Semimicrodetermination of Inorganic Sulfate in Plant Material

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Ion exchange and ultraviolet spectrophotometry are combined to determine sulfate sulfur in plant material. The sulfate in the plant extract is first separated from interfering substances using ion exchange resin IRA 400, and the sulfate in the eluate is then precipitated as benzidine sulfate, the absorbance of which in hydrochloric acid solution is measured at 250 mμ. The method, which has been checked by radioisotope dilution analysis, permits the determination of from 0.1 to 2.4% of sulfate in the sample.

When a crucial role for the part played by inorganic sulfate in the control of copper storage by molybdenum in sheep (Dick, 1953) had been established, the need arose to develop a routine method for the determination of the sulfate sulfur fraction of plant material. As early as 1853 (Fraps, 1903), attempts had been made to differentiate the various forms of sulfur in plants and although many papers appeared during the ensuing century (Evans, 1931; Fraps, 1903; Gortner and Hoffman, 1924; Hall, 1922; Johnson *et al.*, 1943; Johnson and Nishita, 1952; Peterson, 1914; Thomas *et al.*, 1944; Thompson, 1913; Wood and Barrien, 1939) on the topic, the methods which they described for the determination of sulfate sulfur almost invariably depended upon separation as barium sulfate. Although the precipitation of barium sulfate may be satisfactory in inorganic solutions, it is open to question whether this is so in extracts of plants in which a complex mixture of gums, proteins, polyuronides, and sugars exists.

In spite of the recognition of the problems associated with this method of separation, few alternatives have been proposed. Benzidine, which had been demonstrated to be the pre-eminent reagent for the determination of sulfate in plasma (Cuthbertson and Tompsett, 1931) and urine (Owen, 1936) did not find favor (Aitken, 1930) and was abandoned for use with plant extracts because of nonspecific precipitation which occurred (Wood and Barrien, 1939). A significant advance was made when Johnson and Nishita (1952), making use of the reaction by which sulfate may be reduced to sulfide, then to be converted to methylene blue (Gustafsson, 1960), developed a method for the direct determination of low concentrations of sulfate sulfur in plant material. The only source of interference was from nitrate. Hogan and Breen (1960) modified the apparatus of Johnson and Nishita and applied this routinely to pasture samples. In this laboratory, attempts to determine sulfate in extracts of plants by the addition of excess barium chloride and back-titration with EDTA using a photometric determination of the end point were not wholly successful. With low concentrations of sulfate, the end point was attenuated.

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To avoid the necessity for the preparation of the special reducing agent required in the method of Johnson and Nishita, and because procedures involving the separation of barium sulfate were considered to lack specificity and sensitivity when applied to extracts of plants, attention was again directed to the possible use of benzidine as a reagent for the separation of inorganic sulfate from plant extracts. Benzidine forms a sulfate, which in acid solution has a solubility of the same order as barium sulfate, and the value of $E_{1\,cm.}^{1\%}$ of the benzidine at 250 mμ is 1.03×10^3, which is of sufficient magnitude to enable sensitive and quantitative determination of the benzidine moiety of benzidine sulfate and, provided the precipitate of benzidine sulfate is pure, of the amount of sulfate present.

Preliminary experiments showed that the separation of benzidine sulfate from extracts of plant material prepared with aqueous ethanol–trichloroacetic acid mixtures was in the majority of cases quantitative. This was confirmed by means of radioisotope studies with ^{35}S-labeled sulfate. There were, however, certain samples, notably those of gramineous origin, in which the precipitation of benzidine sulfate was completely inhibited unless the inorganic sulfate concentration of the extract were first artificially raised above a threshold concentration equal to 3.6 mmoles. All the sulfate in the extract could then be fully recovered. This indicated that some of the constituents of the plant extract may be acting as peptizing agents, consequently the removal of these would be a necessary preliminary step to any satisfactory method for the direct determination of the sulfate in the extract.

When this extract was passed through an ion exchange resin column consisting of IRA 400 in the chloride form, while the organic constituents remained unabsorbed on the resin column, the sulfate was absorbed and could be determined after elution. This paper describes the application of an ion exchange resin technique and ultraviolet spectrophotometry to the routine determination of sulfate as benzidine sulfate in plant material.

Reagents

Sodium chloride, 3.5% w./v. solution in glass-distilled water.

