Develop the column by washing with four to five 250-ml. portions of 1*N* hydrochloric acid. The dye derived from *o*-aminoacetophenone separates almost immediately, moving ahead of the other dyes, and washes completely through the column. The PCA dye is adsorbed in the lower half of the column and the DCA dye forms a completely separate zone at the top half.

Drain the column, remove the suction, and cut the column appropriately to isolate the desired dyes. Insert new penicillin disks in the shortened sections, and elute the dye by passing 15 to 20 ml. of 1 to 1 (v./v.) mixture of 1*N* hydrochloric acid and glacial acetic acid through the column. Collect the colored solution in a 25-ml. volumetric flask and dilute to volume with the 1 to 1 mixture. Measure absorbance at 560 mμ in 1-cm. cells, using distilled water as the reference solution. Determine micrograms of chlorinated aniline from the appropriate calibration curve and calculate:

$$\text{P.p.m. of monuron} = \frac{\mu\text{g. of PCA in final aliquot} \times 1.56 \times \text{aliquot factor}}{\text{weight of sample in grams}} \quad (1)$$

$$\text{P.p.m. of diuron} = \frac{\mu\text{g. of DCA in final aliquot} \times 1.44 \times \text{aliquot factor}}{\text{weight of sample in grams}} \quad (2)$$

Discussion

Inclusion of a chromatographic cleanup with the direct caustic hydrolysis procedure resulted in a method for determining a few micrograms of monuron and/or diuron residues with quantitative recoveries from a variety of plant tissues. The method is applicable to a wide range of residue concentrations.

Blanks of untreated plant tissues are not a problem, as the azo dye derived from naturally occurring interfering materials is easily eluted from the chromatographic column, resulting in an essentially no-blank method. The dyes derived from *p*-chloroaniline and 3,4-dichloroaniline may also be separated using a longer column (24-inch to replace the 12-inch column) (*1*) and sufficient 1*N* hydrochloric acid to develop the column completely. The *p*-chloroaniline dye is adsorbed in the lower half of the column, and the 3,4-dichloroaniline dye forms a completely separate zone at the top half. Each band may be eluted quantitatively from the column, and the color intensity measured individually and converted to the respective urea herbicide content.

If the sample being analyzed shows only one colored band, the urea herbicide present is determined from the position of this dye on the column. The identity of the compound may be confirmed by forming the dye on a separate aliquot of the aniline solution to which *p*-chloroaniline has been added. The relative position of the dyes on the column will verify the urea herbicide present.

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FEED ADDITIVES ANALYSIS

Field Tests for the Identification of Coccidiostats in Premixes and Finished Feeds

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A series of simple colorimetric procedures has been developed for the identification of the commercial coccidiostats in premixes and finished feeds. These tests can be used in the field to determine which coccidiostat is present in unknown samples of feed. A procedure is described for adapting those tests to the determination of the distribution of Zoamix or other coccidiostats in finished feed which can be used to ascertain how complete the mixing operation has been.

In poultry management, it is often necessary to determine if a given coccidiostat is present in various lots of premix and finished feed, and to ascertain how well the compound is blended in the product. Most of the procedures which have been developed for the analysis of the various coccidiostats in feed product are time-consuming and are designed to be used with samples of more or less known composition. If the composition of the feed is not known, it often requires a considerable amount of time and effort to ascertain which coccidiostat is present using conventional, analytical procedures. In commercial operation, it is desirable to have a simple spot test which may be used in the field to identify the coccidiostat present in the feed.

During the past several years, it has been observed that Zoalene (3,5-dinitro-*o*-toluamide, the Dow Chemical Co. trademark, except in the United States and Canada) could be distinguished from other coccidiostats on the basis of several simple color reactions (*2*, *3*). These reactions have been adapted so that they can be used directly with premixes or finished feed to determine the presence of the compound. The procedures employed may easily be used in the field. It was decided to extend these procedures to determine if a series of color tests could be developed to distinguish between the major coccidiostats now on the market.

The present paper deals with some of the observations which have been made using a series of spot tests. In most cases, the tests employed are modifications of analytical procedures which have been previously developed for the routine analysis of a given coccidiostat. Attempts have been made to distinguish the various coccidiostats on the basis of positive reactions given by each compound.

