An Extraction Procedure for the Determination of Total Calcium, Magnesium, and Potassium in Plant Tissue

THOMAS GREWELING
Agronomy Department,
Cornell University, Ithaca, N. Y.

A method is described for the extraction and flame photometric determination of total calcium, magnesium, and potassium in plant tissue. The tissue is extracted with salts of EDTA; these salts also eliminate anion interferences in the subsequent flame photometric analysis. The results obtained by this method agree well with those obtained by flame and chemical analyses of plant ash solutions.

THE TECHNIQUE which utilizes salts of (ethylenedinitrilo)tetraacetic acid (EDTA) to eliminate anion interference and reduce aluminum interference in the flame photometric determination of calcium and magnesium (*3–8*) produces excellent results when used in the analysis of plant ash solutions (*2*). As a logical extension to this work, an attempt was made to extract total calcium and magnesium from dried plant tissue, thus eliminating the ashing procedure.

Reagents and Apparatus

Tetrasodium EDTA Solution. A 0.1*M* solution was prepared by dissolving 38 grams of the tetrasodium salt of EDTA (Eastman P6253) in distilled water and diluting to 1 liter.

Ammonium EDTA Solution. A 0.1*M* solution was prepared by weighing 29.2 grams of EDTA (Eastman P5416) into a flask containing about 500 ml. of distilled water, adding enough NH_4OH to neutralize the EDTA, and diluting to 1 liter. The pH of this solution is usually about 8.0. Excess NH_4OH can be added to raise the pH if desired.

Calcium Standard Solutions. A solution containing 1000 μg. of Ca per ml. was prepared by dissolving 2.497 grams of $CaCO_3$ in an equivalent amount of HNO_3 and diluting to 1 liter with distilled water. This solution was diluted with the appropriate EDTA solution to produce solutions containing 40, 30, 20, 10, 5, and 0 μg. of Ca per ml. in 0.1*M* EDTA. Storage in polyethylene is recommended for these solutions since prolonged storage in glass causes reduced calcium emission, presumably owing to absorption of calcium by the glass. This effect was found even when the solutions were stored in soft glass volumetric flasks where the opposite effect might be expected.

Magnesium Standard Solutions. A solution containing 5000 μg. of Mg per ml. was prepared by dissolving 52.72 grams of $Mg(NO_3)_2.6H_2O$ in distilled water and diluting to 1 liter in a volumetric flask. By using a fresh bottle of $Mg(NO_3)_2.6H_2O$, a solution was ob-

