interfere when the color was developed in the closed system as recommended (Table I). The only compound which interfered seriously with this method was β-nitropropionic acid, a nitro-aliphatic acid found in a few species of legumes (*10*). The method as given above is not applicable for the determination of nitrates in these particular species of legumes. Occasionally a single treatment with hydrogen peroxide does not completely decolorize an aliquot of leaf extract. This faint residual color does not lead to significant errors when milligram quantities of nitrates are present in the aliquots. However, with microgram quantities, this residual interference must be eliminated by treating the residue a second time with hydrogen peroxide.

The proposed method is not as accurate as some of the more exacting, time-consuming procedures which were developed for industries that must impose a post decimal point stringency on analytical results. Its accuracy of $\pm 4\%$ does, however, meet the requirements of many biological investigations. The rapidity and accuracy of the method make it highly suitable for nutritional studies involving the feeding of fresh forage crops produced under high levels of nitrogen fertilization. Its use permits one to follow very closely the build-up of nitrates in forage crops following the application of nitrogen fertilizers. Correlations between the results of animal feeding tests and the nitrate concentration of the ration being fed can be readily observed. In several cases, the results of animal feeding tests involving toxic quantities of nitrates have been accurately predicted from the results of chemical analyses. It is now possible to make valid recommendations concerning the feeding quality of forage crops insofar as nitrates are concerned (*9*). These recommendations can be made usually within 2 hours after a representative sample is received at the laboratory.

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COUMARIN ASSAY

Determination of Coumarin in the Presence of Sterols

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Colorimetric methods for the determination of coumarin, which involve a color reaction with diazotized *p*-nitroaniline, give inaccurate results when applied to sweet clover and other sterol-containing plant materials. Sterols as well as several other classes of compounds, give the same color reaction as coumarin with this agent. A procedure is described for the quantitative removal of "free" and "combined" coumarin from ground sweet clover seeds and the subsequent spectrophotometric determination of coumarin in the extract.

Coumarin and its derivatives occur abundantly in nature, both in the free state and as glycosides. The principal natural sources are legumes, citrus fruits, orchids, and grasses. Coumarin has a sweet clover odor which is often referred to as the odor of new-mown hay. The tonka bean, a seed of a tropical South American tree, *Dipteryx odorata*, is the richest natural source of coumarin. Natural coumarin has been largely displaced by the cheaper synthetic product. Coumarin, the first natural perfume to be synthesized from coal tar raw materials, is used widely as a perfume in soaps and cosmetics. Until recently it was used as an ingredient of artificial vanilla extracts, but this is no longer permitted in the United States.

The increasing importance of Hubam clover as a southern forage and seed crop has prompted investigations to evaluate it as a potential source of valuable industrial raw materials. Modifications of two methods for the determination of coumarin in Hubam clover seeds were tried. Coumarin was determined colorimetrically by a modification of the method of Roberts and Link (*2*), wherein color is developed by a reaction with diazotized *p*-nitroaniline. This reagent gives the same color reaction with sterols as with coumarin. To overcome this difficulty, a method was developed for the spectrophotometric estimation of coumarin using a Beckman DU ultraviolet spectrophotometer to measure the absorption maxima of coumarin at 275 mμ. The procedure is a modification of that used by Englis and Hanahan (*1*) for the estimation of coumarin in vanilla extracts.

Colorimetric Method

The apparatus and reagents used were similar to those described by Roberts and Link (*2*) except that the Klett-Summerson photoelectric colorimeter, equipped with filter No. 54, and 12 colorimeter tubes calibrated for 5 and 10 ml. were used. A working curve was prepared from data obtained on a series of standardized solutions containing known quantities of coumarin.