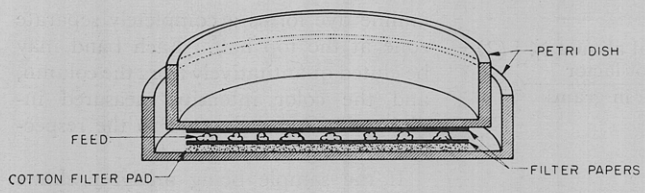

Figure 1. Apparatus for the determination of Zoamix distribution in finished feeds

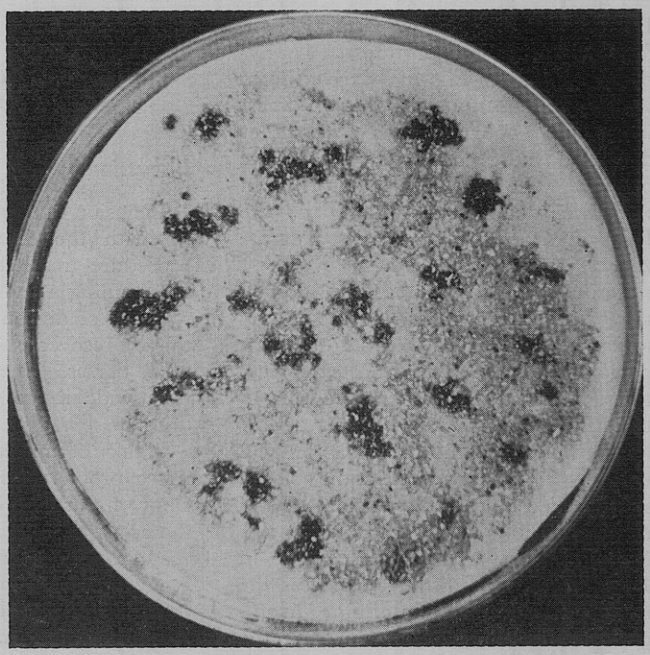

Figure 2. Typical distribution of Zoamix in finished feeds

Methods

Reagents. Dimethylformamide (C.P.).

Sodium methylate, 2*N*. Dissolve 108 grams of sodium methylate (Olin Mathieson Chemical Corp.) in 1 liter of methanol.

Tetramethylammonium Hydroxide. Aqueous solution, 10% (Eastman Organic Chemical No. 1515).

1,3-Diaminopropane (Union Carbide Chemicals Co.).

4-Aminoantipyrine. Aqueous solution, 2%.

Borax Buffer. Dissolve 2.5 grams of sodium borax decahydrate in 100 ml. of 50% methanol.

Potassium Ferricyanide. Aqueous solution, 2%.

Dimethylaminobenzaldehyde Reagent. Dissolve 1 gram of *p*-dimethylaminobenzaldehyde in 30 ml. of ethanol, 30 ml. of concentrated hydrochloric acid, and 180 ml. of 1-butanol.

Starch Iodine Reagent. Dissolve 1 gram of soluble starch in 10 ml. of boiling water and then dilute to 100 ml. with cold water. To this solution add 1 ml. of 0.5% iodine in a 2% potassium iodide solution.

Naphthalenediol Reagent. Mix 90 ml. of a 2,7-naphthalenediol solution (25 mg. per 1000 ml. of methanol) with 5 ml. of a 0.2% solution of potassium ferricyanide $[K_3Fe(CN)_6]$ and 5 ml. of a 1% potassium cyanide solution. After the solution has stood for 30 minutes, add 100 ml. of alcoholic sodium hydroxide solution. Prepare the alcoholic sodium hydroxide solution by diluting 15 ml. of a 10% solution of sodium hydroxide to 200 ml. with methanol.

Coccidiostats. Amprol. (Merck and Co. Inc., Rahway, N. J.)

Arzene. (George Lee Co., Omaha, Neb.)

Borea. (Curts-Folse Laboratories, Kansas City, Kan.)

Glycamide. (Merck and Co., Inc., Rahway, N. J.)

Megasul. (Lederle Laboratories, New York, N. Y.)

Nicarbazin. (Merck and Co., Inc., Rahway, N. J.)

Nitrofurazone. (Dr. Hess and Clark Inc., Ashland, Ohio)

Polystat. (Dr. Salsbury's Laboratories, Charles City, Iowa)

Trithiadol. (Sterwin Chemicals Inc., New York, N. Y.)

Unistat. (Dr. Salsbury's Laboratories, Charles City, Iowa)

Whitsyn-5. (Whitmoyer Laboratories Inc., Meyerstown, Pa.)

