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 (*1, 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-

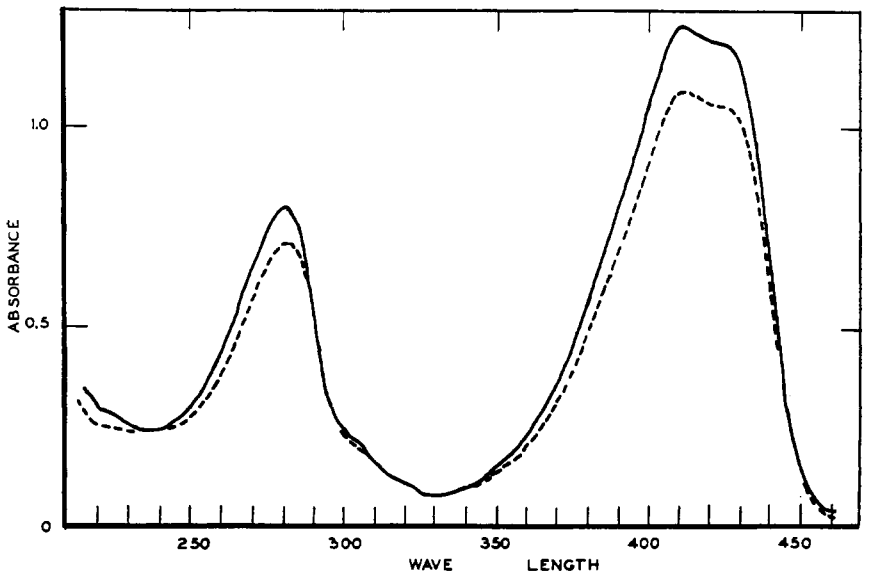

Figure 1. Absorption spectra of gibberellic and gibberellenic acids in 85% sulfuric acid at a concentration of 0.02 mg. per ml.

——Gibberellic acid
– – – Gibberellenic acid

mitted both 366 and 405 mμ. The former wave length was needed to excite the quinine sulfate standard and the latter to provide most of the excitation of the gibberellic acid fluorogen. Several photocell filters (Corning 3389, 3387, or Klett thiamine) gave satisfactorily low blanks. The best filter combination for maximum sensitivity was a Corning 5113 lamp filter and a 3387 photocell filter.

Linearity of response of the fluorescing system was tested with fluorogen equivalent to concentrations of gibberellic acid from 0.00625 γ per ml. to 3.2 γ per ml. The smaller concentrations were obtained by diluting the 3.2-γ-per-ml. solution with 85% acid after the fluorogen was formed. The Fluorimeter scale reading was a linear function of concentration below 1.6 γ per ml. Fluorogen in 85% sulfuric acid was very stable to illumination and to aging in room temperature (22° C.). No decrease in fluorescence was detected during a period of 60 days. The fluorogen from 20 γ of standard when sealed in a cuvette made a satisfactory fluorescent standard for setting the scale of the fluorimeter for a period of a month or two.

Fluorometric Assay

Reagents. Sulfuric acid, 85%. Add 235 grams of ice (or water) to 1 liter of concentrated analytical reagent grade sulfuric acid. Cool to about 12° C. for use. The acid freezes at about 10° C.

Standards. Dissolve gibberellic acid in methanol and dilute to a concentration of 1 mg. per ml. Keep refrigerated. Prepare four working standards by diluting the stock solution with water, acetonitrile, or methanol to give concentrations of 5, 10, 15, and 20 γ per ml. Prepare fresh dilutions each week and keep refrigerated. The gibberellic acid used as standard may contain gibberellenic acid, which gives the same response as gibberellic acid in this assay. In the fluorescence standard, therefore, gibberellenic acid is of no consequence. Correction is made for the water or other solvent in the crystalline gibberellic acid so that all of the assays are reported in terms of the anhydrous free acid.

Prepare a solution of quinine sulfate 0.25 mg. per 100 ml., in 0.1*N* sulfuric acid.

