Indirect Flame Photometric Determination of Total Sulfur in Biological Materials

W. M. SHAW
University of Tennessee Agricultural Experiment Station, Knoxville, Tenn.

A procedure for the flame photometric determination of total sulfur in biological materials consists of the oxidation of the materials in nitric-perchloric acids and determination of the sulfur content by the luminescence of the residual barium after precipitation as barium sulfate. Analysis of representative biological materials, including cystine and methionine, by the flame photometric and gravimetric methods showed differences between methods of ±0.1 to ±0.3 mg. of sulfur for a range of sulfur content of 1 to 26 mg. An aliquot of the perchloric acid solution of the sample may be used for phosphorus determination and the test solution, free of sulfate and phosphate, is also suitable for the flame photometric determination of its calcium, magnesium, and potassium contents without interference from these anions.

TWO PREVIOUS PAPERS have dealt with some of the fundamentals upon which the present procedure is based. One (*5*) described a procedure for the flame photometric determination of sulfate in water; the other (*6*) reported a rapid nitric-perchloric acid procedure for oxidizing biological materials prior to sulfur determination using the gravimetric method. A study of flame photometric determination of sulfate ions was published by Burriel-Marti *et al.* (*1*), who used 873-mμ wave length, oxyacetylene flame, and 0.4-mm. slit width—conditions which do not permit direct correction for cation interference.

The application of the flame method to biological materials necessitates the evaporation of the perchloric acid after completion of the oxidation; for materials devoid of or low in calcium content, the addition of a fixative is required to prevent the loss of sulfuric acid. The effects of fixatives on the accuracy of the flame photometric sulfur determination have not been previously investigated.

The objectives of this study were to adapt the flame photometric procedure (*5*) to sulfur determination in biological materials and organic compounds, following their oxidation in nitric-perchloric acid (*6*), and to investigate the attendant use of calcium as sulfur fixative.

Experimental

Preliminary Test of Photometric Procedure. In the experimental part of this study (Tables I and II), the photometric results are based on calibration curves of standard barium solutions (*5*). The results of tests with representative biological materials and organic compounds ranging in sulfur content from <1 to 26 mg. are given in Table I. The precision of the photometric method, as shown by the standard deviation, compares favorably with the gravimetric method. The most serious negative photometric errors (1 mg. of sulfur) were found with methionine and cystine. The distinguishing characteristics of these two materials were their high sulfur content and high calcium concentration introduced as a sulfate fixative. The factors of sulfate and calcium concentrations, as contributing to a negative error in the photometric method, were investigated further.

Effect of Sulfur Concentration. A series of solutions was set up in which the calcium and potassium were 20 mg. per 100 ml., while the sulfur as ammonium sulfate varied from 1 to 24 mg. The sulfate precipitations and the determinations of residual barium were carried out as described in the procedure for sulfate solutions (*5*) except that the barium standards were compensated for calcium and potassium in the same concentrations as in the unknowns. The variables of the experiment are listed under series I to V (Table II). Four sulfur levels at 1-mg. intervals were within each series.

For sulfur content up to 4 mg., the average sulfur found in the series containing the larger barium excess (IB) deviated positively more than sulfur found in series IA, probably because of the tendency for increased barium chloride coprecipitation with increasing excess of barium.

Effect of Calcium Concentration. For calcium concentrations of 0, 10, 20, 30, 40, and 50 mg. per 100 ml., the deficiencies for 25-mg. sulfur recoveries were 0, 0.70, 0.85, 1.10, 1.00, and 0.80 mg., respectively.

The calcium effect appears somewhat irregular in that the largest negative error, 4.4%, was shown by the addition of 30 mg. of calcium, while the errors from the larger and smaller calcium concentrations were slightly lower.

