Chelometric Titration of Calcium and Magnesium in Plant Tissue. Method for Elimination of Interfering Ions

R. M. CARLSON and
C. M. JOHNSON

Department of Soils and Plant Nutrition, University of California, Berkeley 4, Calif.

Aluminum, copper, iron, manganese, zinc, and phosphate ions in plant tissues interfere with chelometric determinations of calcium and magnesium. A method for separating all of these interfering ions from calcium and magnesium in a single step is presented. The cyclohexanediaminetetraacetic acid chelates of heavy metals are formed, and these chelates, which are anions, are adsorbed along with phosphate ion on an anion exchange column. The method is applicable even to tissues low in calcium and magnesium and high in interfering ions. Neither dry ashing nor perchloric acid wet digestion released all of the calcium and magnesium from a sample of barley straw and it was necessary to remove silica with hydrofluoric acid.

Available methods (*1–3, 5, 9, 11, 15*) for eliminating interference of aluminum, copper, iron, manganese, zinc, and phosphate ions in chelometric calcium and magnesium determinations in plant tissues are complex and under some conditions ineffective. Copper and zinc can be effectively masked with cyanide ion, but iron and manganese, when present in amounts often found in plant tissues, are not satisfactorily masked with either triethanolamine or cyanide ion. The manganese triethanolamine complex reacts with and destroys Eriochrome Black T when manganese exceeds 15 μg. in the sample being titrated. Both iron and manganese triethanolamine complexes are colored, the iron triethanolamine complex being yellow, while that of manganese is green, and the presence of even moderate amounts of these colored complexes makes end point detection difficult.

There is no satisfactory way to mask phosphate ion, which interferes by precipitating calcium phosphate or magnesium ammonium phosphate at the high pH at which titrations must be carried out. It is therefore desirable to remove phosphate ions prior to titrating calcium and magnesium.

Organic solvent extraction of diphenylthiocarbazone or diethyldithiocarbamate complexes of heavy metals is the most commonly employed method of removing heavy metals prior to titration of calcium and magnesium (*3, 5, 9*). Disadvantages of this technique are the high cost of organic solvents and the repeated solvent extractions required when plant tissues with high concentration of iron (>300 p.p.m.) are analyzed.

The proposed method for removing interfering ions is based upon exchange of complex anions on resin columns. Samuelson (*13*) reports the absorption of citrate complexes of some metals by anion exchange resins and Samuelson and Sjöström (*14*) have removed polyvalent cations from solution by exchange adsorption of their ethylenediamine tetraacetate chelates. In the present study, it was found that the ability of anion exchange resins to adsorb metal chelates is related to metal chelate stability and to pH of the solution from which the metal chelate is to be adsorbed. Since aminopolycarboxylate chelators are weak acids, hydrogen ions will successfully compete for ligands at low pH. The pH at which metal aminopolycarboxylate chelates are decomposed is related to their stabilities, the more stable chelate being decomposed at lower pH than the less stable chelates.

With aminopolycarboxylate chelators such as ethylenediamine tetraacetate (EDTA) and cyclohexanediamine tetraacetate (CyDTA), the stability of the metal ion chelates increases according to the order: Mg < Ca < Mm < Al < Zn < Cu < Fe (*7, 10*). The CyDTA chelates are in general more stable than the corresponding EDTA chelates. The difference in stability between the calcium CyDTA chelate and the manganese CyDTA chelate is favorable for selection of pH conditions under which the manganese CyDTA chelate is adsorbed by an anion exchange column, while calcium passes through the column unchelated. Under these pH conditions, all metal ions which form more stable CyDTA chelates than manganese will also be adsorbed as anionic CyDTA chelates by the anion exchange resin.

Careful preparation of the anion exchange resin column and selection of a suitably buffered solution from which the heavy metal CyDTA chelates are adsorbed are necessary to achieve complete separation of calcium and magnesium from interfering ions. Dowex 21K, 50 to 100 mesh, was found to be the most suitable anion exchange resin for the application (*4*). The large effective pore size of this resin favors the adsorption of large anions such as the CyDTA chelates. Greater reaction rates are also a property of the large, effective pore size resins, which allow flow rates of as much as 7 ml. per sq. cm. per minute to be used in this procedure without loss in column efficiency.

