(*18*). Analysis. Calculated for $C_{20}H_{36}O_{12}$: C, 51.27; H, 7.76. Found: C, 51.86; H, 7.77.

**Hydrolysis of Methylated Aldobiouronic Acid and Identification of 2,3,4-Tri-*O*-methyl-D-glucuronic Aidc and 3,4,6-Tri-*O*-methyl-D-mannose.** Fully methylated aldobiouronic acid was heated (sealed tube) with 2% methanolic hydrogen chloride (2 ml.) for 9 hours at 110° C. Neutralization, followed by filtration and evaporation, gave a sirupy product which was treated with methanolic ammonia in the usual way. Removal of solvent gave a crystalline residue, m.p. 223° C. after recrystallization from an ethanol-ether mixture. This appeared to be the amide of the unhydrolyzed, methylated aldobiouronic acid, since the melting point is higher than either that of the $\alpha$- or $\beta$- anomers of methyl 2,3,4-tri-*O*-methyl-D-glucosiduronamide (*19*). The product (3 mg.) was therefore hydrolyzed by heating (sealed tube) for 18 hours at 100° C. with 2*N* sulfuric acid (0.7 ml.). After neutralization ($BaCO_3$), the filtered solution was passed first through a cation (Amberlite IR 120, H form) resin and then through an anion (Duolite $A_4$, OH form) resin. The neutral eluate gave a sirup upon evaporation and after purification by extraction with ether, crystalline 3,4,6-tri-*O*-methyl-D-mannose (1.5 mg.) was obtained [$R_{\text{tetra-O-methyl-D-glucose}}$ 0.86 (solvent A)], m.p. and mixed m.p. 104° C., $[\alpha]_D^{25}$ + 37° in methanol (concentration, 0.4 gram per 100 ml. of solution). The literature (*1*) gives m.p. 102° C., $[\alpha]_D^{22}$ + 36 (methanol). Elution of the anion resin with 0.1*N* sodium hydroxide followed by passage of the eluate through the cation resin gave a solution containing 2,3,4-tri-*O*-methyl-D-glucuronic acid. Removal of solvent followed by treatment first with methanolic hydrogen chloride and then with methanolic ammonia (*18*) yielded crystalline methyl 2,3,4-tri-*O*-methyl-D-glucosiduronamide, m.p. and mixed m.p. 183° C.

**Examination of Fraction 4.** Hydrolysis of this fraction in water with the cation exchange resin for 20 hours at 100° C. (as described for fraction 3) gave components, the chromatographic mobilities (solvent C) of which corresponded to D-glucurono-(6 → 3)-lactone, D-glucuronic acid, D-mannose, 2-*O*-($\beta$-D-glucopyranosyluronic acid)-D-mannose, and the original component. It appears therefore that the fraction is a trisaccharide composed of one mole of D-glucuronic acid and two moles of D-mannose. The mode of attachment between the two D-mannose units remains to be determined.

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*Received for review March 1, 1961. Accepted August 11, 1961. Paper No. 4538 Scientific Journal Series, Minnesota Agricultural Experiment Station.*


**FEED ADDITIVES**

# Nitrofurazone Determination in Poultry Feed by Phenylhydrazine or Alkali Reaction


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NITROFURAZONE, 5 - nitro - 2-furaldehyde semicarbazone (NFZ), is added to poultry feeds in small concentrations as a prophylactic against coccidiosis. For its determination the colorimetric method of Buzard, Ells, and Paul (*3*), which involves conversion to 5-nitro-2-furaldehyde phenylhydrazone, is commonly employed. The main difficulty is in the purification of feed extracts. Other feed compounds also give color reactions with the phenylhydrazine reagent, resulting in more or less extinction at the 440-m$\mu$ wave length, which is characteristic of the 5-nitro-2-furaldehyde phenylhydrazone. Nitrofurazone is insoluble in heptane-$CCl_4$ and hexane. Therefore the authors propose extraction using these solvents. Beckman (*1*) has described an improved method which allows at the same time separation between NFZ and furazolidone. His procedure for determining NFZ by chromatography with an $Al_2O_3$ column was modified by Brüggemann and Bronsch (*2*); these authors determined NFZ either by phenylhydrazine or preferably by reaction with alkali. Our experiments are concerned with critical comparison of both methods applied to commercial feed mixtures and to laboratory preparations as well. They show that column chromatography and reaction of nitrofurazone with alkali are superior to the phenylhydrazine method in selectivity and accuracy.