The photometric error due to calcium is the result of two factors: calcium sul-

Table I. Preliminary Test of Accuracy of Flame Photometric Sulfur Determination

No.	Material[a]	Analysis ± Std. Dev., Mg.[b] Gravimetric	Flame photometric	Photometric-Gravimetric Difference, Mg.	Analytical Error, %
1	Wheat straw	0.90 ± 0.05	0.8 ± 0.00	−0.1	−11.0
2	Soybean hay	2.1 ± 0.10	1.9 ± 0.10	−0.2	− 9.5
3	Rye grass	3.3 ± 0.03	3.4 ± 0.00	0.1	3.0
4	Broccoli	9.4 ± 0.13	9.3 ± 0.14	−0.1	− 1.1
5	Turnip greens	11.5 ± 0.13	11.2 ± 0.10	−0.3	− 2.6
6	Cotton leaves	8.3 ± 0.15	8.3 ± 0.20	0.0	0.0
7	Tobacco	6.3 ± 0.07	6.0 ± 0.20	−0.3	− 4.8
8	Cottonseed meal[c]	5.3 ± 0.06	5.6 ± 0.20	0.3	6.0
9	Blood meal[c]	5.5 ± 0.20	5.7 ± 0.20	0.2	4.0
10	Feather meal[c]	16.6 ± 0.12	16.5 ± 0.14	−0.1	− 0.6
11	Casein[c]	6.8 ± 0.07	7.1 ± 0.20	0.3	4.4
12	Methionine[c]	21.00 ± 0.94	20.00 ± 0.41	−1.0	− 4.8
13	Cystine[c]	26.12 ± 1.30	25.03 ± 0.01	−1.1	− 4.2

[a] Methionine and cystine were 0.1-gram samples; all others were 1 gram.
[b] Represents averages of three samples.
[c] Before evaporation to dryness in perchloric acid, calcium was added as calcium chloride: 10 mg. to Nos. 8, 9, 10, and 11; 40 mg. to No. 12; and 50 mg. to No. 13.

fate coprecipitation and some spectral interference of calcium with the barium flame at 522 mμ (5). The coprecipitation error is negative and amounts to the full equivalent of the coprecipitated calcium. The spectral error is relatively smaller and may be compensated for by the addition of calcium to the standards.

The calcium sulfate coprecipitation also introduces a negative error in the gravimetric determination through the substitution of the lighter calcium ion for the heavier barium ion that amounts to a theoretical 42% of the sulfur affected by the coprecipitation. In the photometric determination, however, the negative error amounts to 100% of the sulfur so affected because the enhancement of barium in solution is equal to the full equivalent of the calcium sulfate coprecipitation.

Additional precipitations of 25 mg. of sulfur as ammonium sulfate made in the absence of added cations, with 40 mg. of calcium and with 40 mg. of potassium, gave the following analytical errors: +0.4, −4.0, and −0.4% for the three conditions, respectively. Parallel determinations of 4 mg. of sulfate sulfur have shown no detectable error in the photometric sulfur recovery in the presence of 10 to 50 mg. of calcium; whereas with 12.5 mg. of sulfur under similar conditions, deficiencies of 0.4 to 0.5 mg. of sulfur resulted.

The extent of the photometric sulfur error is governed primarily by the quantity of the sulfate precipitate and is little affected by the calcium concentration in the range of 10 to 50 mg. per 100 ml.

Correction for Calcium Coprecipitation. In the preliminary studies, calibration graphs were prepared to give the barium luminescence *vs.* barium ion concentration on the assumption of exact equivalence of the barium and sulfate ions in the precipitate. For the correction for the imbalance caused by calcium sulfate coprecipitation, the standards were subjected to the same type of interference (coprecipitation) as the unknowns. Standard sulfate solutions were supplemented with calcium, potassium, and magnesium and processed by the procedure outlined below; barium luminescences *vs.* milligrams of sulfate ion precipitated were plotted as shown in Figure 1. Graph *A* brackets the sulfur contents of 1-gram samples of all forages, field crops, seeds, bloodmeal, and casein; *B* provides for the higher sulfur content of leafy vegetables and feather meal; *C* was prepared especially for the analysis of organic compounds.

Procedure

Although the present procedure ap-

Table II. Effects of Sulfate Concentration on Accuracy of Flame Photometric Determination of Sulfate in Presence of Calcium and Potassium