At flow rates of the order of 5 ml. per sq. cm. per minute, it was found that phosphate ion was not completely removed from solutions of pH 2 to 3 by the anion exchange resin column in the chloride form. This is due to the weakly dissociated nature of phosphoric acid. When the anion exchange columns are initially acetate saturated, no phosphate leakage occurs even at a flow rate of 7 ml. per sq. cm. per minute as long as the exchange capacity of the column is not exceeded by the added phosphate. In this procedure, therefore, the anion exchange columns are used in the acetate form.

Owing to the limited solubility of CyDTA at the pH at which the separation is carried out, the CyDTA is adsorbed, from a solution of its diammonium salt, on top of the column. The sample is then placed on the exchange column and chelation and subsequent absorption of the heavy metal chelates occur on the exchange column.

Hydrolysis of acetate ions displaced from the anion exchange resin causes a rise in pH of the ambient solution. If the pH rises above approximately 4, the calcium CyDTA chelate is partially adsorbed and calcium is then lost from solution. Hence, the sample must be introduced to the exchange column in a solution which is sufficiently buffered against this hydrolysis to prevent loss of calcium from solution. A mixture of acetic acid and monochloroacetic acid which has been partially neutralized with ammonium hydroxide is used to

maintain the pH in the desirable range. A small amount of tartaric acid has been added to this buffered solution to prevent formation of colloidal ferric hydroxide which accumulates at the top of the resin column.

Because the upward shift in pH due to the hydrolysis of acetate ion increases as the amount of acetate displaced from the column increases, it is necessary to set a limit of 5 meq. of total inorganic cation in the sample taken for analysis. If this limit is not exceeded, calcium and magnesium recovery is complete in the described procedure.

Apparatus and Reagents

Anion Exchange Columns. The columns are constructed by sealing a length of 14-mm. borosilicate tubing to a 25 × 150 mm. borosilicate test tube which is used as a reservoir. A 5-mm. borosilicate delivery tube is sealed to the lower end of the 14-mm. tube as shown in Figure 1. The cross-sectional area of this column is approximately 1 sq. cm. A glass wool plug is placed at the bottom of the column, and the column is filled to a depth of 13 cm. with a slurry of acetate-saturated Dowex 21K, 50 to 100 mesh. A plug of either glass wool or glass beads is placed on top of the column to prevent disturbance of the resin, when samples are introduced. After addition of 10 ml. of 0.1*M* $(NH_4)_2$-CyDTA and washing with 2 ml. of water, the column is ready for use. The exchange resin columns are regenerated by eluting with 125 ml. of 2*M* ammonium acetate, followed by a wash with 20 ml. of water to displace excess electrolyte.

Ammonium CyDTA, 0.1*M*. Add 200 ml. of water and 200 ml. of 1*M* NH_4OH to 34.6 grams of cyclohexanediamine tetraacetic acid (CyDTA) (Chel 600, Geigy Chemical Co.) and heat gently while stirring until the solid is dissolved. Cool the solution, filter if turbid, and dilute to 1 liter.

Hydrochloric acid, 1:1 solution.

Buffer Mixture for Solution of Plant Ash. Dissolve 28.4 grams of monochloroacetic acid and 3.0 grams of tartaric acid in 500 ml. of water. Add 153 ml. of glacial acetic acid and 60 ml. of 1*M* ammonium hydroxide and dilute to 1 liter.

Sodium CyDTA, 0.01*N*. Dissolve 1.73 grams of cyclohexanediamine tetraacetic acid in 100 ml. of 0.1*N* sodium hydroxide by gentle heating. Cool and dilute to 1 liter. This solution is standardized against the standard calcium solution.

Standard Calcium Solution. Dry calcium carbonate (Mallinckrodt Chemical Co. No. 4071, Primary Standard grade) overnight at 110° C. Dissolve 0.5004 gram in 30 ml. of 1*M* hydrochloric acid and dilute to 1 liter. This solution is exactly 0.0100*N* in calcium ion.

Potassium Hydroxide, 8*M*.

Potassium Hydroxide, 3*M*.

