Determination of Chlorocholine Chloride Residues in Wheat Grain, Straw, and Green Wheat Foliage

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A method is presented for the chromatographic separation and subsequent colorimetric measurement of chlorocholine chloride (CCC) at residue levels in wheat grain and plants at various stages of growth. Chlorocholine chloride is removed from tissue background by adsorption chromatography on aluminum oxide, and measured colorimetrically as a dipicrylamine–chlorocholine chloride complex at 415 mμ. The average recoveries of CCC from wheat grain, green wheat foliage, and corresponding wheat straw were 76, 86.4, and 90%, respectively, with average control values of 0.10, 0.16, and 0.27 p.p.m. A rate-of-disappearance study at a treatment level of 4 pounds of CCC per acre of wheat showed a biological half life for CCC of 13 days.

Cycocel [chlorocholine chloride (CCC), 2-chloroethyl trimethylammonium chloride] is a plant growth regulant. The physiological and economic importance of this compound, when used to regulate the growth of several major agricultural crops and certain ornamental plants grown commercially, has been documented by numerous investigators.

The effect of CCC on lodging or "hanging" of wheat has been reported (Linser *et al.*, 1961; Primost, 1964a). Lodging results when plant structural tissue has been weakened by such factors as adverse and extreme weather conditions and poor plant nutrition and development. Generous use of nitrogenous fertilizers, necessary to produce high yields of good quality grain, can lead to excessive vegetative growth that also predisposes the plant to the same structural weakening and, consequently, to lodging. In field trials, in which lodging occurred in untreated plots (Primost, 1964b), the grain yield was increased as much as 60% with the use of CCC.

Other physiological advantages reported included increased root growth (Supniewska, 1963), shorter, sturdier plants (Humphries *et al.*, 1965), and increased wilt resistance and resistance to soil pH changes (Miyamoto, 1962). Because of the number and scope of the studies being conducted with CCC, work was initiated on the development of a sensitive and specific analytical procedure for the determination of residues in wheat grain, straw, and green foliage at the 0.1- to 0.2-p.p.m. level.

Several investigators have reported procedures for the determination of CCC in soil or plant tissues. Linser *et al.* (1965) determined the CCC in a soil extract by the ultraviolet measurement of a periodide complex of CCC. The method, sensitive to 5 μg., would not differentiate between CCC and other choline-containing compounds naturally occurring in large quantities in plant and animal tissues.

Jung and Henjes (1964) and Bier and Faust (1965) have reported methods for the determination of CCC residues in plant material in which the CCC was isolated on a column of a strong acid cation exchange resin. Jung and Henjes (1964) subsequently chromatographed the CCC on a thin-layer plate of Kieselguhr G and made a semiquantitative judgment, after spraying with Dragendorff's reagent, by comparing the size and intensity of the spots to standards similarly treated. The method was made quantitative by Bier and Faust (1965) by precipitating the CCC in the column eluate as the Reinecke salt, and determining the nitrogen present by the Kjeldahl procedure.

Both of these methods lacked the quantitative sensitivity desired in a residue procedure as well as simplicity in handling a large number of samples.

Naturally occurring quaternary ammonium compounds, such as choline chloride and other choline derivatives, have been determined in animal tissues (Wacsmuth and Van Koeckhoven, 1959; Vogel and Debusses, 1962) by colorimetric methods using iodine (Hayashi *et al.*, 1962), dye indicators (Auerbach, 1943), or ammonium reineckate as a reagent (Salwin *et al.*, 1958). These methods, in addition to their nonspecificity, lacked the sensitivity desired for a residue method.

Schill (1959) and Schill and Danielsson (1959) reported on the photometric determination of quaternary ammonium compounds with hexanitrodiphenylamine. The procedure is very sensitive and when the proper measurement conditions are used, can detect 0.05 μg. of CCC per ml. of solution.

This report deals with the development of a reproducible, sensitive, and quantitative method for the determination of CCC in wheat tissues at various stages of growth.

EXPERIMENTAL

Reagents. PURIFIED METHYLENE CHLORIDE. Slurry 25 grams of Nuchar C-190N activated carbon (Fisher Scientific Co., Catalog No. C-177) with 1 gallon of methylene chloride, stirring for 15 minutes. Filter through a Whatman No. 12 fluted filter paper, collecting the purified methylene chloride in an amber glass container. Purify all the methylene chloride used in the colorimetric reaction and measurement in this manner.

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DIPICRYLAMINE REAGENT (2,2′,4,4′,6,6′-HEXANITRODIPHENYLAMINE) (Eastman Chemical Co.). Prepare a 0.01% solution in purified methylene chloride.