Series		Components of Mixture[a] SO–S, mg.	Components of Mixture[a] Ba, mg.	Average Values[b] Luminescence, % T	Average Values[b] ± Av. dev., % T	Average Values[b] Residual Ba found, mg.	Sulfur Found, Mg.[c]	Analytical Error Mg.	Analytical Error %
IA		1	5	18.5	0.07	3.82	1.18		
		2	5	13.9	0.07	2.90	2.10		
		3	5	9.0	0.02	1.90	3.10		
		4	5	4.5	0.05	1.00	4.00		
	Av.	2.5	5				2.60	0.10	4.0
IB		1	10	43.1	0.08	8.80	1.20		
		2	10	37.7	1.40	7.70	2.30		
		3	10	33.2	1.70	6.80	3.20		
		4	10	27.9	1.30	5.70	4.30		
	Av.	2.5	10				2.75	0.25	10.0
II		6	10	19.7	0.04	4.05	5.95		
		7	10	15.0	0.05	3.10	6.90		
		8	10	10.6	0.06	2.22	7.78		
		9	10	5.4	0.06	1.20	8.80		
	Av.	7.5	10				7.36	−0.14	−1.9
III		11	15	21.7	0.04	4.48	10.52		
		12	15	16.2	0.06	3.35	11.65		
		13	15	11.9	0.05	2.48	12.52		
		14	15	7.2	0.03	1.55	13.45		
	Av.	12.5	15				12.04	−0.46	−3.7
IV		16	20	22.4	0.04	4.52	15.48		
		17	20	17.6	1.10	3.64	16.36		
		18	20	12.8	0.06	2.68	17.32		
		19	20	7.9	0.09	1.48	18.52		
	Av.	17.5	20				16.92	−0.58	−3.3
V		21	25	24.2	0.06	5.00	20.00		
		22	25	18.7	0.10	3.85	21.15		
		23	25	14.1	0.07	2.95	22.05		
		24	25	7.8	0.02	1.65	23.35		
	Av.	22.5	25				21.64	−0.86	−3.8

[a] In addition to 20 mg. of Ca and 20 mg. of K.
[b] Represents four readings on four different days.
[c] Represents sulfur equivalent of barium.

Table III. Sulfur Recovery from Various Materials by Flame Photometric Procedure

Material	Sample Size Material	Sample Size S content, mg.	Number Processed	Number Readings	S Found, Mean ± Std. Dev. Mg.	S Found, Mean ± Std. Dev. %	Dev. from Gravimetric Mg.	Dev. from Gravimetric %
0.0625*N* H_2SO_4[a]								
+ 2 mg. P as KH_2PO_4	2 ml.	2	1	5	2.05 ± 0.11		0.05	2.5
	4 ml.	4	1	5	4.08 ± 0.08		0.08	2.0
	6 ml.	6	1	5	6.07 ± 0.11		0.07	1.2
	8 ml.	8	1	5	8.00 ± 0.06		0.00	0.0
+ 5 mg. P as KH_2PO_4	2 ml.	2	1	5	2.10 ± 0.10		0.10	5.0
	4 ml.	4	1	5	4.10 ± 0.10		0.10	2.5
	6 ml.	6	1	5	6.10 ± 0.08		0.10	1.7
	8 ml.	8	1	5	8.00 ± 0.06		0.00	0.0
Casein[b] (Labco)	1 g.	6.8[c]	4	3	6.6 ± 0.06	0.66 ± 0.006	−0.20	−3.0
Feather meal[d]	1 g.	16.6[c]	4	3	16.3 ± 0.20	1.63 ± 0.02	−0.30	−1.8
Methionine[e]	0.1 g.	21.0[c]	3	3	21.2 ± 0.10	21.2 ± 0.10	0.20	1.0
Cystine[e]	0.1 g.	26.1[c]	4	3	26.2 ± 0.20	26.2 ± 0.20	0.10	0.4

[a] Contained also 20 mg. of Ca, 20 mg. of K, and 5 mg. of Mg; processed through the entire procedure, including step D.
[b] Received 20 mg. of Ca; processed through the entire procedure, including step D.
[c] From gravimetric determinations (Table I).
[d] Received 15 mg. of Ca; processed through entire procedure, including step D.
[e] Received 50 mg. of Ca; processed through entire procedure, including step alternate D.

