and St. John (*2*), after a very long peried of digestion, were 97.1 and 96.9% for methionine and cystine, respectively.

Adaptations of Procedure

Contrary to the stipulation by Hillebrand *et al.* (*3*) requiring an excess of calcium as a sulfate fixative, it was found that magnesium in 1 to 1 weight ratio to sulfur ensured complete recovery of sulfate upon evaporation in a perchloric acid solution. The use of magnesium instead of calcium might prove advantageous in the analysis of calcium-free sulfur compounds because the coprecipitation and radiation errors from magnesium on barium luminescence are negligibly small. However, natural products are seldom devoid of calcium, so no provision was made for alternative use of magnesium in the procedure.

The use of 10 ml. of 0.0625*N* barium chloride was designed to give a wide latitude in the anticipated sulfur content of the sample. In some borderline cases it may be desirable to know whether this amount is adequate for complete sulfur precipitation and the following simple test for sufficient barium excess has been devised.

After sulfate precipitation with 10 ml. of the barium chloride solution, transfer 2 drops of the clear solution into a 5-ml. beaker, add 2 to 3 ml. of refrigerated 0.2*N* acetic acid–sodium acetate pH 4 buffer solution and about 15 to 20 mg. of tetrahydroxyquinone indicator (measured), and stir. The quick formation of rose-colored crystals indicates sufficient barium; persistence of a yellow color, with only a few rose-colored crystals, shows a need for additional barium. Five milliliters of the barium solution may be added and the test repeated after 15 to 30 minutes.

Where the sulfur content is known to be in the range of 1 to 3 mg., better photometric readings may be obtained with 5 ml. rather than 10 ml. of the standard barium solution. At the higher barium concentration (7 to 9 ml. excess), the galvanometer needle fluctuates badly, necessitating the averaging of several readings for good results. At lower barium concentrations the readings are more stable and easily reproducible.

The ferric chloride addition may be reduced to 2 or 2.5 ml. where the phosphate content is known to be within 2 or 2.5 mg. of phosphorus. In such instances, the departure from the 5-ml. volume should be corrected by a supplementary addition of 1*N* hydrochloric acid.

To reduce the relative error in low-sulfur materials, the sample size may be increased to 2 grams. The increased sample can be managed in the 100-ml. digestion flask, but requires more attention in the early stages of the digestion.

Beakers may be used instead of flasks, but at some loss of precision and accuracy (*6*).

The common metal cations (calcium, magnesium, potassium, and sodium) may be determined flame photometrically in same solution. Phosphorus, may be determined colorimetrically on an aliquot taken at the completion of step A of the procedure.

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GIBBERELLINS DETERMINATION

Fluorometric Assay for Gibberellic Acid

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A fluorometric assay for gibberellic acid (A_3) in the presence of gibberellin A_1 has been developed. At 0° C., gibberellic acid fluoresces strongly when exposed to ultraviolet light after treatment with cold concentrated sulfuric acid; gibberellin A_1 exhibits practically no fluorescence. At this temperature, the assay is reproducible and specific for gibberellic acid (A_3). It agrees well with other quantitative methods for gibberellic acid currently in use and is simple and applicable to process samples.

THE RESPONSE of various plants to the gibberellins is well known (*7*). Neely and Phinney (*6*) have reported a sensitive assay for gibberellins using a mutant dwarf-1 of maize. Arison *et al.* (*1*) have reported on gibberellin plant assays with the Rondo pea plant, Pinto bean, and Avena. Although the plant assay must be used ultimately to establish the biological identity of gibberellin products, for fermentation and process samples it is desirable to have a simple, accurate, and less time-consuming determination.

Cross (*3*) found that in cold concentrated sulfuric acid, gibberellic acid (gibberellin A_3, $C_{19}H_{22}O_6$) gave an intense wine red color with a strong blue fluorescence. Arison *et al.* (*1*) have reported a fluorometric assay for gibberellic acid based on such treatment with sulfuric acid. The specificity of this fluorometric assay method was indicated as not fully established. Recently, Kavanagh and Kuzel (*5*) also reported a fluorometric assay for both purified and process samples of gibberellic acid; no mention was made of the effect of the presence in such samples of gibberellins other than gibberellic acid. The present authors report here a fluorometric assay procedure specific for gibberellic acid in the presence of gibberellin A_1.