Table I. Results Obtained in Analysis of Plant Materials

Lab. No.	Material	Calcium[a] EDTA Extraction Na^+	Calcium[a] EDTA Extraction NH_4^+	Calcium[a] Ashing EDTA Flame Na^+	Calcium[a] Ashing EDTA Flame NH_4^+	Calcium[a] Ashing Precipitation oxalate	Magnesium[a] EDTA Extraction Na^+	Magnesium[a] EDTA Extraction NH_4^+	Magnesium[a] Ashing EDTA Flame Na^+	Magnesium[a] Ashing EDTA Flame NH_4^+	Magnesium[a] Ashing Precipitation oxyquinolate	Potassium[a] EDTA Extraction, NH_4^+	Potassium[a] Ashing EDTA Flame NH_4^+	Potassium[a] Ashing EDTA Flame H_2O
6a	Corn grain	0.02	0.02	...	0.02	0.02	0.12	0.08	0.12	0.08	0.08	0.33	0.33	0.30
5	Red pine	0.19	0.11(0.23)[b]	0.24	0.25	0.20	0.09	0.06	0.07	0.05	0.06	0.38	0.37	0.37
4a	Fescue	0.26	0.20	0.24	0.25	0.20	0.20	0.18	0.18	0.12	0.17	2.00	2.06	1.88
6	Red pine	0.22	0.12(0.24)[b]	0.20	0.20	0.23	0.06	0.03	0.06	0.04	0.05	0.60	0.69	0.57
G-2	Orchard grass	0.30	0.22	0.24	0.26	0.25	0.18	0.17	0.21	0.17	0.17	3.15	2.94	3.00
3	Red pine	0.27	0.16(0.29)[b]	0.24	0.25	0.29	0.09	0.09	0.10	0.07	0.06	0.29	0.34	0.31
5a	Sorghum	0.45	0.34	0.36	0.38	0.34	0.25	0.22	0.26	0.21	0.24	1.35	1.35	1.28
4	Red pine	0.33	0.18(0.38)[b]	0.34	0.32	0.35	0.09	0.06	0.10	0.09	0.09	0.25	0.31	0.31
G-1	Brome grass	0.34	0.34	0.36	0.37	0.35	0.14	0.14	0.14	0.14	0.11	3.50	3.56	3.50
G-3	Timothy	0.50	0.36	0.37	0.40	0.40	0.18	0.15	0.20	0.16	0.16	3.75	3.62	3.62
3a	Corn leaf	0.66	0.60	0.58	0.65	0.66	0.53	0.45	0.50	0.46	0.47	2.13	2.16	2.03
10	Apple leaf	0.83	0.72	0.67	0.80	0.76	0.25	0.26	0.23	0.20	0.19	1.83	1.88	1.75
7	Oats	0.85	0.72	0.84	0.83	0.79	0.30	0.27	0.34	0.29	0.31	3.14	3.12	3.25
8	Oats	0.78	0.66	0.80	0.78	0.79	0.29	0.27	0.31	0.31	0.20	3.10	3.25	3.31
9	Corn leaf	0.78	0.73	0.80	0.79	0.85	0.49	0.46	0.52	0.48	0.45	1.20	1.19	1.12
42	Ladino clover	1.56	1.36	1.43	1.48	1.40	0.36	0.33	0.36	0.33	0.32	2.30	2.38	2.50
55	Ladino clover	1.46	1.35	1.40	1.50	1.48	0.41	0.35	0.40	0.36	0.34	1.55	1.56	1.62
49	Alfalfa	1.34	1.35	1.44	1.53	1.48	0.40	0.30	0.38	0.31	0.32	1.50	1.50	1.62
11	Sour cherry leaf	2.30	2.25	2.08	2.45	2.10	0.80	0.66	0.69	0.55	0.59	1.50	1.56	1.44
1	Turnip greens	2.50	2.25	2.32	2.22	2.27	0.37	0.39	0.37	0.37	0.33	4.30	4.19	4.25
2	Turnip greens	2.71	2.38	2.57	2.46	2.58	0.34	0.39	0.36	0.34	0.32	3.55	3.56	3.75

[a] Per cent by weight in the dried plant material; average of two or more determinations.
[b] ( ) Overnight extraction.

Table II. Precision of the Method Given in Per Cent

	Ca	Mg	K		Ca	Mg	K
	Sample A				Sample B		
1	0.17	0.14	3.12	1	1.30	0.39	1.61
2	0.18	0.14	3.12	2	1.28	0.37	1.62
3	0.18	0.14	3.12	3	1.32	0.38	1.61
4	0.18	0.14	3.14	4	1.30	0.37	1.53
5	0.18	0.14	3.12	5	1.30	0.37	1.61
6	0.17	0.15	3.10	6	1.30	0.38	1.67
7	0.17	0.14	3.10	7	1.29	0.38	1.67
8	0.17	0.15	3.10	8	1.29	0.38	1.68
9	0.17	0.15	3.08	9	1.30	0.39	1.67
10	0.17	0.14	3.05	10	1.29	0.38	1.68
11	0.17	0.14	3.05	11	1.29	0.39	1.67
12	0.17	0.14	3.08	12	1.30	0.38	1.68
13	0.17	0.15	3.05	13	1.29	0.39	1.67
14	0.17	0.15	3.08	14	1.31	0.39	1.67
15	0.17	0.15	3.08	15	1.29	0.40	1.68
Std. dev.	0.0052	0.0063	0.0278		0.0115	0.0089	0.0278
Results by dry ashing	0.19	0.17	2.94		1.39	0.36	1.56

