The Microdiffusion Separation and Determination of Microgram Quantities of Thiocyanate in Corn

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Amitrol-T, an approximately equimolar mixture of 3-amino-1,2,4-triazole and ammonium thiocyanate, has produced nearly complete control of quackgrass in corn. A method was developed to determine the presence of any thiocyanate residues in the crop. Aqueous extraction is followed by conversion to cyanogen chloride, microdiffusion separation from interferences, and simultaneous color formation with a pyridine-pyrazolone system. The calibration curve is linear to 7 μg., and the limit of detection in corn is approximately 0.2 μg. of thiocyanate. No thiocyanate residues were found attributable to the herbicide mixture. Naturally occurring thiocyanate reached 80 p.p.m.

An approximately equimolar mixture of 3-amino-1,2,4-triazole and ammonium thiocyanate (Amitrol-T, Amchem Products, Inc.) has been used successfully to control quackgrass in corn fields (*9*, *13*). A 4-pound per acre application plowed under before planting provides 85 to 90% control of quackgrass without further cultivation during the growing season. A 2-pound per acre treatment combined with normal cultivation affords very nearly the same degree of control as the higher rate.

It was necessary to determine if any residue of thiocyanate occurred in corn grown in treated fields and the amount of any residue found. It had previously been determined by Rogers (*11*) that 3-amino-1,2,4-triazole used in a preemergence treatment at these or double these concentrations produced a residue of less than 0.25 p.p.m. in corn. The mechanism of conversion of thiocyanate with chloramine-T to cyanogen chloride and the subsequent reaction with a pyridine-pyrazolone system to produce a colored complex stabilized by bis(pyrazolone) has been described (*5*) and further developed as an analytical method by others (*1*, *8*). Colorimetric development yields identical results for cyanide but previous work (*3*, *14*) indicated that any cyanide present would be at least two and probably three orders of magnitude smaller than the thiocyanate content. Consequently no attempt was made to distinguish between thiocyanate and cyanide.

It was felt that a method should be developed which did not employ the usual precipitation removal of protein and consequently potential losses of thiocyanate. By first converting thiocyanate to the very volatile cyanogen chloride and then employing the elegant microdiffusion apparatus developed by Conway (*4*) it was possible first to treat the thiocyanate in the presence of protein and then separate the cyanogen chloride formed for an interference-free development with the colorimetric reagent.

Experimental

Apparatus. Grinding mill, capable of reducing 50-gram loads of hard, dry corn to 20 mesh or smaller.

Waring, commercial, Blendor (Model CB-4) or equivalent, 1.5 hp., 14,000, 17,000, 19,000 r.p.m. with Eberbach Adaptor No. 77-855C to accommodate 1-quart, stainless steel container (press-fit lid). If a mill capable of grinding corn to a very fine meal is available, a common blender could be used.

Centrifuge (International, SBV-1) with head and cups for 250-ml. bottles.

Bottles, wide-mouthed, 8-ounce, with screw cap to fit 250-ml. centrifuge cups (Armstrong Co., Lancaster, Pa.).

Conway microdiffusion dishes. Modified, 68 mm.-type (Aloe Scientific Co. 12005B or Scientific Products 53614A).

Shaking Apparatus. Reciprocating type, variable speed (Arthur H. Thomas Co. 8917-A).

Beckman DU Spectrophotometer with 1-cm. cells.

Reagents. Sodium Acid Succinate. Buffer, 0.01*M*, pH 4.7 ± 0.2. The following are dissolved in 1 liter of distilled water: 0.5903 gram of succinic acid (Eastman 237) and 0.8102 gram of succinic acid, disodium salt (Eastman 1219).

Saturated Pyrazolone Solution. Five hundred milligrams of 3-methyl-1-phenyl-2-pyrazolin-5-one (Eastman 1397) are dissolved in 200 ml. of water at 85° C. (some insoluble residue may remain) and are cooled in a dark place. The solution is filtered through medium speed paper and stored in a dark bottle. It is prepared fresh every few weeks.

Bis(pyrazolone), 0.25% Solution. Twenty-five milligrams of 3,3′-dimethyl-1,1′-diphenyl-(4,4′-bis-2-pyrazoline)-5,5′-dione (Eastman 6969) are dissolved in 25 ml. of purified pyridine, with considerable shaking. This solution is prepared daily and protected from strong light until used.

Mixed Pyrazolones. A sufficient volume of five parts pyrazolone solution with one part bispyrazolone is mixed shortly before use.