Instrumentation and Reagents

The Aminco-Bowman spectrophotofluorometer is used for the characterization of the gibberellic acid activation and fluorescence spectra. The Coleman photofluorometer Model 12 C

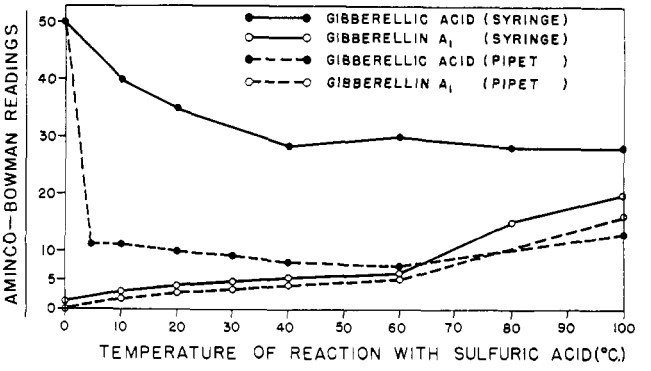

Figure 1. Effect of temperature of fluorescence activation on density of fluorescence of gibberellic acid (A_3) and gibberellin A_1

is used for the routine assay with a B-1 filter for the primary or activation filter and a PC-1 filter for the secondary or fluorescence filter.

Reagents. Concentrated sulfuric acid, reagent C.P. grade sulfuric acid containing 0.01% stannous chloride.

Ethyl acetate, analytical reagent grade.

Monobasic potassium phosphate, 0.5% aqueous solution, adjusted to pH 3.0 with phosphoric acid.

Gibberellic acid (gibberellin A_3). The gibberellic acid standard used assayed 99% pure by infrared analysis.

Quantitative Fluorometric Determination of Gibberellic Acid

Procedure. To prepare the standard curve, a stock solution of 100.0 μg. per ml. of gibberellic acid standard is made up in distilled water. Some warming may be necessary. This solution is stored in the refrigerator and is stable for one month. From this stock solution are made individual dilutions ranging from 1.0 to 8.0 μg. per ml. at intervals of 1.0 μg. per ml. One-milliliter aliquots of each dilution are pipetted into three replicate 10.0-ml. glass-stoppered volumetric flasks. One milliliter of distilled water is pipetted into another flask for the blank.

Concentrated sulfuric acid is cooled to approximately 1.0° C. The standards and blank are rapidly frozen in an isopropyl alcohol–dry ice bath and then placed in an aqueous ice bath. Four milliliters of the cold sulfuric acid are added rapidly from a 5.0-ml. syringe while the flasks are partially submerged in an aqueous ice bath. The flasks are swirled to ensure complete mixing and are allowed to stand in the aqueous ice bath for 10 minutes. Uniformity of acid addition from sample to sample is important. The concentrated sulfuric acid may be added quickly or slowly, but should be added in the same manner for all of a particular batch of samples. Four milliliters of cool 5*N* sulfuric acid (10° to 15° C.) are then added to each flask while partially submerged in the aqueous ice bath. The flasks are again swirled and placed in the ice bath for an additional 5 minutes. The use of sulfuric acid of lower normality, while slightly reducing the absolute quantitative fluorescent response, has no effect on the relative quantitative response and, in addition, facilitates operations during the routine assay. The flasks are finally removed from the ice bath and diluted to volume with cool 5*N* sulfuric acid. The standards and blank are transferred to cuvettes and read in either the Aminco-Bowman spectrophotofluorometer or the Coleman photofluorometer. With the Aminco-Bowman spectrophotofluorometer, the maximum activating wave length is found at 405 mμ and the maximum fluorescent wave length at 455 mμ. With the Coleman photofluorometer, the B-1 filter is used for activation and the PC-1 filter is used for the fluorescence measurement. The Coleman photofluorometer is adjusted so that the reading with the 4.0-μg. per ml. standard (mid-point of the standard curve) is 40.0.