ALUMINUM OXIDE. Fisher Scientific Co., Catalog No. A-540, adsorption alumina for chromatographic analysis.

AMBERLITE LA-2 LIQUID ANION EXCHANGE RESIN (Rohm and Haas Co., Philadelphia, Pa.). Prepare a 10% solution in carbon tetrachloride.

CYCOCEL (2-CHLOROETHYL TRIMETHYLAMMONIUM CHLORIDE) STANDARD (American Cyanamid Co., Princeton, N. J.). Prepare standard solutions in methanol containing 10 and 1.0 μg. of CCC per ml. of solution.

Apparatus. CHROMATOGRAPHIC COLUMNS, 10 × 450 mm., with 250-ml. reservoir. Kontes Glass Co., Vineland, N. J., Catalog No. 42028-MA3, 450 mm. (special item).

FLASKS, PEAR-SHAPED, 100- and 250-ml. capacity, 24/40 joint, Kontes Catalog No. K-60870. Also, 10-ml. capacity, Catalog No. K-29425, with connecting adaptors, Catalog No. K-27475.

SEPARATORY FUNNELS, 60- and 250-ml. capacity, with polytetrafluoroethylene stopcocks.

BUFFALO FOOD CUTTER MODEL 111-E, John E. Smith's Sons Co., 50 Broadway, Buffalo 3, N.Y.

Analytical Procedure, CALIBRATION CURVE. Pipet aliquots of the standard solution, containing 1, 3, 5, 8, and 10 μg. of CCC, into 50-ml. beakers and evaporate to dryness on a steam bath. Allow the beakers to cool to room temperature. Add 10 ml. of 0.03*N* sodium hydroxide and mix by swirling. Allow to stand for 5 minutes with occasional mixing. Transfer the solutions to 60-ml. separatory funnels. Rinse the contents of the beakers into the funnels with an additional 5 ml. of 0.03*N* sodium hydroxide.

Add, by pipet, 15 ml. of dipicrylamine reagent to the separatory funnels. Stopper and shake vigorously for 30 seconds. Allow the layers to separate. Draw the lower layers (methylene chloride) into second 60-ml. separatory funnels. Add 5 ml. of 0.03*N* sodium hydroxide to the second funnels and shake vigorously for 15 seconds. Allow the layers to separate. Draw the lower layers into small funnels containing a pledget of glass wool in the stems and collect the filtrates in 25-ml. volumetric flasks. Simultaneously run a reagent blank on 15 ml. of 0.03*N* sodium hydroxide beginning with, "Add, by pipet, 15 ml. of dipicrylamine reagent . . . ."

Measure the absorbance of the solutions on a spectrophotometer at 415 mμ in 5-cm. Corex cells using the reagent blank as a reference. Plot the curve of the absorbance *vs.* the micrograms of CCC per 15 ml. of solution on linear graph paper.

Analysis of Wheat Grain. Pass a portion of wheat in excess of 100 grams through a Wiley mill, or its equivalent, fitted with a screen of 1-mm. diameter openings. Transfer 100 grams of ground wheat to a 1000-ml. screw-cap bottle fitted with a vinyl-lined cap. Add 250 ml. of methanol and extract for 16 hours (overnight) on a reciprocating shaker at a slow to moderate speed. Transfer the mixture to a 250-ml. centrifuge bottle, and centrifuge for 15 minutes at 1500 r.p.m. Decant the supernatant into a 250-ml. Erlenmeyer flask and stopper. Place a glass wool plug in the bottom of a 10 × 450 mm. chromatographic column, and fill the barrel of the column with acetone. Slowly add alumina to the column, and allow it to settle, using constant tapping with a spatula or vibrator to ensure uniform packing. Pack the column to a height of 100 mm. Open the stopcock, and allow the acetone to percolate through the alumina. Wash the column with an additional 20 ml. of acetone. When the acetone has reached the level of the alumina bed, stop the column flow. Do not allow the alumina to go dry. Pipet a 25-ml. aliquot of the sample extract into the reservoir. Allow it to percolate through the column, collecting the effluent in a 100-ml. pear-shaped flask. When the sample extract has reached the level of the alumina, rinse the reservoir of the column with 10 ml. of methanol. Repeat the washing procedure with an additional 15 ml. of methanol. Evaporate the combined effluent and washing solutions to dryness on a rotating film evaporator using a 40° C. water bath. Add 2 ml. of 50% methanol in acetone to the residue and swirl the flask until the contents are completely dissolved. Transfer the solution to a 10-ml. pear-shaped flask. Rinse the contents of the 100-ml. flask into the smaller flask with two additional 2-ml. portions of 50% methanol in acetone. Evaporate to dryness on a rotary film evaporator with a 25° to 30° C. water bath.