### Methods

**Method 1.** Modification of the Buzard, Ells, and Paul Method for Nitrofurazone Determination (*3*).

A. Weigh 5 grams of feed and fill into a coarse fritted-disk funnel. Apply suction and extract successively with the following solvents previously warmed to 50° to 60° C.:

1. Heptane, 30 ml.
2. Heptane-$CCl_4$ (1:1), 15 ml.
3. $CCl_4$, 25 ml.
4. Heptane-$CCl_4$ (1:1), 15 ml.
5. Hexane, 15 ml.

Dry by use of full vacuum and transfer the sample to a 100-ml. Erlenmeyer flask. Add exactly 50 ml. of dimethylformamide (DMF). Shake for 30 minutes in a water bath heated to 70° C. protected from light and filter through a paper filter (Schleicher & Schüll No. 588). Cool and add 25 ml. of water to 25 ml. of the DMF extract. Return to

**Nitrofurazone is a widely used additive in chicken feed, with prophylactic value against coccidiosis. Its determination as a 5-nitro-2-furaldehyde phenylhydrazone in pre-extracted feed is unsatisfactory because of interfering substances. The method of Beckman, which includes adsorption of NFZ on an $Al_2O_3$ column after pre-extraction of the feed extract, was modified. NFZ is measurable in the eluate by reaction with alkali. The recovery has been 99.5 ± 0.83% with complete elimination of interfering substances from the feed. The method described here is the only one used in West Germany for NFZ determination in feed and offers optimal results in the presence of alfalfa or fish meal.**

room temperature. Pipet 5-ml. portions into each of two test tubes and add to one 5 to 10 mg. of sodium dithionite ($Na_2S_2O_4$). The nitrofurazone is reduced to detect interfering chromogens in the blank. Add 5 ml. of a freshly prepared solution of phenylhydrazine to both test tubes (dissolve 0.5 gram of phenylhydrazine hydrochloride in 67 ml. of $H_2O$, and add 50 ml. of 5*N* HCl to 50 ml. of this solution). Insert both test tubes for 25 minutes into a 70° C. water bath to develop the color of the 5-nitro-2-furaldehyde phenylhydrazone. Cool and extract with exactly 10 ml. of toluene. Centrifuge and read the extinction of the toluene layer in a spectrophotometer at 440 mμ or in a suitable electric colorimeter (Zeiss & Co., Elko II, Filter S 45, $d$ = 1.0 cm.). Use the reduced sample as blank and calculate the nitrofurazone content from a standard curve.

B. This method gives accurate results ony if a control sample of feed without nitrofurazone is available. This feed blank allows determination and calculation of interfering substances with uncontrolled color reactions. If no unmedicated feed is available, pre-extraction with 60 ml. of warm hexane and $Al_2O_3$ column chromatography of the feed extract (diluted with equal parts of water) have been recommended. Pelleted feeds are extracted with DMF in a 90° C. water bath, powdered feeds only at room temperature for 15 minutes.

**Method 2.** Nitrofurazone Determination by Beckman's Method (*1*), Modified by Brüggemann and Bronsch.

Pre-extract 5 grams of feed as in Method 1. Transfer to an Erlenmeyer flask and add exactly 50 ml. of DMF. Shake mechanically for 30 minutes in a warm water bath (70° C.). Filter the suspension through a paper filter (Schleicher & Schüll No. 588). Meanwhile fill fritted-glass columns for chromatography (diameter 27 mm. or less) with 50 grams of $Al_2O_3$ (Brockman for chromatography, Merck), which has been heated to 750° C. for 12 hours and sprayed after cooling with 5% (w./w.) $H_2O$ for deactivation. Allow to stand 4 hours at room temperature before use. ($Al_2O_3$ thus prepared is suitable for several days.) Then add 10 ml. of pure DMF to the filled column, followed by 10 ml. of the filtered DMF extract. Rinse another 20 ml. of pure DMF into the column without applying suction and without running the $Al_2O_3$ dry.

