RESEARCH BULLETIN 570 OCTOBER, 1954

UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE
AGRICULTURAL EXPERIMENT STATION
J. H. Longwell, *Director*

Direct Ethylenediaminetetraacetate Titration Procedure for Calcium In Biological Substances

Anion Exchange Separation of Phosphate

CHARLES W. GEHRKE, HAROLD E. AFFSPRUNG AND YUNG C. LEE

(Publication authorized October 14, 1954)

COLUMBIA, MISSOURI

TABLE OF CONTENTS

Report on Department of Agricultural Chemistry Research
Project 132, "Analytical Services"

Direct Ethylenediaminetetraacetate Titration Procedure for Calcium In Biological Substances Anion Exchange Separation of Phosphate

CHARLES W. GEHRKE, HAROLD E. AFFSPRUNG AND YUNG C. LEE

INTRODUCTION

Methods have been developed, based upon the original work of Schwarzenbach and co-workers (6, 16, 17), for the analysis of calcium and magnesium in water (4, 5, 9, 10), limestone and soils (2, 7, 8). Banks (3) determined the calcium and magnesium content of coal ash and ceramic materials, and Mattock and Hernandez (15) used disodium dihydrogen ethylenediaminetetraacetate (ETA) for the determination of calcium in pharmaceutical products. Stephens (18) used an excess of a much stronger solution of ETA than most investigators and then back-titrated with standard calcium and magnesium solutions. He applied the method for the analysis of calcium and magnesium in pharmaceuticals, calcium gluconate, calcium sulfate, and phosphate. Attempts have been made to apply the methods for the determination of calcium in plant materials (7, 11, 19). In nearly all cases, the procedures developed require two titrations, a titration for calcium plus magnesium using Eriochrome Black T as the indicator, and a titration for calcium with murexide as the indicator, the magnesium being calculated as the difference between the two titrations. Difficulties are encountered in the calcium titration when orthophosphate ions are present. A direct method has been developed for calcium in milk and milk fractions (13). A method (12) has also been developed for the direct determination of magnesium in limestone, employing a separation of calcium as calcium sulfite; this method is also applicable to some biological materials. Mason (14) worked out a method in which calcium was separated from phosphate by a cation exchanger. The calcium was then eluted from the exchanger and titrated with ETA in the effluent.

A definite need was realized for a direct titration procedure for calcium in biological materials which would be free of interference due to phosphate ions. This bulletin describes a rapid and accurate "anion-exchange separation—direct titration procedure."

APPARATUS AND REAGENTS

Artificial light source, such as Precision Scientific Co., Titra lite. Cat. No. 9555.

pHydrion indicator paper.

Microburets, 5 and 10 ml.

Volumetric flasks and pipets.

Anion exchange resin. Amberlite IR-4B, an anion exchanger with a high capacity for phosphate was used. The resin particles are 0.4 to 0.6 mm. in size, and approximately 20-50 mesh. This resin can be obtained from the Rohm and Haas Co., Philadelphia, Pennsylvania.

Anion exchange column. Regenerate the resin to the chloride form by the batch process. First exhaust it with three separate portions of 5% sodium carbonate or sodium hydroxide. Wash until all excess base is removed. Then treat the resin with at least three separate portions of 5% hydrochloric acid with stirring. Rinse the resin with distilled water until there is no further color throw. Prepare a glass column approximately 23 cm. long and 2 cm. in diameter. Divide the column 5 cm. from the bottom by sealing in a coarse porosity sintered glass plate. Attach a 2mm. two-way stopcock to the bottom of the column for regulating the flow of solution through the column. Place 30-50 grams of IR-4B-C1 in the column. The column of resin is about 9 cm. in height. Stir the resin to remove air bubbles and maintain the water level above the resin surface.

Calcium indicator. Intimately mix 40 grams of C. P. potassium sulfate and 0.2 gram of murexide powder (Eastman Kodak Product) in a mortar (5).

Potassium hydroxide solution, 10%. Dissolve 10 grams of reagent grade potassium hydroxide in 100 ml. of distilled water.

