Specific Determination of Nitrofurans in Feeds Containing Interfering Pigments

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A precise method for the quantitative estimation of nitrofurans in highly pigmented feeds (standard deviation less than 5%, accuracy ±3%) is based on the conversion of nitrofurans into 5-nitrofurfuraldehyde phenylhydrazone, followed by toluene extraction and then double chromatographic purification through superposed columns of silicic acid, then alumina. The blue coloration developed by potassium hydroxide in dimethylformamide is measured at 630 mμ. A new method for the specific estimation of nitrofurans consists of making iodine react in warm alkaline medium on nitrofurans with a semicarbazide function. Formation of the phenylhydrazone compound is thus no longer possible. By using this method, combined with the previous, furazolidone and nitrofurazone may be estimated concurrently in highly pigmented feeds, with precision within 7% and accuracy within ±6%.

The quantitative estimation of nitrofurans, in feeds with a high pigment content, presents difficulties. The need to extract in dimethylformamide (DMF), a strongly polar solvent, in order to obtain a good recovery, involves the presence of many interfering substances with an adverse effect on the determination. The official AOAC method becomes imprecise, if not inapplicable, because of the strong absorbance of the blanks. The method proposed by Brüggemann (*3*), which consists of pre-extracting the pigments using apolar solvents, then extracting the nitrofuran in DMF, and finally developing a coloration with potassium hydroxide, has been applied only to nitrofurazone and does not provide for either the individual or the total estimation of a mixture of two nitrofurans. The method proposed by Stone gives good results for furazolidone (*5*), but presents difficulties in the case of nitrofurazone (*6*).

We have obtained good results by using the reaction giving the formation of the 5-nitro-2-furfuraldehyde phenylhydrazone, applicable to all nitrofurans. After the highly pigmented feed has been extracted in DMF, the filtered extract is treated with phenylhydrazine in acid medium containing oxalic acid, a reducing agent which controls this reaction in complex media. The phenylhydrazone is extracted in toluene and purified chromatographically by passing through a silicic acid column and then an alumina column superposed by means of a standard taper joint. It is eluted from the silicic acid by toluene and absorbed into the alumina, from which, in turn, it is eluted by DMF. Most of the interfering substances remain absorbed in the silicic acid, and a small proportion remains in the alumina. Thus, the absorption spectrum of the eluate exhibits few impurities (Figure 1, upper). The alumina column enables the almost total elimination of the toluene and

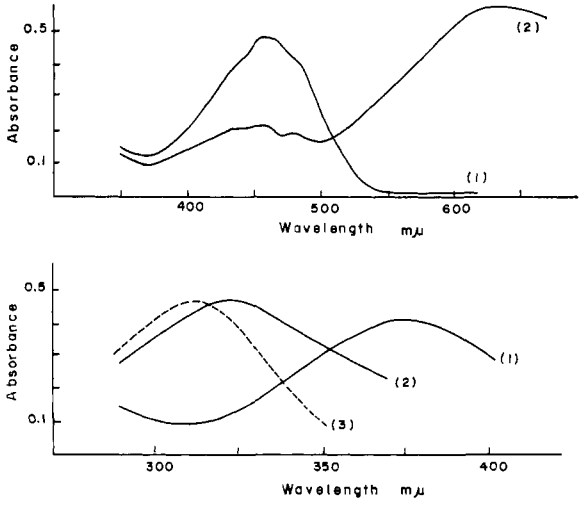

Figure 1. Absorption spectra

Upper
1. Chromatographic effluent
2. Same effluent treated by potassium hydroxide

Lower
1. Nitrofurazone in water
2. Nitrofurazone treated by iodine
3. 5-Nitrofurfuraldehyde in water

recovery of the phenylhydrazone in DMF. The potassium hydroxide, in this solvent, gives a blue coloration (*4*), absorbing at 630 mμ and remaining stable for at least 15 minutes and independent of the proportion of toluene carried through by the DMF during elution. An extract of feed rich in pigments, treated in the same way, exhibits negligible absorption. Since the conversion into phenylhydrazone is incomplete in complex organic media, but reproducible in the presence of oxalic acid under identical conditions of acidity, temperature, and time, we include an internal standard.