The standard curve is plotted as micrograms per milliliter of gibberellic acid against readings of the instrument. The fluorescent response is directly proportional to the concentration of gibberellic acid between 1.0 and 8.0 μg. per ml. In the routine assay, only the mid-point of the standard curve is used, and five samples are run to establish this point.

Aqueous samples or samples dissolved in water-miscible solvents should be filtered if not clear, and then diluted to approximately 4.0 μg. per ml. with 0.5% potassium phosphate buffer. One milliliter of the diluted sample is pipetted into a tube and extracted three times, with separate 3.0-ml. aliquots of ethyl acetate. The ethyl acetate extracts are combined and evaporated to dryness in a 10.0-ml. volumetric flask by blowing filtered air on the surface of the solvent. Evaporation of the solvent at temperatures higher than 30° C. causes fluorescence quenching. One milliliter of distilled water is added to the dried residue in the flask, and the fluorescence is developed as stated for standards.

Samples dissolved in water-immiscible solvents are diluted to approximately 4.0 μg. per ml. with the same solvent. One milliliter of the diluted sample is evaporated to dryness, and the residue is dissolved in 1.0 ml. of distilled water. Again, the fluorescence is developed in the same manner as with the gibberellic acid standards.

Calculations. Results for unknown samples are determined directly from the standard curve with appropriate allowances for blanks and the necessary calculations for dilutions.

Experimental

Preliminary Studies with Gibberellic Acid and Gibberellin A_1. Early experiments involved dissolving pure gibberellic acid and gibberellin A_1 in distilled water separately and studying the quantitative fluorescent response resulting from treatment with concentrated sulfuric acid. The gibberellic acid used was the standard material described previously under reagents. The gibberellin A_1 was crystalline material which assayed 98% pure by infrared analysis. Unless indicated otherwise below, these early studies were carried out under the standard conditions outlined under "Quantitative Fluorometric Determination of Gibberellic Acid." One-milliliter aliquots of the dilutions were each treated with 4.0 ml. of room-temperature concentrated sulfuric acid. The fluorescent responses obtained for both gibberellins, while somewhat erratic, were directly proportional to concentration.

The inconsistency in the quantitative fluorescent response of gibberellic acid under the conditions described above was found to be due to at least two different variables. The first was the inconsistent fluorescent response obtained with different lots of concentrated sulfuric acid. This was corrected by adding stannous chloride to all lots of acid used. On the basis of the corrective action of stannous chloride, the inconsistency of most sulfuric acid was, and is, believed to be due to high variable concentrations of nitrate ion (*7*).

The second variable involved consistency of concentrated sulfuric acid addition. Although it was found that consistency of acid addition from sample to sample was essential, the method of adding the acid could vary considerably. The syringe is routinely used because of ease in handling the concentrated sulfuric acid. If desired, however, the acid may be added rapidly or by gravity flow from a calibrated pipet with the same precision as the syringe.

At this time, the effect of various tem-

peratures of fluorescence activation on gibberellic acid and gibberellin A_1 was taken under consideration. The pure gibberellins were dissolved in 1.0 ml. of distilled water (10.0 μg. per ml.), and 4.0 ml. of room-temperature concentrated sulfuric acid were added rapidly by two methods, from a graduated pipet and from a 5.0-ml. syringe. For 100° C., the samples were reacted at room temperature and then boiled in a water bath for 10 minutes. For 60° and 80° C., the samples were reacted at room temperature and heated to the appropriate temperature for 10 minutes. After the addition of acid, all samples were allowed to stand 10 minutes, diluted to 10.0 ml. with 5*N* sulfuric acid, and then read in the Aminco-Bowman spectrophotofluorometer.

The fluorescent response of the two gibberellins at various temperatures of fluorescence activation is shown in Figure 1. All points plotted are average values of triplicate flasks and represent maximum activation and fluorescence peaks of the two gibberellins at the temperature indicated. The fluorescence of gibberellic acid is at a maximum at 0° C. and gradually decreases with increase in temperature; this confirms data published by Kavanagh and Kuzel (*5*). In addition, Figure 1 also shows the fluorescence of gibberellin A_1 to be minimal at 0° C. and to increase gradually with temperature rise. It appears, however, that the fluorescent moiety formed is the same for both gibberellins, since the maximum activation and fluorescence peaks were found to be identical at all temperatures for both gibberellins.