Procedure. Add 1.00 ml. of a standard solution or of the unknown diluted to a concentration between 5 and 20 γ per ml. to a 50-ml. Erlenmeyer flask. Standards and sample must be dissolved in the same solvent (water, methanol, or acetonitrile). Cool the flask in an ice bath and add 25 ml. of chilled 85% sulfuric acid, with continuous shaking of the flask. Allow the acid to flow as rapidly as it will from the pipet. The tips of the 25-ml. transfer pipets were enlarged to give identical delivery times of 9 seconds for chilled acid. Let the solutions stand for at least an hour at room temperature before measuring the fluorescence.

Prepare the standard curve by measuring the four concentrations of gibberellic acid standard and an acid blank. Set the Fluorimeter potentiometer scale to some reference value with the quinine standard or gibberellic acid–fluorogen standard. A quinine setting of 160 with the 5970/3389 filter combination gives a potentiometer reading of about 170 for 20 γ of gibberellic acid. Measure the sample and obtain the apparent gibberellic acid concentration from the calibration curve, which should be linear and pass through the origin when correction is made for blank.

Preparation of Samples for Fluorometry

Purification by Column Chromatography. Many samples need purification prior to fluorometric determination because of interference by contaminants with the photometric measurement of gibberellenic acid. Chromatography on a potassium bicarbonate column separates gibberellic acid from gibberellenic acid (*5*) and other contaminants including dyes. Approximately 95 to 98% of the gibberellic acid put on the column was recovered free from gibberellenic acid.

Reagents and Apparatus. Chromatography column (20 mm. inside diameter, 250 mm. long, fitted with a stopcock and 6-mm. exit orifice).

Acetonitrile, add 1% v./v. of distilled water to technical grade.

Potassium bicarbonate, granular, analytical reagent grade, Mallinckrodt.

Sodium sulfate, anhydrous, analytical reagent grade.

Standard. Prepare the reference standard gibberellic acid at 0.5 mg. per ml. in water or suitable buffers just before using.

Procedure. Add a mixture of 50 grams of granular potassium bicarbonate and 20 grams of anhydrous sodium sulfate to a chromatographic column. Add the salts in small lots with gentle tapping to obtain uniform packing. Add 5 ml. of aqueous solution of gibberellic acid (2 to 3 mg.) to a column, let stand for 10 minutes, add about 10 grams of potassium bicarbonate to the column, and place a pledget of glass wool on the bicarbonate. Elute the column at a rate of about 3 ml. per minute with acetonitrile solution until a total of 200 ml. of effluent is collected. Determine the amount of gibberellic acid in 1 ml. of effluent by the fluorometric method. The standard for the fluorometric assay is carried through the chromatographic procedure and the value obtained on this standard is used for purposes of calculation.

Gibberellenic acid and many other impurities may be removed from the column by subsequent elution with methanol.

Dilution with Water or Methanol. For rapid estimation of the gibberellic acid in fermentation products and process samples, a direct fluorometric method can be used with suitable correction for gibberellenic acid.

Mix the whole broth from the fermenter with an equal volume of methanol before filtering to obtain a clear filtrate. Make further dilutions with water or methanol if the methanol does not cause the formation of a precipitate. If processing samples are of low concentration or in a solvent likely to interfere, evaporate a small measured volume to dryness, dissolve the residue in a small

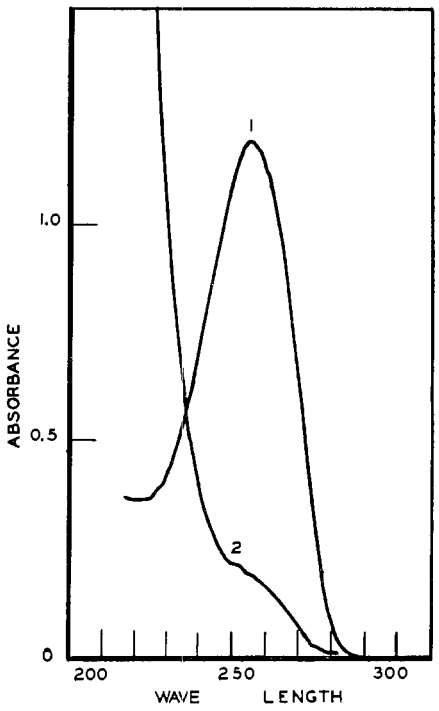

Figure 2. Absorption spectra of gibberellic and gibberellenic acids in methanol

1. Gibberellic acid (0.15% gibberellenic acid), 2 mg. per ml.
2. Gibberellenic acid, 0.02 mg. per ml.

amount of methanol (5 ml. of warm methanol will dissolve about 1 gram of gibberellic acid) and dilute with water or methanol to 20 γ per ml. Determine the apparent concentration of gibberellic acid in 1 ml. of diluted sample by the fluorometric assay.