Existing methods for the specific estimation of nitrofurans consist in the determination of a mixture of nitrofurazone and furazolidone (Bifuran) after chromato-

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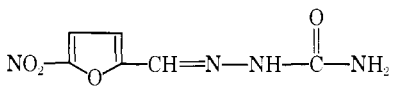
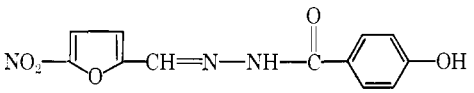
graphic separation on alumina. The method of Zwingelstein and Jouanneteau (*7*), involving an extraction in acetone, allows a good evaluation of each of the constituents, but a bad recovery. The Beckman method (*1*) has not been applied to highly pigmented feeds; pre-extraction was modified by Brüggemann and Bronsch (*2*) to obtain a better estimation of the nitrofurazone. The solution proposed by Stone (*6*) presents difficulties in the complete recovery of nitrofurazone. A more general and yet specific method has therefore been developed. The nitrofurans with a semicarbazide function—e.g., nitrofurazone, nifuroxazide, or (5-nitro - 2 - furfurylidene) - 4 - hydroxybenzhydrazide—react with iodine in warm alkaline medium (85° C., for 5 minutes, at pH 8).



Nitrofurazone



Nifuroxazide

This type of nitrofurans, after reaction, neither liberates the 5-nitro-2-furfuraldehyde by acid hydrolysis (Figure 1, lower), nor therefore produces phenylhydrazone. Other nitrofurans do not react with iodine and continue to give the 5-nitro-2-furfuraldehyde phenylhydrazone. It is therefore possible to estimate a mixture of two nitrofurans, belonging to each of the two groups, by comparing the difference in the coloration of the phenylhydrazone obtained before and after the action of iodine. Application of this specific method to the quantitative estimation in feeds is adversely affected by incomplete conversion into the phenylhydrazone of the sample treated by iodine. We have modified this reaction so that it is comparable to that without previous treatment with iodine, by extracting the feed in a mixture of DMF and water (2:1, v./v.). This leaves a much lower proportion of pigments than when DMF is used, but ensures a good recovery.

Instruments and Reagents

Instruments. Superposed chromatographic tubes connected by means of a standard taper joint (Figure 2). Centrifuge tubes graduated to 100 ml., also used as test tubes. A Jobin and Yvon spectrophotometer.

Reagents. Potassium hydroxide, 2% in methanol. Iodine, 1.7% in DMF.

Phenylhydrazine hydrochloride, 1.5% in water, freshly prepared every day, and stored in the cold.

Citric acid and disodium phosphate buffer solution, pH 8 (for 500 ml., 270 mg. of citric acid + 13.8 grams of disodium phosphate).

Aluminum oxide Merck, treated as follows: Wash 500 grams of alumina with 1 liter of water, followed by two lots of 1 liter of 0.1*N* hydrochloric acid, dry on a Büchner with acetone, and then activate overnight at 105° C.

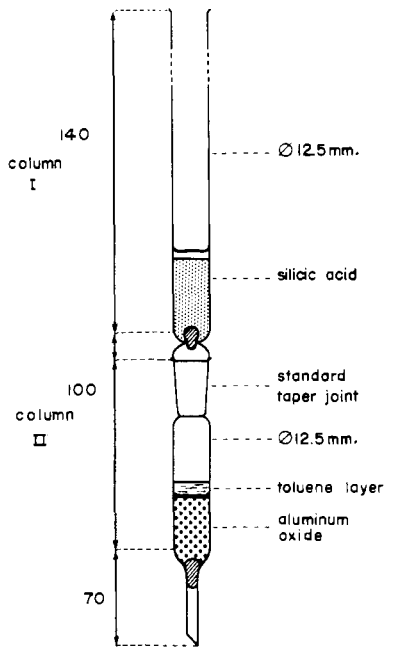


Figure 2. Superposed chromatographic columns

Silicic acid Mallinckrodt, 100-mesh, treated as follows: Wash 300 grams as for alumina, eliminating fine particles unsuitable for chromatographic elution and then activate for 2 hours at 105° C.

