Estimation of Gibberellic Acid Residue in Rhubarb

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A relatively rapid and specific method for gibberellic acid (GA3) sensitive to 3 p.p.b. in rhubarb is based on the adsorption of GA3 on aluminum oxide from a large volume of ethyl acetate, subsequent quantitative elution with a small volume of methanol, and thin layer chromatography. Initial separation from rhubarb is by aqueous extraction at pH 8, followed by extraction at pH 2 into ethyl acetate. Final estimation is by visual comparison.

A reasonably rapid, specific, and sensitive analytical procedure was required for determination of gibberellic acid (GA3) residue in rhubarb stalks, for obtaining U. S. Department of Agriculture approval in the use of GA3 on commercial rhubarb crowns. Methods for GA3 are numerous. Exclusive of bioassay procedures, they can be classified as chromatographic methods, which include paper (*2*, *13*), thin layer (*6*, *10*, *14*), and gas (*5*), photometric methods (*3*, *4*, *11*, *18*), including fluorometry (*7*, *12*, *17*) and infrared (*19*), and isotope methods (*1*, *9*, *20*). Most of these procedures are insensitive from the residue work standpoint and all, except the thin layer methods, are either insensitive below 20 p.p.b. or otherwise not suitable for residue study. The highest level encountered in treated rhubarb was on the order of 10 p.p.b. Thus, the thin layer chromatographic technique (*15*, *16*), because of its extreme sensitivity as pointed out by MacMillan and Suter (*10*), provided an ideal analytical approach. Under conditions for rhubarb analysis some sensitivity was lost; however, the resulting sensitivity of 3 p.p.b. was satisfactory.

Reagents and Apparatus

All reagents were of ACS grade unless otherwise specified.

Gibberellic acid (Merck & Co., 98.8% GA3). The working standards contained 10 μg. per ml. of ethyl acetate or methanol.

Aluminum oxide, reagent grade powder (Matheson, Coleman, and Bell, Catalog No. AX710).

Silica gel G (according to Stahl, E. Merck, Brinkmann Instruments, Inc.).

Chromatographic solvent mixture. Ethyl acetate–acetic acid, 95:5. Other mixtures described by MacMillan and Suter (*10*) and Sembdner *et al.* (*14*).

Toledo food chopper, Model 5 120-0-009.

VirTis 45 homogenizer, with 250-ml. capacity flasks (No. 16-094).

Thin layer chromatographic apparatus.

Microsyringe, 100-μl. capacity.

Column. Seal an 80 × 0.5 mm. i.d. capillary tubing to a 300 × 20 mm. o.d. borosilicate glass tubing. Pack the column with 6.5 grams (3 cm.) of aluminum oxide using a 2-cm.-diameter disk of glass filter paper below and above the adsorbent.

Ultraviolet light source. Mineralight was used. Excitation wave length for GA3 is given by Kavanagh and Kuzel (*7*) as any one or combination of the mercury lines, 366, 405, and 436 mμ.

Method

Preparation of Sample and Extraction. Pass frozen samples of rhubarb rapidly through an electric food chopper, collect in a plastic bag, and mix. Weigh 70 grams of this into a 250-ml. homogenizing flask, add 77 ml. of 2% sodium carbonate solution, and immediately homogenize, slowly at first, then at full speed for 10 minutes. The pH of the homogenate should be 7 to 8. Transfer the homogenate to a 250-ml. centrifuge tube and centrifuge at 2000 r.p.m. for 15 minutes. Decant the liquid into a second 250-ml. centrifuge tube. Mix residual solids with 50 ml. of water, recentrifuge, decant combining liquids, and adjust to pH 1.8 to 2.0 by dropwise addition of 18*N* sulfuric acid with mixing. Divide the pH-adjusted extract between two 250-ml. centrifuge tubes. Add 100 ml. of ethyl acetate, stopper (with polyethylene), shake vigorously for 5 minutes, and centrifuge for 10 to 15 minutes. Siphon the ethyl acetate phase into a 500-ml. container (a 1-pint medicine bottle is satisfactory). Re-extract the aqueous phase with 50 to 75 ml. of ethyl acetate. Combine the extracts.

