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Recent developments in ruminant nutrition by using estrogenic additives to stimulate the rate of gain and feed efficiency of beef cattle (Burroughs et al. 1954) indicated the important role of feeds and forages containing estrogenic substances. Forages such as clover and alfalfa have been shown to contain small amounts of estrogenic substances (Cheng et al. 1953a). An isoflavone derivative, genistein, isolated from soybean oil meal was found to be estrogenic by the mouse uterine weight assay (Cheng et al. 1953b). Several isoflavone compounds have been synthesized chemically (Yoder et al. 1954) and they have been shown to be estrogenic also (Cheng et al. 1954). It appears then the total content of isoflavones in plant material may be related to its estrogenic potency. Unfortunately, there are no satisfactory chemical methods available for the estimation of isoflavones in feeds. The objective of this communication is to describe a procedure in which the total isoflavone content of a plant can be determined chemically.

A chemical property common to all isoflavones prepared from soybean oil meal was described in detail by Walz (1931). He found that 30% potassium hydroxide at the reflux temperature would split the pyrone ring of the isoflavone at carbon-1 position to form an equivalent of potassium formate. The formate formed could be steam distilled and then determined by a microprocedure of Perlin (1954) by the oxidation of formic acid with lead tetra-acetate and measuring the carbon dioxide evolved in a Warburg respirometer.

Experimental Procedure

In a typical experiment 27 mg. of genistein (4',5,7-Trihydroxyisoflavone) was hydrolyzed with 5 ml. of 30% KOH by refluxing with one ml. of butanol for 30 minutes. The hydrolysate was transferred to a beaker and evaporated on a steam bath to almost dryness. Twenty ml. of water was added to dissolve the residue and the solution was acidified with 6.5 ml. of 5 N H_2SO_4. Formic acid formed in the hydrolysate was distilled until one liter of distillate was collected. The distillate was made alkaline with NaOH and evaporated to almost dryness. The residue was dissolved in 10 ml. of 90% acetic acid. Aliquots of 0.5 to 1.0 ml. were taken for the de-

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termination of formic acid by the method of Perlin (1954) in a Warburg respirometer using lead tetra-acetate as the oxidizing agent. The carbon dioxide produced was calibrated with standard amounts of formic acid used. The results obtained with several synthetic isoflavones are shown in Table 1.

Table 1

Determination of Pure Isoflavone Derivatives

Compounds	mg.	Formic acid produced mg.	Conversion factor	Isoflavin recovered mg.	% recovery
Genistein	27.0	4.55	5.87	26.7	98.9
Daidzein	25.4	4.55	5.52	25.1	98.8
Formononetin	27.0	5.06	5.83	29.5	109.3
Biochanin A	27.0	4.42	6.17	27.3	101.1

Since the formic acid produced was in molecular equivalent of the isoflavone used the amount of isoflavone can be calculated by multiplying the amount of formic acid produced by a conversion factor, i.e., the ratio of molecular weights of the isoflavone and formic acid. Among the four synthetic compounds determined, the recovery varied from 99 and 109%. This method appears to be satisfactory for determining pure isoflavone compounds.

However, some difficulties were encountered in applying this procedure to the estimation of isoflavones from feeds due to the presence of interfering substances. It was found that the presence of carbohydrates greatly increased the value of isoflavones determined. This was to be expected since some carbohydrates were known to produce formic acid upon alkaline hydrolysis. Therefore, some modifications were necessary to adopt this method for the determination of isoflavones in feeds. The modifications adopted were: (1) the sample was extracted with ethanol and (2) it was necessary to separate carbohydrates from the alcohol extract by precipitating the isoflavones from the alcohol extract with water and then extracting them with ether before submitting them to alkaline hydrolysis.

In a typical experiment, one gram of finely ground air-dry hay was extracted with 50 ml. of 95% ethanol in a Goldfisch Extractor for five hours. The ethanol was then evaporated to a volume of about 5 ml. Twenty ml. of water was added. This water suspension was then extracted three times with 25 ml. of ether each. The ether extracts were pooled and evaporated to dryness. The residue was then used for alkaline hydrolysis as outlined earlier.

Some forages were submitted to analysis by this procedure. The results are shown in Table 2. The total isoflavone contents of these forages were expressed in terms of genistein. The range of the total

isoflavones in these forages varied from 2.8 to 4.3 mg. per gram of air-dry forages.

Table 2

Total Isoflavone Contents of Forages

	Formic Acid Produced mg.	Genistein Equivalent mg.
Ladino clover, 1 g	0.68	3.99
Red clover, 1 g.	0.55	3.23
Brome grass, 1 g.	0.48	2.82
Wheat, 1 g.	0.74	4.34

Discussion

The estimation of estrogenic substances in feeds and forages has usually been carried out with a biological assay procedure using laboratory animals. When genistein was identified as a compound responsible for the major part of the estrogenic activity of plants, attempts were made to develop a method for its chemical determination. It was known that isoflavones gave a color reaction with acid methanolic ferric chloride. The color produced was unstable and changed with pH (Pope and Wright, 1954). A paper chromatographic method was developed by Curnow (1954) for the determination of genistein. This method, however, was not quantitative. The present chemical method outlined here using alkaline hydrolysis with subsequent determination of the formic acid produced was found to be satisfactory for samples as small as one gram. This method will be useful in estimating the total isoflavone contents in feeds. However, it is impossible to identify the specific isoflavones present. Another limitation in the present method is that it is uncertain whether the total isoflavone content thus obtained is correlated with the estrogenic activity of the forages. Further investigation will be necessary to substantiate the usefulness of this method.

Summary

A method was developed for the chemical determination of total isoflavone contents in feeds and forages. When isoflavones were hydrolyzed in 30% potassium hydroxide, an equivalent of potassium formate was obtained. The formate was then steam distilled and its amount quantitatively determined by measuring the carbon dioxide evolved upon oxidation with lead tetra-acetate in a Warburg respirometer. This method was sensitive to determine isoflavones from samples as small as one gram of forage.

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