Enzymatic Determination of Citrate in Detergent Products

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ABSTRACT

A routine analytical procedure employing enzyme methodology has been developed for the determination of the citrate content of commercial detergent products. The enzyme system used is based upon the selective cleavage of citrate by citrate lyase (citrate oxaloacetate lyase; EC 4.1.3.6). One of the products, oxaloacetate, is reduced to malate by malic dehydrogenase (EC 1.1.1.37) with the simultaneous oxidation of reduced β-nicotinamide adenine dinucleotide to β-nicotinamide adenine dinucleotide, oxidized form. The course of the reaction is measured spectrophotometrically. The decrease in absorbance at 340 nm caused by the formation of β-nicotinamide adenine dinucleotide, oxidized form, is directly proportional to the concentration of citrate. The accuracy of the method which was determined by recovering citrate spikes into citrate-free detergent formulations is excellent. The precision of the method (±1.4% [two standard deviations relative to the average]) is adequate for an analysis of this type. Several commercial detergent products containing 10-20% trisodium citrate were assayed by this method.

INTRODUCTION

Trisodium citrate appears to be a very promising substitute for phosphates in detergent products because of its building power and rapid biodegradability. In certain areas of the country where phosphate-free detergents are mandatory, citrate-containing detergents are available. Laboratory (1) and consumer testing (H.L. Gewanter, private communication and 2) have shown that the cleaning performance of certain citrate-built detergents is comparable to typical phosphate-containing products. Citrates are common components in almost all living systems. It is, therefore, not at all surprising that this material is easily biodegraded. The biodegradability of citrate has been studied in both sewage plant sludge systems and in natural waterways.

To evaluate the biodegradability of citrates in sewage plant sludge, one experiment (3) employed Swisher columns. These columns inoculated with sewage plant sludge were fed continuously with 1% synthetic sewage solution (4) and a solution of sodium citrate or citric acid. In all tests, over 90% degradation of citrate was seen. In most cases, over 95% of the citrate introduced to the column was degraded, even when the concentration of the citrate was very high (3300 ppm). Similar results were obtained using draw-and-fill columns (3).

TABLE I

Assay Specificity Challenge

Compound	Amount assayed (μg)	Assay response (Δ absorbance)
Water blank		0.016
Citric acid	42.6	0.440
Citric acid	85.3	0.874
cis-Aconitic acid	51.6	0.015
trans-Aconitic acid	51.1	0.016
d,l-Isocitric acid	51.8	0.015
α-Ketoglutaric acid	50.8	0.014
Oxalic acid	53.4	0.014
Succinic acid	50.3	0.016
Tartaric acid	50.8	0.016
Glucose	51.2	0.020
Raffinose	51.7	0.018

In natural water, citrate degrades very rapidly. In fish tanks, it is difficult to maintain citrate concentrations for toxicology work. While feeding 950 ml/hr of 48 ppm citric acid into a 3 liter tank, the concentration of citric acid in the tank is only 7.5-11 ppm. River water dieaway tests have shown citric acid concentrations dropping from 10 to 0.1 ppm in a day and sometimes within 8 hr (3).

This potential widespread use of citrates in detergents has prompted the need for a fast, simple, and specific method for determining the citrate content of detergent products. Numerous methods exist for determining bulk citrate and citrate in various matrices; however, little or no work has been done on the assay of citrate in a detergent formulation. Titration methods, most of which are based upon either nonselective oxidation reactions (5) or complex formations (6) would appear to be prone to interferences from the various detergent components and thus would be nonspecific. Colorimetric methods are also relatively nonspecific. The widely used Fuerth and Herrmann reaction (7) with pyridine and acetic anhydride has been applied to citrate in detergents with only limited success. Gas liquid chromatography of the methyl ester of citric acid has been attempted unsuccessfully in this laboratory. Interferences during the extraction step was the primary difficulty. The gravimetric pentabromoacetone method (8) has been the assay of choice; however, this method usually is avoided due to the length and difficulty of the analysis.

The enzymatic method described in this paper is simple, fast, and specific. With the highly purified enzymes and coenzymes now commercially available, the method does not involve lengthy enzyme preparations and requires only a UV spectrophotometer which is now a standard piece of instrumentation in most laboratories. The high specificity of the method was demonstrated by challenging it with a series of compounds, some of which are closely related to citric acid (Table I). At concentrations equivalent to citric acid, these compounds gave essentially no response.