Standard Thiocyanate Solutions. At least 99% pure ammonium thiocyanate is used to prepare a 10-gram per liter CNS standard. This solution may be stored in a refrigerator for months and appropriate dilutions made from it; 40 μg. per ml. and 4 μg. per ml. dilutions were found satisfactory. If desired, the 10-gram per liter standard can be checked by titration using one of the common external standards or with β-methylumbelliferone (0.1% in very dilute sodium hydroxide solution) as an internal fluorescence indicator for titration with silver nitrate under an ultraviolet lamp. The end point is indicated by a decrease in the intensity of the blue fluorescence.

Procedure. A curve may be prepared to satisfy the analyst that the absorbance rise is linear with concentra-

Table I. Analysis of Corn Samples

Sample	Sample Conditions	CNS, P.P.M.[a]
A-1	Treated plot[b]	70, 81[c]
A-2	Treated plot[b]	64, 65
A-3	Treated plot[b]	72, 76
A-4	Treated plot[b]	62, 61
		Av. 69
B-1	Untreated check plot	65, 73
B-2	Untreated check plot	56, 62
B-3	Untreated check plot	69, 72
B-4	Untreated check plot	79, 83
		Av. 70
C-1	Fresh frozen corn[d]	61, 58
R-1	Sample B-1 plus 20 p.p.m. CNS added	Recovery 107%
R-2	B-2 plus 20 p.p.m. CNS added	Recovery 98%
R-3	C-1 plus 20 p.p.m. CNS added	Recovery 100%

[a] Calculated on dry basis after moisture determination.
[b] Amitrol-T used.
[c] All duplicate analyses are for samples carried through analysis simultaneously, but independently.
[d] Corn purchased locally.

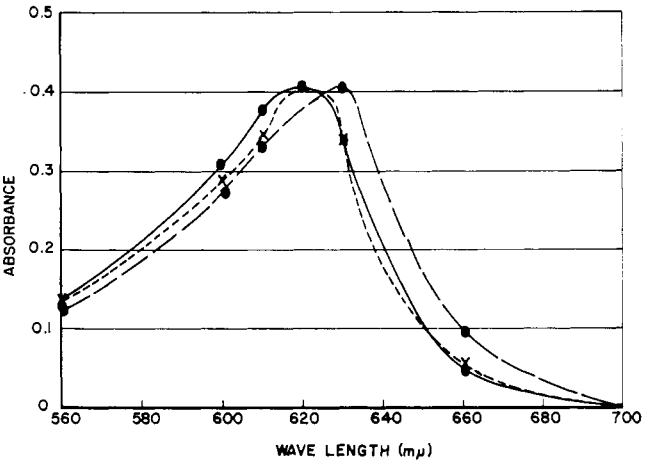

Figure 1. Comparison of spectra of colored reaction products obtained from 0.1 gram of corn, by microdiffusion

×. CNS treated with chloramine-T and color developed in same beaker
●. CNS in water by microdiffusion
All values were scaled to yield equal absorbance at 620 mμ.

tion from 0 to 7 μg. of CNS and this curve might be used for approximate determinations. However, the average slope of 0.14 will be found to vary by about ±10% from day to day and it has been found necessary to run one or two standards and reagent blanks along with each batch of samples. Precision for duplicate runs on the same day is ±1%. Treatment of the standards is the same as for samples, described below.

Extraction. Approximately 50 grams of dry corn kernels are ground to 20-mesh or finer and the ground sample weighed accurately. The entire sample is transferred into an 8-ounce bottle with 150 ml. of water, covered with a double layer of Saran wrap and a screw cap, and placed on the shaking machine for 1 hour. [For fresh corn take an appropriate weight, according to moisture content (*2*), which will contain 30 to 50 grams of dry material and proceed from this point.] The sample is transferred to the blender and reduced to as homogeneous a slurry as practical. The slurry is returned to the 8-ounce bottle and shaken for 30 minutes (fresh corn) to 2 hours (dry corn). The samples are centrifuged at 280 × G for several minutes and the milky supernatant decanted. The process of resuspending in a small volume of water, shaking briefly, centrifuging, and decanting is repeated several times interspersed by a few periods of prolonged (30 minutes) equilibration. The combined decanted solution is diluted that a 1-ml. aliquot will represent 0.05 to 0.08 gram of corn on a dry basis.

