CHROM. 3372

A quantitative indicator for the paper chromatographic or electrophoretic determination of polyaminocarboxylic acids

The increasing use of polyaminocarboxylic acids as complexing agents have necessitated the development of better methods of analysis. Paper chromatography and electrophoresis are potentially useful tools for the analysis of chelating agents in biological systems. For example, HILL-COTTINGHAM AND LLOYD-JONES[1] have separated EDTA, CDTA, and iron chelates by paper chromatography. The chelating agents were observed by staining with nickel dimethylglyoxime (Ni-DMG) producing white spots, and iron chelates observed by viewing under ultra-violet light. HILL-COTTINGHAM[2] found that the DARBEY reaction[3], utilizing Ni-DMG, produced white and pale-yellow spots with many complexing agents, while ninhydrin indicated relatively few. A note by BLASS AND VICAIGNE[4] reports the use of the Folin reagent for the detection of phenols.

With the exception of Ni-DMG, the detection methods are limited and restricted to only a small number of complexing agents. Although Ni-DMG is a general indicator for polyaminocarboxylic acids, the white or pale-yellow color produced on paper cannot be used for quantitative determinations requiring densitometry or other light-absorption methods. A general reagent which would enable the ready quantitative detection of polyaminocarboxylic acids is yet to be reported.

We found that phosphomolybdotungstic acid (the Folin–Ciocalteau reagent) possessed stable color characteristics with certain polyaminocarboxylic acids. Since these color reactions have not been characterized, quantitative applications of this reagent were tested. Polyaminocarboxylic acids in an Arizona soil were resolved with paper electrophoresis using the Folin reagent as an indicator to illustrate the application of this reagent to a biological system.

Experimental

Paper. Beckman R paper strips No. 320046 measuring 3.0 × 30.6 cm made for use with the Beckman Model R Durrum type paper electrophoresis system were used in testing the detection reagent for the polyaminocarboxylic acids and in the electrophoresis study of the complexing agents.

Detection reagent. Phosphomolybdotungstic acid 1 *N* (the Folin–Ciocalteau reagent), obtained from the Fisher Scientific Co., Fair Lawn, N. J., U.S.A. was used as the indicator for the polyaminocarboxylic acids. The reagent may be easily prepared according to the method described by COLOWICK AND KAPLAN[5].

Detection on paper. The detection procedure developed is as follows:

(1) The moist paper strips from the electrophoresis cell are removed and allowed to dry.

(2) The paper is then sprayed with 1 *N* Folin–Ciocalteau reagent until moist and yellow in color.

(3) This is followed by a second spraying with 2.8 *N* Na_2CO_3 (under a ventilating hood). A blue color indicating the chelating agent will begin to appear within a minute or two. Spraying of Na_2CO_3 is continued until the yellow color of the Folin reagent has nearly disappeared.

(4) The paper is then suspended in a closed container for about 15 min to allow the blue color of the chelate to fully develop and the yellow background color of the Folin reagent to fade away before being air-dried.

Polyaminocarboxylic acids and complexing agents not containing amino-carboxylic acid groups listed in Tables I and II, respectively, were applied to the paper and allowed to dry before testing for color reaction by the procedure above.

Electrophoresis of polyaminocarboxylic acids in soil solution extracts. Columns of 2.03 kg of Mohave sandy loam, a semiarid calcareous soil from southern Arizona, were wetted with 1.25 l of 8×10^{-2} *M* amounts of EDTA, EGTA, DCTA, HEEDTA, and DTPA in five separate treatments. Soil solution extracts were obtained from the five separate treatments and 30 λ from each were applied to five separate electrophoretic paper strips. Soil solution extract from untreated soil was applied to a sixth strip as a control. The electrophoresis buffer media having a pH and ionic strength of 8.2 and

