tions are, however, slightly skewed with a deficit of low values, which also is apparent from the summarized data in Table XIV of (*3*).

The slightly nonsymmetrical frequency distributions observed in histograms *a* and *b* in Figure 5 are based on data underlying Table VI. An attempt is made to normalize the data by using logarithms, histograms *c* and *d*. The latter histograms are more symmetrical, which raises the question of whether normalization of primary data in this way would be a preferred route. Use of logarithms may, furthermore, diminish the differences between the R- and N-kernel populations to the extent that separation into two populations may even no longer be necessary in case of whole kernel assay, where differences between R- and N-kernels are moderate.

Table VIII illustrates these questions. The coefficients of variation reported here are based on: primary N-kernel data; primary (R+N)-kernel data; logarithms of N-kernel data; and logarithms of (R+N)-kernel data taken from the experiments in Tables V and VI. (The totals quoted in the bottom row of the table have no physical meaning but are introduced as a guide for comparison between the studied statistical procedures.) The primary N-kernel statistics show again minimum variation for $A/W^{1/3}$. Differences between statistics based on A, $A/W^{1/3}$, and $A/W^{2/3}$ are small, whereas A/W gives a higher variancy.

The practical conclusion of this is that although $A/W^{1/3}$ is the most correct choice, primary analytical data, A γ Hg/kernel, may be used as well in practice. Statistics based on A/W—e.g., p.p.m. figures—should, however, not be used. Logarithms give more symmetric distributions but do not reduce the coefficients of variation. The coefficient of variation reported in Table VIII for logarithims is the antilogarithm minus 1. (In this example an increase is actually observed due to the more sharply peaked distribution with a broad base indicated by the histograms *c* and *d* in Figure 5.) Statistics based on the combined (R + N)-kernel populations give much larger coefficients of variation, and consequently the separation into two populations seems justified also for populations based on whole kernel assay.

Conclusions

Distribution statistics based on whole kernel assay differs slightly from statistics based on the previous beta counting technique, but the same approach involving two populations, R- and N-kernels, should be used. Coefficients of variation for the N-kernel population, "spreading error," are about the same with the two methods, whereas R-kernel statistics exhibit larger differences with higher readings for beta counting R-kernels. Only a weak correlation exists between fungicide content and kernel size. The fungicide content is roughly proportional to the cube root of the kernel weight and not to the two thirds power as might be expected. In practice primary analytical results—e.g., γ Hg/kernel—may be used as well as data corrected for kernel size, in view of the weak correlation between size and mercurial uptake.

The results of the earlier radioactive distribution studies were confirmed by the present studies. Further proof is presented that the mercurial fungicide and the dye are distributed in different ways. The impression of dye uniformity is governed very much by the amount of dye used, which has no connection with the mercurial distribution. Whole kernel assay studies could also demonstrate that the small liquid dosages characteristic of the Panogen process can be reduced further, well below present volume rates, with no adverse effects on the mercurial distribution. This indicates a considerable margin of safety with the method. Laboratory treatment and large scale commercial treatment give similar distribution patterns because of the vapor action of these mercurial fungicides.

The spreading errors for the commercially treated seeds are of the order of 0.2 to 0.3, which indicates a sufficiently uniform distribution to produce but a negligible drop in disinfection efficiency (*4*). Estimates of the spreading error are furthermore conservative, as this statistics is actually mainly governed by kernels with larger amounts of mercurial. The distribution curves have no "tail" with deficient kernels.

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FEED ADDITIVES

Determination of 3,5 Dinitro-*o*-toluamide (Zoalene) in Feed Concentrates

GRANT N. SMITH

The Dow Chemical Co., Midland, Mich.

Premix concentrates containing 25% 3,5-dinitro-*o*-toluamide (zoalene) with soybean meal are being used in the feed to aid in the prevention of cecal and intestinal coccidiosis in chickens. In the manufacture of such a premix concentrate it is essential to have a rapid easy-to-handle analytical method, with a high degree of accuracy, reproducibility, and specificity, for quality control.

The method which has been developed, using the colorimetric procedure previously described (*1*), is based on the extraction of the 3,5-dinitro-*o*-toluamide from the feed concentrate with dimethylformamide. This solution is then mixed with a solution of methylamine. The reaction results in the formation of a purple-colored complex, the absorbance of which can be read at wave length 550 mμ in a Beckman spectrophotometer. The intensity of the color is proportional to the concentra-

A rapid analytical method is described for the routine determination of 3,5-dinitro-o-toluamide in premix concentrates containing 25% active ingredient. It is based on the reaction of methylamine with dimethylformamide and 3,5-dinitro-o-toluamide. This reaction results in the formation of a purple complex, the intensity of which at 550 mμ is proportional to the concentration of the 3,5-dinitro-o-toluamide present in the solution.

tion of 3,5-dinitro-*o*-toluamide in the solution.