To obtain the true concentration of gibberellic acid in the sample, correct for the amount of gibberellenic acid present by subtracting its concentration, as determined by ultraviolet spectrophotometry, from the apparent gibberellic acid concentration.

Determination of Gibberellenic Acid

Gibberellenic acid (*4*), in contrast to gibberellic acid (Figure 2), has an adsorption peak at about 254 mμ, which can be used to measure its concentration in the absence of interfering substances such as ketones and products of the fermentation. Direct spectrophotometry may be satisfactory for crude crystalline or purer samples of gibberellic acid.

Many of the substances in filtered broths that interfered with direct spectrophotometry were extracted into *n*-butyl alcohol along with gibberellenic acid but were not extracted by pH 7.5 buffer from the alcohol along with the acid. Such a purification step enabled an estimate to be made of the concentration of gibberellenic acid in fermentation broths and early process material. The buffer solution of gibberellenic acid was scanned from 220 to 270 mμ on a Carey Model 11 spectrophotometer and the maximum height of the absorption peak of the curve was taken as a measure of the concentration of the acid. A spectrophotometer such as a Beckman Model DU could, of course, be used; but an absorption curve was of value in detecting unexpected interfering substances. All solutions were measured in 1-cm. cuvettes.

Reagents. Methanol.

Butyl alcohol, *n*-butyl alcohol equilibrated with the acid buffer.

Acid buffer, pH 1.1 (50 grams of sodium chloride plus 8 ml. of concentrated hydrochloric acid diluted to 1 liter).

Phosphate buffer, pH 7.5 (20 grams of potassium phosphate, monobasic, plus 2.5 grams of potassium phosphate, dibasic, diluted to 1 liter) saturated with *n*-butyl alcohol.

Standard. Dissolve gibberellenic acid in methanol to give a concentration of 0.2 mg. per ml. The solution is stable for at least 3 months when kept in the refrigerator at 4° C. as judged by constancy of absorption spectrum. A working standard is not essential to the successful application of the method.

Procedure. Mix whole broth with an equal volume of methanol and filter to obtain a clear sample. If a sample contains a ketone or other interfering solvent, evaporate a measured volume to dryness at room temperature and dissolve the residue in methanol. Place 1.00 ml. of filtered broth or other sample which contains less than 200 γ of gibberellenic acid in a 25-ml. cylindrical separatory funnel (reaction vessel, Conner and Straub; Scientific Glass Apparatus Co. J-6025), add 5 ml. of acid buffer and 10 ml. of *n*-butyl alcohol, shake, let the layers separate (centrifuge if necessary), draw off and discard the lower (aqueous) layer. Do this separation sharply so as to leave very little aqueous solution in the funnel or suspended in the alcohol. Add 10.0 ml. of phosphate buffer, shake, let the two layers separate completely, draw off all of the lower layer into a small flask and add 1 ml. of methanol to it. The methanol prevents the separation of butyl alcohol, which otherwise causes turbidity if the aqueous solution warms slightly. Obtain the spectrophotometric curve of the phosphate buffer solution of gibberellenic acid in the wave length interval from 240 to 270 mμ. Use a mixture of 10 ml. of phosphate buffer and 1 ml. of methanol in the reference beam of the instrument.

An alternate procedure, suitable for certain samples obtained during preparation of crystalline gibberellic acid, omits the step with the acid buffer. Dilute the sample with pH 7.5 buffer, shake 10 ml. of the solution with butyl alcohol to remove colored substances, remove the aqueous layer, add 1 ml. of methanol to it, and obtain the absorption curve as above.

Examples. The effect of the purification procedure upon the absorption spectrum of a culture filtrate can be seen by comparing curves 1 and 4 in Figure 3. Apparently the culture filtrate contained less gibberellenic acid than it did of a neutral substance with an absorption spectrum similar to gibberellenic acid. Solutions 1 and 3, but not 4, were put through the purification steps. All three had initially received 1 ml. of a 1 to 1 diluted culture filtrate. Solution 2 was a gibberellenic acid standard. The undiluted culture filtrate contained about 0.16 mg. per ml. of gibberellenic acid. Recovery of gibberellenic acid added to solution 3 was 95%.