Prepare a 10 × 450 mm. column and pack with alumina to a depth of 250 mm., using the technique described above. Add 0.5 ml. of 50% methanol in acetone to the 10-ml. pear-shaped flask and rotate to dissolve the residue. Using a hypodermic syringe, transfer the solution to the barrel of the column. Open the stopcock and allow the solution to percolate through the column until it reaches the level of the alumina. Close the stopcock. Add a second 0.5 ml. of 50% methanol in acetone to the flask and repeat as described above. Add 1.0 ml. of 18% methanol in acetone to the flask and swirl to rinse the sides. Transfer the solution to the column, rinsing the walls o the column barrel. Allow the solution to percolate through the column until it reaches the level of the alumina. Add 30 ml. of 18% methanol in acetone to the column, and allow it to percolate through the alumina at a rate of 80 to 100 drops per minute until it reaches the level of the alumina. Discard eluate. Percolate an additional 60 ml. of 18% methanol in acetone through the column and collect the eluate in a clean, dry 150-ml. beaker. Evaporate solution to dryness on a steam bath.

Proceed with the colorimetric reaction and quantitative measurement beginning with, "Allow the beakers to cool to room temperature," under the preparation of the calibration curve.

NOTE. If the color is too intense to read accurately, dilute the sample with purified methylene chloride to an appropriate volume for measurement and make adjustments in the calculation accordingly.

Determine the concentration of CCC in the solutions from the standard curve.

Analysis of Wheat Straw. Pulverize sufficient dry ice in a bowl of a Buffalo food chopper to aid in the preparation of the sample. Slowly add wheat straw in excess of 50 grams to the dry ice, and continue chopping until the sample is reduced to a fine powder. Distribute the sample on a sheet of paper, and store in a freezer until the CO_2 has completely dissipated.

Transfer 50 grams of ground wheat straw to a 1000-ml.

screw-cap bottle fitted with a vinyl-lined cap. Add 500 ml. of methanolic 0.01*N* hydrochloric acid, and extract for 16 hours (overnight) on a reciprocating shaker at a slow to moderate speed. Filter the mixture through a Whatman No. 12 fluted filter paper, or its equivalent.

Prepare the first alumina column as described under the analysis of wheat grain, beginning with, "Place a glass wool plug...." Pass a 50-ml. aliquot of sample through the column, washing the column as described in the grain procedure, beginning with, "When the sample extract has reached the level of the alumina...." Collect the effluent in a 250-ml. pear-shaped flask and evaporate to dryness using a rotary film evaporator and a 40° C. water bath.

Add, by pipet, 2.5 ml. of methanol to the residue in the flask, and swirl to rinse the sides of the flask. Add 20 ml. of methylene chloride and again swirl. Transfer the solution to a 60-ml. separatory funnel. Repeat the transfer with an additional 2.5 ml. of methanol and 20 ml. of methylene chloride. Add, by pipet, 5 ml. of distilled water to the funnel, and shake vigorously for 30 seconds. Allow the layers to separate completely, and draw the lower (methylene chloride) layer into a second 60-ml. separatory funnel. Add 5 ml. of methanol to the second funnel, and swirl to mix well. Add 5 ml. of water to the funnel, and repeat the extraction of the methylene chloride solution. Discard the lower layer, and combine the aqueous methanol extracts in the first funnel. Wash the extract by shaking vigorously for 15 seconds with 50 ml. of methylene chloride. Discard the wash, and draw the upper layer into a 100-ml. pear-shaped flask.

Continue with the analysis as under the determination of CCC in wheat grain beginning with, "Evaporate the combined effluent and washes...."

Analysis of Green Wheat Foliage. Prepare the sample of frozen green wheat foliage as in the procedure for the analysis of wheat straw beginning with, "Pulverize sufficient dry ice...." Transfer 50 grams of chopped foliage to a 1000-ml. screw-cap bottle fitted with a vinyl-lined cap. Add 400 ml. of methanol, and extract for 16 hours (overnight) on a reciprocating shaker at a slow to moderate speed. Filter the mixture, using vacuum, through a fritted glass funnel of medium porosity. Slurry the cake remaining on the filter with 100 ml. of methanol and filter to dryness. Transfer the filtrate to a 500-ml. volumetric flask. Rinse the contents of the filter flask into the volumetric flask with methanol. Dilute to volume with methanol and mix well.