Concentration of GA3. Transfer the extracts to a 500-ml. separatory funnel and shake with 10 volume % of water until the wash water is pH 3. After removal of as much water as possible, dry the ethyl acetate phase using powdered anhydrous sodium sulfate at the rate of 4 grams per 100 ml. of solution. Pass the solution through the alumina column at a maximum flow rate of 10 ml. per minute to adsorb GA3 from the solution. After the last of the solution and washings reaches the level of the adsorbent, wash down the sides of the column with ethyl acetate. Wash the adsorbent with 20 ml. of ethyl acetate. Wash the sides of the column with 4 ml. of methanol. After the methanol reaches the level of the adsorbent, change the receiver to a 150 × 18 mm. test tube, add 15 ml. more of methanol, and collect until the flow ceases. Evaporate the methanol to dryness at room temperature in the collection test tube, using a stream of dry, filtered, compressed air delivered inside the test tube by a 4-mm. o.d. glass tubing.

Further Purification of Concentrate. Add 5 ml. of water to the residue, mix, then add one drop of 9*N* sulfuric acid with mixing. The pH should be 1.8 to 2.0. Add 6 ml. of ethyl acetate, stopper (with polyethylene), and shake 1 minute. Carefully remove and rinse stoppers with ethyl acetate, and centrifuge. Siphon the ethyl acetate phase containing the GA3 into a second test tube. Dilute the residual ethyl acetate phase with an additional 2 ml. and resiphon. Repeat twice more, combining all ethyl acetate fractions in the second test tube. Evaporate extracts to dryness as before. Add 0.20 ml. of ethyl acetate to the test tube just before chromatography and stopper. Carefully dissolve all solids.

Chromatography. Spot appropriate aliquots 2 cm. apart on an activated silica gel chromatoplate using a microsyringe. Spot appropriate volumes of ethyl acetate standard between the samples. Develop plates until the solvent front is 15 cm. from the starting point. Dry and check the plates for fluorescent spots under ultraviolet light. Spray plates with 70% aqueous sulfuric acid for 1 minute. Immediately compare fluorescent spots corresponding to GA3 visually with known standards. The R_f of the GA3 in the sample, if any, and that of the adjacent standards should always correspond.

Results and Discussion

Table I summarizes the recovery of added GA3 from rhubarb. Recovery is indicated to be about 80%. A limiting factor in the method was a slight fluorescent streaking which occurred occasionally after acid spraying. However, when an aliquot corresponding to 35 grams of rhubarb was used, 0.1 μg. of GA3 was clearly visible through this background. Unavoidable errors associated with visual estimation are expressed in Table II. With practice

Table I. Recovery of Added GA3 from Rhubarb

Ground Rhubarb, G.	Added Methanolic GA3		Aliquot Spotted, Ml.	Recovered from Aliquot, μG.	Total GA3 Recovered	
	μG.	P.p.b.			μG.	P.p.b.
70	1.0	14	0.050/0.200	0.2	0.8	11
70	0.5	7	0.100/0.200	0.2	0.4	6
70	0.2	3	0.100/0.200	<0.1	<0.2	<3
70	0.0	0	0.100/0.200	0.0	0.0	0

Table II. Unavoidable Errors in Visual Estimation of GA3

Spot[a] Estimated, μG.	Probable Range, μG.	Probable Range, P.P.B. (35-G. Aliquot)	Spot Estimated, P.P.B. (35 G. Aliquot)	Comments
0.00	0.00–0.02	0.0–0.6	0.0 to 0.6	(When clearly defined)
0.05	0.03–0.07	0.9–2.0	1.4 ± 0.6 (40%)	(When clearly defined)
0.10	0.07–0.13	2.0–3.7	2.9 ± 0.9 (30%)	Definite GA3
0.20	0.15–0.25	4.3–7.1	5.7 ± 1.4 (25%)	Definite GA3
0.30	0.25–0.35	7.1–10.0	8.6 ± 1.4 (17%)	Definite GA3
0.40	0.35–0.45	10.0–12.9	11.4 ± 1.4 (13%)	Definite GA3

[a] Spots obviously intermediate between two levels estimated as such (0.15, 0.25, 0.35, etc.).

and careful estimation, values obtained should fall within these estimated ranges.