Zoamix. (The Dow Chemical Co., Midland, Mich.)

Spot Tests for Coccidiostats in Premixes. In testing premixes for coccidiostats, place approximately 20 to 50 mg. of the premix in a test tube and perform the appropriate tests indicated below. The exact amount of compound used is not important, since the coccidiostat is generally present in sufficient quantity to give a positive test always. It is important to use a small enough sample of the premix so that the color obtained will not be too dark to ascertain its true shade. In many cases, the premixes will be colored themselves and will impart some color to the test solutions. Under these conditions, the test is considered positive only when the color obtained is characteristic enough to distinguish it from the color of the premix itself.

The tests employed are as follows:

Test 1. Shake the sample with 10 ml. of dimethylformamide and note the color (Table I).

Test 2. Shake the sample with 10 ml. of dimethylformamide, add three to five drops of 2*N* sodium methylate solution, and note the color (Table I).

Test 3. Shake the sample with 10 ml. of dimethylformamide, add three to five drops of tetramethylammonium hydroxide, and note the color (Table I).

Test 4. Shake the sample with 10 ml. of dimethylformamide, add 5 ml. of 1,3-diaminopropane, and note the color (Table I).

Test 5. Shake the sample with 10 ml. of dimethylformamide, add 5 ml. of 1,3-diaminopropane, and dilute the sample with 15 ml. of water. Note the change in color (Table I).

Test 6. Mix the sample with 10 ml. of borax buffer and 1 ml. of 2% potassium ferricyanide solution, then add 0.5 ml. of 2% 4-aminoantipyrine solution. This is a test for Trithiadol and a purple color indicates a positive test.

Test 7. Shake the sample with 5 ml. of the dimethylaminobenzaldehyde reagent and then add 10 ml. of water. A distinct golden-yellow layer indicates a positive test.

Test 8. Suspend the sample in 10 ml. of water and add 0.5 ml. of starch iodine solution. With the coccidiostat Arzene, the purple starch-iodine color rapidly disappears.

Test 9. Suspend the sample in 5 ml. of 65% methanol and add 10 ml. of naphthalenediol reagent. A purple color indicates a positive test for Amprol coccidiostat.

In the spot tests for coccidiostats in finished feed, use approximately 2 to 3 grams or about one half of a teaspoon of feed in each test. Most finished feeds will contribute a significant amount of yellow color to the test solutions. Caution must be exercised in distinguishing the color produced by the coccidiostat from the color produced by the feed ingredients.

Distribution Test. To determine the distribution of Zoamix in finished feeds, the apparatus shown in Figure 1 is employed. This consists of a 9-cm. Petri dish containing a filter pad. This pad is saturated with a solution containing 1 ml. of 2*N* sodium methylate and 10 ml. of dimethylformamide. Spread approximately one half teaspoon of feed uniformly between two Whatman No. 1, 9-cm. filter papers. Gently press the filter papers and feed onto the saturated filter pad, using the bottom of the Petri dish to exert uniform pressure. The Zoamix appears as green spots on a yellowish background.

Results and Discussion

The tests described above were developed for rapid identification of the

Table. I Spot Tests for Determination of Coccidiostats in Premixes and in Finished Feeds

Spot Test No. and Reagents	Coccidiostat and Color of Spot Test											
	Amprol	Arzene	Borea	Glycamide	Megasul	Nicarbazin	Nitrofurazone	Polystat	Trithiadol	Unistat	Whitsyn-5	Zoamix
1. Dimethylformamide, 10 ml.	–	–	–	–	–	–	Y	Y	–	Y	–	–
2. Dimethylformamide, 10 ml., sodium methylate, 3–5 drops	–	–	–	–	R[a]	P	R	Y	–	R	–	G
3. Dimethylformamide, 10 ml., tetramethylammonium hydroxide, 3–5 drops	–	–	–	–	R-B	P[a]	P	Y	–	R	–	G
4. Dimethylformamide, 10 ml., 1,3-diaminopropane, 5 ml.	–	–	–	–	R[b]	Y	P[a]	Y	–	R	–	P
5. Dimethylformamide, 10 ml., 1,3-diaminopropane, 5 ml., water, 15 ml.	–	–	–	–	Y	Y	R	Y	–	Y	–	G
6. Borax buffer, 10 ml., 2% potassium ferricyanide solution, 1 ml., 2% 4-aminoantipyrine solution, 0.5 ml.	–	–	–	–	–	–	–	–	P	–	–	–
7. Dimethylaminobenzaldehyde reagent, 5 ml., water, 10 ml.	–	–	–	–	–	+	–	+	–	+	+	–
8. Water, 10 ml., starch-iodine solution, 0.5 ml.	–	+[c]	–	–	–	–	–	–	–	–	–	–
9. 65% methanol, 10 ml., naphthalenediol reagent, 5 ml.	P[c]	–	–	–	–	–	–	–	–	–	–	–

G = green. B = brown. P = purple. R = red. Y = yellow.

