Fluorometric Determination of Gibberellic Acid

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Microgram quantities of gibberellin A_3 were determined by separating it from plant materials with solvent extraction and thin-layer chromatography. The gibberellin was eluted from the plate, made to fluoresce in dilute sulfuric acid, and measured fluorometrically.

The growth response of some plants to gibberellins has been known for some time (Stowe and Yamaki, 1957). Of the known gibberellins, gibberellin A_3 is one of the most active biologically (Schmidt, 1961) and appears to exert its effect by increasing cell growth, not by cell division. In recent years, the use of gibberellin A_3 (gibberellic acid) has become important in the production of various crops. This has required the development of a practicable method of analysis for gibberellic acid in or on various plant materials.

MacMillan and Suter (1963) separated the gibberellins by thin-layer chromatography and located the zones by spraying with sulfuric acid solutions and heating. The gibberellic acid appeared as a bright blue fluorescent spot. Kavanagh and Kuzel (1958) determined gibberellic acid after purifying with a potassium bicarbonate column by measuring the fluorescence developed in 85% sulfuric acid. Theriault *et al.* (1961) added stannous chloride to the sulfuric acid solution to correct inconsistencies in the quality of fluorescence from different lots of sulfuric acid. Kimura and Miller (1965) estimated gibberellic acid in rhubarb as low as 3 p.p.b. with a combination of column and thin-layer chromatography. The quantitation was by visual comparison of the intensities of the fluorescent zones. Holzer and Kuzel (1960) determined microgram amounts of gibberellic acid in malt. Separation was made by dialysis, which is time-consuming.

The object of this work was to develop an analytical method which would combine the advantages of the above methods and be suitable for determining gibberellic acid at very low concentrations in a variety of plant materials. The following procedure was developed and has been used in this laboratory for a number of years.

REAGENTS

Buffer solution, pH 7. Dissolve 6.7 grams of reagent grade KH_2PO_4 and 1.2 grams of reagent grade sodium hydroxide in water to make 1 liter.

Florisil, TLC with binder (Floridin Co., Hancock, W. Va.). Suspend 100 grams in 300 ml. of methanol and 30 ml. of concentrated hydrochloric acid for 30 minutes. Allow the solids to settle and carefully decant the liquid. Resuspend in 100 ml. of methanol and decant. Repeat five times. Filter off on a Büchner funnel and wash with three 50-ml. portions of methanol. Dry in a 110° C. oven for 2 hours and sieve through a 200-mesh screen.

Amdal Co., Agricultural Division, Abbott Laboratories, North Chicago, Ill. 60064

Mobile solvent. Mix 75 ml. of chloroform, 24 ml. of acetic acid, and 3 ml. of methanol.

Gibberellic acid standard solution. 1 μg. per ml. in water.

Sulfuric acid, 85%–stannous chloride reagent. Dissolve 1 gram of reagent grade stannous chloride $\cdot 2H_2O$ in 235 ml. of water. Chill in an ice bath and carefully add 1 liter of sulfuric acid.

APPARATUS

Omnimixer (Sorvall), Waring Blendor, or equivalent.

Rinco evaporator or equivalent.

Spectrofluorophotometer with 1-cm. square silica cells. Aminco Bowman or equivalent (American Instrument Co., Silver Spring, Md.)

Thin-layer plates, Florisil. Disperse 25 grams of prepared Florisil and 2.5 grams of calcium sulfate in 65 ml. of water. Spread a 250-micron layer on 5 × 20 cm. glass plates. Air-dry 15 minutes and activate by heating $1\frac{1}{4}$ hours at 110° C.

PROCEDURE

Grind a 100-gram sample in 125 ml. of acetone and 20 ml. of water in an Omnimixer or Waring Blendor. In recovery studies add the standard gibberellic acid at this point.

Filter off the pulp on a Büchner funnel through Whatman No. 1 paper. Return the pulp to the Omnimixer, add 125 ml. of fresh acetone, and grind again. Filter again. Wash the mixer jar with 50 ml. of acetone and add to the filter. Wash the filter cake with another 100-ml. portion of acetone.