Calcein Indicator Solution. Dissolve 0.2 gram of Calcein (G. Frederick Smith Chemical Co., No. 222) in 25 ml. of 0.1*N* sodium hyroxide and dilute to 100 ml. This solution should be stored in the dark.

Ammonium Chloride–Ammonium Hydroxide Buffer Solution. Dissolve 67.5 grams of ammonium chloride in 570 ml. of concentrated ammonium hydroxide. Dilute to 1 liter.

Methyl Red Indicator, 0.1%. Dissolve 0.1 gram of methyl red in 100 ml. of 50% ethanol.

Eriochrome Black T Indicator. Dissolve 0.5 gram of Eriochrome Black T and 4.5 grams of hydroxylamine hydrochloride ($H_2NOHHCl$) in 100 ml. of 95% ethanol. Prepare fresh solution at monthly intervals.

Calmagite Indicator Solution. Dissolve 0.2 gram of Calmagite (G. Frederick Smith Chemical Co., No. 278) in 100 ml. of water.

Ammonium Acetate, 2*M*. Mix 114 ml. of glacial acetic acid with 500 ml. of water. Add 132 ml. of concentrated ammonium hydroxide (sp. gr. 0.90), allow to cool, and dilute to 1 liter.

Procedure

Dry Ashing of the Sample. Transfer a sample of plant tissue that contains not more than 5 meq. of total inorganic cations to a porcelain casserole or evaporating dish. Place the sample on Nichrome wire gauze in a cold electric furnace. Slowly raise the furnace temperature until 550° C. is reached and maintain this temperature until no carbon remains in the sample. Cool the sample and then moisten the ash with a few drops of water. Cover with a watch glass and add 10 ml. of 1:1 hydrochloric acid. After all effervescence has ceased, remove the watch glass and evaporate the sample to dryness on a steam bath.

Dry Ashing Followed by Removal of Silica. Ash the sample as above, but use platinum dishes or crucibles. After the sample has been removed from the electric furnace and cooled, add 1 ml. of 60% perchloric acid and 5 ml. of concentrated hydrofluoric acid. Place the sample on a sand bath or hot plate and heat until all of the perchloric acid has been fumed off.

Wet-Ashing Procedure. Place the sample of plant tissue in a 125-ml. Phillips beaker and add 10 ml. of concentrated nitric acid, and 1 ml. of 60% perchloric acid per gram of sample taken. (If less than 1 gram is taken, add the amounts required for 1 gram.) Cover with a watch glass, and allow to stand for 1 hour or until the danger of foaming is over. Heat moderately on an electric hot plate until dense brown fumes no longer exist above the solution, then remove the watch glass and continue to heat until the sample has evaporated to dryness and all of the perchloric acid has been fumed off.

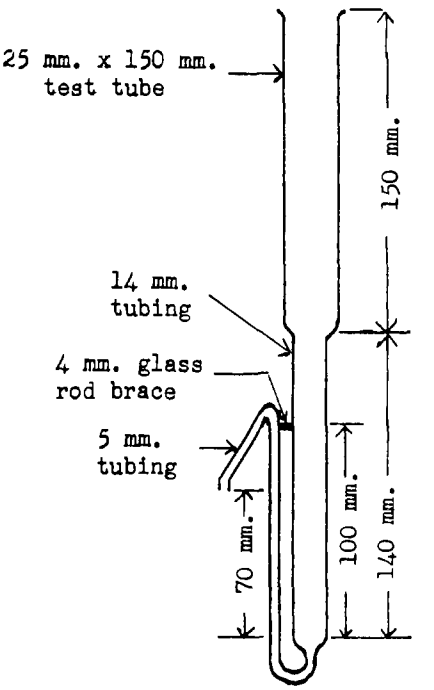

Figure 1. Ion exchange column

Separation of Calcium and Magnesium from Interfering Ions. Add 10 ml. of the acetic acid–monochloroacetic acid buffer to the residue of the sample ashed by any of the above procedures. Cover the sample and heat it on a steam bath for 20 to 30 minutes, then filter to remove silica. Transfer the sample to the anion exchange column and collect the elute from the column in a volumetric flask. After all of the sample has flowed into the column, wash the column with 30 ml. of water. When this wash has flowed through the column, add another 30 ml. of water. Make up the sample and combined washings to volume.