TABLE I

POLYAMINOCARBOXYLIC ACIDS STUDIED

No.	*Chelating agents*	*Abbreviations*	*Amount tested* (μg)	*Purity* (%)
1	Ethylenediaminetetraacetic acid	EDTA	1.46	99.0
2	Ethyleneglycol bis(β-aminoethyl ether) N,N'-tetraacetic acid	EGTA	1.90	> 98
3	1,2-Diaminocyclohexanetetraacetic acid	DCTA	1.73	> 93
4	N-2-Hydroxyethyl-ethylenediamine-N,N',N'-triacetic acid	HEEDTA	1.39	> 98
5	Diethylenetriaminepentaacetic acid	DTPA	1.97	> 98
6	Uramil-N,N-diacetic acid	UDA	1.39	> 98
7	β-Alanine-N,N-diacetic acid	ADA	1.04	—
8	N,N'-Ethylenebis[2-(*o*-hydroxyphenyl)]-glycine	EDDHA	1.71	94.8
9	N,N-Bis(carboxymethyl)anthranilic acid	CMA	1.26	—
10	N,N-Di-2-hydroxyethyl-glycine	DHEG	0.82	—
11	Diaminoethyl ether tetraacetic acid	chel Me[a]	1.68	—
12	Ethylenediaminedi-*o*-hydroxyphenylacetic acid	EDHPA	1.66	> 95
13	N-Methyliminodiacetic acid	MeIDA	1.47	> 95
14	N-2-Hydroxyethyl-iminodiacetic acid	HEIDA	1.77	> 95
15	Nitrilotriacetic acid	NTA	15.28	—
16	Diaminopropanetetraacetic acid	DPTA	12.24	—
17	Ethylenediamine-N,N'-diacetic acid	EDDA	3.52	> 95

[a] Trademark of Geigy Chemical Company.

TABLE II

COMPLEXONES GIVING NO COLOR REACTION WITH THE FOLIN REAGENT

No.	*Chelating agent*	*Abbreviation*	*Amount tested* (μg)	*Purity* (%)
1	5-Sulfosalicylic acid	SSA	50.84	99.7
2	DL-Malic acid	—	13.41	> 99
3	D-Tartaric acid	—	15.01	99.0
4	Adipic acid	—	14.61	—
5	Itaconic acid	—	13.01	—
6	Adenosine-5'-phosphate	AMP	36.42	99
7	Adenosine-5'-triphosphate	ATP	60.50	99

0.30, respectively, consisted of 13.00 ml of 0.20 *M* $NaH_2PO_4 \cdot H_2O$ and 488.5 ml of 0.20 *M* $Na_2HPO_4 \cdot 7H_2O$ diluted to 1 l with deionized water. A potential difference and current intensity of 75 V and 5.3 mA, respectively, was applied for a duration of 8.5 h after which the strips were air-dried and sprayed with the Folin reagent and Na_2CO_3 as described.

Densitometric determination of DTPA. Applications of 10, 20, 30, 40, 50, 60, and 70 mμ moles of DTPA were spotted on a paper strip, dried and color-developed. A standard extinction curve was drawn by use of a Spinco Model RB "Analytrol" Densitometer (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif. U.S.A.) which measures and records the light absorbed by colored material distributed on a paper strip placed before a measuring photocell according to the Lambert-Beer Law:

$$E = \log \frac{I}{I_0} = K\frac{a}{f} = kcd$$

where E is the extinction or light absorbed; I_0 and I, the light intensity transmitted through the blank paper and through the colored spot, respectively; K and k, proportionality constants; a, the weight of material on the paper; f, the area of the strip perpendicular to the transmitted light beam; d, the thickness of the layer; and c, the color concentration.

The instrument contains an integrator which automatically produces saw-tooth patterns representing the areas or extinctions under the densitometric curves. These objective values of the area were plotted against concentration of the separate DTPA fractions to yield a standard extinction curve.

Results and discussion

A blue spot showing the presence of the chelating agents on paper was produced when sprayed with the Folin reagent, with all polyaminocarboxylic acids listed in Table I. The color reaction occurred with all polyaminocarboxylic acids examined. Table II lists complexing agents examined which did not give any color with the Folin reagent.

Fig. 1 illustrates the result of electrophoresis of soil extracts and spraying with the Folin reagent. Strips numbered 1 through 5 represent electrophoresis of soil extracts from soils treated with EDTA, EGTA, DCTA, HEEDTA, and DTPA, respectively. Strip No. 6 represents a control, the electrophoresis of extract obtained from untreated soil.

The Folin reagent served as an excellent indicator in the above case, showing the movement of complexones toward the positive pole due to the negative charge on the chelate complexes.