GA3 spots appeared under ultraviolet light in characteristic fluorescent color which varied with the amount of GA3 and time lapse after spraying with sulfuric acid. For more than 1.0 μg. the color is blue-green; for less than 1.0 μg. the blue-green is not always apparent. Color in these cases may best be described as whitish. The greatest intensity of the GA3 spots occurred during the first few minutes after spraying. The samples and standards were compared rapidly during this period. Faded spots were useful in comparing spots > 0.1 μg. Spots of 0.02 and 0.05 μg. of GA3 faded completely within 5 minutes after spraying. Spot diameters were ≦5 mm. on one-dimensional chromatoplates developed in ethyl acetate–acetic acid. Spotting of GA3 from ethyl acetate solutions led to tight spots about a third of the diameter of the solvent spot. Spots obtained from methanol solution were as large as the solvent spots. Addition of a small amount of ethyl acetate to the carbonate extraction mixture caused foaming. For these reasons the methanolic standard was used for fortifying samples and the ethyl acetate solution for the chromatographic standard.

Two-dimensional chromatography (Figure 1) was done on fortified and unfortified extracts for GA3 identification. The first solvent was ethyl acetate–acetic acid and the second, after thorough evaporation of the first solvent mixture from the plate, was isopropyl ether–acetic acid (*10*). Numerous fluorescent spots were observed before and after acid spraying. These spots, however, were not observed consistently but varied from sample to sample. The general location of GA3 on the plate, in both one- and two-dimensional chromatograms, was vacant when the untreated sample was unfortified. R_f values for

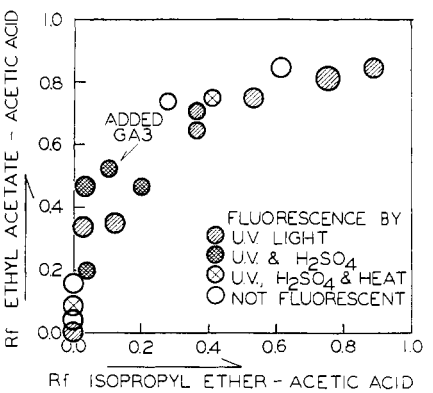

Figure 1. Two-dimensional thin layer chromatogram of rhubarb extract

GA3 were 0.49 (±0.02) in the first solvent and 0.10 in the second. The known R_f value for GA3 in the second solvent is 0.11. GA1, which has an R_f identical to that of GA3 in the second solvent, does not fluoresce after spraying with 70% aqueous sulfuric acid (*2, 10*). Sensitivity was lost during two-dimensional chromatography. It is apparent from Figure 1 that a one-dimensional separation in ethyl acetate–acetic acid is adequate for GA3 separation in rhubarb. Endogenous GA3 was not detectable in mature rhubarb stalks.

The stability of GA3 under acidic and basic conditions is discussed briefly by Kavanagh and Kuzel (*7*) and Kuhr (*8*). Although the degradation of GA3 appears to be slow at room temperature, solutions during extractions were kept at extreme pH as short a time as possible as a precaution. Whenever work was carried over to the following day they were stored at −17° C. in the water-washed ethyl acetate state or in the methanol effluent state.

Solvent systems of MacMillan and Suter generally gave low R_f's for GA3 and for numerous fluorescent materials in the rhubarb extract, causing overlapping of the GA3 with intensely fluorescent areas. Estimation of GA3 under these conditions was not possible. However, in gibberellin identification in impure extracts, these solvents and that of Sembdner *et al.* were useful as second solvents when chromatography on rhubarb extracts was performed in the second dimension after initial separation with ethyl acetate–acetic acid.

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