Transfer, by pipet, 50 ml. of the sample extract to a 250-ml. separatory funnel. Add 10 ml. of aqueous 0.2*N* hydrochloric acid and mix well. Add 100 ml. of carbon tetrachloride to the funnel, and shake vigorously for 30 seconds. Allow the layers to separate, and discard the lower (carbon tetrachloride) layer. Repeat the above wash with another 100 ml. of carbon tetrachloride. Add 50 ml. of 10% liquid anion exchange resin (LA-2) to the separatory funnel containing the aqueous methanol layer, and invert the funnel five or six times. Allow the layers to separate, and discard the lower (resin) layer. Wash the aqueous methanol layer with 100 ml. of carbon tetrachloride as described above. Filter the aqueous methanol layer through glass fiber filter paper into a 100-ml. pear-shaped flask. Rinse the contents of the funnel into the filter paper with 5 ml. of 20% aqueous methanol. Evaporate the extract to dryness using a rotary film evaporator and a 40° C. water bath.

Prepare the first alumina column as under the procedure for the analysis of CCC in wheat grain beginning with "Place a glass wool plug...."

Add 10 ml. of methanol to the flask and swirl to dissolve the residue. Transfer the solution to the column, and collect the effluent in a 100-ml. pear-shaped flask. When the sample extract has reached the level of the alumina, rinse the contents of the flask into the column with 10 ml. of methanol. When the wash has reached the level of the alumina, rinse the column reservoir with 10 ml. of methanol. Repeat the washing procedure with an additional 15 ml. of methanol. Continue with the analysis as under the procedure for the determination of CCC in wheat grain beginning with "Evaporate the combined effluent and washes...."

CALCULATION

$$\frac{\mu\text{g. from standard curve} \times \text{dilution factor}}{\dfrac{\text{wt. of sample in grams}}{\text{volume of extract}} \times \text{volume of aliquot of extract}} = \text{p.p.m. apparent CCC}$$

where dilution factor equals

$$\frac{\text{Final diluted volume}}{\text{15 ml. of dipicrylamine reagent}}$$

Recovery Determinations. Recovery and control determinations were conducted on grain samples of the Genesee variety of soft white winter wheat. Green wheat foliage and straw samples, obtained from a local farm, were of an undetermined variety. The samples were fortified with methanolic solutions of CCC at varying concentrations, and allowed to equilibrate for 4 hours prior to extraction. The results of recovery and control analyses are presented in Table I.

Table I. Recovery of CCC from Wheat Tissue

Tissue	P.P.M. Added	P.P.M. Found[a]	% Average Recovery[a]	Number of Determinations
Wheat grain[b]	0	0.10 ± 0.070[c]	...	8
	0.2	0.152	76	4
	0.5	0.385	77	8
	1.0	0.740	74	4
	2.0	1.50	75	4
Green wheat foliage[d]	0	0.16 ± 0.066[c]	...	8
	0.5	0.60	120	2
	1.0	0.77	77	2
	2.0	1.62	81	4
	5.0	3.95	79	2
	10.0	7.96	80	2
Straw[d]	0	0.27 ± 0.090[c]	...	11
	0.5	0.61	122	2
	1.0	1.01	101	2
	2.0	1.72	86	3
	5.0	3.62	72	2
	10.0	8.33	83	2
	20.0	15.68	78	2

[a] Corrected for average control.
[b] Aliquot taken for analysis equivalent to 10 grams of wheat grain.
[c] Average control and standard deviation (2σ).
[d] Aliquot taken for analysis equivalent to 5 grams of wheat foliage and straw.

A number of different varieties of control wheat grain were analyzed to determine the variations which might be encountered on the analysis of numerous types of wheat (Table II).

Field Experiment. An experiment was designed to follow the rate of disappearance of CCC from wheat plants at various stages of growth and from wheat grain. Winter wheat of the Redcoat variety was treated at the first joint stage (wheat at full tiller near first joint, approximately 8 inches tall). The CCC was applied as an aqueous solution at 4 pounds of real CCC per acre in 40 gallons of spray per acre. Plot size was 10 × 40 feet. An accurate account of the monthly rainfall was recorded from the treatment date to the day of harvest which covered the period from April 23 to July 23. The initial residue sample, April 23, was taken immediately after the deposit dried.

Table II. Apparent CCC in Various Types of Wheat Grain

Variety	Type	Source	Apparent P.P.M. CCC
Genessee	White Winter	USA	0.10
Gage	White Winter	USA	0.14
Odin	Winter	Sweden	0.02
Ring	Spring	Sweden	0.02
Starke	Winter	Sweden	0.05

Table III. Analysis of Wheat Plants and Grain Treated with 4 Pounds of CCC per Acre

Sampling Date	Days Post-treatment	Moisture Content of Tissue	P.P.M. CCC (Dry-Weight Basis)	Rainfall Month	(Total) Inches
4/23	0	82	218	April	2.63
5/14	21	78	125	May	1.29
6/4	42	59	20	June	2.89
6/26 (straw)	64	37	5.6	...	...
7/23 (straw)	91	18	2.8	July	6.97
7/23 (grain) control	91		0.15 (6 samples)	...	...
7/23 (grain) treated	91		1.4 (6 samples)	...	...