Nitrofuran standard solution, containing one of the nitrofurans to be estimated, is prepared in the usual way by diluting a stock solution of a pure nitrofuran in DMF with water. It must be protected from light. Five milliliters of the final dilution should contain approximately half of the nitrofuran content of 5 ml. of the filtered feed extract.

Procedure

Estimation of Total Nitrofuran Content (one, or a mixture of two). In an Erlenmeyer flask, weigh between 10 and 15 grams of feed containing 0.01% nitrofurans, add 100 ml. of DMF, place in a boiling water bath for 5 minutes, remove and shake for a further 5 minutes, then cool, and filter through folded paper. Place two lots of 5 ml. of the extract in two 100-ml. centrifuge tubes, and add 5 ml. of standard solution to tube A and 5 ml. of water to tube B. Then add to each tube 1.5 grams of oxalic acid, 10 ml. of phenylhydrazine solution, and 5 ml. of concentrated hydrochloric acid. Heat in a constant temperature bath at 75° C. for 20 minutes, shaking occasionally. After cooling, pipet 8 ml. of toluene into each tube, stopper, and shake vigorously for 1 minute. Centrifuge for a few minutes and then place 5 ml. of the clarified phase on the top of the silicic acid column.

Set up chromatographic columns (Figure 1). Plug column II with glass wool and introduce 2.5 cm. of dry alumina, cover with a disk of filter paper to protect the surface, and then add toluene to slurry pack (the column can be allowed to dry). Place column I on top of column II, plug with glass wool, fill with a slurry of 3.5

to 4 cm. of silicic acid in toluene, and place a protective disk of filter paper above the silicic acid slurry. Connect II on a vacuum flask, remove I to add toluene to a level of 1 cm. above the alumina, replace I, add 5 ml. of the toluene phase obtained by centrifuging, and then set up a weak vacuum. After passing the 5 ml., elute with toluene: A red band gradually elutes and passes into II, where it remains fixed at the top when all the band has passed. Disconnect the vacuum and remove I. Elute II with DMF. When the orange band has reached the middle of the column, receive the drainage in a 20-ml. volumetric flask, and complete the elution. Add 1 ml. of potassium hydroxide in methanol, shake to obtain a deep blue coloration, and make up to 20 ml. with DMF. After 5 minutes, read at 630 mμ in comparison with a blank consisting of 19 ml. of DMF and 1 ml. of potassium hydroxide in methanol.

Specific Estimation of Nitrofurans. Extract as previously, but in 100 ml. of a mixture of DMF and water (2:1, v./v.). Place 5 ml. of filtered extract in each of three tubes A, B, and C, add 5 ml. of standard solution to A and B. Add 5 ml. of buffer solution and 2 ml. of iodine solution to B, heat for 5 minutes at 85° C., and cool. Make all three tubes up to the same volume with distilled water. Add oxalic acid, phenylhydrazine, and hydrochloric acid to the three tubes, as in the method just described, and carry out the determination in the same way.

Calculations. The aliquot part of 5 ml. of filtered extract is termed E.

Determination of One Nitrofuran or a Mixture of Two. Let Q be the amount of nitrofurans in E and q the amount of nitrofuran in 5 ml. of standard solution.

$$\text{Let} \quad r = \frac{\text{molecular weight of standard nitrofuran}}{\text{molecular weight of other nitrofuran}}$$

Table I. Total Determination of Nitrofurans

(Nitrofurans added, 10 g./100 kg.)