Determination of Thiocyanate. A 1.0-ml. aliquot of the extract is placed in a 5-ml. stoppered cylinder with 0.2 ml. of a solution containing 100 p.p.m. of $FeCl_3$ and 1.0 ml. of buffer and then chilled in an ice bath. One milliliter of 1% chloramine-T solution is added, and the cylinder is tightly stoppered, and allowed to stand at room temperature for 3.5 hours. The sample is chilled in a 0° C. ice-salt bath, 0.45 ml. of a 4% sodium arsenite solution is added, and allowed to stand in the ice bath for an additional 5 minutes. Using the test tube funnel, the sample is transferred,with a few small washings, to the evolution ring of the microdiffusion unit. The absorption ring of the unit is loaded with 2.0 ml. of freshly prepared mixed pyrazolone solution shortly before the sample is transferred to the evolution ring; the microdiffusion unit is promptly sealed and placed on the shaking machine at its slowest speed (58 strokes per minute, 4-cm. stroke) for 30 minutes. The colored reaction product formed in the center ring is transferred, with small washings, to a 5-ml. graduate, using a dropper pipet, and is diluted to 3.0 ml. The absorbance of standards, samples, and reagent blanks is read at 620 mμ *vs.* any background at 700 mμ. Standards and samples are corrected for the reagent blank and samples are evaluated against a calibration curve constructed from the standards. Micrograms of thiocyanate found, divided by equivalent grams of sample in the aliquot taken (corrected for moisture content), yield thiocyanate concentration in parts per million, on a dry basis.

Results and Discussion

An aqueous extraction of thiocyanate seemed a good approach, since thiocyanates generally have high aqueous solubilities. Apparently, the thiocyanate in this case is strongly bound to some constituent of corn and many equilibrations with water are needed to achieve reasonable recoveries of added reagent. If ammonium thiocyanate is added to corn and an attempt made to recover it 3 hours later, approximately 78% is recovered. If a similar spiked sample is left overnight before analysis, recovery drops to 60%, while analysis made within 0.5 hour of CNS addition is essentially quantitative. Additions of sodium hydroxide, magnesium sulfate, and a mixture of magnesium sulfate and colloidal iron as suggested by Gemeinhardt (*7*) only hindered extraction. Centrifuging and decanting the cloudy, supernatant liquid gave almost identical values as did filtering through moderately retentive filter paper. The former procedure was then chosen as being more convenient.

The problem of the proper size aliquot of extract to be analyzed was an interesting one, since the smaller the aliquot chosen, the larger became the apparent thiocyanate concentration for a given extract. A dilutable interference was found to be mostly due to incomplete conversion of thiocyanate to cyanogen chloride present in larger samples, when the problem of complete reaction time was considered more carefully. In early work, treatment with chloramine-T was carried out entirely in an ice bath and it took about 6 hours to treat a 0.1-gram sample completely, while smaller samples apparently required less time.

Equal 0.1-gram samples developed for increasing times with chloramine-T gave increasing values of developed color which approached asymptotically to a value near that for a 6-hour reaction time. Reaction times of 1, 2, and 4 hours produced, respectively, 69, 91, and 97% of the value produced after 6 hours. The final procedure, using low temperature reagent additions and sample transfer, combined with room temperature de-

velopment was safe from volatility losses and saved reaction time. In all probability, higher chloramine-T concentrations, coupled with appropriate adjustments in the amount of arsenite used, would allow complete chlorination in a much shorter time, perhaps as little as 30 minutes.

Sodium arsenite had to be introduced to reduce a significant reagent blank apparently caused by excess chloramine-T. [Sodium arsenite has been used previously (*12*) as a titrant to determine chloramine-T, using starch–potassium iodide paper as an external indicator.] Reduction in background had to be balanced against a reduction in developable color, since it was found that a twofold excess of arsenite started to cause reductions in developed colors. Color reduction was probably caused by attack on the intermediate cyanogen chloride already formed. With longer times allowed for reaction of chloramine-T with samples, the reagent blank diminished, but was still worth correcting for. As demonstrated by Epstein (*5*), ferric chloride acts as a catalyst in the conversion of thiocyanate to cyanogen chloride. In early experiments with corn samples to which thiocyanate had been added, those chlorinated in the presence of ferric chloride gave two and a half times the color density of those treated without this catalyst. Large quantities had no more effect than the indicated amount of ferric chloride.

Sodium acid succinate buffer used to alter the sample from a very slight acid condition (pH 6.5 to 6.8) to pH 4.7 was successful in giving an absorbance increase of 22% for thiocyanate standards. A potassium acid phthalate buffer of very close pH (4.2) produced very little enhancement.

At first, microdiffusion was permitted to proceed with agitation once every few minutes and this appeared to give maximum transfer in 30 minutes. Later work showed that continuous, gentle rocking doubled the slope of the calibration curve for the same 30-minute transfer period. Because of the beginning of a slow decomposition of the developed color after about 40 minutes, it was not possible to determine how long complete transfer, without agitation, would take. A value extrapolated from "stagnant" microdiffusion experiments would be between 70 and 110 minutes for complete transfer.