**Table I. Examination of Feed Extracts without NFZ by Phenylhydrazine (Method 1A) and Alkali (Method 2) Reaction**

| | Simulated NFZ Content, Mg. % | | | |
|---|---|---|---|---|
| | Method 1A without pre-extraction | Method 1B with pre-extraction | Method 2 | |
| | | | Without pre-extraction | With pre-extraction |
| Wheat | 1.56 | 0.96 | 0.00 | 0.00 |
| Barley | 0.32 | 0.32 | 0.00 | 0.00 |
| Oats | 0.40 | 0.32 | 0.00 | 0.00 |
| Corn | 1.06 | 0.90 | 1.16 | 0.50 |
| Alfalfa meal | 18.16 | 16.60 | 7.95 | 2.45 |
| Fish meal | 3.60 | 1.62 | 1.89 | 1.00 |
| Feed A | 1.56 | 1.57 | 2.70 | 0.00 |
| Feed B | 2.52 | 2.04 | 1.67 | 0.00 |
| Feed C | 2.12 | 2.00 | 1.46 | 0.00 |
| Feed D | 3.28 | 2.48 | 0.25 | 0.00 |
| Feed E | 2.32 | 2.24 | 0.63 | 0.00 |
| Feed F | 2.91 | 2.64 | 0.88 | 0.00 |
| Feed G | 2.04 | 1.80 | 0.50 | 0.00 |

Elute with 80% (v./v.) ethanol-$H_2O$ with light suction if necessary. Discard about 25 ml. of the first green-yellow DMF fraction. Transfer the following 50-ml. portion of fraction to a volumetric flask. Mix and pipet 6 ml. of this eluate into each of two 25-ml. volumetric flasks. To one flask add 2 ml. of 1*N* KOH (contains 1 equivalent of KOH per liter dissolved in a mixture of equal volumes of ethanol and $H_2O$) and make up to volume with 80% (v./v.) ethanol. Read within 60 seconds against pure ethanol as blank in a spectrophotometer at 470 mμ in 5-cm. cuvettes or in the colorimeter (Zeiss, Elko II, Filter S 47, $d$ = 5 cm.). Fill the second 25-ml. flask to volume with 80% (v./v.) ethanol without addition of alkali and read extinction of the blank sample against pure ethanol. Calculate the NFZ concentration from a standard curve, after blank correction.

The recovery of NFZ from well mixed and finely screened samples is 99.5 ± 0.83%. For all routine analysis of commercial feeds the reproducibility is within ±5%. The method is suitable for samples containing between 0.007 and 0.02% NFZ, or 0.005% NFZ by changing the reference standard curve. The reaction of NFZ with alkali is not specific. Similar light absorption can be produced by reaction of molecules with equal π-electron systems and single electron pairs with alkali. Such substances could not be detected in feeds. Interfering light absorption of unknown origin is produced mainly by alfalfa meal and fish meal.

### Experimental Feeds

Different feed mixtures have been prepared in the laboratory for comparison (A to C). Others were commercially manufactured (D to G) without NFZ. Percentage composition for feed mixtures is shown on page 110.

### Results and Discussion

The commercial and experimental feed mixtures without NFZ were analyzed by the above methods. Table I shows that alfalfa meal and fish meal give considerable color reactions. These additional extinctions simulate a higher amount of NFZ at the peak of the NFZ absorption. When the phenylhydrazine method is applied, interference is larger than when the reaction is carried out with alkali. The influence of the pre-extraction with heptane–carbon tetrachloride and hexane on the extinction of feed extracts without NFZ is not quite specific: Not only substances which increase the blank are removed, but also others which give additional color reactions. Ingredients which are soluble in DMF or alcohol and react like NFZ cannot be removed completely from most feeds.