Distribution Coefficients. The distribution coefficient of gibberellenic acid between *n*-butyl alcohol and the pH 7.5 buffer was about 100 so that about 99% of the gibberellenic acid was extracted into the buffer. The distribution coefficient for gibberellic acid under the same conditions was about 3. About 99% of the gibberellenic acid, and even more of the gibberellic acid, was extracted by the butyl alcohol from the solution at

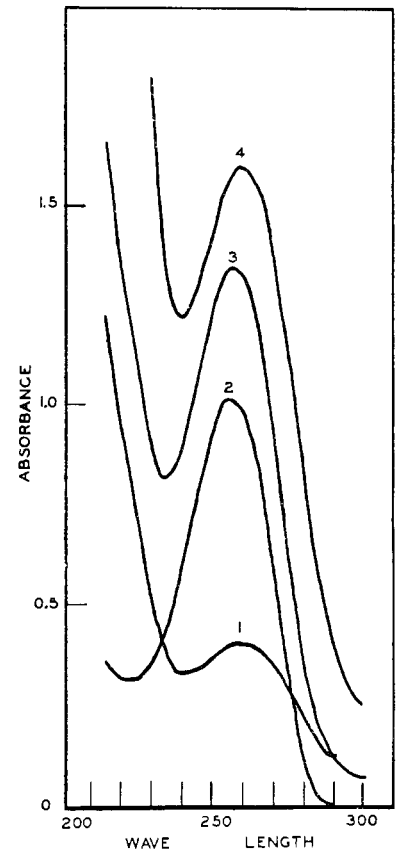

Figure 3. Absorption spectra of solutions of gibberellenic acid in pH 7.5 buffer

1. Culture filtrate, purified
2. Gibberellenic acid, 0.2 mg.
3. Culture filtrate plus 0.2 mg. gibberellenic acid and purification steps
4. Culture filtrate, not purified

pH 1.1. Recovery of added gibberellenic acid was good because of the favorable distributions at each step.

Absorption Curves of Gibberellenic and Gibberellic Acids. Gibberellenic acid at the concentrations used in the assay procedure had an absorption curve with a single peak in the vicinity of 254 mμ. The exact position of the peak and the absorbance depended upon the pH and the solvent. The positions of the maximum points of the curves and the absorbances for a concentration of 18.2 γ per ml. are given in Table I. Gibberellic acid purified by distribution between *n*-butyl alcohol and pH 7.5 buffer and measured immediately had an absorbance at 254 mμ of about 0.01 for a concentration of about 1 mg. per ml. Consequently, no correction need be made for absorption by gibberellic acid in the gibberellenic acid solutions.

Stability of Gibberellic and Gibberellenic Acids. Gibberellic acid was converted into gibberellenic acid and other substances in both the pH 1.1 and the pH 7.5 buffers. As gibberellic acid usually was present in much greater concentration than gibberellenic acid, conversion of a small proportion of it would cause a large error in the amount of gibberellenic acid found. A solution of 1 mg. of gibberellic acid in 10 ml. of the pH 1.1 buffer increased in gibberellenic acid by 30 γ upon standing 6.6 hours at 22° C. A solution of gibberellic acid at pH 7.5 increased in gibberellenic acid concentration from an initial 3% to a value of 12% in 12 days at 22° C. These rates of formation are slow enough so that a significant amount of gibberellenic acid would not be formed in the few minutes required for a determination. Evidence obtained from the absorption spectrum in the ultraviolet and from paper chromatography indicated that gibberellenic acid in pH 1.1 solution was converted in a few days at room temperature into gibberic acid. Gibberellenic acid seemed to be stable in pH 7.5 buffer solution at 22° C. for at least 4 months.