Standard calcium solution. Dissolve 2.4972 grams of reagent grade calcium carbonate, previously dried at 110° C., in dilute hydrochloric acid. Dilute to 1 liter with double distilled water. This solution contains 1 mg. of calcium per milliliter.

Standard solutions of disodium dihydrogen ethylenediaminetetraacetate (ETA), 0.4 and 0.1%. Dissolve 20 grams, or 5 grams of the reagent in double distilled water, and dilute to 5 liters. Standardize the solution against a standard calcium solution. The titers are approximately 0.44 and 0.11 mg. of calcium per milliliter, respectively.

Standard phosphate solution. Dissolve 2.8658 grams of C. P. potassium dihydrogen phosphate in double distilled water, and make to 1 liter. This solution contains 2.00 mg. of phosphate ion (PO_4) per milliliter.

ANALYTICAL PROCEDURE

Preparation of Sample

Ash a 10 gram sample of the plant material overnight in a quartz crucible at 550° C. Place the sample in a cold furnace and gradually increase

the temperature to 550° C. Add 75 ml. of 1:1 hydrochloric acid and 2 drops of concentrated nitric acid. Digest the sample on a hot plate or steam bath for 2 hours and finally take to dryness. Add about 3 ml. of 1:1 hydrochloric acid to the residue and approximately 100 ml. of distilled water and digest for about 1 hour. Allow the solution to cool and transfer quantitatively to a 250 ml. volumetric flask. Fill to the mark with distilled water and mix thoroughly.

Removal of Phosphate

Transfer a 25 ml. aliquot of the sample solution to a 250 ml. beaker. The aliquot should contain between 10 and 15 mg. of calcium. Neutralize the solution to a pH of 3 to 4 with a 10% solution of potassium hydroxide using pHydrion indicator paper. Pass the sample solution through the column of anion exchanger in the chloride form, and collect the effluent in a 300 ml. Erlenmeyer flask. Control the rate of flow to about 2 to 3 ml. per minute. Wash the resin column thoroughly with 150 ml. of distilled water in three separate portions. Pass the first portion of 50 ml. through at the same rate as the sample. Pass the second portion through at a rate of about 9 ml. per minute. Allow the final portion to pass through the column freely. Twelve samples can be handled at one time using a bank of 12 columns. The exchange capacity for phosphate of one column containing 30 grams of resin is about 1800 mg. Thus it is possible to pass a large number of aliquots through a column before regeneration of the resin is necessary.

Titration of Calcium

Add 5 ml. of 10 per cent potassium hydroxide and about 50 mg. of murexide indicator to the effluent and washings in the Erlenmeyer flask. Titrate the sample, with swirling, using a standard solution of ETA of the desired titer. The end point is from salmon pink to a deep purple. At the end point no pink coloration is observed upon viewing the solution against a background of artificial light. It is always preferable to titrate with an artificial light, and a blank is used for comparison.

Calculations

The calcium content may be calculated as follows when a 10-gram sample is ashed, made to 250 ml. volume, and a 25 ml. aliquot is used for titration.

$$\frac{AxB}{10} = \% \text{ calcium}$$

A = milligrams of calcium/ml. of titrant
B = milliliters of titrant

RESULTS

The results of a series of determinations in which increasing amounts of phosphate were added to known amounts of calcium are shown in Table 1. These known solutions were prepared to give ratios of phosphate to calcium from less than 1:1 to 20:1. After the solutions were passed through the anion exchange column, the calcium was titrated with ETA, using murexide as the indicator. The effluents were also analyzed for phosphorus by the volumetric A. O. A. C. method (1); none was found. The recovery of calcium was excellent for the solutions containing a small amount as well as a large amount of phosphate. The standard deviation was found to be ±0.017 for 12 samples containing 10 mg. of calcium and different amounts of phosphate ranging from 6 to 100 mg.