Trichloroacetic acid, 10% w./v. solution in glass-distilled water. The analytical grade is usually free of sulfate. When tests show the presence of appreciable amounts of sulfate, the reagent should be purified by

redistillation under vacuum in the presence of a little barium chloride.

Benzidine, 1% w./v. solution in ethanol. This reagent should be purified if it is not near-white in color. This is done by boiling a 10% alcoholic solution with charcoal, and pouring the filtered solution into ice-cold glass-distilled water. The purified reagent is filtered off, washed with water, and dried at 37° C. in the dark and subsequently in a desiccator over calcium chloride.

Standard potassium sulfate solution, 0.1810 gram of dried analytical grade potassium sulfate is dissolved in water and then made up to 100 ml. to provide a stock solution of 1.0 mg. per ml. from which suitable substandards may be prepared.

Hydrochloric acid, redistilled, is diluted sixfold to yield an approximately 1*N* solution, and 600-fold for a 0.01*N* solution.

Apparatus

The ion exchange column consists of a glass tube 8 to 10 cm. in length and 5 to 6 mm. in diameter, fitted with an outlet tap and surmounted by a reservoir about 2 cm. in diameter and 3 cm. in length. A column of IRA 400 resin of 20 to 50 mesh, 5 to 6 cm. in length, is contained within the tube, and a glass wool pad at either end serves to maintain the column. Before use, the resin is converted into the chloride form by the passage of 6 ml. of 3.5% sodium chloride solution followed by water until tests for chloride in the eluate are negative. The resin column has a capacity of approximately 1 meq., and the rate of flow should be controlled so that the outflow of more than 0.2 ml. per cc. of resin per minute is not exceeded. The Beckman Model DU Spectrophotometer was fitted with quartz cells of 1 cm. light path for measurement of absorbance at 250 mμ.

Method

A 1-gram sample of air-dried plant material ground to pass a 0.5-mm. screen is weighed into an 8 × 1 inch borosilicate glass digestion tube fitted with a standard tapered joint, and 15 ml. of 0.01*N* hydrochloric acid is added. The mixture is gently boiled under reflux for 10 minutes, and after being cooled is centrifuged to remove suspended particles. An aliquot, usually 4 ml. of the supernatant, is transferred to a column of IRA 400 ion exchange resin in the chloride form. The rate of flow is adjusted and the aliquot passed through the column, followed by two flushes with 4 ml. of water each. The sulfate is now eluted from the column by passing 6 ml. of 3.5% sodium chloride at the same rate as that used during the adsorption step. The column is then rinsed with 4 ml. of water, the combined eluate is collected in a small beaker, and the contents are carefully taken to dryness on a hot plate. The residue is dissolved in approximately 1 ml. of 10% trichloroacetic acid and the solution transferred to a pointed centrifuge tube. Using a further 1 ml. of 10% trichloroacetic acid, the beaker is rinsed, and these rinsings are added to the centrifuge tube, after which 4 ml. of 1% benzidine in ethanol are added with mixing. The tubes are capped and set aside overnight at 4° C., and then the benzidine sulfate precipitate is separated by centrifugation at 750 G for 10 minutes. The supernatant liquid containing excess benzidine and trichloroacetic acid is poured off, the tube inverted and drained on filter paper, and the walls and mouth are rinsed with 50% ethanol delivered from a wash bottle with a fine jet. The tube is again allowed to drain on filter paper and the precipitate then resuspended in 5 ml. of ethanol. After centrifugation, the wash liquid is poured off, the tube inverted and rinsed with ethanol. The benzidine sulfate is then dissolved in 5 ml. of warm 1*N* hydrochloric acid and this solution rinsed into a 100-ml. volumetric flask. The tube is further rinsed with portions of 1*N* hydrochloric acid, and the washings are added to the volumetric flask. The flask is then made up to the mark with 1*N* hydrochloric acid, and the absorbance at 250 mμ measured in a 1-cm. quartz cell.

Alternatively, if ultraviolet absorbance measuring equipment is not available, an aliquot of the solution may be diazotized and coupled with thymol according to the method of Cuthbertson and Tompsett (1931) with the provision that the diazotization reaction be stopped with 2.5% sulfamic acid solution. In the absence of this precaution, spurious yellow colors are often produced when the alkaline thymol solution is added.