Three separate blue bands were observed on strip No. 5. Since only DTPA was added, we suggest that the three color bands represent a separation of three distinct metal-chelate complexes of DTPA. (These were not identified.) No colored regions were observed with the control strip No. 6.

Fig. 2 shows the absorption peaks plotted by the "Analytrol" densitometer corresponding to blue spots of 10, 20, 30, 40, 50, 60, and 70 mμ moles of DTPA developed by the Folin reagent. The saw-tooth patterns below each spot represent the areas under the absorption peaks determined by the "Analytrol" integrator. Other complexones were not tested quantitatively so far.

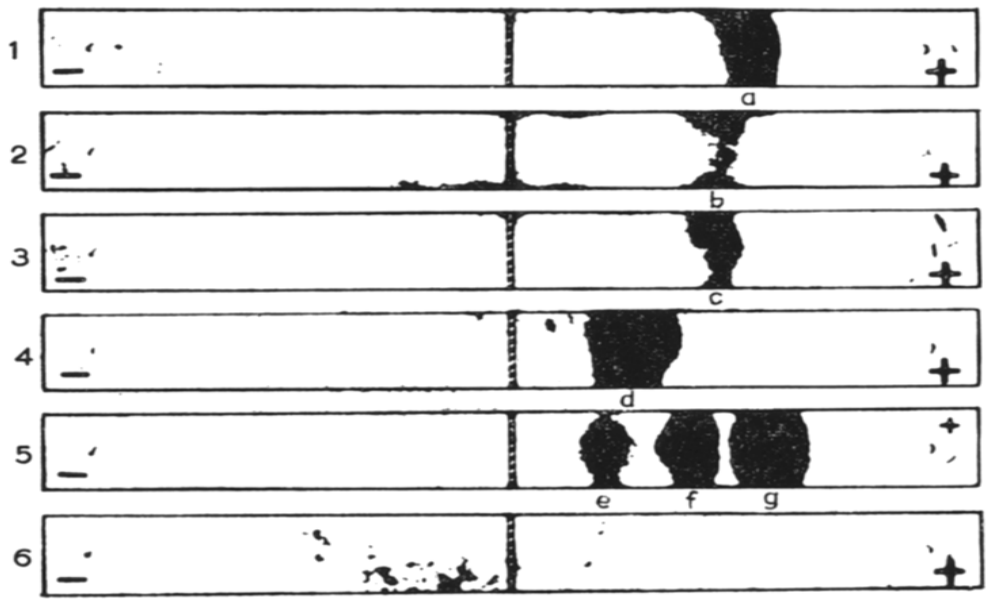

Fig. 1. Electrophoretic movement of polyaminocarboxylic acids from soil solution extracts after treatment with the Folin reagent. Strips numbered 1 through 6 represent soils containing EDTA, EGTA, DCTA, HEEDTA, DTPA, and control, respectively.