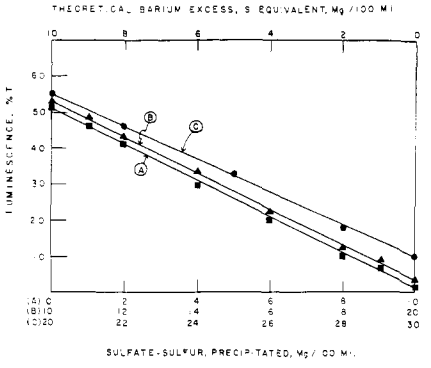

Figure 1. Calibration curves for barium luminescence (% *T* readings at 515 mμ minus those at 522 mμ) vs. varying sulfate content precipitated with a constant of barium chloride

- *A.* For sulfur content of 0 to 10 mg. with 10 ml. of 0.0625*N* $BaCl_2$ plus extraneous cations (20 mg. Ca, 20 mg. K, and 5 mg. Mg)
- *B.* For sulfur content of 10 to 20 mg. with 20 ml. of 0.0625*N* $BaCl_2$ plus extraneous cations (30 mg. Ca, 20 mg. K, and 5 mg. Mg)
- *C.* For sulfur content of 20 to 30 mg. with 30 ml. of 0.0625*N* $BaCl_2$ plus extraneous cation (50 mg. Ca, for organic compounds only)

pears similar in outline to the one presented previously (*5*), in actual performance the conditions are too dissimilar for the use of cross references for its description.

Reagents. Oxidation Mixture. Mix equal volumes of 70% nitric acid and 70% perchloric acid.

Standard Sulfate Solution. Prepare a 0.0625*N* sulfuric acid solution; 1 ml. is equivalent to 1 mg. of sulfur.

Standard Barium Chloride Solution. Prepare a 0.0625*N* solution of reagent grade barium chloride dihydrate; 1 ml. is equivalent to 1 mg. of sulfur.

Acetate Buffer, 2*N*. Mix 115 ml. of glacial acetic acid with about 800 ml. of water; adjust to pH 4 with 1 + 1 ammonium hydroxide and dilute to 1 liter.

Ferric Chloride Solution. Prepare a 0.5*N* ferric chloride hexahydrate solution in 0.5*N* hydrochloric acid.

Stock solutions of calcium, magnesium, and potassium, used primarily as additives, were prepared as neutral chlorides from C.P. chemicals in the concentration of 1 mg. of the metal per 1 ml. of solution. Precipitated calcium carbonate (low in alkali), magnesium ribbon, and potassium chloride were used as sources of the three elements.

Preparation of Test Solution. A. Oxidation of Organic Matter. Weigh a 1-gram sample of plant or animal tissue or 0.1 gram of organic sulfur compounds. Transfer to a 100-ml. Kohlrausch-type flask of borosilicate glass; wash down dust particles with a little water. Introduce 10 ml. of the oxidation mixture and swirl contents until completely wetted. Place flasks on the edge of the hot plate, and apply heat cautiously so that foaming does not get out of control. When foaming has subsided, increase the heat so that the digestion is maintained at brisk boiling. Maintain at boiling temperature for 1 hour after condensate in the expanded portion of the neck has completely disappeared. Remove the flasks from the hot plate, dilute with 10 ml. of water, and carefully transfer digests into 250-ml. beakers, rinsing the flasks with water three times.

B. Perchloric Acid Evaporation. To digests of animal products and amino acids, add calcium as calcium chloride in the proportion of at least 1.5 mg. per each mg. of sulfur expected or a minimum of 20 mg. Evaporate to dryness on a hot plate, taking precautions against spattering. Drive off the condensed acid on the sides of the beaker either by prolonged heating on the hot plate or, more conveniently, by heating for 5 minutes in a muffle furnace set at 190° to 200° C.

C. Sulfate Precipitation. Dissolve the cooled residue in 5 ml. of 1 + 1 hydrochloric acid and evaporate to dryness at below boiling heat. Add 5 ml. of 1*N* hydrochloric acid and warm on the hot plate for 5 minutes in a covered beaker; dilute with about 100 ml. of water, and heat at gentle boiling for 30 minutes to ensure that all the calcium sulfate has dissolved. Precipitate the sulfate by dropwise addition of 10, 20, or 30 ml. of the standard barium chloride solution—according to the sulfur range expected—from a pipet, while stirring. (Wipe off the condensate on the pipet before reintroducing into the standard barium chloride solution.) Digest the precipitate on the hot plate for about 2 hours, during which time the volume should be reduced to about 70 ml. Cool to room temperature. For very low sulfur content, leave the precipitate overnight at room temperature; otherwise proceed with the next step.