Experimental Results

Ultraviolet Absorption Spectrum of Benzidine in Hydrochloric Acid. The spectral absorption curve of benzidine dissolved in hydrochloric acid was examined and the maximum was at 250 mμ, which confirms the observation of Andersen (1953) for this substance. When benzidine sulfate is dissolved in hydrochloric acid, the absorbance maximum is the same.

Calibration Procedure. The absorbance-concentration relationship was examined for a range of concentrations of solutions of benzidine in hydrochloric acid and for solutions of benzidine sulfate which had been precipitated from trichloroacetic acid solutions in the presence and absence of 0.6*M* sodium chloride. Similar measurements were made on solutions in hydrochloric acid of benzidine sulfate which had been precipitated from the same quantities of sulfate after adsorption and elution from the IRA 400 resin column. The results of these calibration procedures are summarized in Table I from which the curve presented in Figure 1 has been constructed.

Sodium Chloride Concentration Required for Elution of Sulfate from the Resin Column. To determine the sodium chloride concentration necessary for quantitative elution of the adsorbed sulfate from the resin, tests were carried out with three different concentrations to determine their efficiency as eluting agents. When 0.60*M* sodium chloride was passed through the column, 100% of the sulfate adsorbed was eluted, with 0.30*M* sodium chloride only 71%, and with 0.15*M* sodium chloride no sulfate was eluted. For routine purposes, therefore, 0.60*M* sodium chloride was used.

Solubility of Benzidine Sulfate in Alcoholic Trichloroacetic Acid. Owen (1936) determined that benzidine sulfate had minimal solubility at pH 2.75 in aqueous solutions, but no data were available on the solubility

Table I. Absorbance of Benzidine and of Its Sulfate

Sulfate Precipitated, μg.	Absorbance[a]			
	Benzidine Sulfate[b]			
	NaCl absent	NaCl present	Resin eluate	Benzidine[c]
125	0.226, 0.229, 0.243	0.243, 0.248, 0.254	0.248, 0.256	0.244
250	0.491, 0.493, 0.502	0.516, 0.527, 0.528	0.501, 0.514	0.488
500	0.985, 0.987, 0.989	1.045, 1.046, 1.053	0.983, 0.986	0.976

[a] Absorbance at 250 mμ, 1-cm. quartz cell, 25° C.
[b] Benzidine sulfate precipitated in the presence or absence of sodium chloride and then dissolved in 100 ml. of 1*N* HCl or similarly precipitated and dissolved after adsorption and elution from IRA 400.
[c] Benzidine in stoichiometric amount equivalent to sulfate in benzidine sulfate.

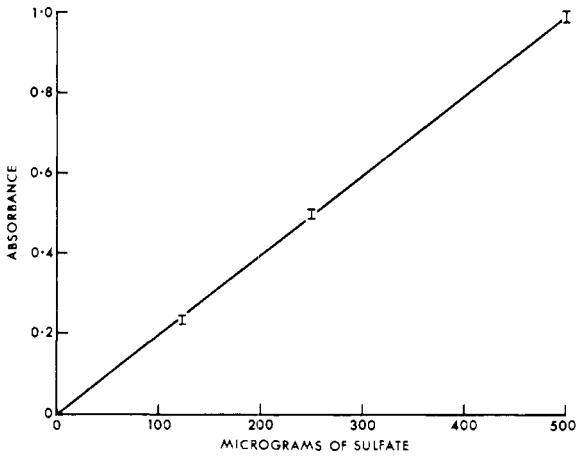

Figure 1. Calibration curve

Absorbance-concentration relationship of sulfate measured as benzidine derived from benzidine sulfate dissolved in 100 ml. of 1*N* HCl with ranges of triplicate determinations

of benzidine sulfate in alcoholic trichloroacetic acid. This was therefore determined using ^{35}S-labeled sulfate.

Amounts of 67, 102, and 138 μg. of sulfate containing the ^{35}S-isotope at high specific activity were precipitated as benzidine sulfate from a solution of 3% trichloroacetic acid in 70% ethanol. After standing overnight, the precipitate was separated by centrifugation, and a measured quantity of the supernatant was removed, to which further sulfate was added and benzidine sulfate again precipitated. The precipitate was then thoroughly washed and the radioactivity of the dried sample measured as an infinitely thick layer using an end-window G.M. counter. From the radioactivity measured and the weight of benzidine sulfate, the amount of radioactive sulfate remaining in the supernatant in the first precipitation could be calculated and hence the solubility of benzidine sulfate. This was 0.13 μg. per ml. at 4° C.