Collect the filtrate in a 1-liter round-bottomed flask and evaporate under reduced pressure on a Rinco evaporator. Warm the flask in a water bath at 30° C. Stop evaporating when only the aqueous solution is left.

Filter the solution through paper, rinse the flask with a 50-ml. and two 10-ml. portions of water, and pass each through the filter.

Adjust to pH 2.5 (±0.2) with 50% sulfuric acid. Extract in a separatory funnel with three 50-ml. portions of ethyl acetate.

Collect the solvent and extract with three 50-ml. portions of pH 7 buffer solution. If this forms an emulsion, separate by centrifuging at 2500 r.p.m. in 250-ml. plastic centrifuge tubes.

Adjust the collected aqueous solution to pH 2.5 (±0.2) and extract with three 50-ml. portions of ethyl acetate. Filter the collected solvent through paper and evaporate to dryness on a Rinco evaporator.

Dissolve the residue in 1.0 ml. of ethyl acetate. (If the rest of the procedure cannot be completed during the same day, store the extract overnight in a refrigerator at this point.) Apply a 50-μl. portion in a streak 2 cm. from the lower edge of the Florisil plate.

Develop the plate with freshly prepared mobile solvent 15 cm. beyond the origin. At the same time develop a second plate which has 500 μg. of standard gibberellic acid at the origin. Allow both to air-dry.

Spray the latter with ethanol–sulfuric acid (9 to 1) and heat 10 minutes at 110° C. Cool and examine under an ultraviolet lamp which has principal emission at 365 mμ. The gibberellin shows a blue fluorescence at R_f 0.50.

Scrape the corresponding zone from the sample plate and extract in a 1-cm. medium porosity glass filter crucible with 25 ml. of chloroform-methanol (2 to 1) added in small portions. Evaporate the extract to dryness in a round-bottomed flask on a Rinco evaporator.

Dissolve the residue in 5.0 ml. of water. Pipet 2.0 ml. into a 22 × 150 mm. test tube. Into a second test tube pipet 2.0 ml. of water which contains 2.0 μg. of gibberellic acid. Chill the tubes in an ice bath for 5 minutes and add 10.0 ml. of sulfuric acid–stannous chloride reagent. An all-glass, 10-ml. syringe works best in making this addition. Keep the tubes in the ice bath 10 minutes longer. Remove and allow to stand at ambient temperature 1 hour.

Measure the fluorescence of each solution at 455 mμ when excited at 410 mμ.

Calibrate the fluorophotometer with the standard solution to give 100 scale divisions deflection with the incident monochromator at 410 mμ and the emergent at 455 mμ. Determine the deflection of the sample solution. Avoid leaving the solutions in the cell holder for long periods; warming decreases fluorescence.

Table I. Recovery of Gibberellic Acid from Various Plant Materials

	Gibberellin A_3 Added, P.P.M.	Fluorescence (Std. = 100)	Total Gibberellin A_3 Found, P.P.M.	% Recovered
Artichokes	0	8.0	0.08	...
	0.1	16.0	0.16	80
	0.3	38.0	0.38	100
	0.7	84.0	0.84	97
Celery	0	6.8	0.07	...
	0.1	17.5	0.18	110
	0.3	45.2	0.45	138
	0.7	88.4	0.88	115
Cherries	0	1.2	0.01	...
	0.1	14.0	0.14	130
	0.3	30.5	0.31	100
	0.7	72.6	0.73	103
Grapes, seedless	0	5.0	0.05	...
	0.1	12.0	0.12	70
	0.3	30.0	0.30	83
	0.7	65.0	0.65	86
Limes, pulp	0	24.0	0.24	...
	0.1	34.0	0.34	100
	0.3	56.0	0.56	107
	0.7	97.0	0.97	104
Limes, rind	0	23.0	0.23	...
	0.1	34.0	0.34	110
	0.3	58.0	0.58	83
	0.7	99.0	0.99	108
Prunes	0	4.9	0.05	...
	0.1	12.0	0.12	70
	0.3	32.6	0.33	93
	0.7	73.9	0.74	97
Tangelos Unripe pulp	0	4.2	0.04	...
	0.1	17.2	0.17	130
	0.3	31.7	0.32	93
	0.7	66.5	0.67	90
Rind	0	6.6	0.07	...
	0.1	14.5	0.15	80
	0.3	33.0	0.33	88
	0.7	69.4	0.69	89