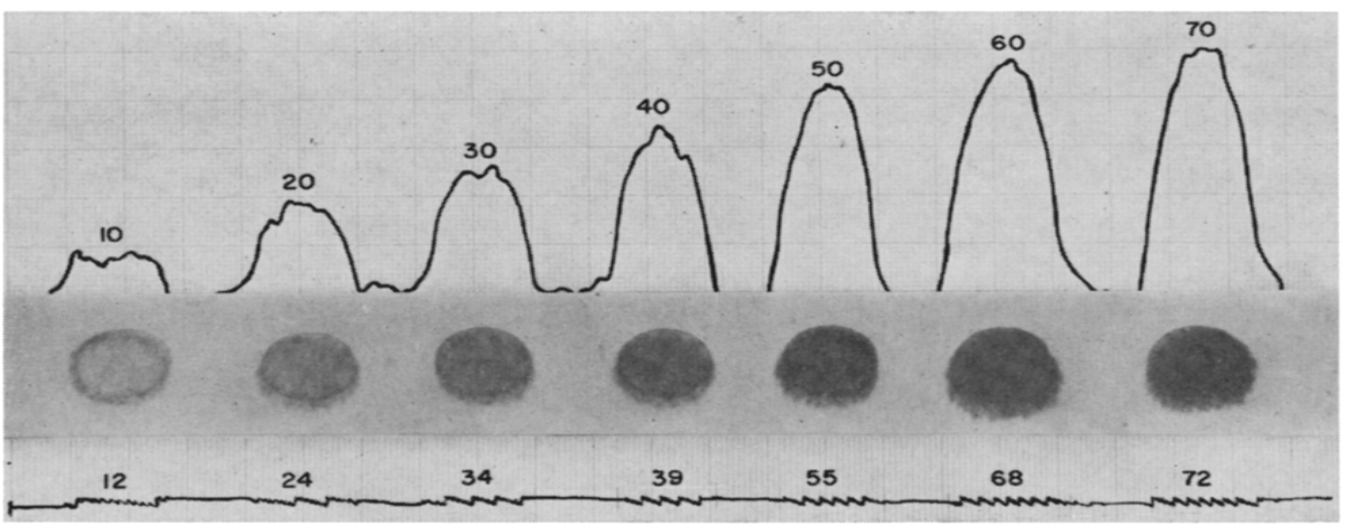

Fig. 2. Absorption peaks of blue DTPA spots plotted by a densitometer with a wavelength of 6000 Å. The figures above each peak from left to right represent increasing amounts of DTPA in mμmoles. Below each spot is given the total number of saw-tooth patterns or area under each curve.

The extinction of area under each of the above peaks plotted against amount of DTPA produced the standard curve shown in Fig. 3. The points were linear from 0–70 mμ moles of DTPA. A definite negative deviation from linearity occurred with amounts $>$ 70 mμ moles.

The color reactions for the quantitative detection of certain complexones, which

utilizes phosphomolybdotungstic acid as an indicator for paper chromatographic and electrophoretic techniques, were found to be stable and quantitatively measurable with a densitometer.

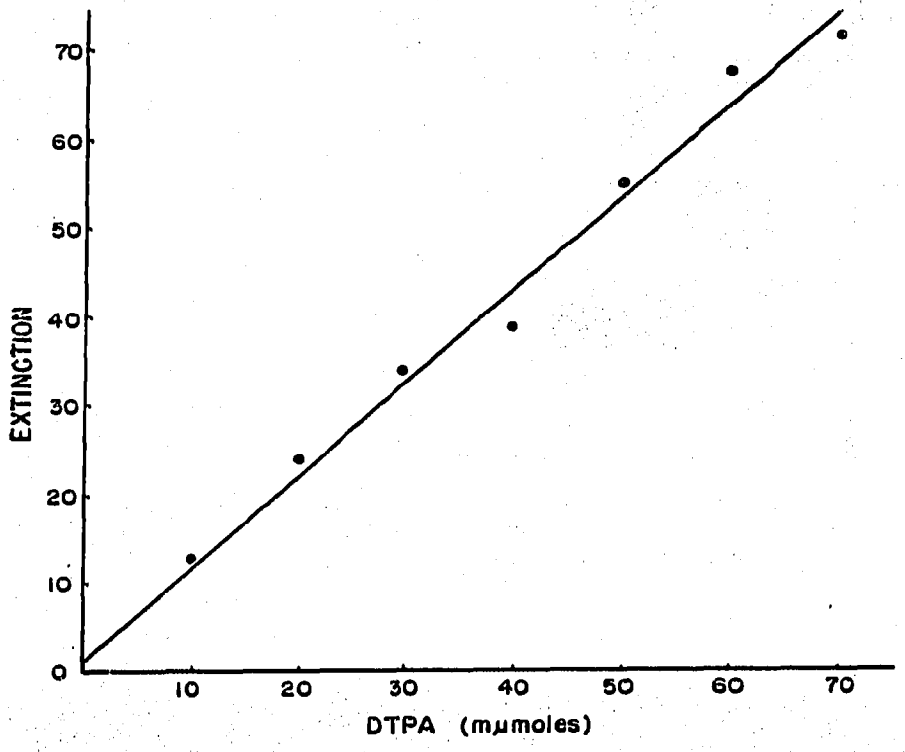

Fig. 3. A standard absorption curve for DTPA with extinction (area under absorption curve) plotted against increasing amounts of DTPA. The values γ and y represent the product-moment coefficient of linear correlation and the least-square equation of the line, respectively. $\gamma = 0.9991$; $y = 1.044x + 1.333$.

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Department of Agricultural Chemistry and Soils, University of Arizona, Tucson, Ariz. 1292 (U.S.A.)

M. F. L'ANNUNZIATA
W. H. FULLER

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