TABLE 1 -- RECOVERY OF CALCIUM FROM STANDARD SOLUTIONS CONTAINING PHOSPHATE

Phosphate[a] ($PO_4^{\equiv}$) Added (Mg.)	Milligrams of Calcium		
	Added	Found	Difference
6.00	10.00	10.00	0.00
10.00	10.00	10.02	+0.02
16.00	10.00	10.02	+0.02
20.00	10.00	10.02	+0.02
26.00	10.00	10.02	+0.02
36.00	10.00	9.99	-0.01
40.00	10.00	9.99	-0.01
46.00	10.00	9.97	-0.03
50.00	10.00	9.98	-0.02
56.00	10.00	9.99	-0.01
60.00	10.00	10.00	0.00
80.00	10.00	9.98	-0.02
70.00	5.00	5.00	0.00
90.00	5.00	5.00	0.00
100.00	5.00	4.99	-0.01
Standard deviation = ± 0.017			

[a] Phosphate added as potassium dihydrogen phosphate.

The data obtained upon analyzing various forage and feed samples are presented in Table 2. The samples were chosen to have a range in phosphate content, as well as of calcium. The determinations were conducted by both the classical oxalate method, as described in the Official Agricultural Chemists (1), and the proposed "ion-exchange—ETA titration procedure." For samples 1, 2, 3, 4, and 5, three independent ashings were made at different times. For each ashing, two or three replicate determinations were made by both methods. For sample No. 6 only one ashing was made and a triplicate determination was conducted by both methods. The phosphate (PO_4) content of these samples ranged from 0.54 per cent to 6.48 per cent. It was observed that the phosphate ions were effectively removed by

TABLE 2 -- DETERMINATION OF CALCIUM IN FORAGES BY THE CLASSICAL OXALATE AND ION-EXCHANGE SEPARATION-ETA TITRATION METHODS.

Sample Number	$(PO_4^{\equiv})$[a] Phosphate	% Calcium			Deviation	
		Oxalate Method	ETA Method	Diff.	Oxalate Method	ETA Method
1	0.80	0.200	0.244		-.023	+.002
		0.227	0.240		+.004	-.002
(Forage)		0.268	0.242		+.045	0.000
		0.213	0.240		-.010	-.002
		0.222	0.240		-.001	-.002
		0.209	0.244		-.014	+.002
		0.207	0.240		-.016	-.002
		0.234	0.242		+.011	0.000
			0.244			+.002
	Average =	0.223	0.242	+0.019		
	Standard Deviation =				0.020	0.0017
2	0.60	0.847	0.836		0.000	+.009
		0.833	0.818		-.014	-.009
(Forage)		0.847	0.826		0.000	-.001
		0.861	0.827		+.014	0.000
		0.864	0.820		+.017	-.007
		0.850	0.822		+.003	-.005
		0.840	0.836		-.007	+.009
		0.830	0.827		-.017	0.000
			0.833			+.006
	Average =	0.847	0.827	-0.020		
	Standard Deviation =				0.0111	0.0063
3	0.54	1.177	1.106		+.012	+.003
		1.170	1.102		+.005	-.001
(Forage)		1.177	1.104		+.012	+.001
		1.154	1.102		-.011	-.001
		1.156	1.104		-.009	+.001
		1.156	1.098		-.009	-.005
	Average =	1.165	1.103	-0.062		
	Standard Deviation =				0.0100	0.0025
4	6.48	1.693	1.787		-.059	+.029
		1.693	1.787		-.059	+.029
(small animal ration)		1.644	1.778		-.108	+.020
		1.793	1.747		+.041	-.011
		1.814	1.747		+.062	-.011
		1.809	1.742		+.057	-.016
		1.783	1.744		+.031	-.014
		1.784	1.747		+.032	-.011
			1.747			-.011
	Average =	1.752	1.758	+0.006		
	Standard Deviation =				0.0605	0.0183
5	1.47	0.812	0.879		-.055	-.001
		0.840	0.889		-.027	+.009
(Dehydrated spinach)		0.848	0.880		-.019	0.000
		0.898	0.880		+.031	0.000
		0.902	0.880		+.035	0.000
		0.883	0.882		+.016	+.002
		0.888	0.880		+.021	0.000
			0.871			-.009
			0.880			0.000
	Average =	0.867	0.880	+0.013		
	Standard Deviation =				0.0316	0.0044
6	0.86	1.280	1.251		+.004	-.001
		1.280	1.253		+.004	+.001
(Forage)		1.267	1.251		-.009	-.001
	Average =	1.276	1.252	-0.024		
	Standard Deviation =				0.0053	0.001
Average Standard Deviation =					±0.023	±0.0057