At 60° C. or above, when the acid is added by pipet, the fluorescence of the two gibberellins is approximately equal. At 0° C. the fluorescence of gibberellin A_1 is practically nonexistent, while that of gibberellic acid is at a maximum regardless of the method of acid addition. Temperatures lower than 0° C. obtained by diluting the aqueous samples with ethyl alcohol did not increase the quantitative amount of fluorescence obtained with gibberellic acid significantly over that obtained at 0° C. In addition to the possibility of a fluorometric assay for total gibberellin activity (A_3 and A_1) using a fluorescence activation temperature of 60° C. or above, these results also indicated the possibility of a specific fluorometric assay for gibberellic acid in the presence of gibberellin A_1 using a fluorescence activation temperature of 0° C.

Studies on Process Samples. The addition of concentrated sulfuric acid to gibberellin process samples containing small amounts of sugars, proteins, and other media ingredients caused severe charring and almost total fluorescence quenching. These interfering substances were eliminated by the ethyl acetate extraction procedure described. It was also noted, when gibberellic acid standard was used, that fluorescence quenching resulted from the effect of sulfuric acid on residual solvents after extraction. This was eliminated by dissolving the dry residue from the extraction in 1.0 ml. of distilled water prior to the addition of sulfuric acid.

In support of the quantitativeness of the adopted ethyl acetate extraction procedure are the data shown in Table I. These data show the percentage recovery of added pure gibberellic acid from gibberellin process samples. All readings are average values of duplicate samples. The percentage recovery of gibberellic acid from all samples was within 8% of the theoretical.

Evaluation of Method

Statistical Evaluation. To determine the precision and reproducibility of the assay, 20 replicate 10.0-ml. volumetric flasks each containing 3.0 μg. per ml. of gibberellic acid were reacted with sulfuric acid according to the standard assay conditions.

The diluted samples were read in a Coleman photofluorometer, with the instrument adjusted to read 50.0 with one of the samples. The mean was 50.2 and the standard deviation of the distribution of observations was found to be 0.831.

Comparative Evaluation. The fluorometric assay for gibberellic acid was further evaluated by comparative studies with other gibberellin assays currently in use. These assays included a dwarf pea biological assay (*4*) and a radioisotopic chromatographic assay (*2*), both of which can be used for either purified or process samples, and an infrared analytical method (*8*) which is normally used only for purified samples.

Purified and process samples analyzed by the fluorometric and dwarf pea methods agreed well. With gibberellin A_1 the fluorometric value was 2.0% of that reported by the pea assay. With gibberellic acid, however, the two assays agreed within 5.0%.

Comparative data for the radioisotopic chromatographic assay and the fluorometric assay have been reported by Baumgartner *et al.* (*2*). The lack of interference of gibberellin A_1 was indicated by the close agreement for gibberellic acid (A_3) by the two methods.

The percentages of gibberellic acid in lots of crystalline material isolated from fermentation beers found by infrared analysis and the fluorometric assay are shown in Table II. With the exception of one lot, the two assays agreed within 3.0%.

Since the development of the above method, the interference by gibberellenic acid has been reported by Kavanagh and Kuzel (*5*). This method does not correct for gibberellenic acid.

Table I. Recovery of Added Gibberellic Acid Standard from Fermentation Beers Using Fluorometric Assay

Fermentation Beer	Gibberellic Acid, μg./Ml. Found	Gibberellic Acid, μg./Ml. Standard added	Gibberellic Acid Recovery μg./ml.	Gibberellic Acid Recovery %
1	2.00	5.0	6.9	98
2	1.25	5.0	5.9	93
3	1.50	5.0	6.9	108
4	2.35	5.0	7.1	95

Table II. Gibberellic Acid (A_3) Present in Lots of Crystalline Material

Sample No.	Gibberellic Acid Content, % Infrared Analysis A_3	Infrared Analysis A_1	Fluorometric assay, A_3
1	25	75	26
2	39	56	36
3	38	56	39
4	57	38	60
5	62	26	60
6	68	26	63
7	68	22	70
8	82	9	85

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