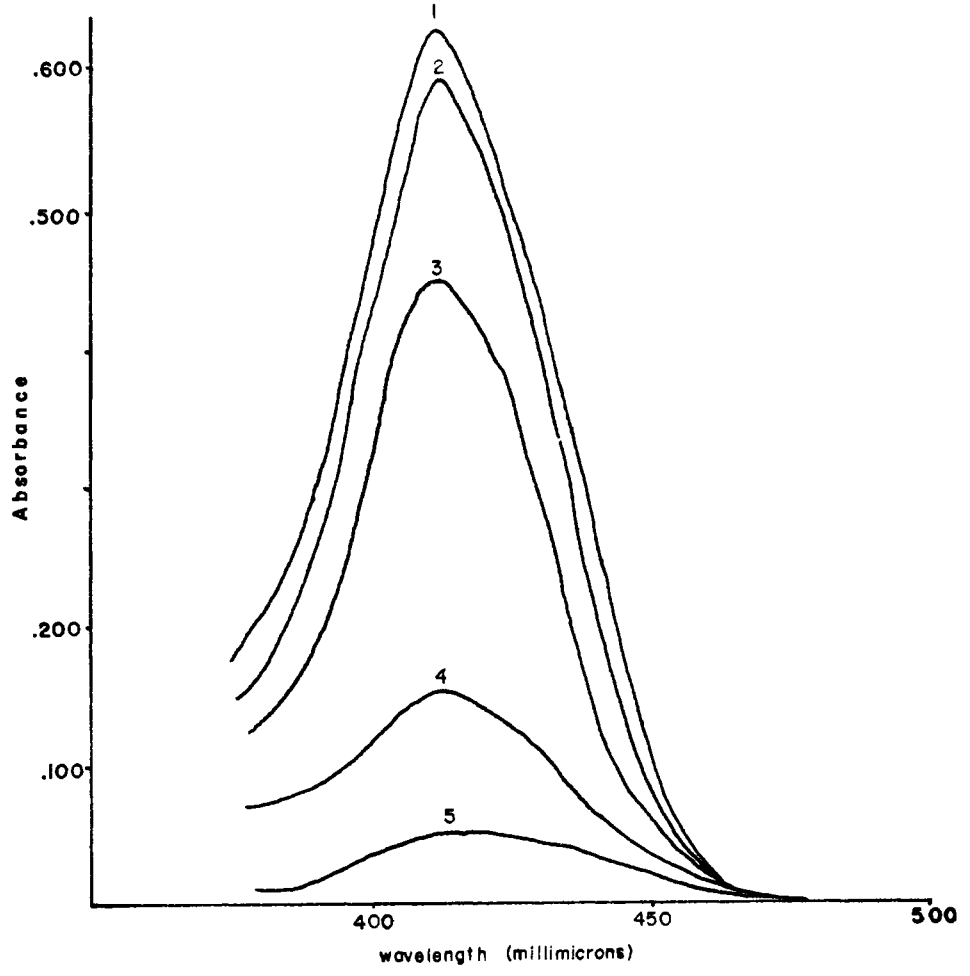

Figure 1. Relationship of color intensity to sodium hydroxide concentration and effect of alkaline wash on reagent blank

1. 0.03*N* NaOH
2. 0.30*N* NaOH
3. 3.00*N* NaOH
4. Reagent blank, 0.03*N* NaOH, no NaOH wash
5. Reagent blank, 0.03*N* NaOH, with NaOH wash

Analysis of Tissues. The results of the experiment are summarized in Table III, which includes moisture values for the tissues examined and the rainfall for that period. Since the moisture content of the plant tissue varies with the growth stage and differs considerably from a young green wheat plant to wheat straw, it was necessary to calculate the CCC concentrations of a dry-weight basis for a more accurate presentation of the disappearance of the compounds. A plot of the concentration of CCC in plant tissue (dry-weight basis) *vs.* days after application indicates that CCC had a biological half life of 13 days.

DISCUSSION OF ANALYTICAL PROCEDURE

Colorimetric Reaction. Schill (1959) and Schill and Danielsson (1959) reported on the reaction of 2,2′,4,4′,6,6′-hexanitrodiphenylamine (dipicrylamine) with quaternary ammonium compounds for the quantitative measurement of these substances. The reaction involved the formation of an alkali insoluble salt of the quaternary compound with the dipicrylamine acting as an anion. This salt was soluble in methylene chloride, while the unreacted reagent was partitioned into sodium hydroxide.