Replicate Determinations and Recovery of Added Sulfate from Oaten and Lucerne Hay. Dried samples of chaffed oaten and chaffed lucerne hay were extracted by the method given, and different aliquots were taken representing different weights of the sample. Triplicate aliquots were taken at each of two levels and analyzed with and without the addition of 100 and 25 μg. of sulfate (Table II).

In addition, a sample of oaten hay, low in sulfate, was analyzed in replicate, and a value of 0.089 ± 0.002% of sulfate found. Various aliquots from different extracts were then taken to represent different weights of sample and 110 μg. of sulfate were added. Each aliquot was then analyzed for sulfate (Table III).

Table II. Replication of Values and Recoveries of Added Sulfate in Oaten and Lucerne Hay

Nature of Sample	Wt. of Sample, Gram	Sulfate Added, μg.	Sulfate Found	
			μg.	Mean[a]
Oaten hay	0.206	0	286, 289, 297	291
		25	325, 331, 346	334
		100	387, 394, 396	393
Oaten hay	0.103	0	147, 155, 157	153
		25	145, 172, 180	166
		100	254, 262, 264	260
Lucerne hay	0.198	0	790, 800, 800	796
		25	820, 828, 830	826
		100	890, 900, 902	897
Lucerne hay	0.099	0	390, 393, 405	396
		25	402, 404, 412	406
		100	488, 490, 490	490

[a] Standard error of means of three = 4.6. Average recovery (4 samples) = 101.1% with S.E. = 5.85. (Recoveries do not differ significantly between hays and sample weights.)

Check Analysis by Radioisotope Dilution Procedure. Samples of oaten hay and subterranean clover were analyzed for sulfate by the method proposed and contained 0.15% (0.15, 0.15) and 2.41% (2.34, 2.43, 2.46), respectively. The same samples were then analyzed by a radioisotope dilution technique using ^{35}S-labeled sulfate. The values for these samples using this procedure were 0.18% (0.15, 0.21) and 2.43% (2.37, 2.49), respectively.

Discussion

Attempts to develop satisfactory methods for the determination of inorganic sulfate in plant material have been hampered by the presence in the extract of compounds which interfere in the quantitative recovery of sulfate, whether this is attempted by precipitation with barium chloride or benzidine or by the conversion of the sulfate to sulfide, which is then determined as methylene blue.

The actual nature of the compounds which inhibit

Table III. Recovery of Added Sulfate from Different Weights of Oaten Hay[a]

(110 μg. of SO_4 added to each sample)

Sample weight, g.	0.077	0.103	0.168	0.181	0.256	0.261
Sulfate found, μg.	180	213	258	276	354	345

[a] Average recovery, 101.8%, S.E. 7.20. Replicate analyses without added sulfate—910, 880, 910, 870 μg./gram; mean 893, S.E. 10.3

precipitation is unknown. The use of the protein precipitants trichloroacetic acid and alcohol do not completely remove them since benzidine fails to precipitate sulfate in some plant extracts so prepared.

With the advent of ion exchange resins, however, the separation of inorganic constituents from complex organic mixtures has become relatively simple, and, as in this case, by the selection of an appropriate ion exchange resin (IRA 400), the sulfate in the plant extract has been readily separated from the polyuronides, gums, and sugars which accompany it.

When the sulfate has been adsorbed onto the resin and the column washed, the sulfate is eluted with sodium chloride solution, which must not be less than 0.6*M* in concentration. The sulfate eluted must therefore be precipitated in the presence of a relatively high concentration of chloride. Care must be taken that the final alcohol concentration does not exceed 66%; otherwise benzidine hydrochloride is likely to be coprecipitated, and its subsequent removal from benzidine sulfate may be difficult.

The determination of sulfate through the benzidine moiety by measuring the absorbance at 250 mμ requires that there be a strict stoichiometric relationship between the sulfate present and the benzidine determined. Because trichloroacetic acid also absorbs in the ultraviolet region of the spectrum (Ogur and Rosen, 1950), both this and excess benzidine must be completely washed from the benzidine sulfate as is demonstrated by the data of Table I.

The method has been applied to a wide range of pasture samples having sulfate concentrations from under 0.1% to over 2.4%. The restoration of the resin columns is readily arranged, and the solutions of benzidine sulfate in hydrochloric acid store without appreciable change for indefinite periods.

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