Table I. Effect of pH and Solvent upon Absorbance of Gibberellenic Acid at a Concentration of 18.2 γ per Ml. in 1-Cm. Cuvette

		Maximum		Minimum	
Solvent	pH	Wave length, mμ	Absorb.	Wave length, mμ	Absorb.
Methanol	..	254	1.18	220	0.34
HCl-NaCl	1.1	252	1.18	220	0.34
Phosphate	5.0	254	1.14	220	0.34
Phosphate	7.6	256	1.10	224	0.35
Bicarbonate	9.0	256	1.00	225	0.30

Discussion

Interferences. The only blank in this method of assay is the reagent blank which does not correct for fluorescence from substances other than gibberellic acid. The blank should be a sample from which gibberellic acid had been specifically removed or destroyed. However, as a method for doing this was not known, extensive dilution of the samples was assumed to be sufficient to reduce interference by fluorogens, other than gibberellic acid, to an insignificant level. The best indication of lack of interference was the good agreement of the amount of gibberellic acid in the purification steps, as indicated by the assay, with the amount obtained as the crystalline acid.

As fluorometric methods in general are subject to interference by substances that enhance or quench fluorescence, several solvents likely to be used in isolating and purifying gibberellic acid were tested for interference with the assay. The numbers in Table II are fluorescence values of a sample in the solvents compared to the same sample in water. The low fluorescence of the sample in methyl isobutyl ketone may be in part a result of partial absorption of the exciting light by the yellow solution. The interference of a solvent may be eliminated by sufficient dilution, by evaporation of the sample and taking up in a noninterfering solvent, or by incorporating an identical amount of interfering solvent in the standard solutions.

Evidence for quenching by dissolved oxygen was sought but not found.

Fluorescence decreased about 2% for each 10° C. rise of the temperature at which the sample was measured over the range from 20° to 60° C.; consequently, care should be taken to have all of the solutions at the same temperature at the time of measurement.

Accuracy. Fluorometric methods in general are less accurate and precise than photometric methods and require meticulous attention to details to obtain a precision that by the standards of photometry would be considered low.

To obtain information on the precision of the assay, a set of three solutions was measured in duplicate on seven successive working days. The samples were prepared by the water or methanol method. A diluted filtrate of the culture fluid from a fermenter was chosen as test solution 1. It was diluted further with an equal volume of water to form solution 2. Solution 3 was gibberellic acid at a concentration of 400 γ per ml. in water. The results of the assay are given in Table III. A standard curve was made each day. The intercepts on the 20 γ gibberellic acid abscissa ranged from 161 to 178 potentiometer divisions.

The reproducibility of this assay is especially sensitive to the way the sulfuric acid is added. The cold acid must be added to the cold sample in a manner that does not vary from flask to flask, and the flasks must be agitated the same each time. The fluorescence will vary with the time used to add the acid. For example, very rapid pouring of the cold acid into the gibberellic acid sample gave a fluorometer reading of 183 compared with 165 obtained when the acid was added from a pipet. When more than one pipet is used, the delivery times must be the same or excessive variation will be introduced. Such sensitivity to technique is not unexpected in a test in which the product being measured is not the principal one formed. With practice and attention to details, an operator can reproduce his assays with a standard deviation of from 3 to 5%. Variability of the assay was slightly less for standard and samples in methanol than for aqueous solutions.

Table II. Effect of Several Solvents and Two Concentrations of Sulfuric Acid upon Development of Fluorescence of Gibberellic Acid

	Relative Fluorescence	
Solvent	85%	96%
Water	100	37
Methanol in water		
10%	103	...
20%	107	...
50%	112	...
Methanol	113	86
Ethyl alcohol	113	...
n-Butyl alcohol	113	...
Acetone	115	...
Methyl isobutyl ketone	77	...
Ethyl acetate	130	...
Acetonitrile	77	56

Table III. Assay of Three Solutions for Gibberellic Acid

Solution No.	Average[a], γ/Ml.	Std. Deviation
1	292	14
2	152	5
3	404	12

[a] Duplicate assays done on 7 consecutive working days.