Titration of Calcium. Transfer an aliquot of the sample containing from 0.02 to 0.10 meq. of calcium to a 150-ml. beaker. Add 5 ml. of 8*M* potassium hydroxide and 1 drop of calcein indicator solution, and dilute to 80 to 100 ml. Titrate with standardized 0.01*N* disodium CyDTA until the green fluorescence just disappears. A reagent blank should be titrated and this value subtracted from the sample and standard solution titrations. If the magnesium in the sample is large compared to the calcium, it may be necessary to add sodium carboxymethylcellulose (*17*) or some other protective colloid to prevent formation of magnesium hydroxide floccules which interfere by adsorbing calcium calcein complex.

Table I. Recovery of Calcium and Magnesium from Standard Solutions after Removal of Added Interfering Ions

In all cases, 7.96 mg. of calcium and 4.85 mg. of magnesium were taken

Interfering Ion Added, Mg.						Recovered, Mg.	
Al	Cu	Fe	Mn	Zn	P	Ca	Mg
2.00	...	...	...	...	...	7.94	4.83
...	2.00	...	...	...	...	7.96	4.84
...	...	3.00	...	...	...	7.94	4.84
...	...	...	3.00	...	...	7.92	4.84
...	...	...	...	2.00	...	7.94	4.84
2.00	2.00	3.00	3.00	2.00	...	7.92	4.83
...	...	...	...	...	30.0	7.94	4.83

Table II. Analysis of Plant Tissue

Material	Calcium, Mg./Gram					Magnesium, Mg./Gram				
	A	σ	B	C	σ	A	σ	B	C	σ
Alfalfa	11.17	0.08	11.28	11.36		4.20	0.03	4.23	4.18	
Barley straw	4.68	0.12	3.85	5.25	0.09	2.70	0.04	2.82	2.96	0.03
Lettuce	6.09	0.10	6.17	6.00		2.78	0.04	2.80	2.71	
Range grasses	5.36	0.05	5.36	5.34		1.59	0.02	1.62	1.58	
Red clover	9.41	0.07	9.45	9.51		3.88	0.06	3.93	3.88	

A. Dry ashing without silica removed.
B. Wet digestion.
C. Dry ashing with silica removed.
σ. Standard deviation of individual determinations. All values followed by σ are means of 11 replicates; other figures are single determinations.

Titration of Calcium plus Magnesium. Transfer an aliquot containing 0.02 to 0.10 meq. of calcium plus magnesium to a 150-ml. borosilicate glass beaker. Add 5 ml. of the ammonium chloride–ammonium hydroxide buffer solution and stir the solution before adding 1 ml. of 3*M* potassium hydroxide for each 0.1 of sample taken for titration. Addition of potassium hydroxide at this rate just neutralizes the acetic acid in which the sample is dissolved. Add 6 drops of Eriochrome Black T indicator solution and 6 drops of methyl red indicator solution and titrate with the 0.01*N* disodium CyDTA until the color just changes from orange red to green. (If Calmagite is used as the indicator, add 4 drops of Calmagite solution and 5 drops of methyl red and titrate the solution from orange red to green.) Reagent blanks should be titrated and the calcium plus magnesium titration corrected accordingly. The magnesium is obtained by subtracting the milliequivalents of calcium per unit of plant tissue from the milliequivalents of calcium plus magnesium per unit of plant tissue.

Results and Discussion

The described method was tested by analysis of standard solutions with added interfering ions and by analysis of five plant samples. Recovery of calcium and magnesium after the removal of added interfering ions was complete to within 0.5% of the added amounts (Table I). The amounts of interfering ions added are equivalent to very high concentrations of these elements in 1-gram samples taken for analysis.

Five plant tissue samples were analyzed for calcium and magnesium in 11 replicates, each using the dry-ashing technique. Mean values found and standard deviations of individual determinations are presented in Table II. In these analyses, calcium plus magnesium were titrated using Eriochrome Black T.

Calmagite indicator was brought to the attention of the authors at this time (*8*). It was tested by running six replicate analyses on the alfalfa and the Romaine lettuce and computing the standard deviation of individual determinations. Magnesium found was 4.21 mg. per gram of alfalfa with a standard deviation of 0.03 and 2.75 mg. per gram of lettuce with a standard deviation of 0.03. The values obtained agree well with those in Table II, and since Calmagite solution is more stable than Eriochrome Black T, it is recommended.