The concentration of sodium hydroxide at which the reaction and partitioning were carried out was critical, and varied for different compounds.

Figure 1 demonstrates the relationship of the color intensity produced to the concentration of sodium hydroxide used in the reaction. The CCC-dipicrylamine salt was recovered quantitatively in one 15-ml. extraction from 0.03*N* sodium hydroxide. Multiple extractions were required to recover the CCC-dipicrylamine salt completely from higher normality caustic.

The standard curve of the CCC-dipicrylamine salt, using 0.03*N* sodium hydroxide as the reaction medium, is linear and follows Beer's law from 0 to 15 μg. of CCC. The slope of the calibration curve for the CCC-dipicrylamine salt is 0.100 absorbance unit per 1.7 μg. of CCC in 15 ml. of methylene chloride measured in a cell with a 5-cm. light path length.

The methylene chloride used in the preparation of the reagent must be purified because of high blanks encountered when the reagent was prepared in untreated solvent. Nuchar C-190N activated carbon adequately removed impurities in the solvent that caused high reagent blanks.

The reagent blank was further reduced by washing the methylene chloride solution of the CCC-dipicrylamine salt with 0.03*N* sodium hydroxide. This step removed the unreacted reagent which was dispersed in the solvent layer, and reduced the reagent blank reading as much as 70%. Figure 1 demonstrates the effect of an alkaline wash on the reagent blank.

Extraction. The solubility of CCC limited to water, methanol, and ethanol precluded the use of nonpolar or water-immiscible solvents for extraction. The extraction of CCC was best accomplished with methanol, although