[a] R-B for feeds.
[b] B for feeds.
[c] Tests must be conducted with feed extracts.

major coccidiostats now on the market. They were tested with various commercial premixes containing the coccidiostats listed (Table I). If a positive test was obtained, it is indicated in the table either by a + sign or by a letter designating the color obtained. This table shows that some of the tests are specific for a given coccidiostat. Under these conditions, it is possible to identify the coccidiostat by a single color reaction. In other cases, it is necessary to use several tests to identify the compound positively.

Amprol can be identified by Test 9, which is a modification of the colorimetric procedure developed for the determination of this compound (*1*). The test appears to be specific for Amprol. The reagent is very unstable and must be prepared each time it is employed and can be used only for about 2 hours before it deteriorates. The color develops very slowly and at least 30 minutes are required before it can be ascertained if a positive reaction is obtained.

The procedure used to detect Arzene (Test 8) is based on the reaction of Arzene with iodine to form HI. With the loss of free iodine, the starch-iodine color disappears rapidly. This reaction is specific for Arzene and can therefore be used to distinguish it from the other coccidiostats.

No satisfactory color tests could be found for Borea or Glycamide; hence the compounds could not be identified in premixes or finished feeds.

For positive identification of Megasul, it is necessary to use a series of color tests. By employing Tests 1, 2, and 4 it is possible to distinguish Megasul from the coccidiostats. In cases where several coccidiostats give similar color reactions, it is desirable to use all the spot tests to ensure positive identification.

Nicarbazin can generally be identified by Test 2. In some cases, however, it is difficult to distinguish between a purple and a red color. For this reason, it is desirable to substantiate the identification of Nicarbazin by employing Tests 3, 4, 5, and 7.

Nitrofurazone can be detected by use of Tests 1, 4, and 5, with Tests 2, 3, and 7 being employed for additional proof of identity.

No specific test was found for Polystat, but this coccidiostat can be identified by the yellow color obtained in Tests 1 to 5 and 7.

Test 6 is a modification of the colorimetric procedure which has been described for the determination of Trithiadol (*4*). This test appears to be specific for the compound. The reagents employed for this test are not completely stable and must be prepared fresh each week.

Tests 1 to 5 are used to distinguish Unistat from the other coccidiostats. The colors obtained with Unistat are generally more intense than those obtained with the other coccidiostats.

Whitsyn-5 gives a positive test with the dimethylaminobenzaldehyde reaction (Test 7). By combining this test with Tests 1, 2, and 3 it is possible to distinguish Whitsyn-5 from other coccidiostats which give positive reactions with the dimethylaminobenzaldehyde reagent.

Zoamix can easily be detected with Tests 2 or 3. Both of these are specific for Zoamix and positive results are easily obtained (*2, 3*). In general, Test 2 is preferred because of the stability of the reagents.

When the tests described above were applied to finished feeds, the results obtained were similar to those obtained with premixes (Table I). In general, the tests were, however, not as distinct and in many cases the color produced was difficult to determine. The tests for Zoamix were all clear-cut, and distinct colors were obtained. No difficulty was encountered in identifying this coccidiostat in various lots of finished feed.

The test for Arzene was very poor with finished feeds. In many cases, a distinct reaction was not obtained. In general, the only satisfactory test that could be found for Arzene in finished feed consists of combusting the sample and then determining the arsenic. This type of procedure cannot be adapted to a field spot test.

The tests for Whitsyn-5, Nitrofurazone, and Megasul were distinct enough to obtain an indication that these compounds were present in the finished feed.

The tests for Unistat, Nicarbazin, and Polystat were satisfactory for routine operations and could be used as field tests. The color obtained with Unistat is redder than that obtained with Megasul, so it is possible to distinguish them, when the two compounds are run together.

The test for Amprol cannot be run directly on the finished feed. It is necessary to extract the compound with 65% methanol and filter the extract.