The enzyme system employed in this method is summarized by the following set of reactions which are catalyzed by citrate lyase and malic dehydrogenase (MDH):

$$\text{Citrate} \xrightarrow{\text{Citrate Lyase}} \text{Oxaloacetate} + \text{Acetate} \qquad \text{(I)}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+ \qquad \text{(II)}$$

The enzyme citrate lyase originally was described by Dagley (9). His enzyme system employed additional enzymes, oxaloacetate decarboxylase, which is present in the citrate lyase to convert oxaloacetate to pyruvate and lactic dehydrogenase (EC 1.1.1.27) to convert the pyruvate to lactate. As was clearly pointed out by Moellering and Gruber (10), the dependence of the Dagley method on the oxaloacetate decarboxylase being present in the citrate lyase extract was an uncertainty that should be avoided. Not all citrate lyase preparations contain oxaloacetate decarboxylase. In the Moellering and Gruber method, both MDH and lactic dehydrogenase (LDH) are coupled with the citrate lyase. The oxaloacetate is assayed by the first enzyme as in equation II above, and any pyruvate formed from the oxaloacetate is assyed by the second as follows:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+ \qquad \text{(III)}$$

For the experimental conditions employed by Moellering and Gruber, these authors considered the conversion of oxaloacetate into pyruvate a potential problem. They, therefore, used LDH as shown above to compensate for this

side reaction. For the application described in this procedure, the LDH was found to be unnecessary for two reasons. First, the citrate lyase purchased from Sigma Chemical Co. (St. Louis, Mo.) was sufficiently purified to eliminate any oxaloacetate decarboxylase, and, second, the detergent matrix did not cause any nonenzymic conversion of oxaloacetate to pyruvate. This second point was confirmed by the excellent recoveries of the citrate spikes into the citrate-free detergent formulations.

EXPERIMENTAL PROCEDURE

Apparatus

Micropipettes: Micropipettes were 10 μliter and 100 μliter, e.g. Cole-Parmer (Chicago, Ill.) micropipettes with disposable tips, catalog nos. 7839-04 and 7839-14.

Curvettes: These were 1 cm quartz cells with tapered Teflon stoppers, dimensions 12.5 x 50 mm, e.g. SGA Scientific, Bloomfield, N.J., catalog no. 4820-5.

Interval timer: The timer was from Arthur H. Thomas Co., Philadelphia, Pa., catalog no. 3045-J10.

Suitable spectrophotometer: This was a Beckman DU.

Reagents

0.1M Triethanolamine buffer, pH 7.6: Dissolve 6.65 ml triethanolamine in ca. 250 ml distilled water. Adjust to pH 7.6 with 1N HCl and dilute to 500 ml with distilled water.

0.003M Zinc chloride: Dissolve 41 mg zinc chloride in sufficient distilled water to make 100 ml.

0.032 β-Nicotinamide adenine dinucleotide, reduced form (NADH): Add 4.0 ml distilled water to a premeasured vial containing 10 mg NADH. Wrap vial in aluminum foil (NADH is light sensitive) and keep refrigerated (an ice bath is suitable for the duration of the analyses). Prepare fresh daily. Sigma Chemical Co., stock no. 340-110.

MDH: Add 1.6 ml distilled water to a premeasured vial containing 5 mg enzyme protein (activity ~1000 μ molar units/mg) suspended in 2.8M ammonium sulfate. Keep vial refrigerated (an ice bath is suitable for the duration of the analyses). This reagent may be stored under refrigeration for ca. 1 week. Sigma Chemical Co., stock no. 410-13.

Citrate lyase: Add 2.0 ml distilled water to a premeasured vial containing 5 mg enzyme protein (activity ~8 units/mg). Keep vial refrigerated (an ice bath is suitable for the duration of the analyses). This reagent may be stored under refrigeration for ca. 1 week. Sigma Chemical Co., stock no. C1132.

Trisodium citrate dihydrate: Suitable standard material.

Standard Preparation

Dissolve an accurately weighed sample (~150 mg) of trisodium citrate dihydrate in sufficient distilled water to make 100 ml. Dilute 2.0 ml and 4.0 ml aliquots of the resulting solution to 100 ml with distilled water. These are the trisodium citrate working standards (~30 μg/ml and ~60 μg/ml, respectively). Calculate the actual concentration of trisodium citrate dihydrate in μg/ml.

Sample Preparation

Dissolve an accurately weighed sample equivalent to ~300 mg trisodium citrate dihydrate in sufficient distilled water to make 200 ml. Dilute a 3.0 ml aliquot to 100 ml with distilled water.