Table IV. Relative Fluorescences of Aqueous Solutions of Gibberellic and Gibberellenic Acids Developed in Various Concentrations of Sulfuric Acid

	Sulfuric Acid, %				
Acid	70	80	85	90	96
Gibberellic	28	85	100	80	25
Gibberellenic	31	83	100	73	20

Concentration of Sulfuric Acid. The effect of concentration of sulfuric acid upon the fluorescence was investigated with the maximum formed with about 85% sulfuric acid by weight as is shown in Table IV. The fluorescence values were the result of two opposite effects of the sulfuric acid concentration: one on the formation of the fluorogen, and the other on the intensity of the fluorescence of a given amount of fluorogen. Maximum fluorescence of the fluorogen occurred at some acid concentration between 20 and 70%. The formation of fluorogen by 70 and 96% acids was lower than with 85% acid. The solvent influenced the relative effects obtained from 85 and 96% sulfuric acid (Table II). The sample in methanol gave about twice as much fluorescence as the sample in water when development was by 96% acid in contrast to a 13% increase when 85% acid was used. Greater fluorescence was obtained from the 85% acid than the 96% regardless of the solvent in which gibberellic acid was dissolved.

Fluorogen. Two different principal substances were formed by the reaction between gibberellic acid and 85% sulfuric acid. The major product was a yellow, acidic substance with a golden fluorescence under ultraviolet on air-dried paper chromatograms and a faint pinkish fluorescence when 85% sulfuric acid was applied to the paper. The minor substance had a blue fluorescence on paper, both before and after wetting with 85% sulfuric acid. Presumably, the substance with the blue fluorescence was the one measured in assay. When the isolated fluorogenic mixture was chromatographed in the butanol-ammonia system used for gibberellic acid (*1*, *6*), about 10 spots appeared in addition to the two principle ones. Three of the spots were yellow with yellow fluorescence and behaved toward sulfuric acid exactly as did the principle yellow material (R_f = 0). One spot (R_f 0.63) had green fluorescence before and after the acid was added. The remainder of the spots had blue fluorescence which was intensified when acid was added. The largest of these had an R_f of 0.16. The two principal spots reduced permanganate just as gibberellic and gibberellenic acids did (*1*).

Temperature of Sulfuric Acid. The higher the temperature of the acid when it was added to the sample, the lower was the subsequent fluorescence. There was a limit to the increase obtained by lowering the temperature of the acid. Rapid addition of acid supercooled to minus 5° C. to a frozen sample increased the fluorescence very little over that obtained when acid at 12° C. was added equally as fast. Acid added at 25° C. gave a fluorescence only two thirds as great as that obtained with chilled acid.

Acknowledgment

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INSECTICIDE EVALUATION

Determination of Allethrin Residues in Milk and Meats

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A method accurate to 0.1 p.p.m. is presented to determine allethrin in the milk or meat tissues of domestic farm animals, which have been subjected to a concentrated allethrin spray. The determination involves solvent extraction of the tissues, concentration, and reaction with highly acidic mercuric oxide–sulfuric acid reagent to produce a red color. The color developed follows Beer's law and can be measured colorimetrically. Allethrin was not found in the milk of dairy cows, which had been sprayed daily for 3 weeks, or in the meat tissues of a female goat that had been sprayed daily for 5 weeks—all with a large overdose of the spray.

THE EXPERIMENTS presented determine whether or not allethrin (*dl*-2-allyl-4-hydroxy-3-methyl-2-cyclopentene-1-one ester of *cis-trans-dl*-chrysanthemummonocarboxylic acid) will appear in the milk of lactating cows or in the meat tissues of farm animals, which have been sprayed for extended periods with concentrated oil sprays of allethrin. For this determination, a modification of the method of Schreiber and McClellan (*3*) was used, which was suggested by the color reaction produced by the Deniges reagent in the determination of pyrethrins by the Association of Official Agricultural Chemists method (*1*) and by Fischer's method (*2*). The reagent used differs from that of AOAC and Fischer by its degree of concentration and acidity.

Chemical Analysis

The method of analysis, which was developed in the laboratory of McLaughlin Gormley King Co., involves the separation of the allethrin from the material in question by solvent extraction and the reaction of the concentrated extract with the mercuric oxide–sulfuric acid reagent. Allethrin will react with the reagent to give a pronounced red color. If the chrysanthemummonocarboxylic acid component of the allethrin is present in the free state, a pink to purple color will develop. The absence of these colors will show an absence of either of these compounds. The colors developed, which follow Beer's law, can be measured on a Klett-Summerson or other photoelectric colorimeter with suitable filters. The method is sensitive to as low as 10 γ of allethrin.