is an aliquot of the whole extract.

In cases where it is not convenient to perform the assay immediately, the fresh plant material may be stored conveniently in a sealed container with alcohol, in the proportions described above, for periods up to at least 3 months with no measurable loss of coumestrol.

DRIED ALFALFA. A 20-gram sample of the dried forage, ground to a fine meal, is rehydrated with 80 ml. of water in a 500-ml. Erlenmeyer flask for 1 hour at room temperature. Three hundred and twenty milliliters of 95% alcohol is then added and, after gently swirling to assure mixing, the flask is stored in the dark at room temperature for 24 hours. At the end of this time the mixture is filtered by the procedure used for the extract of the fresh sample, and an aliquot obtained as before for purification and analysis.

Purification. SOLVENT SEPARATION. A 100-ml. aliquot of the alcohol extract from either the fresh or dried alfalfa is shaken with four successive 35-ml. portions of petroleum ether, in a 250-ml. separatory funnel. The petroleum ether removes most of the waxes and fatty materials which interfere with later paper chromatography. The alcohol-water phase is then concentrated in a rotary evaporator at reduced pressure to a volume of about 20 to 25 ml. The remaining concentrated aqueous mixture is transferred to a 125-ml. separatory funnel and extracted successively with one 15-ml. and three 8-ml. portions of ethyl ether. The water phase is discarded and the combined ether extracts are concentrated in a 50-ml. round-

Table I. Comparison of Solvent Extraction Procedures for Coumestrol

Solvent	Procedure	Apparent Coumestrol, Mg./Kg. of Meal				
		1 hr.	24 hr.	48 hr.	72 hr.	96 hr.
Acetone	Soxhlet	..	86	94	98	99
95% alcohol	Soxhlet	..	87	93	99	100
70% alcohol	Rehydrate and soak	..	98	..	98	..
70% alcohol	Rehydrate, 3-minute blend and soak	101	..	..	..	..

Table II. Recovery of Added Coumestrol from a Known Alfalfa Extract

Coumestrol, Mg.		Recovery, %
Added	Recovered	
0.17	0.16	94
0.17	0.16	94
0.34	0.34	100
0.34	0.32	94
0.34	0.32	94
0.34	0.36	101
0.34	0.31	91
0.34	0.32	94
0.68	0.64	94
0.68	0.65	96
0.68	0.65	95
0.68	0.63	93
0.68	0.65	95

Table III. Comparison of Assay Procedures for Coumestrol in Alfalfa Meal

Procedure	Apparent Coumestrol, Mg./Kg. of Meal
Fluorometric on paper	98 ± 3[a]
Silicic acid chromatostrip	89 ± 10[b]
Direct spectrophotometric on paper	110 ± 20[b]

[a] Standard deviation based on 10 analyses.
[b] Estimate based on two analyses.

bottomed flask on a rotary evaporator to a volume of 7 to 8 ml. The concentrate is then rinsed carefully into a 10-ml. volumetric flask with absolute alcohol and adjusted to the mark.

PAPER CHROMATOGRAPHY. The techniques used in the preparation of paper chromatograms and their evaluation after development were described earlier (7). The instrument used to read the fluorescence of the coumestrol spots was designed by Bailey (7). All of the fluorescent measurements were made with a 30-mm. aperture over the photocell.

Alfalfa extracts are applied to the papers with a 2-μl. pipet. The papers are the same as those used in preparing a standard coumestrol curve; five spots of extract and five of standard are applied alternately on each sheet. The volume of extract applied to each spot is selected to give a coumestrol level that will fall within the range of the standard.

The chromatograms are then developed by the ascending technique in a mixture of acetic acid, water, and hydrochloric acid (50:35:15). This solvent mixture was more satisfactory for extract solutions than the acetic acid and water (1 to 1) previously used for pure coumestrol (7). The fluorescent values of at least four of the coumestrol spots from each extract are averaged, and the coumestrol concentration is determined from the standard curve. That of the original sample is then calculated on a dry weight basis as milligrams of coumestrol per kilogram of meal.

Results and Discussion

Preparation of Extracts. Previous bioassay procedures at this laboratory for dried samples were concerned solely with total estrogenic activity and employed a Soxhlet extraction procedure (2). However, our present studies indicate that only about 86 to 87% of the coumestrol is extracted during the first 24 hours with either acetone or alcohol under the described conditions (Table I). Since none of the other solvents tested were more efficient than either of these two, it would be necessary to extract for at least 72 hours in order to obtain a quantitative measurement of coumestrol. Two alternative rehydration procedures were therefore devised which require less time for sample preparation. The more rapid extraction procedure involves a rehydration of the meal followed by a 3-minute blend with alcohol, and a soaking period of 1 hour before filtration (Table I). For routine analysis of a large number of samples, overnight soaking of a rehydrated sample with alcohol is more suitable.

Earlier studies from this and other laboratories (2) have suggested that estrogenic compounds may be unstable during drying. We have now confirmed this and have found that drying at 80° C. in a forced air oven destroyed about 25% of the coumestrol. The most accurate estimation of coumestrol content of alfalfa will therefore be made by the extraction of the fresh plant material.

Purification. After preparation of the extract by either the fresh or rehydration procedure, the purification techniques are the same. Since the whole extract contains impurities which interfere with the paper chromatographic purification of coumestrol, it is first necessary to purify the extract partially by distribution with petroleum ether in a separatory funnel. To determine a any coumestrol were removed also, if solution of pure coumestrol in alcohol-water (7 to 3) was shaken in a separatory funnel with petroleum ether. Examination by paper chromatography and analysis showed that no coumestrol was extracted by the petroleum ether. During the purification procedure slight mechanical losses may occur because of emulsion formation unless care is exercised. In a typical recovery experiment, a known concentration of pure coumestrol in alcohol was added to a low-activity fresh alfalfa extract, and an average value of about 95% recovery was found as shown in Table II.