To obtain better information about the influence of these effects, we prepared and analyzed feed samples without coccidiostatics prepared according to current diets. Table I shows some examples and confirms that when the

| | | | |
|---|---|---|---|
| | Mixture A | | |
| Barley | 25 | Minerals | 5 |
| Oats | 20 | Alfalfa meal | 5 |
| Wheat bran | 20 | Cornstarch | 4 |
| Corn | 20 | Vitamin concentrate | 1 |
| | Mixture B | | |
| Barley | 25 | Minerals | 5 |
| Oats | 20 | Vitamin concentrate | 1 |
| Wheat bran | 20 | Alfalfa meal | 5 |
| Fish meal | 20 | Cornstarch | 4 |
| | Mixture C | | |
| Rye | 20 | Corn gluten | 10 |
| Barley | 12 | Wheat bran | 11.79 |
| Corn | 6 | Minerals | 2 |
| Milo | 6 | Terramycin TM 5 | 0.2 |
| Fish meal | 17 | Endobion vitamin B complex | 0.01 |
| Soybean meal | 15 | absorbed in wheat bran | |
| | Mixture D | | |
| Wheat | 20 | Fish solubles | 1 |
| Corn | 20 | Whey powder | 1 |
| Rye | 10 | $CaCO_3$ | 6 |
| Tapioca meal | 6 | $CaHPO_4$ | 3.6 |
| Soybean meal | 20 | Iodized salt | 0.4 |
| Alfalfa meal | 3 | Vitamin mixture | 1 |
| Wheat middlings | 8 | | |
| | Mixture E | | |
| Feed cereals (10% wheat, 10% barley, 10% oats) | 30 | Tapioca meal | 7 |
| | | Cornstarch | 6 |
| Wheat middlings | 20 | Wheat germ | 5 |
| Protein concentrate (45% fish meal, 22.5% soybean meal, 20% meat meal, 10% yeast, 2.5% vitamin mixture) | 20 | Alfalfa meal | 5 |
| | | Malt sprouts | 5 |
| | | Minerals | 2 |
| | Mixture F | | |
| Feed cereals (20% corn, 15% oats, 10% wheat, 5% barley) | 50 | Wheat bran | 9 |
| | | Malt sprouts | 5 |
| Protein concentrate (40% fish meal, 37% soybean meal, 8% bone meal, 5% meat meal, 5% whey powder, 5% peanut meal) | 23 | Alfalfa meal | 5 |
| | | Vitamin mixture | 4 |
| | | Wheat germ | 2 |
| | | Minerals | 2 |
| | Mixture G | | |
| Wheat bran | 20 | Wheat | 15 |
| Protein concentrate (30% fish meal, 15% dried yeast, 10% meat meal, 10% dried skim milk, 10% soybean meal, 5% peanut meal, 7.5% alfalfa meal, 10% bone meal, 2.5% vitamin mixture) | 20 | Wheat meal | 10 |
| | | Oats | 10 |
| | | Tapioca meal | 10 |
| | | Corn | 8 |
| | | Wheat germ | 5 |
| | | Minerals | 2 |

phenylhydrazine method is used the feed without NFZ gives erratic blanks in concentrations between 1.47 and 2.64 mg.% of NFZ. The results of 54 analyzed samples of unmedicated poultry feeds show erratic values averaging 2.1 mg.% of NFZ.

In Method 1B we extracted at room temperature and got smaller blanks, but noticed that this procedure is still not quantitative for each kind of sample (*2*). An extraction at 90° C.—as recommended for pelleted feeds—gives extinctions for feeds without NFZ simulating an average of 1.3 mg.% of NFZ. Even the warm extraction cannot meet the conditions for a reliable control test, especially for NFZ, as the difference between the effective and harmful dose is relatively small.