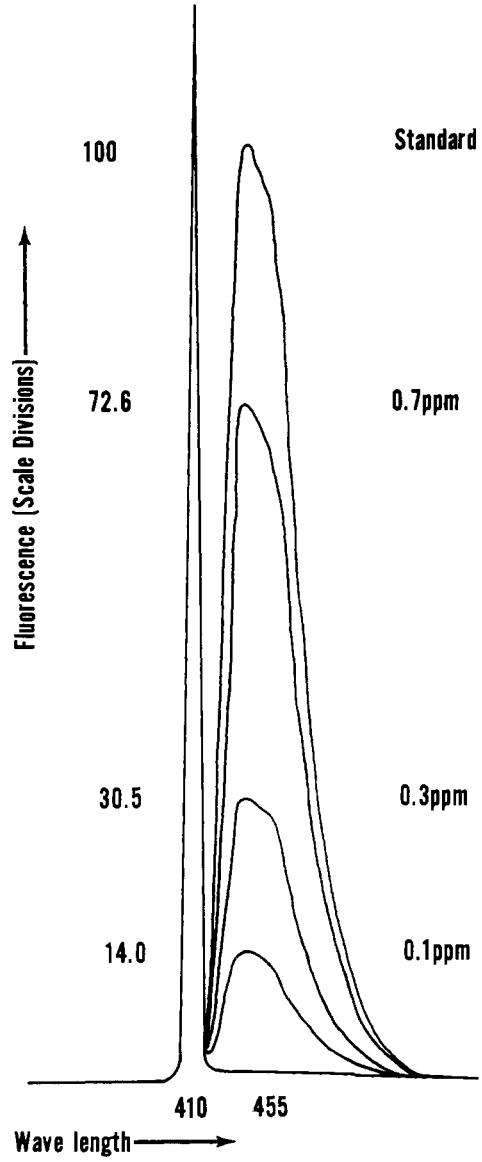

Figure 1. Fluorescence of gibberellic acid recovered from sweet cherries

CALCULATIONS:

$$2.0 \times 10^{-6} \times \frac{\text{fluorescence sample}}{\text{fluorescence standard}} \times \frac{50}{100} \times 10^{6} = \text{p.p.m. gibberellic acid}$$

EXPERIMENTAL

Small amounts of gibberellic acid were added to plant samples known not to be treated with gibberellic acid. This addition was made to the acetone in the first homogenate of the sample equivalent to 0, 0.1, 0.3, and 0.7 part of gibberellic acid per million parts of sample.

Each of the four portions of sample was carried through the whole procedure and the resulting fluorescence was compared to a standard described in the procedure which contained 1 μg. of gibberellic acid.

The results are reported in Table I. Figure 1 illustrates the fluorescence obtained with sweet cherries.

DISCUSSION

The R_f value of gibberellic acid on freshly prepared plates was 0.40, but after the plates aged for a week or more the value increased. In each determination a plate of standard gibberellic acid alone was developed and used to find the gibberellic acid on the sample plate.

The Florisil as received did not separate the gibberellic acid from interfering materials. It was necessary to wash it with hydrochloric acid to get the desired resolution. The calcium sulfate in the Florisil TLC was partially removed by this treatment and must be replaced to get firm plates. The authors were unable to get satisfactory separation with alumina G, silica gel G, or kieselguhr G plates.

The ethyl acetate solution applied to the Florisil plate in the case of artichokes contained considerable resinous material. As these zones dried, they had a distinct inclination to curl and even flake off completely. This can be overcome by making harder TLC plates by adding more calcium sulfate during the plate preparation.

In all instances, the temperatures were kept at 30° C. or below. If the grinding in the mixer was lengthy, there was some heating. The greatest possibility of losing gibberellic acid from overwarming was during the evaporations.

There was some heating of the final fluorescent sample in the spectrofluorophotometer from the intense light of the xenon lamp. Fluorescence decreased with temperature rise. Cuvettes should be exposed in the instrument for short periods.

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