D. Removal of Phosphate and R_2O_3. Add 10 ml. of the acetate buffer, 5 ml. of the ferric chloride, and 1*N* ammonium hydroxide in the equivalent of 10 ml. of 1*N* hydrochloric acid. Digest at boiling temperature in covered beakers for 15 minutes. Transfer the suspension into a 100-ml. volumetric flask, rinsing the flask three times with water. Cool the flask rapidly in running cold water, fill to the mark with distilled water, mix, and filter on a Whatman No. 12, 12.5-cm. folded paper, or its equivalent, catching the filtrate in a polyethylene bottle. Store in a cool, dark place until ready to analyze.

(Alternate D.) For standard sulfate solutions and for phosphate-free organic compounds, omit the ferric chloride and the boiling; instead, add 5 ml. of 1*N* hydrochloric acid, and otherwise proceed as in D.

Flame Photometric Determination. Calibration Standards. Into a series of 250-ml. beakers introduce 0, 2, 4, 6, 8, and 10 ml. of the 0.0625*N* sulfuric acid, and add 20 mg. of calcium, 10 mg. of potassium, 5 mg. of magnesium, and 1 ml. of perchloric acid. Evaporate to dryness and proceed as described under B, C, and alternate D. The extraneous cations are added to compensate for slight spectral and radiation effects.

Using the oxyhydrogen flame of the Beckman DU spectrophotometer with photomultiplier attachment, adjust the instrument to give for the zero-sulfur standard a 100% *T* value of 515-mμ wave length. The approximate settings should be: 10, 1.5, 3, 0.04, 1.0 ± 0.5, denoting oxygen and hydrogen pressure in pounds per square inch; photomultiplier switch position; slit width in millimeters; and sensitivity in number of turns from counterclockwise limit, in the order given. The selector switch is always on 0.1. Repeat readings of the zero-sulfur standard, with intervening rinsings with water until a stable 100% *T* value is obtained; then turn the wave length dial to 522 mμ and obtain the background reading. Subtract this reading from the one at 515 mμ and denote the differences as the "luminescence," expressed in % *T*. Plot the luminescences of the several standards against the sulfur content in milligrams, as indicated in Figure 1, *A*. The 100% *T* value at 515 mμ for the zero-sulfur standard also serves as a pivotal point for the calibrations of the higher ranges of sulfur content (graphs *B* and *C*).

The luminescences of the unknowns are obtained in the same manner as described for the standards, and the sulfur contents are read from the calibration graphs. To prevent cumulative errors, aspirate water between readings, maintain fuel pressure, and frequently check dark current and zero-sulfur standard for 100% *T* value at 515 mμ.

Test of Over-All Procedure

A final test of the flame photometric procedure was made by the analysis of sulfate-phosphate mixtures, casein, feather meal, methionine, and cystine; and by comparison of these results with those obtained gravimetrically (Table III). The deviations from gravimetric results of the sulfate-phosphate mixtures are accounted for by the sulfur content (0.09 mg.) of the ferric chloride additive, as calculated from the manufacturer's analysis.

As per cent of theoretical ± standard deviation, the sulfur recoveries for methionine and cystine were 98.8 ± 0.5% and 97.8 ± 0.8%, respectively. These compare favorably with McChesney and Banks' (*4*) recoveries of 98.2 ± 4.6% for methionine and 98.6 ± 1.1% for cystine. The sulfur recoveries by Evans

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.

Literature Cited

(1) Burriel-Marti, F., Ramirez-Munoz J., Rexach, M. L., de Lizarduy, M., *Anal. Chim. Acta* **17,** 559 (1957).
(2) Evans, R. J., St. John, J. L., *Ind. Eng. Chem., Anal. Ed.* **16,** 630 (1944).
(3) Hillebrand, W. F., Lundell, G. E. F., Bright, H. A., Hoffman, J. I., "Applied Inorganic Analysis," 2nd ed., p. 714, Wiley, New York, 1953.
(4) McChesney, E. W., Banks, W. F., *Anal. Chem.* **27,** 987 (1955).
(5) Shaw, W. M., *Ibid.*, **30,** 1682 (1958).
(6) Shaw, W. M., J. AGR. FOOD CHEM. **7,** 843 (1959).

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GIBBERELLINS DETERMINATION

Fluorometric Assay for Gibberellic Acid

ROBERT J. THERIAULT, WALDO C. FRIEDLAND, MERLIN H. PETERSON, and J. C. SYLVESTER

Department of Microbial Physiology, Research Division, Abbott Laboratories, North Chicago, Ill.

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