In general, the dimethylformamide–sodium methylate test was most satisfactory as a field procedure for detection of Zoamix in either premixes or finished feed. The reagents are very stable and can be used for long periods of time. The sodium methylate test was therefore adapted to the determination of the distribution of Zoamix in the finished feed. In the preparation of Zoamix, the chemical is coated on small particles of soybean meal. When the meal is mixed with the other feed ingredients, these particles are distributed throughout the feed. When a small quantity of the feed is spread uniformly between two pieces of filter paper and is pressed on a pad moist with dimethylformamide and sodium methylate, the compound appears as green areas. The color is transferred to the paper when pressure is exerted on the system, and visual indication of the distribution of Zoamix is obtained (Figure 2). This test is simple to operate and the equipment—i.e., Petri dishes, test tubes, etc.—can be used over and over again by simply washing and drying it between each test. This procedure has been employed to study the distribution of Zoamix in various lots of commercially prepared feed and has been very helpful in determining when the sample is well mixed.

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FEED ADDITIVES

Colorimetric Determination of Procaine Penicillin in Premixes

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A colorimetric method has been developed for determining procaine penicillin in premix materials. It is based upon the conversion by base of penicillins to penicilloic acids. The penicilloic acids yield penaldic acid derivatives and penicillamine when treated with mercuric ions. Penicillamine is oxidized to the disulfide by phosphomolybdic acid which is converted into molybdenum blue. The intensity of the blue color is proportional to the concentration of penicillin present. Laboratory prepared feed supplements showed average recoveries of 93.9 to 104.5% of the theoretical with coefficients of variation ranging from 3.4 to 6.1%. Commercial premixes showed average recoveries of 98.8 to 110.3% of tag guarantees with coefficients of variation ranging from 2.8 to 4.3%.

When used in animal nutrition, procaine penicillin is ordinarily available in supplement form, consisting of a uniform mixture of procaine penicillin in a suitable carrier. The use of premixes permits greater accuracy and makes for greater ease in handling the small amounts of procaine penicillin required.

At present, procaine penicillin in premixes is determined by a microbiological cylinder-plate procedure (*4*). This method yields results that range from 84.0 to 115.2% of procaine penicillin added, based on 95% confidence limits and takes 18 to 24 hours for completion. The chemical method of analysis proposed herein offers a more rapid and accurate means of determining procaine penicillin in premix materials. This method is based upon the Pan procedure (*3*) of simultaneously determining penicillins and penicilloic acids. It is well established that penicillins yield penicilloic acids when treated with base, Equation 1 (*2*). When treated with mercuric ions, the penicilloic acids or alkali-inactivated penicillins form penaldic acid derivatives and penicillamine, Equation 2 (*1*). Since penicillamine is a mercaptan, it is oxidized to the disulfide by phosphomolybdic acid which gives molybdenum blue (*1*). The intensity of the molybdenum blue is proportional to the concentration of penicillin present.

Reagents

Nelson's Color Reagent A. Dissolve 25 grams of ammonium molybdate in 450 ml. of distilled water, add 21 ml. of concentrated sulfuric acid, and mix. Add 3 grams of disodium arsenate, $Na_2HAsO_4 . 7H_2O$, dissolved in 25 ml. of water. Mix and place in an incubator at 37° C. for 24 to 48 hours.

Mercuric Chloride Solution B. Dissolve 0.7 gram of reagent grade mercuric chloride per liter of distilled water.

Mixed Color Reagent. Mix 3.5 ml. of reagent A, 4.0 ml. of reagent B, and 2.5 ml. of water. Prepare daily.

Procedure

Establishment of Calibration Curve. Weigh sufficient procaine penicillin standard into a 100-ml. volumetric flask so that the resulting solution will contain 100 μg. of procaine penicillin per ml. (1 mg. of procaine penicillin is equivalent to 0.6 mg. of penicillin G, master standard). Pipet aliquots of the standard solution (in the range of 100 to 1000 μg.) into 25-ml. volumetric flasks. Add 1 ml. of *1N* sodium hydroxide to each 25-ml. volumetric flask, shake, and allow to stand for at least 15 minutes. Acidify with 1 ml. of *2N* sulfuric acid and mix well. Add 1 ml. of the mixed color reagent and mix well; bring to volume and allow to stand for 30 minutes. Measure the intensity of the resulting blue color at 740 mμ with a Beckman DU spectrophotometer or any other suitable spectrophotometer using a rea-