Procedure

To a 1 cm quartz cuvette, add 1.0 ml of either distilled water, working standard solution, or sample solution. Add 2.0 ml triethanolamine buffer, pH 7.6, 100 μliter NADH solution and 100 μliter 0.003M zinc chloride solution. Introduce 10 μliter MDH below the solution surface, and start timer. Immediately stopper the cuvette and mix by inverting the cuvette several times. Replace the pipette tip after each addition to avoid contamination of reagents. After 2.0 min, read the absorbance (A_1) at 340 nm vs water. After an additional 1.0 min, introduce 10 μliter citrate lyase below the solution surface, stopper, and mix by inverting. After an additional 3.0 min, ready the absorbance (A_2) at 340 nm vs water. Run each solution in duplicate.

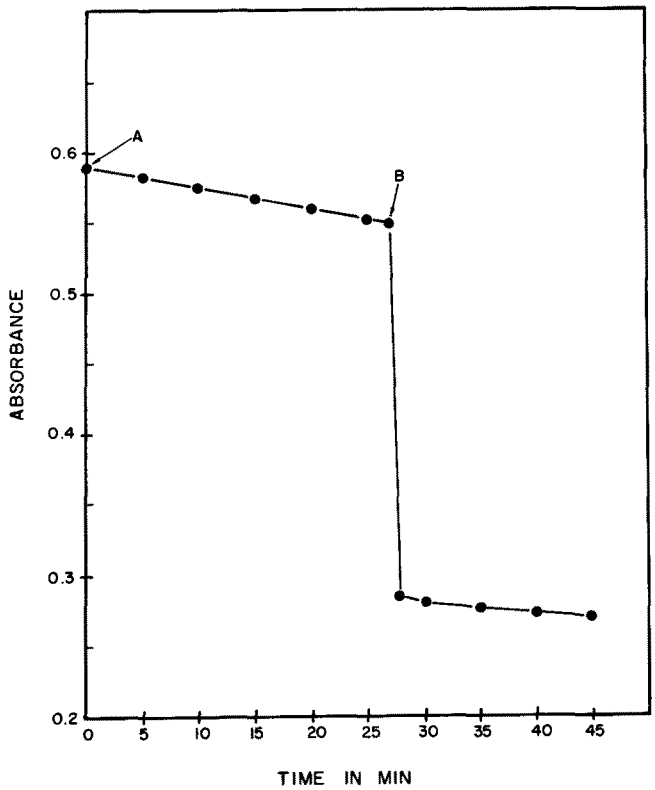

FIG. 1. The reaction curve showing the gradual "creep" in absorbance upon addition of the malic dehydrogenase (A), as well as the very fast reaction rate upon addition of the citrate lyase (B).

TABLE II

Experimental Detergent Formulations

Formulation and components	%
Machine dishwasher detergent (MDD-8)	
Trisodium citrate·$2H_2O$	20.0[a]
Hydrous sodium silicate L-20 L-20 (Philadelphia Quartz)	2.5
Hydrous sodium silicate H-20 (Philadelphia Quartz)	27.5
Nonionic surfactant plurafac RA-43 (BASF Wyandotte)	3.0
Sodium dichloroisocyanurate CDB-63 (FMC)	1.7
Sodium sulfate	43.8
Micro-Cel C (Johns Manville)	1.5
Hard surface cleaner 192-248A	
Trisodium citrate·$2H_2O$	18.0[b]
Sodium xylene sulfonate	4.0
Linear alkyl benzene sulfonate	8.0
Sodium carbonate	0.5
Water	69.5

[a]In the case of the blank formulation, the trisodium citrate was replaced with an equal wt of sodium sulfate.

[b]In the case of the blank formulation, the trisodium citrate was replaced with an equal wt of water.

Calculations

Calculate the trisodium citrate standard factor (F) for each of the working standards as follows:

$$F = (\Delta A_{Std} - \Delta A_B)/C$$

TABLE III

Multiple Analyses of Various Citrate Containing Detergents and Blank Formulations

Sample	Percent trisodium citrate dihydrate						
	Label claim	Day 1	Day 2	Day 3	Day 4	Day 5	Average
MDD-8 "blank"	0	0.00	0.18	0.14	0.17	0.00	0.10
Hard surface cleaner "blank" 192-248B	0	0.28	0.04	-0.12	0.18	0.00	0.08
MDD-8	20	18.42	18.50	18.06	18.24	18.19	18.28
Hard surface cleaner 192-248A	18	18.74	18.94	18.63	18.44	18.59	18.67
Wisk (Sample A)	--	10.14	10.16	10.25	10.29	10.20	10.21
Wisk (Sample B)	21	20.75	20.88	20.99	20.65	20.75	20.80
Liquid All	20	20.30	20.21	20.09	20.16	20.09	20.17
Mr. Clean	--	17.21	17.28	17.31	17.23	17.33	17.27