tained which assayed 4980 μg. of Mg per ml. when tested by oxyquinolate precipitation. Magnesium sulfate was not used as a standard because the sulfate ion exerted a depressing effect on the flame spectra of magnesium which was not completely removed by $(NH_4)_4$-EDTA, although it was removed by Na_4EDTA. The concentrated solution of magnesium was diluted with the appropriate EDTA salt to produce solutions containing 1000 μg. of Mg per ml. These solutions were further diluted with their respective EDTA salts to produce solutions containing 30, 25, 20, 15, 10, 5, and 0 μg. of Mg per ml. in 0.1*M* EDTA.

Potassium Standard Solutions. A solution containing 1000 μg. of K per ml. was prepared by dissolving 1.907 grams of KCl in distilled water and diluting to 1 liter in a volumetric flask. This solution was diluted with the appropriate EDTA salt to prepare solutions containing 80, 60, 40, 20, 10, and 0 μg. of K per ml. in 0.1*M* EDTA.

Flame Photometer. The instrument used was a Beckman Model DU spectrophotometer equipped with a Beckman Model 9220 gas regulator, oxygen-hydrogen atomizer-burner, and a Beckman Model 4300 multiplier phototube.

Procedure

Extraction of Plant Tissue. The plant tissue was dried at 70° C., ground to pass a 20-mesh sieve, and dried again just before sampling. A 0.4-gram sample was weighed into a 125-ml. Erlenmeyer flask, and 80 ml. of 0.1*M* EDTA solution was added. The mixture was shaken for 30 minutes on a reciprocating shaking machine before being filtered through Whatman No. 31 filter paper. This extract was diluted as necessary with EDTA solutions before flame analysis.

Ashing of Plant Tissue. A 1-gram sample of dried, ground plant tissue was dry ashed at 550° C., evaporated to dryness with HCl to dehydrate silica, and was taken up in 25 ml. of 0.2*N* HNO_3. This solution was diluted as necessary with EDTA solutions or with water before flame analysis.

Calcium Determination. Flame Photometric Determination. The plant extract was diluted with the appropriate amount of EDTA solution to bring the calcium content within the range of the dilute calcium standard solutions. The efficiency of EDTA in eliminating anion interference decreases as calcium concentration increases. Early experiments showed that failure to dilute the plant extracts to a calcium concentration of less than 40 μg. of Ca per ml. led to erroneous results due to incomplete elimination of anion interferences. The intensity of the calcium emission was measured at 422 mμ.

Chemical Determination. An aliquot of the plant ash solution was treated to remove iron, aluminum, manganese, and phosphate prior to precipitation of calcium oxalate using the method described by Peech (*5*) for analysis of soil extracts. Calcium oxalate was dissolved in dilute HCl and calcium determined on the flame photometer *vs.* calcium standards in dilute HCl.

Magnesium Determination. Flame Photometric Determination. The plant extract was analyzed without dilution and compared to standards in the appropriate EDTA solution. When analyzing sodium EDTA solutions for magnesium, it was necessary to use the zero suppressor attachment of the multiplier phototube. For this reason, analysis of ammonium EDTA extracts was less tedious and more reproducible. The intensity of the magnesium emission was measured at 285.2 mμ.

Chemical Determination. The method described by Peech (*5*) was used to precipitate magnesium in the filtrate obtained after the separation of manganese, iron, aluminum, phosphate, and calcium from the plant ash solution. The oxyquinolate was dissolved in dilute HCl and measured in a spectrophotometer at 360 mμ.