	Mixture A		Mixture B	
	Found	Diff., %	Found	Diff., %
	10.35	+3.5	10.20	+2.0
	10.15	+1.5	10.00	0
	10.10	+1.0	9.75	−2.5
	9.95	−0.5	10.45	+4.5
	9.80	−2.0	10.00	0
	10.30	+3.0	10.45	+4.5
	9.85	−1.5	10.85	+8.5
	10.05	+0.5	9.65	−3.5
	9.95	−0.5	10.30	+3.0
	9.70	+3.0	10.40	+4.0
Av.	10.02	±1.7	10.20	±3.25
Std. dev., %	2.10		3.70	

Table II. Specific Determination of Bifuran

[Nitrofurans added. Furazolidone (FZ) 5 g./100 kg. + nitrofurazone (NF) 5 g./100 kg.]

	FZ		NF		Total	
	Found	Diff.	Found	Diff.	Found	Diff.
Mixture A	4.90	− 2	5.05	+ 1	9.95	−0.5
	5.05	+ 1	4.70	− 6	9.75	−2.5
	5.30	+ 6	5.50	+10	10.80	+8.0
	5.10	+ 2	4.70	− 6	9.80	−2.0
	4.50	−10	4.70	− 6	9.20	−8.0
	4.40	−12	5.30	+ 6	9.70	−3.0
	4.80	− 4	4.70	− 6	9.50	−5.0
	4.65	− 7	5.35	+ 7	10.00	0
	4.60	− 8	5.30	+ 6	9.90	−1.0
	4.75	− 5	5.10	+ 2	9.85	−1.5
Av.	4.81	± 5.7	5.04	± 5.6	9.85	±3.15
Std. dev., %	6.72		6.35		4.1	
Mixture B	5.10	+ 2	4.75	− 5	9.85	−1.5
	5.00	0	4.85	− 3	9.85	−1.5
	4.35	−13	5.25	+ 5	9.60	−4.0
	5.40	+ 8	4.45	−11	9.85	−1.5
	4.90	− 2	5.15	+ 3	10.05	+0.5
	4.60	− 8	5.00	0	9.60	−4.0
	4.55	− 9	4.80	− 4	9.35	−6.5
	4.80	− 4	4.80	− 4	9.65	−3.5
	4.90	− 2	4.60	− 8	9.50	−5.0
	4.45	−11	5.05	+ 1	9.50	−5.0
Av.	4.81	± 5.9	4.87	± 4.4	9.68	±3.3
Std. dev., %	6.45		4.7		2.1	

Let $$R = \frac{\%\text{ standard nitrofuran}}{\%\text{ other nitrofuran}}$$

(ratio of relative proportions in feed)

Then $$Q = q.r.R\,\frac{\text{absorbance of tube B}}{\text{abs. A}-\text{abs. B}}$$

SPECIFIC DETERMINATION. Let Q (amount of nitrofuran in E) $= Q_1 + Q_2$, and let q, r, and R, be defined as previously. Then:

$$Q_1 + Q_2 = q.r.R\,\frac{\text{abs. C}}{\text{abs. A}-\text{abs. C}}$$

Hence $$Q_1 = q\,\frac{\text{abs. B}}{\text{abs. A}-\text{abs. C}}$$

If R is unknown, give it an initial value of 1, and calculate Q_1 and Q_2. $R' = Q_1/Q_2$. Repeat the calculation with R' and proceed by successive approximations.

Results and Discussion

Methods of total determination and separate determination have been applied to two mixtures containing 0.005% furazolidone + 0.005% nitrofurazone.

Mixture A, a chicken feed with the following formula: corn 64%, soybean cake 10%, peanut cake 7%, sunflower oil 5%, fish meal 5%, peanut oil 3%, alfalfa meal 1%, salts, and vitamins.

Mixture B, a highly pigmented mixture with the following formula: alfalfa meal 20%, fish meal 20%, meat meal 20%, corn meal 20%, soybean cake 20%.

Ten replicates were carried out (extraction and determination). Tables I and II show that for the total determination, the results for highly pigmented mixture B were just as good as for feed A. However, in the specific determination, results for both A and B were only half as good because of calculating by difference.

These two methods have a considerable specific and general advantage over existing methods. The main inconvenience is the time required to carry out the determination, at least as long as the official AOAC method ($2^1/_4$ hours for one determination and $3^1/_4$ hours for two concurrent determinations).

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