A standard aqueous solution of chemically pure coumarin was prepared and measured into a series of colorimeter tubes so that the quantity of coumarin increased through the series in 25-γ increments—the first tube contained none and the last contained 225 γ. Exactly 1 ml. of 1% sodium carbonate solution was added to each tube and then enough

distilled water to bring the volume to 5 ml. After the contents were mixed, the tubes were placed in a water bath at 85° C. for 5 minutes, then cooled to room temperature. To each sample, 2 ml. of the diazotized *p*-nitroaniline reagent were added, drop by drop, with constant mixing. The volume in each tube was then made up to 10 ml. with distilled water and mixed. Readings were taken immediately with the photoelectric colorimeter. From these data the calibration or working curve was constructed.

Standards were run with each group of samples analyzed because of the number of factors causing variations in the intensity of the color obtained. By this procedure, Hubam clover seeds were shown to contain approximately 2.8% coumarin; however, as the sterols in these seeds give the same color reaction as coumarin with diazotized *p*-nitroaniline, probably less coumarin than the test indicates is present.

Ultraviolet Absorption Method

To overcome problems of estimating coumarin in the presence of sterols, a spectrophotometric method was developed by the modification of a procedure by Englis and Hanahan (*1*). With a Beckman DU ultraviolet spectrophotometer, coumarin was estimated on the basis of its absorption maximum at 275 mμ.

Establishment of Standards. The two curves in Figure 1 were constructed from data obtained on samples of pure coumarin, one a sample of c.p. coumarin (Eastman Reagent 79) and the other a purified sample isolated from Hubam clover seeds. In each case, readings were taken on samples dissolved in 95% ethyl alcohol (1 mg. in 100 ml.). These absorption-coefficient *vs.* wave-length curves show a sharp absorption maximum at 275 mμ and another distinct maximum at 310 mμ.

Curve *A* of Figure 2 shows the absorption of an ethyl alcohol extract of Hubam clover seeds—also the two maxima at 275 and 310 mμ, typical of coumarin. These maxima have nearly the same ratio to each other as those for pure coumarin shown in Figure 1. Therefore, the maximum at 275 mμ, curve *A*, is probably due entirely to coumarin.

To strengthen this assumption, a sample of finely ground Hubam clover seeds was moistened with water for 1 hour at room temperature and dried to constant weight in a circulating drying oven at 110° C. This treatment completely volatilized the coumarin. An ethyl alcohol extract of this material showed no maxima at 275 or 310 mμ (Figure 2, curve *B*). Only traces of substances remained that influenced the absorption maxima of coumarin; hence, the absorption maxima obtained at 275 mμ can be used for the estimation of coumarin in Hubam clover seeds. The only question about this assumption is the possible presence of some substance so similar to coumarin in properties that it affects both the absorption maxima of coumarin in the same ratio as coumarin and is, also, completely volatilized at 110° C.

Careful examination of curves *A* and *B* show that other ultraviolet absorbing compounds are present in the ethyl alcohol extract of Hubam clover seed meal. Ratios of absorption coefficients (minimum 240 mμ to maximum 275 mμ and maximum 310 mμ to maximum 275 mμ), differences between spectra at 232 mμ and at wave lengths over 320 mμ, and differences in the spectra on either side of 275 mμ indicate that such is the case. However, these differences are of a very minor nature and are not serious enough to offset the merits of this procedure.

A calibration curve was constructed from absorbance measurements made at 275 mμ on a series of dilutions of an ethyl alcohol solution of pure coumarin. Concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg. per 100 ml. were used. A straight-line relationship was obtained. Amounts of coumarin in ethyl alcohol extracts of Hubam clover seeds were then read from this curve on the basis of their conductance readings at 275 mμ.

Procedure. Ground Hubam clover seeds were moistened with distilled water and left for 1 hour at room temperature to allow liberation of coumarin from its combined forms by enzymic hydrolysis. The moistened meal was then dried in a desiccator to minimize vaporization of the coumarin. An accurately weighed sample (around 20 mg.) of the dried meal was extracted over a 16-hour period with about 50 ml. of 95% ethyl alcohol using a small scale Soxhlet extractor. The extract was diluted to 100 ml. with the same strength alcohol and the conductance read at 275 mμ. The coumarin content was obtained by conversion, using the calibration curve referred to above and expressed as percentage of the sample weight.