Analytical Procedure

A 1-gram sample of premix concentrate is suspended in 200 ml. of dimethylformamide in a 600-ml. beaker. The suspension is warmed slightly on a hot plate and stirred continuously for 5 minutes to ensure complete solution of the 3,5-dinitro-*o*-toluamide. The warm solution is transferred to a 1-liter volumetric flask using dimethylformamide to wash the beaker and transfer the feed residue. The solution is cooled to 20° C. and diluted to volume with dimethylformamide. The solution is thoroughly mixed, and the insoluble feed particles are allowed to settle. A 10-ml. aliquot of the extract is then transferred to a 100-ml. volumetric flask, and the sample is diluted to 100 ml. with dimethylformamide.

After thorough mixing, a 10-ml. aliquot of the diluted extract is transferred to a 25-ml. volumetric flask and the sample is diluted to volume with a 40% aqueous solution of methylamine. The methylamine solution is previously cooled and maintained at 3° C. After thorough mixing, the absorbance of the sample is determined in a Beckman spectrophotometer at a wave length of 550 mμ. The sample should be read exactly 3 minutes after the methylamine solution is added to develop the color.

A reference standard and feed blank are run with each set of determinations. A reference standard solution is prepared by dissolving 250 mg. of a reference standard of 3,5-dinitro-*o*-toluamide in 1 liter of dimethylformamide previously adjusted to 20° C. A 10-ml. aliquot of the reference solution is transferred to a 100-ml. volumetric flask and diluted to volume with dimethylformamide (20° C.). After thorough mixing, a 10-ml. aliquot of the diluted reference standard solution is transferred to a 25-ml. volumetric flask and diluted to volume with the methylamine solution (3° C.). The absorbance of the solution is then determined in a spectrophotometer at a wave length of 550 mμ. The solution should be read exactly 3 minutes after the methylamine solution is added to develop the color.

A feed blank should be prepared by extracting a 750-mg. sample of the soybean meal present in the premix concentrate. The feed blank sample is handled in the same manner as described above for the determination of 3,5-dinitro-*o*-toluamide in the sample of premix concentrate.

The absorbances of the feed blank solution and the standard reference solution should be determined at the same time the absorbances of the various solutions of the premix concentrate are being determined.

Calculation of Percentage of 3,5-Dinitro-o-toluamide in Premix Concentrate

In the procedure described above, the percentage of 3,5-dinitro-*o*-toluamide in the premix concentrate is determined by comparison with a standard reference sample.

Manufacturing specifications for the premix concentrate prescribe that it contain 25% of 3,5-dinitro-*o*-toluamide. Therefore, a 1-gram sample of the concentrate should contain 250 mg. of the drug. In the procedure described above, the 3,5-dinitro-*o*-toluamide in a 1-gram sample of the concentrate is extracted from the soybean meal and carried through three dilution steps to give a concentration of approximately 10 γ per ml. in the final solution, the absorbance of which is read in the spectrophotometer. The reference standard and the feed blank are handled in a similar manner.

The percentage of 3,5-dinitro-*o*-toluamide in the premix concentrate samples can therefore be calculated from the following equation:

$$\% \text{ 3,5-dinitro-}o\text{-toluamide} = \frac{A - B}{C} \times 25$$

A = absorbance of the premix concentrate sample
B = absorbance of the feed blank
C = absorbance of the reference sample

Discussion and Experimental Results

The three general types of reactions proposed for the determination of aryl dinitro compounds described previously (*1*) were tested with 3,5-dinitro-*o*-toluamide in premix concentrates. The reduction reaction gave erratic results because of the difficulties in controlling the reduction of the compound. Apparently the amide group was destroyed at the same time that the nitro groups were being reduced. The free carboxyl group could then react with the amino groups as they were formed to produce a compound which could no longer be diazotized and coupled. Furthermore, most of the reduction reactions produced a mixture of mono- and diamino toluamides and toluic acids.

Treatment of 3,5-dinitro-*o*-toluamide with acetone and alkali resulted in the formation of a green complex which exhibited an absorption peak at 640 mμ. The color was very intense but extremely transient. Because of the evanescent properties of this complex it was impossible to measure the intensity of the solution. Similar reactions could be obtained with ethyl alcohol, methyl ethyl ketone, acetonitrile, pyridine, and dimethylformamide. The dimethylformamide gave the most stable complex, but here again the color was not stable enough to be used as a colorimetric procedure.

The potassium cyanide reaction gave a yellow complex which exhibited an absorption peak at 360 mμ. The color produced was very stable and not greatly influenced by environmental conditions. Two disadvantages were found with this reaction. Many of the premix concentrates contained plant pigments which had absorption peaks in the same regions of the spectrum as did the cyanide complex (300 to 400 mμ). In addition, the test was not specific for the dinitro-*o*-toluamide but would give a positive test with all the closely related compounds of the dinitro-*o*-toluamide series.

The reaction of 3,5-dinitro-*o*-toluamide with methylamine in the presence of dimethylformamide to form an intense purple complex previously described (*1*) had three distinct advantages as the basis for a colorimetric procedure for the determination of 3,5-dinitro-*o*-toluamide in premixes. The reaction was specific for the 3,5-dinitro-*o*-toluamide. None of the possible decomposition products of the drug would give a positive reaction. The complex had a very intense color which permitted the detection of microgram quantities of the drug. The absorption peak exhibited by the complex was in a portion of the visible spectrum in which plant pigments did not exhibit absorption bands.