[a] Phosphate expressed as percent PO_4

the anion exchanger. The difference in the averages of the per cent calcium between the oxalate and ETA methods ranged from -0.062 to +0.019 per cent, which is within the analytical tolerance usually allowed for the classical method of analysis for calcium. The precision of the ETA method was excellent and much better than for the oxalate method; however, the precision of the oxalate method was also considered good. Refer to Table 2 for comparisons.

Recovery experiments were made using various types of forages, such as ladino clover, orchard grass, red clover, soybeans, and alfalfa. Known amounts of calcium were added as the chloride to aliquots of the original ash solution of the samples. The final concentration of calcium in the aliquot was between 10 and 16 mg., as this quantity gave the smallest titration error. The solutions were adjusted to a pH of 4 to 5, then passed through the anion exchange column. The recoveries of calcium ranged from 97.8 to 100.6 per cent, with an average recovery of 99.3 per cent. These recoveries were considered satisfactory.

A study was made to determine the extent of the titration error when using ETA as the titrant and murexide as the indicator (Table 3). All of

TABLE 3 -- RECOVERY OF CALCIUM FROM A STANDARD SOLUTION OF CALCIUM CHLORIDE; TITRATION ERROR

Milligrams of calcium		
Added	Found[a]	Difference
1.00	1.00	-----
2.00	2.00	-----
5.00	5.02	+0.02
10.00	10.04	+0.04
15.00	15.04	+0.04
20.00	20.40	+0.40
25.00	25.61	+0.61
30.00	30.76	+0.76
40.00	40.80	+0.80
50.00	50.98	+0.98

[a] Each value is an average of two independent determinations.

the volumetric glassware was calibrated and corrections applied when necessary. The results show that a significant positive error occurred when the quantity of calcium exceeded 20 mg. in the solution being titrated. No significant error was found when smaller amounts of calcium were titrated. The end point was usually elusive, due partly to the competition of the ETA and the murexide for the calcium and also perhaps to air oxidation of the indicator. The pink color returns to the solution a few seconds after the stoichiometric point is reached. The end point used was that point at which a complete discharge of the pink color was observed. With experience and a good artificial light source one can become proficient in detecting the purple end point. To avoid a titration error in routine application of the

method, the aliquots were always chosen so as to contain 15 mg. or less of calcium.

DISCUSSION

The "anion-exchange separation-ETA titration method" is well suited to the routine analysis of all types of biological materials. At the Missouri Agricultural Experiment Station during the past two years, hundreds of analyses have been made by this method on plant materials, milk, urine, serum, experimental rations, grains, and other substances in which phosphate interferes with the titration.

The results obtained by the proposed method are superior in accuracy to those for the accepted classical oxalate method, and the procedure is much less time consuming. In this study, the number of analyses made by each method on forages and rations was approximately 50. The average difference in per cent calcium was found to be 0.024 for all the results by the proposed method, compared to the result by the oxalate method. In an overall evaluation, the ETA values were slightly lower than the oxalate values. This average difference, 0.024, was nearly equal to the average standard deviation of the oxalate method which was $\pm$ 0.023. These data show that the results of the proposed method are in good agreement with those of the classical oxalate procedure. The small average standard deviation of $\pm$ 0.0057 for the proposed procedure indicates that the reproducibility is excellent.