Experimental Results

The following percentage values for coumarin, on a dry-weight basis, in Hubam clover represent the average of at least five determinations by each method: by adaptation of the colorimetric method of Roberts and Link (*2*) on meal dampened before extraction,

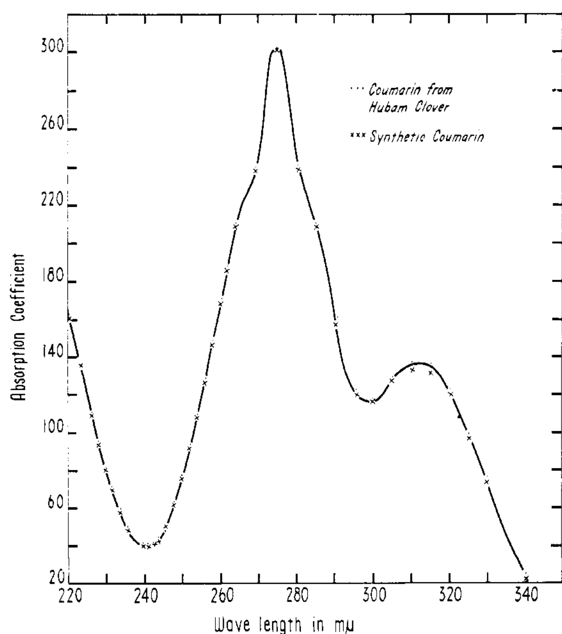

Figure 1. Ultraviolet absorption curve for coumarin

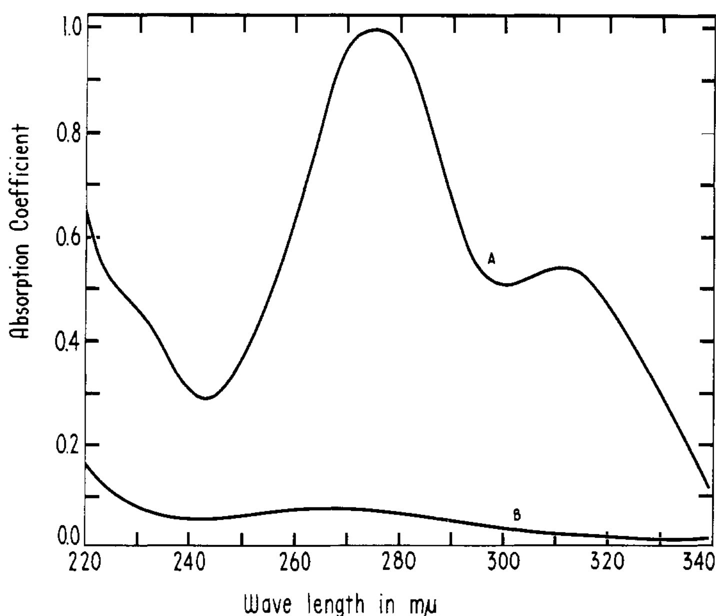

Figure 2. Ethyl alcohol extract of Hubam clover seed meal

2.80%; by spectrophotometric measurement of percentage transmittance at 275 mμ of ethyl alcohol extract of meal dampened before extraction, 2.18%; by spectrophotometric measurement of percentage transmittance at 275 mμ of ethyl alcohol extract of meal not dampened before extraction, 0.75%; and by spectrophotometric measurement, as in the second method, on meal allowed to stand several months after grinding, 0.88%.

Discussion

By the spectrophotometric procedure, results could be duplicated within ±0.1%. The coumarin value of 2.18% obtained by this method is lower than the 2.80% given by the colorimetric procedure because it is not augmented by the presence of sterols.