Potassium Determination. Potassium was determined on the flame photometer after proper dilution of the plant extract or the plant ash solution. For some samples, the same dilution could be used for determination of calcium. When analyzing sodium EDTA extracts, it was necessary to keep the sodium concentration identical in samples and in standards; small variations in EDTA concentrations were less critical when the ammonium salt was used. The intensity of the potassium emission was measured at 769.9 mμ.

Results and Discussion

Calcium. As shown in Table I, sodium EDTA satisfactorily extracted calcium from all samples with a tendency toward high results on some samples. The pH of the sodium EDTA extracting solution was 9.8. Ammonium EDTA satisfactorily extracted calcium from all samples except the four samples of red pine needles. Identical results were obtained with extraction times of 30 and 60 minutes and with ammonium EDTA at pH 8.0 and 9.3. Overnight extraction at pH 9.3, however, completely extracted calcium from the pine needles. This difference in solubility might be useful in determing the calcium compounds present in pine needles. Since ammonium EDTA has a low flame background and causes less clogging of the atomizer-burner than the sodium salt, the ammonium salt is preferred for extraction when the time of extraction can be kept conveniently short.

Magnesium. Table I indicates that either the sodium or ammonium salt of EDTA satisfactorily extracts magnesium from the plant tissue tested. There is some tendency toward high results when sodium EDTA is used for extraction.

Potassium. The ammonium salt of EDTA satisfactorily extracts potassium from the tissue tested. Attempts to flame 0.1*M* Na_4EDTA solutions for potassium caused erratic behavior of the flame photometer probably due to high flame background and to atomizer clogging. If potassium is to be determined on sodium EDTA extracts, it would be best to make any necessary dilutions with water before analysis. Standard solutions must, of course, be made in solutions having the same sodium content as the samples.

Precision. To determine the precision of this method, 15 replicates of two

different samples were extracted and analyzed. Results are recorded in Table II.

Other Elements. Manganese is probably completely extracted from plant tissue by salts of EDTA, but attempts to use the 403-mμ Mn line for flame analysis were unsuccessful owing to the proximity of the 404-mμ potassium line. Sodium analysis by this procedure is not recommended, since the calcium normally present in plant tissue causes a positive error in the flame analysis of sodium. Sodium is more conveniently extracted from plant tissue with ammonium oxalate (*4*) which simultaneously precipitates calcium.

Phosphorus is not completely extracted by this procedure. A nearly colorless extract is obtained by addition of activated carbon. EDTA in low concentrations does not interfere with the molybdovanadophosphoric acid method for phosphorus so that "soluble" phosphorus might be determined by this method. A test of some samples showed that approximately 65% of the total phosphorus was extracted by ammonium EDTA at pH 8.0.

No attempt was made to extract other elements, although presumably any metals complexed by EDTA should be extractable. Since EDTA is used in the determination of copper (*1*), that element might be determined by this procedure if the organic matter extracted by EDTA does not interfere with the copper determination.

The simplicity and rapidity of this procedure suggest that it might be useful as the basis of a quick test system of plant analysis.

Literature Cited

(1) Cheng, K. L., Bray, R. H., *Anal. Chem.* **25,** 655 (1953).
(2) Greweling, Thomas, *Chemist Analyst* **50,** 40 (1961).
(3) Herrman, R., Rick, W., *Naturw.* **46,** 492 (1959).
(4) Mathis, W. T., *J. Assoc. Offic. Agr. Chemists* **39,** 419 (1956).
(5) Peech, Michael, *Ind. Eng. Chem., Anal. Ed.* **13,** 436 (1941).
(6) West, A. C., Cooke, W. D., *Anal. Chem.* **32,** 1471 (1960).
(7) Willis, J. B., *Spectrochim. Acta* **16,** 259, 273 (1960).
(8) Wirtschafter, J. D., *Science* **125,** 603 (1957).