If only a small amount of sample solution is available, the ion exchange column can be made proportionally smaller. It is important to keep the quantity of calcium below 20 mg. in the sample solution being titrated, as a significant titration error occurs when attempts are made to titrate larger amounts. Large amounts of potassium chloride were found to have no effect on the analysis for calcium.

Dry ashing was preferred over the various wet ashing procedures for the removal of organic material in the forage samples. The dry ashing procedure is rapid and convenient. When wet ashing is used the anion exchange columns become exhausted more quickly as a result of the removal of sulfate, perchlorate, and nitrate anions used in the wet ashing method. As a result more time is consumed in regenerating the resin and refilling the columns. Several hundred samples of feces, foods, and urine were wet ashed using nitric, sulfuric, and perchloric acids. When aliquots of these solutions were passed through the columns for phosphate removal, a more frequent change of resin was needed as the aliquots contained large quantities of anions. Such difficulties were not experienced when the same samples were dry ashed. When a column containing 30 grams of resin is used, a large number of aliquots can be passed before regeneration is necessary.

LITERATURE CITED

1. Assoc. of Official Agric. Chemists, "Book of Methods of Analysis," 7th Ed., (1950). Box 540, Benjamin Franklin Station Washington 4, D. C.
2. Banewiz, J. J., and Kenner, C. T., Determination of Calcium and Magnesium in Limestones and Dolomites, Anal. Chem., 24, 1186 (1952).
3. Banks, J., The Volumetric Determination of Calcium and Magnesium by the Ethylenediaminetetraacetate Method, Analyst, 77, 484 (1952).
4. Betz, J. D., and Noll, C. A., Total-Hardness Determination by Direct Colorimetric Titration, J. Am. Water Works Assoc., 42, 49 (1950).
5. Ibid., p. 749.
6. Biedermann, W., and Schwarzenbach, G., Complexons XI. The Complexometric Titration of Alkaline Earths and Some Other Metals with Eriochrome Black T., Chima Prague, 2, 56 (1948).
7. Cheng, K. L., and Bray, R. H., Determination of Calcium and Magnesium in Soil and Plant Material, Soil Sci., 72, 449 (1951).
8. Cheng, K. L., Kurtz, T., and Bray, R. H., Determination of Calcium, Magnesium, and Iron in Limestone, Anal. Chem., 24, 1640 (1952).
9. Connors, J. J., Advances in Chemical and Colorimetric Methods, J. Am. Water Works Assoc., 42, 33 (1950).
10. Diehl, H., Goetz, C. A., and Hach, C. C., The Versenate Titration for Total Hardness, Ibid., p. 40 (1950).
11. Gastler, G. F., Proc. S. Dakota Acad. Sci., 28, 77 (1949).
12. Gehrke, C. W., Affsprung, H. E., and Lee, Y. C., A Direct Disodium Dihydrogen Ethylenediaminetetraacetate Titration Procedure for Magnesium in Limestone, Anal. Chem., Submitted for publication (1953).
13. Jenness, R., Titration of Calcium and Magnesium in Milk and Milk Fractions with Ethylenediaminetetraacetate, Anal. Chem., 25, 966 (1953).
14. Mason, A. S., The Determination of Small Amounts of Calcium in Plant Materials, Analyst, 77, 529 (1952).
15. Mattocks, A. M., and Hernandez, H. R., Assay of Official Calcium Products by the Schwarzenbach Method, J. Am. Pharm. Ass'n., Sci. Ed., 39, 519 (1950).
16. Schwarzenbach, G., and Ackermann, H., Complexons V. Ethylenediaminetetraacetic Acid, Helv. Chim. Acta, 30, 1798 (1947).
17. Schwarzenbach, G., Biedermann, W., and Bangerter, F., Complexons VI. New Simple Titrating Methods for Determining the Hardness of Water, Ibid., 29, 811 (1946).
18. Stephens, R. L., The Volumetric Determination of Calcium and Magnesium, J. of Pharm. and Pharmacology, 5, 709 (1953).
19. Willson, A. E., Volumetric Determination of Calcium and Magnesium in Leaf Tissue, Anal. Chem., 22, 1571 (1950).