Approximately two thirds of the coumarin present in Hubam clover seeds is in a combined form, probably as a glycoside. Moistening the ground meal with water causes liberation of the "bound" coumarin by glycosidase enzymes.

Coumarin analyses must be conducted on freshly ground seeds. According to spectrophotometric measurements, ground Hubam clover seeds will lose more than half of their coumarin, over a period of 6 to 10 months.

The spectrophotometric procedure described above was used extensively in the writer's investigations. While to some this method may appear somewhat empirical, there is no apparent evidence of interfering substances (Figure 2, curve *A*).

The ultraviolet absorption method presented here is not equally free of interfering substances when applied to all types of plant material. However, the convenience, time-saving features, and accuracy of readings make this method an inviting choice when an ultraviolet spectrophotometer is available for use.

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GIBBERELLINS ASSAY

Fluorometric Determination of Gibberellic and Gibberellenic Acids in Fermentation Products, Commercial Formulations, and Purified Materials

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A fluorometric method for determining gibberellic and gibberellenic acids is described. However, the method does not distinguish between the acids. Gibberellenic can be detected by its absorption at 254 mμ and can be corrected for in the fluorometric method. The samples can be passed over a potassium bicarbonate column to separate gibberellic acid from interfering impurities. Certain samples can be measured without purification and correction made for gibberellenic acid.

Gibberellic acid, a product of the metabolism of *Gibberella fujikuroi* (imperfect stage, *Fusarium moniliforme*), is a growth substance for plants. Its effect on the growth rate of plants, flower induction, and morphology of plants has induced several chemical and pharmaceutical companies to manufacture it. The wide use of this substance necessitated an assay for it in fermentation products and various commercial formulations.

Gibberellic acid is a colorless, polycyclic, unsaturated, monobasic acid containing a lactone group, having a molecular weight of 346 (*3*), and only end absorption in the ultraviolet region (*4, 6*). It has no intrinsic properties which aid in its direct determination during production and purification. Cross (*2*) reported that treatment of gibberellic acid with concentrated sulfuric acid produced a wine-colored solution with blue fluorescence. Conversion of gibberellic acid into a fluorogen in 85% sulfuric acid is the basis of the method of assay to be described. The method was particularly useful for assaying fermentation beers and samples taken from the various stages of purification.

Of nine decomposition products of gibberellic acid, three, including gibberic and allogibberic acids, did not fluoresce in 85% sulfuric acid. Five of the six fluorescent decomposition products were found in very small amounts except in solutions which had stood for a considerable time at high or low pH. The most important decomposition product was gibberellenic acid (*4*), which might occur in concentrations as great as 4% in crystalline gibberellic acid, as great as 10% in commercial fermentations, and in higher concentrations in mother liquors obtained during purification of gibberellic acid.

As gibberellenic acid has little or no biological activity (*4*), it is an undesired impurity in crystalline gibberellic acid. The fluorometric method does not distinguish between them; consequently the total fluorogen measured as gibberellic acid must be corrected for the amount of gibberellenic acid in the sample. This correction can be avoided by suitable preparation of the sample as described later. Gibberellenic acid may be determined by means of its absorption in the ultraviolet.

Fluorometry

Most of the measurements of fluorescence were made with a Klett-Fluorimeter equipped with a Minneapolis-Honeywell null indicator, Model 104 W1G, in place of the usual direct current galvanometer. The null indicator was slightly more sensitive and, because of its short period (1 second), was much more convenient to use than the usual galvanometer.

Absorption spectra of the reaction products of 85% sulfuric acid with gibberellic and gibberellenic acids are given in Figure 1. A scan with the Aminco-Bowman spectrofluorometer showed that they probably were also the excitation spectra of the fluorescing substance. The fluorescent light was emitted principally between the wave lengths from 440 to 550 mμ with the peak between 450 and 480 mμ. Fluorescence was excited by any one or combination of the three mercury lines, 366, 405, and 436 mμ. The usual lamp filter was a Corning 5970 which trans-