To obtain a sufficiently concentrated coumestrol solution for spotting on paper it is necessary to concentrate the alcohol-water solution about fourfold. During the concentration, most of the alcohol evaporates and many of the solids are in suspension, so that it is desirable to extract the coumestrol from the aqueous mixture with ether. By concentrating the ether solution to 10 ml., multiple spots can be applied so that levels of coumestrol as low as 1 mg. per kg. of alfalfa meal will be discernible on the developed paper chromatogram.

In an analytical procedure employing the direct estimation of the quantity of material from the intensity of fluorescence of a zone on paper, the purity of the material must be ascertained in the zone. To verify this, a large number of fluorescing zones corresponding to coumestrol were cut out from several developed chromatograms, the coumestrol was eluted with alcohol, and, after concentration, developed on silicic acid chromatostrips. The chromatostrips were developed in three different solvent systems: ethyl ether and petroleum ether (7 to 3), petroleum ether and ethyl acetate (7 to 3), and chloroform and ethyl ether (1 to 1). The developed strips were dried and observed under ultraviolet light. Only coumestrol was observed on the strips.

The coumestrol zones from developed chromatograms of extracts of red clover, subterranean clover, and ladino clover contained other fluorescing impurities. Therefore, before applying the method of analysis to plant material other than alfalfa, the purity of the coumestrol zone for each plant source studied must be determined.

Estimation. In an earlier paper the authors described in detail the variation in the apparent coumestrol content with the size of aperture in the fluorometer (7). Our final choice of aperture was based on correlation of the values obtained with the alternative silicic acid chromatostrip and paper spectrophotometric methods (Table III). These methods are much more tedious and are impractical for large numbers of

Table IV. Precision of Method, Coumestrol Estimated as Milligrams per Kilogram of Meal

Detn.	Dried Alfalfa	Fresh Alfalfa
1	85	55
2	86	56
3	80	56
4	86	58
Mean	84	56
Std. dev.	±2.88	±1.29
Coeff. of variation %	3.4	2.3

assays. Table IV shows the precision of the fluorometric method for both fresh and dried alfalfa. The accuracy is shown from the coefficient of variation, ranging from 2.3% for the fresh alfalfa to 3.4% for the dried alfalfa. Each determination is the average coumestrol value obtained from two aliquots of an extract of a sample of meal.

Evaluation by Alternate Procedures. CHROMATOSTRIP METHOD. The preparation of the chromatostrips and details of their usage were described in an earlier paper (*8*).

Aliquots of the purified extract are applied to a large number of the chromatostrips. The strips are then developed four successive times in a mixture of ether and petroleum (7 to 3), with air drying between developments. After the final drying the strips are placed under an ultraviolet lamp and the fluorescent zone corresponding to coumestrol is carved out with a spatula. The coumestrol is eluted from the silicic acid adsorbent mixture with methanol, and after filtration the solution is read at 352 mμ in an ultraviolet spectrophotometer. The concentration of coumestrol in the sample is then calculated with absorbance of a solution of pure coumestrol as the standard. The procedure was proved to be reliable by the quantitative recovery of pure coumestrol which was similarly added to the chromatostrips. Accuracy, based upon two analyses, is estimated as about ±10%.

PAPER CHROMATOGRAPHIC–SPECTROPHOTOMETRIC ASSAY. The fluorescent zones corresponding to coumestrol on the developed paper chromatograms prepared as described previously are cut out and their absorbances measured on the ultraviolet spectrophotometer. The concentrations of coumestrol in the unknown extracts are then calculated from a standard curve prepared in the same manner from the fluorescent spots of pure coumestrol.

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SUGAR—STARCH TRANSFORMATIONS IN PEAS

Relation between Changes in Glucose, Fructose, Galactose, Sucrose, and Stachyose, and the Formation of Starch in Peas

R. S. SHALLENBERGER and J. C. MOYER

New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y.

Data on the accumulation of individual sugars in processing peas during maturation were collected in an attempt to elaborate on sugar content as related to pea sweetness and starch content. Peas may contain considerable amounts of stachyose, which explains why pea sweetness bears an uncertain relation to total sugar (sucrose) content. During starch accumulation, sucrose is inversely related to total starch and amylose content, while stachyose is directly related to pea amylopectin content. Attempts to relate pea sugars to starch content and sweetness require attention to individual starch fractions and individual sugars.

PROCESSING pea quality attributes, such as sweetness and tenderness for canning or freezing, or the rehydration rate in the case of dried peas, is related to the pea sugar and starch content, which changes as peas mature. As peas mature, the sieve size increases (*7*), and starch is formed at the expense of the sugars (*1, 2–4, 11, 12*). Pea sugars are of interest, since nonreducing sugar determinations, usually calculated as sucrose and accounting for 95% of the total pea sugar (*1*), bear uncertain relation to organoleptic ratings of pea sweetness.

The accumulation of starch is also of interest since the amylose-amylopectin ratio of starch also increases (*7*). Amylopectin and amylose have distinctly different physicochemical properties, and changes in the proportion of these starch fractions influence pea tenderness, pea dehydration, and dried pea rehydration rates, and perhaps even frozen pea texture. A study was initiated therefore in an attempt to elaborate upon the relation between starch-sugar transformations and pea processing quality. This report describes changes observed in individual sugars in peas of varying