While chloramine-T and bis(pyrazolone) solutions can be successfully stored in a very cold refrigerator (30° F.) for several days, the over-all sensitivity will suffer somewhat. The bis(pyrazolone) preserves the colored reaction product of thiocyanate, pyridine, and pyrazolone; however, the unreacted mixed pyrazolones reagent deteriorates quickly and should be mixed within 0.5 hour of use. Refrigeration does not seem to help and reagent blanks are needed to correct for the build-up of background color even when used as suggested. After microdiffusion is completed the color is stable for at least 15 minutes and slow detectable fading is evident only after 25 minutes. In order to minimize the above variables, two standards and two reagent blanks were run with each batch of five or six samples.

The sensitivity of the method is readily increased by an order of magnitude by extracting the developed color into *n*-butyl alcohol (*1*), but this seemed unnecessary because of the large quantities of naturally occurring thiocyanate found in corn.

Evidence that the substance being determined is actually thiocyanate is available from a comparison of spectra (Figure 1) of the colored reaction products produced by pure thiocyanate in water and by untreated corn. The original use (*5*, *8*) of the pyridine-pyrazolone system was for color development directly in the same solution in which the thiocyanate had been treated with chloramine-T. The spectrum of such a reaction is also shown for comparison. The small deviations in the shape of these curves as well as the minor shift in peak location are unexplained.

The analytical peak had been reported at both 620 (*8*) and 630 mμ (*5*) by different workers.

Corn Grown on Amitrol-T Treated Plots. Experimental plots were treated with 4 pounds per acre of Amitrol-T, 1 month before planting and plowed 3 days before planting. Corn from these plots as well as from duplicate, untreated plots was harvested about 15 weeks after planting. Analyses of these samples (Table I) showed no detectable increase of thiocyanate over the naturally occurring levels. Use of the 2-pound rate should provide an even smaller opportunity for residue pickup.

A sample of fresh frozen corn purchased at a local food market showed a thiocyanate level in the same 60 to 80 p.p.m. range as did the test samples.

The natural occurrence of thiocyanate in plants has been recorded before as has been the occurrence of thiocyanate in normal human beings: blood (1.6 to 15 p.p.m.) (*10*), urine (8 to 20 p.p.m.) (*3*), gastric juice (11 to 40 p.p.m.) (*3*), spinal fluid (0.06 to 0.2 p.p.m.) (*3*), saliva (mean value 117 p.p.m.) (*6*), and breast milk (4 to 5 p.p.m.) (*14*). Over 100 samples of milk from Jersey cows showed 4 to 5 p.p.m. (*14*). Gemeinhardt (*7*) reported 0.03 to 9.5 p.p.m. of thiocyanate in 55 different plants, mostly common fruits, vegetables, and grains. His values are reported on an as received basis and make no allowance for moisture content. It would appear from the above that small quantities of thiocyanate are a normal component of our diet and of our bodies.

Acknowledgment

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Literature Cited

(1) Am. Public Health Assoc., "Standard Methods," 10th ed., p. 299, 1955.
(2) Assoc. Offic. Agr. Chemists, Washington, D. C., "Official Methods," p. 367, 1955.
(3) Boxer, G. E., Rickards, J. C., *Arch. Biochem. Biophys.* **39,** 292 (1952).
(4) Conway, E. J., "Microdiffusion Analysis and Volumetric Error," 4th ed., Crosby Lockwood and Son, London, 1957.
(5) Epstein, J., *Anal. Chem.* **19,** 272 (1947).
(6) Fischmann, E. J., Fischmann, A., *J. Lab. Clin. Med.* **33,** 772 (1948).
(7) Gemeinhardt, K., *Ber. deut. botan. Ges.* **56,** 275 (1938).
(8) Kruse, J. M., Mellon, M. G., *Anal. Chem.* **25,** 446 (1953).
(9) Raleigh, S. M., Proc. Notheast. Weed Control Conf., 13th Meeting, p. 441, New York, January 1959.
(10) Ramos, F. H., Visca, P., *Arch. uruguay. med. cir. y especial.* (*Montevideo*) **26,** 185 (1945).
(11) Rogers, B. J., "Application for Registration of Aminotriazole Weedkiller on Corn, Soybeans, Apples and Cranberries," submitted to U. S. Dept. Agr., February 19, 1957.
(12) Suter, C. M., "Organic Chemistry of Sulfur," p. 613, Wiley, New York, 1944.
(13) Tafuro, A. J., Proc. Northeast. Weed Control Conf., 13th Meeting, p. 423, New York, January 1959.
(14) Wolkes, F., Congr. intern. biochim, 2e Congr., p. 378, Paris, 1952.

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Correction

Determination of Heptachlor Epoxide in Fat and Milk

In this article by Charles F. Meyer, Marshall A. Malina, and Percy B. Polen [J. Agr. Food Chem. **8**, 183 (1960)], under the Reagents section, the 25th line of column two on page 183 should read "Add an equal volume of butyl Cellosolve." The word Cellulose was incorrectly used in place of Cellosolve.