On the other hand, obviously better results are obtained by intermediate column chromatography with $Al_2O_3$. Without pre-extraction some blank extinction values may occur with unmedicated feeds (Table I, feed mixture A), but after washing with heptane, carbon tetrachloride, and hexane interferences are eliminated completely.

NFZ may be determined colorimetrically by using phenylhydrazine after column chromatography, but the alkali reaction requires less time and reagents and the determination of the coccidiostat is quantitative in every case. Comparing experiments revealed that only 92% of the NFZ could be recovered after column chromatography and subsequent conversion to the phenylhydrazone compound.

**Table II. Detection of NFZ in Commercial Feeds**

(10 mg. % added, blank subtracted)

| | | Method 2 | |
|---|---|---|---|
| Feed | Method 1A with Pre-extraction | Without pre-extraction | With pre-extraction |
| D | 10.96 | 10.20 | 10.00 |
| | 8.48 | 9.95 | 10.00 |
| E | 11.04 | 10.52 | 9.86 |
| | 8.80 | 9.89 | 9.86 |
| F | 12.00 | 10.80 | 10.04 |
| | 9.36 | 9.92 | 10.04 |
| G | 10.76 | 10.63 | 9.91 |
| | 8.96 | 10.13 | 9.91 |

This effect and the results of our experiments on unmedicated feeds were investigated by addition of 10 mg.% of NFZ to the feed mixture and subsequent analysis. Examples of such experimental addition of pure NFZ are given in Table II. Following Method 1A interferences are found even after pre-extraction, simulating the presence of more or less NFZ. After the blank extinctions of unmedicated feeds are subtracted, the total added and carefully homogenized NFZ is not recovered.

The same result is confirmed by Method 1 with an extraction temperature of 90° C. Only 91% of the added NFZ is found as an average of all experiments, if the blank extinction of unmedicated feed is subtracted.

Considering these results, the phenylhydrazine reaction does not seem to be satisfactory for the determination of NFZ under present conditions. The influence of interfering substances on the extinctions may in several cases have compensated erratically for the losses of NFZ caused by the method of analysis. The nonuniformity of both factors has been misleading for a long time. Possibly a remarkable amount of commercial feed mixtures has been misjudged in the past. Method 2 may be more satisfactory without applying pre-extraction but the blank extinctions of unmedicated feeds should be known; in fact, unmedicated samples are not normally available. The only possibility left is pre-extraction with heptane, carbon tetrachloride, and hexane (Table II). If the medicated samples do not contain extreme amounts of alfalfa meal and fish meal, 99.5 ± 0.83% of the NFZ can be recovered, as shown by our analytical series.

As the influence of interfering substances in commercial feeds and their possible overlapping is unknown, an extreme deviation of ±5% has been roughly estimated for the method.

If NFZ premixtures (2% NFZ) in wheat middlings are to be analyzed purification by column chromatog-

raphy and alkali reaction may be applied, omitting the special pre-extraction. In premixes the NFZ will be recovered with an accuracy of 99.8 ± 0.42%.


### Summary

In the determination of NFZ in feed the main problem is purification of the extracts. Suitable conditions for color reactions are obtained only by pre-extraction using 60° C. heptane, carbon tetrachloride, and hexane, followed by absorption of the NFZ on $Al_2O_3$ chromatography columns and elution with 80% (v./v.) ethanol. If this method is applied, the determination of blanks by analysis of unmedicated feed mixtures is not necessary.

For colorimetric determination the reaction with alkali is preferable. The method allows detection of 99.5 ± 0.83% of NFZ. Reaction with phenylhydrazine is not strictly quantitative in samples where the overlapping error due to unknown interfering substances cannot be exactly controlled.