To ascertain complete solution of calcium and magnesium following the dry-ashing procedure, the five plant tissue samples were wet-ashed according to the procedure described above. Results are presented in Table II. The values obtained for all of the samples except the barley straw are in good agreement with the values obtained by the dry-ashing technique. Wet digestion yields a much lower value for the concentration of calcium in the barley straw than does the dry-ashing procedure.

Analysis of barley straw using wet oxidation with perchloric acid was repeated several times with low and erratic results ranging to only 60% of the mean figure obtained by dry ashing. Even treating the sample with the nitric acid–perchloric acid mixture and fuming to dryness three times did not release all of the calcium. Since a treatment as drastic as digestion with nitric acid–perchloric acid mixture will certainly completely oxidize all of the organic matter, it was assumed that silica was most probably responsible for the loss of calcium.

To test the hypothesis that silica is involved in the low calcium recovery, samples of barley straw were dry-ashed in platinum crucibles and then treated with perchloric acid and hydrofluoric acid according to the procedure described above. Mean calcium and magnesium concentrations along with standard deviations of individual determinations are given in Table II. Both calcium and magnesium found by this procedure are greater than those found in barley straw by either wet oxidation or dry ashing without removal of silica. These results were entirely unexpected. Piper (*12*) and Thiers (*16*) discuss the loss of trace elements in silica when biological tissues are dry-ashed, but there are apparently no previous reports of losses of calcium and magnesium. It is generally held that wet digestion with a nitric–perchloric acid mixture completely releases all of the metal ions from biological materials (*6*). While wet digestion did release more magnesium from barley straw than dry ashing, magnesium is still less than 95% of the mean value obtained by treating the ash with hydrofluoric acid.

This result led to suspicion of the data obtained on the other four samples, so they were analyzed using the dry ash–perchloric acid–hydrofluoric acid treatment. The values found on these materials agree well with data obtained by the two other ashing techniques described, and will also be found in Table II.

In view of these results, it is recommended that ashing procedures be evaluated by comparison of analysis with data obtained by the dry ash–perchloric acid–hydrofluoric acid procedure described above. Incomplete recovery of calcium and magnesium, and perhaps of other elements, without removal of silica by hydrofluoric treatment should be especially anticipated in samples of mature plant tissue which contain large amounts of silica such as cereal grain straw.

Literature Cited

(1) Cheng, H. H., Kurtz, L. T., J. Agr. Food Chem. **8,** 24 (1960).
(2) Cheng, K. L., Bray, R. H., *Soil Sci.* **72,** 449 (1951).
(3) Cheng, K. L., Melsted, S. W., Pray, R. H., *Ibid.*, **75,** 37 (1953).
(4) The Dow Chemical Co., Midland, Mich., "Dowex, Ion Exchange," 1959.

(5) Forster, W. A., *Analyst* **78,** 179 (1953).
(6) Gorsuch, T. T., *Ibid.*, **84,** 135 (1959).
(7) Holloway, J. H., Reilley, C. N., *Anal. Chem.* **32,** 249 (1960).
(8) Lindstrom, F., Diehl, H., *Ibid.*, **32,** 1123 (1960).
(9) Malmstadt, H. V., Hadjiioannou, T. P., J. AGR. FOOD CHEM. **7,** 418 (1959).
(10) Martell, A. E., Calvin, M., "Chemistry of the Metal Chelate Compounds," pp. 536–7, Prentice-Hall, New York, 1952.
(11) Mason, A. C., *Analyst* **77,** 529 (1952).
(12) Piper, C. S., "Soil and Plant Analysis," pp. 258–75, Interscience, New York, 1950.
(13) Samuelson, O., "Ion Exchangers in Analytical Chemistry," p. 156, Wiley, New York, 1953.
(14) Samuelson, O., Sjöström, E., *Anal. Chem.* **26,** 1908 (1954).
(15) Smith, A. M., McCallum, E. S. R., *Analyst* **81,** 160 (1956).
(16) Thiers, R. E., *Methods of Biochem. Anal.* **5,** 284–93 (1957).
(17) Van Schouwenburg, J. Ch., *Anal. Chem.* **32,** 709 (1960).