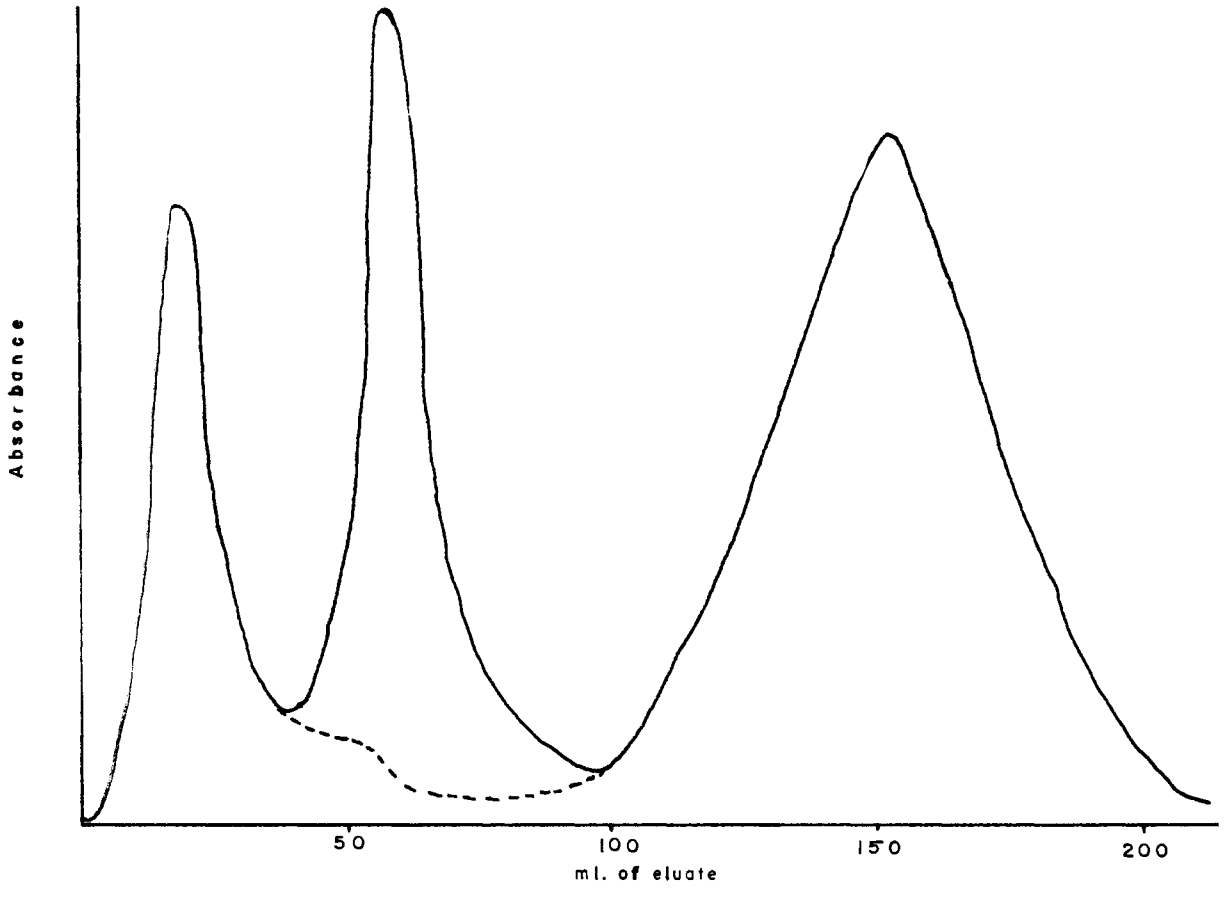

Figure 2. **Elution pattern of various components of control and CCC-fortified wheat grain extract**

---- Control wheat extract —— CCC-fortified wheat extract

different treatments were required for each of the various types of tissues studied. The CCC in a 100-gram sample of ground wheat grain was extracted quantitatively with 250 ml. of methanol, a solvent-to-sample ratio of 2.5 to 1, whereas it required 500 ml. of methanol to penetrate the extract CCC from a 50-gram sample of green wheat foliage, a ratio of 10 to 1. When smaller volumes of methanol were used, only 60% of CCC was recovered from the foliage.

Wheat straw extracted with methanol gave poor recovery at a cumbersome solvent-to-sample ratio of 20 to 1. The addition of wetting agents to the sample to aid in the penetration of the solvent was ineffective. When methanolic hydrochloric acid was used, the recovery increased. The recovery of CCC was consistent (85 to 90%) when the concentration of acid was varied from 0.01 to 0.5*N*. The control value, however, increased from 0.26 p.p.m. at 0.01*N* to 0.60 p.p.m. at 0.5*N* hydrochloric acid.

CCC was extracted from the wheat tissue by either blending in a Sorvall or Waring Blendor for 30 minutes or by overnight extraction on a reciprocating shaker. The latter method was chosen as a matter of convenience in analyzing large numbers of samples.

Extraction of Background Interferences. Each type of tissue required its own individual cleanup procedure prior to the column chromatography step. The carbon tetrachloride extraction described by Blinn (1967) for the removal of chlorophyllic materials from an acidified plant extract was used for green wheat foliage. Excess acid, found detrimental in the chromatography steps, was removed from the extract by shaking with a 10% solution of Amberlite LA-2 liquid anion exchange resin. The use of this resin permitted the neutralization of the acid without the formation of salts in the extract. The removal of these chlorophyllic materials was performed prior to passing the extract through the first column. The extract, after this partition step, was evaporated to dryness and then carried through the two-column cleanup and colorimetric measurement.

In the analysis of wheat straw, a similar extraction was employed after the first alumina column. Water was used in place of the acid, and the need for the liquid anion exchange resin thus was eliminated. Radiometric analysis demonstrated that CCC was recovered quantitatively in this step.