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*Received for review March 6, 1961. Accepted June 22, 1961.*


**FOOD ADDITIVE SAFETY**

# Metabolism of Glyceryl Lactate-$C^{14}$ Palmitate by Rats


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**Glyceryl lactate palmitate has been found useful in baking, and therefore studies to prove its safety were conducted. The ester was readily hydrolyzed in vitro by a lipolytic enzyme to its components: lactic acid, palmitic acid, and glycerol. When administered orally to rats in different vehicles, the lactate moiety of glyceryl lactate (2,3-$C^{14}$) palmitate was metabolized as rapidly as and in a manner similar to 2,3-$C^{14}$ lactic acid, which was largely absorbed, readily stored and oxidized by the liver, and randomly distributed throughout the body without high localization in any organ when equilibrium was attained. This ester was easily hydrolyzed to compounds which are natural and accepted as safe, and since the lactate moiety of the ester was metabolized like free lactic acid, this glyceryl lactate palmitate is believed safe for use in shortening.**


GLYCERYL LACTATE palmitate has been useful in shortening for cake mixes and other bakery goods.

Lactic acid, palmitic acid, glycerol, and glyceryl monopalmitate are accepted as safe for use in foods. If glyceryl lactate palmitate hydrolyzed readily to these materials and the fate of the lactate moiety of the ester was metabolically similar to that of free lactic acid, its use in shortening would be safe. To establish its safety, the hydrolysis of the nonlabeled ester by a lipolytic enzyme was studied.

Two metabolic balance studies in fasted rats were conducted with both 2,3-$C^{14}$ lactic acid (L.*A) in the presence of glyceryl palmitate (GP) and glyceryl lactate palmitate (GL*P) containing the comparable labeled lactic acid. In one study (series 1), the labeled ester was intubated as an emulsion in water–propylene glycol (PG) containing carboxymethylcellulose; the labeled lactic acid was given in water, which was preceded immediately by warmed glyceryl palmitate, administered in a water–propylene glycol suspension containing carboxymethylcellulose. During the analytical determination, oxidation was performed by wet combustion and activity of a Hyamine solution was counted by liquid scintillation. In the other metabolic study (series 2), the tagged ester or the labeled lactic acid and glyceryl palmitate were given as an aqueous emulsion that contained sodium caseinate and sucrose. In series 2a, one rat was given the ester in the vehicle employed for series 1. For analyses, oxidation was by dry combustion and activity of barium carbonate was counted by a flow gas counter.

Although the quantity of lactic acid or ester given in both series was of the same order of magnitude, the concentration of activity was much greater in the first series (Table I).

### Methods

**Enzymatic Study.** To 200 mg. of nonlabeled glyceryl lactate palmitate and 50 mg. of sodium taurocholate were added 200 ml. of water. This mixture was heated to 65° C. and shaken vigorously to emulsify the GLP. After the emulsion had been cooled (with shaking) to about 40° C. 2.0 ml. of a buffer solution (66 ml. of 1*N* $NH_4OH$ and 134 ml. of 1*N* $NH_4Cl$) and 50 mg. of a lipase enzyme (a whole hog pancreas preparation, Viokase, Viobin Corp., Monticello, Ill.) were added. The final mixture was vigorously shaken for about 1 minute and then placed immediately in a shaking water bath at 37° C. Upon completion of incubation (1 to 22 hours), the flask was removed from the bath and diluted with 100 ml. of 3A alcohol (95.2% ethyl alcohol; 5 gallons of commercially pure methanol added to every 100 gallons of ethyl alcohol).

The hydrolyzed mixture was made acidic and extracted with 25 ml. of *n*-hexane. The hexane phase was washed three times with 25 ml. of water. Each water phase was extracted with 25 ml. of *n*-hexane. All of the hexane phases were combined and evaporated to dryness over a steam bath. To determine free palmitic acid, the residue was dissolved in alcohol, and titrated with dilute alcoholic KOH, using phenolphthalein as an indicator. (Good recovery of a known amount of palmitic acid from a synthetic mixture approximating that of the enzyme hydrolyzed mixture had been shown previously.)

The free lactic acid was determined on the above combined three water phases. The lactic acid was oxidized to acetaldehyde with a potassium permanganate–manganous sulfate system.

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