With a large concentration of 3,5-dinitro-*o*-toluamide in the premix (25%) and a very sensitive colorimetric method, it was possible to dilute the premix extract to such an extent (the equivalent

of 1 gram of premix in 25 liters of solution) that the pigments and other components of the premix would not contribute any significant amount of color to the final solution.

It was possible to analyze the premix for 3,5-dinitro-*o*-toluamide by simply extracting the drug from the feed with dimethylformamide and reacting a diluted aliquot of the extract with methylamine or 1,3-propanediamine.

To determine if the proposed method would be reproducible, three premixes were prepared and analyzed for the drug. Each sample was analyzed on three successive days using different reagents each time to be sure that the same results would be obtained under various laboratory conditions. The data in Table I indicated that reproducible results can be obtained with this method. Preliminary experience with the method indicated that any variations that might be observed could generally be ascribed to dilution error or lack of precision in the development of the colored complex and measurement of its absorbance. The factors influencing color formation and the stability of the complex have been described in great detail in a previous paper of this series (*1*) and will not be elaborated on here. To obtain reproducible results, it is absolutely essential that the color development steps be carried out precisely as described.

A standard curve was prepared using the procedure described and, when plotted, gave a straight line having a slope of 0.056 ± 0.002 absorbance unit per microgram of 3,5-dinitro-*o*-toluamide per milliliter of solution. Because of the various factors, such as temperature, etc., which might influence this determination, it is advisable to run a reference standard with each set of determinations and to calculate the concentration of the drug in the unknown samples of premix in terms of the reference standard rather than using a standard curve.

The recovery of 3,5-dinitro-*o*-toluamide from various premix concentrates was determined by analyzing a series of feed concentrates of known composition. This was done by mixing from 200 to 350 mg. of 3,5-dinitro-*o*-toluamide with sufficient soybean meal to give 1-gram samples. The samples were handled in such a manner that there could not be any loss of the drug in mixing or transferring the samples before they were analyzed. The absorbances of the samples were compared with those of the premix feed blank and the reference standard (Table II). The feed blank generally was in the range of 0.005 to 0.009 absorbance units when compared with water. When compared with the reagent blank, the absorbance of the feed blank was insignificant and could be ignored.

Table I. Reproducibility of Analytical Procedure for Assay of 3,5-Dinitro-o-toluamide in Premix Concentrate

	3,5-Dinitro-o-Toluamide, %			
		Found		
Sample	Added to sample	1st analysis	2nd analysis	3rd analysis
1	25.2	25.2	25.0	25.0
2	24.0	24.0	24.1	24.0
3	26.2	26.2	26.0	26.1

Table II. Recovery of 3,5-Dinitro-o-toluamide from Premix Concentrate

Sample	Concn. of 3,5-Dinitro-o-toluamide in Premix, %	3,5-Dinitro-o-toluamide per Gram Sample of Premix, Mg.		Recovery, %
		Added	Found	
1	21.5	215.0	213.0	99.1
2	22.4	224.5	217.5	96.9
3	23.7	237.5	219.8	92.5
4	25.3	253.2	243.2	96.0
5	25.6	256.0	256.8	100.3
6	26.1	261.0	260.8	99.9
7	26.5	264.8	250.5	94.6
8	26.7	267.5	252.8	94.5
9	27.7	277.0	266.8	96.3
10	28.3	282.8	279.0	98.6
11	28.5	285.0	282.8	99.2
12	29.1	290.8	294.0	101.1
13	30.4	304.0	295.0	97.0
14	31.5	315.0	304.0	96.5
15	32.9	329.0	316.0	96.0
				Av. 97.2 ± 2.6

The results in Table II indicate an average recovery of 97.2 ± 2.6%, which is satisfactory for the determination of 3,5-dinitro-*o*-toluamide in premix concentrates.

Table III shows the results obtained by analyzing several lots of commercially prepared premix concentrates.

During this investigation, it was observed that 1,3-propanediamine could be substituted for the methylamine used in this reaction and the same results would be obtained. The diamine gave a more stable complex and more intense color. The use of the diamine, however, has been limited because of its availability and cost. Recently it has been indicated that the 1,3-propanediamine will be produced commercially in the near future by the Union Carbide Chemicals Co. at a price which will permit its use in routine analysis (*2*). When this material becomes available it would be desirable to use the 1,3-propanediamine in place of the methylamine and to measure the absorbance of the colored complex at 560 mμ.

Table III. Recovery of 3,5-Dinitro-o-toluamide from Commercially Prepared Samples of Premix Concentrate

	3,5-Dinitro-o-toluamide %	
Lot	Theoretical	Found
1	25.0	25.2
2	25.0	25.0
3	25.0	24.6
4	25.0	25.3
5	25.0	24.8

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