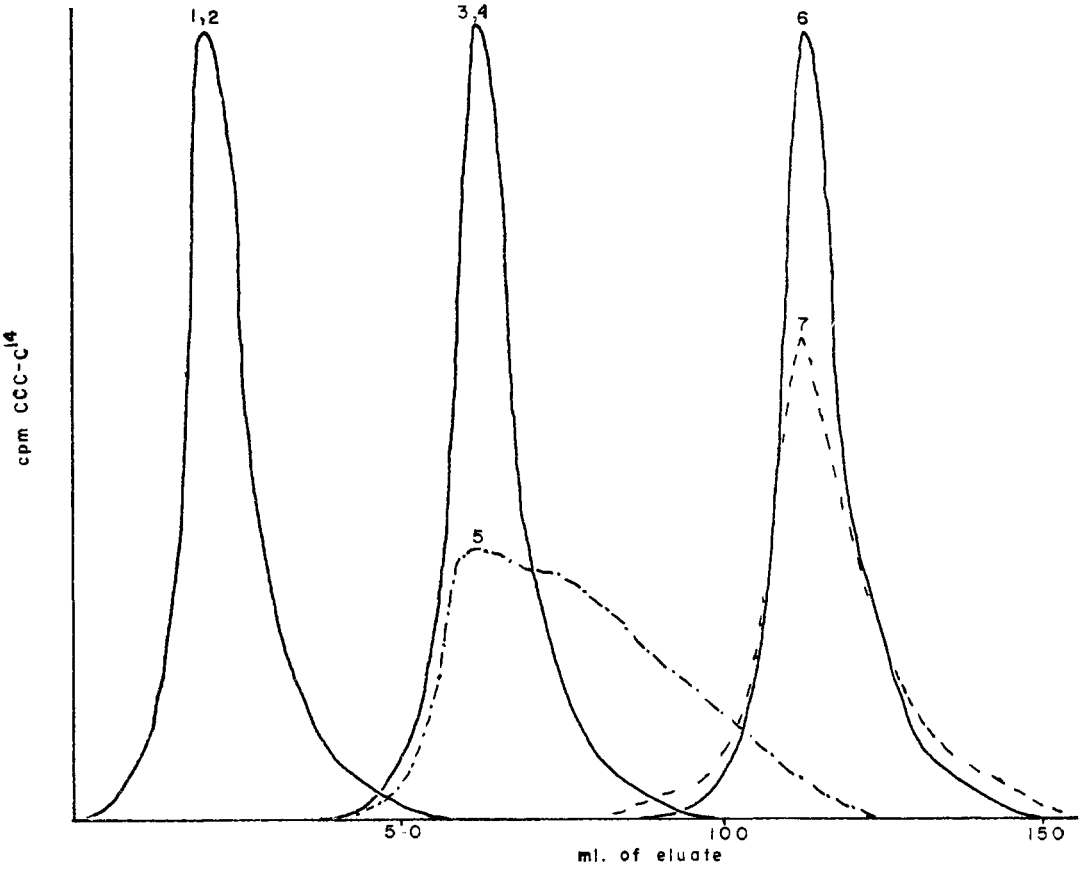

Figure 3. Elution of CCC (C^{14}) from various alumina columns

1. Fisher acid alumina
2. Fisher neutral alumina
3. Alcoa alumina F-20
4. Fisher alumina for chromatography
5. Fisher alumina for chromatography activity grade V
6. Woelm basic alumina
7. Merck chromatographic alumina

Eluate. 18% methanol in acetone

Column Adsorption Chromatography. The isolation of CCC from other components of the methanolic extract was attempted first by thin-layer chromatography using alumina and cellulose as the adsorbents. A complete separation of the CCC from other coextractives was not obtained because of the lack of capacity of the plates to handle the large amount of material in the extract. Although thin-layer chromatography proved impractical for a residue method, the studies conducted did indicate a possible adsorbent and solvent system for the separation of CCC by column chromatography.

CCC was isolated from the other extractives in the methanol by a two-column adsorption chromatographic system. The two columns, both utilizing aluminum oxide as the adsorbent but different solvent systems, performed different functions. The first column removed a large quantity of material from the methanolic extract which later coprecipitated with CCC or interfered with the final quantitative isolation of the CCC. The CCC was subsequently separated from the nonionic oils and other choline derivatives on the second alumina column, using a less polar solvent system.

CCC was separated preferentially on the second alumina column with 18% methanol in acetone as the eluting solvent. A column of adsorbent 25 cm. high and 1 cm. in diameter was used. This column and the conditions presented in the recommended procedure were critical, and were the result of a study of various column sizes and the polarity of the eluting solution.

Increasing the polarity and/or decreasing column length resulted in high recoveries and also high apparent CCC values for control tissue. Decreasing the polarity and/or increasing the column length resulted in low recoveries and low apparent CCC control values. The more polar systems gave poor resolution of CCC from natural components that react with dipicrylamine reagent, while the latter systems indicated inadequate elution of CCC from the adsorbent. The recommended system can be considered a "compromise" of all the systems studied.

The method of introducing the sample on the column also was critical, and affected the reproducibility of the separation. Adding the sample to the column in the smallest practical volume allowed the components to develop in compact bands, affording the best and most consistent separation possible.

The average control and standard deviation was 0.10 ± 0.07 p.p.m. when the sample was introduced into the column in 0.5 ml. of solvent, and 0.15 ± 0.50 p.p.m. when 2.5 ml. of solvent was used.

Figure 2 is a graphic representation of the elution of various components of a fortified wheat sample from the column measured colorimetrically. Neutral oils, a visible yellow color, and other components that interfered in the colorimetric determination were eluted in the first 30 ml. of eluate. CCC was eluted in the 30- to 90-ml. fraction and other more polar compounds, choline and choline chloride, eluted after the CCC.

The elution patterns of ^{14}C-labeled CCC (Figure 3) show considerable variation among different types of alumina and alumina from various suppliers. An effort was made to determine the nature of the adsorption of CCC in order to be able to standardize the alumina from the various suppliers. A study was conducted on the effect of activation, deactivation, particle size, and alkalinity on the adsorption of the CCC. None of the treatments resulted in what could be considered a standardized alumina.

The adsorbent used in the recommended procedure is limited to that supplied by the Fisher Scientific Co. because of its availability, cost, and reproducibility of the results obtained, and because resolution of CCC from other wheat extractives was adequate without the long elution time required by the others studied.

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