Received for review March 1, 1961. Accepted June 9, 1961. Agronomy Paper No. 535, Agronomy Department, Cornell University, Ithaca, N. Y.

CASTOR BEAN COMPONENTS

The Chemistry of Allergens. Inactivation of the Castor Bean Allergens and Ricin by Heating with Aqueous Calcium Hydroxide

JOSEPH R. SPIES, E. J. COULSON HARRY S. BERNTON, P. A. WELLS, and HENRY STEVENS

Allergens Laboratory, U. S. Department of Agriculture, Washington 25, D. C.

Castor oil with its derivatives has scores of industrial uses, while the pomace is used only as a fertilizer and this usage has some hazard because of the potent allergens it contains (*11, 16*). Maximum safe utilization of castor bean pomace, as fertilizer and even more so as livestock feed, requires inactivation of not only the allergens and ricin, the two principal harmful components, but also a substance which causes necrosis on injection of castor bean extracts. The toxicity of ricin is destroyed when its water solution is heated to boiling (*6*) or even to the coagulation temperature of the protein (*5*). However, the principal castor bean allergen retains immune-precipitating and allergenic properties after heating at 100° C. even in alkaline solution (*7, 16*).

The isolation and chemical and immunological properties of the principal allergen or allergens of castor beans, CB-1A, have been described in previous papers from this laboratory (*2, 4, 15, 17–19*). CB-1A is a mixture of proteins and polysaccharidic proteins, classified as natural proteoses. Layton, Moss, and DeEds (*10*) have described separation of six components of CB-1A by ion exchange chromatography and further study of relationships of antigenic and allergenic specificities of these components is in progress. Defatted, domestic castor beans and a commercial pomace yielded 1.8 and 0.33% of CB-1A, respectively. Isolation methods give minimal yields, and the allergen contents of several varieties of castor bean meals ranged from 6 to 9% as determined by a quantitative precipitin method (*3*). The allergenic and antigenic specificities of the components of CB-1A are attributed to the protein components (*4, 17*). CB-1A is soluble in water and in basic lead acetate solution but is precipitated by 75% ethyl alcohol. CB-1A is composed of amino acids with relatively high arginine and glutamic acid contents and no tryptophan (*18*). CB-1A contains no ricin and is nontoxic.

In a previous report the effects of heating the castor bean allergen in solutions of pH 4 to 10 for various times on its immune-precipitating and reagin-neutralizing properties were determined (*16*). Gardner and associates (*7*) made practical exploratory experiments with several chemicals on the detoxification and deallergenization of castor beans. They determined destruction of ricin toxicity by agglutination, and allergen content of treated material by an antigen dilution method with rabbit antiserum. Alkaline heat treatments were effective in destroying the precipitating property of the allergen, but no tests of products of inactivation were made with a castor bean–sensitive person either directly or with serum by the passive-transfer method. Gardner worked mostly with castor bean meats (undefatted) of low moisture content; since he gave no pH values, comparison with the present work is not possible. Calcium hydroxide was used by Gardner in one experiment, but under conditions of the test it was not as effective as sodium hydroxide in reducing allergen content. Kodras, Whitehair, and MacVicar (*9*) studied the effects of heating castor bean pomace with water, sodium hydroxide, and hydrochloric acid slurries on the oral toxicity to rats and chicks.

This paper describes the conditions of temperature, time, and pH for the inactivation of the castor bean allergen, ricin, and the necrotizing agent (*4*) by heating with aqueous calcium hydroxide. After inactivation, excess calcium hydroxide is neutralized with phosphoric acid to yield a safe-to-handle mixture suitable for plant nutrition and possibly for livestock feed. Both castor bean meal and the isolated allergen were studied. Inactivation of ricin was determined by toxicity tests with guinea pigs. Destruction of immune-precipitating property of the allergen was determined with rabbit antiserum. Destruction of the allergenic properties of reagin neutralization and skin reactivity were determined with