TABLE IV

Recovery Study

Blank formulation used	Percent trisodium citrate dihydrate		
	Added	Found	Recovered
MDD-8	20.45	20.54	100.42
MDD-8	20.15	20.32	100.81
MDD-8	19.87	20.10	101.10
Hard surface cleaner 192-248B	16.96	17.37	102.42
Hard surface cleaner 192-248B	19.84	19.83	99.93
Hard surface cleaner 192-248B	19.44	19.48	100.23

where, $\Delta A_{Std} = (A_1 - A_2)$ = decrease in absorbance due to citrate content of the working standard; ΔA_B = decrease in absorbance for a 1.0 ml aliquot of distilled water taken through the procedure; and C = concentration in μg/ml of the trisodium citrate working standard. Calculate the trisodium citrate content of the sample as follows:

$$\% \text{ Trisodium citrate dihydrate} = (\Delta A_{Sample} - \Delta A_B)/(W \times F_{avg} \times 1.5)$$

where, $\Delta A_{Sample} = (A_1 - A_2)$ = decrease in absorbance due to citrate content of the sample; ΔA_B = decrease in absorbance for a 1.0 ml aliquot of distilled water taken through the procedure; W = sample wt in g; F_{avg} = average of the factors calculated for the working standards (as shown above); and 1.5 = conversion factor.

RESULTS AND DISCUSSION

Using the above procedure, the reaction rate of this enzyme system is extremely fast, as is illustrated in Figure 1. The MDH is added first and a short time allowed to pass in case there are any MDH reactive substrates present in the sample. There is a very slight linear decrease in absorbance that occurs after the MDH addition. The cause of this absorbance change or "creep" (11) has not been explained; however, it is a common effect which might be attributable, e.g. to trace contaminating enzymes in the enzyme preparation which react slowly with substrates in the sample. Upon addition of the citrate lyase, the absorbance decrease proceeds rapidly to a near constant value, except again for a slow decrease similar to that occurring after the MDH addition. The exact timing of the enzyme additions and spectrophotometric readings was incorporated into the procedure to compensate for these near constant drifts in absorbance.

The function of the zinc as an activator for the citrate lyase and its superiority to magnesium were discussed at length by Moellering and Gruber (10). Zinc was maintained in this procedure solely on this basis. Although no experimentation was conducted, it is felt that the superior activation has enabled us to use a minimum amount of enzyme/analysis. The enzymes, citrate lyase and MDH, were serially diluted and used in the assay until the reaction failed to proceed to completion within the time spans given in the procedure. This resulted in a reasonably fast assay taking 10 min once all solutions were prepared and at a moderate cost of ~$.15/assay (at date of publication).

The detergent formulations of known composition used in this study are given in Table II. These formulations were indicated to be somewhat representative of the detergent products on the market and were supplied to us with and without citrate. The citrate-free (blank) formulations were run as is and also spiked with accurately known amounts of trisodium citrate dihydrate. The blank samples gave a slight response in the assay equivalent to 0.10% trisodium citrate dihydrate (Table III). This response is negligible with respect to the 10-20% trisodium citrate dihydrate found in typical citrate-containing detergents and also falls well within the precision of the method. This slight blank could be an acceleration of the previously mentioned absorbance "creep" caused by one or more of the components in the formulation. These blank formulations also were used in spiking experiments, the results of which are summarized in Table IV. In every case the recoveries were very close to 100%, clearly supporting the accuracy of the method. The citrate-containing formulations corresponding to these blank samples also were assayed (Table III). The results agreed satisfactorily with the label claims, except in the case of the MDD-8. It is felt that the discrepancy encountered on this sample was due to an error in preparing the formulation and was not caused by an interference from any of the components in the detergent matrix. This reasoning is clearly supported by the previously mentioned spking experiment (Table IV) in which excellent recoveries were obtained using a similar MDD-8 formulation (less citrate) as the spiking matrix.

The citrate content of several products purchased in a part of the country that requires phosphate-free detergents was determined by this enzymatic method (Table III). The label claims for some of these products were available; the levels of citrate found for the others were in the range anticipated for these types of detergent products.

The precision of the method was determined from the 6 citrate containing samples (Table III) which were run on 5 separate days. Calculation of the standard deviation(s) was according to $S = \bar{R}/d_2$, were $\bar{R}$ is the average of the ranges and d_2 is a tabulated value associated with the distribution of the average range. For a single run, the precision of the method is ±1.9% (expressed as two standard deviations relative to the average value) and for a duplicate run, ±1.4%. A run is defined here as the taking of the sample and standard solutions through the reaction and UV measurement part of the assay. The advantage in running each sample and standard in duplicate is a gain in precision of ~ ±0.5%. Depending upon the precision required, the need to make duplicate determinations rests with the individual laboratory. All data presented in this paper were gathered by making duplicate runs as described in the procedure.

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