Received for review December 15, 1960. Accepted April 17, 1961.

ROLE OF SILICA IN PLANTS

Absorption and Deposition of Silica by Four Varieties of Sorghum

F. C. LANNING and YU-YEN LINKO
Department of Chemistry, Kansas State University, Manhattan, Kan.

To study absorption and deposition of silica by four varieties of sorghum, plants were grown in an open field and gathered at regular 3-week intervals throughout the growing season. Silica content of leaf sheaths and leaves of all varieties continuously increased throughout the season. Stems and seeds remained low and constant in silica content. The silica content of roots decreased during the first 3 to 6 weeks; thereafter a slight increase was observed. Generally, roots had a higher silica content than had been expected. There was a considerable variation in rate and amount of silica absorbed by the four varieties. Spur feterita absorbed the most and Dwarf yellow milo the least. Plants resistant to insects or diseases had a higher silica content at most stages than corresponding susceptible varieties.

EARLY INVESTIGATORS thought silicon essential for plants, since it occurs in them in relatively large amounts. Later observations indicated that this element may be essential only to barley, sunflower, and beets (*10, 16*). There appears to be a relationship between silicon and phosphorus metabolism (*12*).

Wittenberger (*18*) and Cooper (*1*) showed that maximum absorption of soluble silica by certain plants was favored by neutrality (pH 7.1) and that grasses readily absorb silica at pH's between 4.74 and 7.64. Imaizumi and Yoshida (*4*) have shown that an available silica content of 13 mg. per 100 grams of soil, or above, is favorable for maximum absorption of silica by rice.

In general, the more water absorbed by a plant, the greater the amount of silica deposited; Laiseca (*8*) showed that the silica content (by weight) of the ash of beech leaves increased continuously from 1.2% in May to 24.4% of SiO_2 in November.

As early as 1913 Lundie (*11*) concluded that silicon would protect against fungal diseases. Palladin (*14*) recorded that wheat and rye grown in nutrient solutions deficient in silicic acid suffered severely from rusts. Since 1934, Japanese scientists have indicated that silicon is essential for normal growth of rice (*5, 13*).

The quantity of "dilute acid-soluble silicon" in soils was correlated by Imaizumi and Yoshida (*4*) with uptake of silica by the rice plant. The presence of free organic acids increases the availability of silicon. Field tests indicated that application of suitable silicon compounds to soil greatly diminished the appearance of blast and brown spot diseases. These results were confirmed by Ishibashi and Kawano (*6*).

Recently, Yoshida, Ohnishi, and Kitagishi (*19*) showed that silicon deficiency in rice increases susceptibility to diseases or insects. Ponnaiya (*15*) observed that irregularly shaped silica deposits in sorghum varieties resistant to *Antherigona indica M.* appeared in the leaf sheath epidermis at an earlier time than in nonresistant varieties. Palladin (*14*) reported that *Lithospermum arvense* grown without silica was badly attacked by plant lice.

The great economic losses in the United States due to attack on sorghum and corn by chinch bugs (*Blissus leucopterus*, Say) have long been known (*2*). The present work has been initiated to study absorption and deposition of silica by four different varieties of sorghum over the growing season as well as the possible relationship of silica to the susceptibility of sorghum to fungal diseases and chinch bugs.

Materials and Methods

The following four varieties of sorghum (*Sorghum subglabrascens*) were studied: Pink kafir, Spur feterita, Atlas, and Dwarf yellow milo. These varieties were chosen because of their resistance or lack of resistance to disease or insect pests (Table I). Sorghum was planted on June 9, 1959, in an open field in which the available silica content was approximately 20 mg. per 100 grams of soil. The pH of the soil was 5.2 at 1 to 1 dilution. Plants were collected during the growing season at 3-week intervals starting on June 29. Dwarf yellow milo showed typical chinch bug damage early in the season. No other insect damage or disease was observed.

Table I. Resistances of Sorghum Varieties Studied

	Resistance to Diseases and Insects		
Variety	Smut	Milo disease	Chinch bugs
Spur feterita	High		
Pink kafir	Very low	High	
Atlas			High
Dwarf yellow milo		Very low	Very low