Fluorometric Method for Determining Residues of Terephthalic Acid in Chicken Tissues

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A sensitive fluorometric method has been developed for the estimation of terephthalic acid, TPA, residues present in chicken tissues. The tissue sample is cut into small pieces and extracted in a blender with an ammonium hydroxide–methanol mixture. Interfering substances are removed by precipitation with barium chloride from alkaline solution and filtration followed by extraction of the filtrate with chloroform and ethyl ether. After acidification of the solution, the TPA is extracted. The TPA is then nitrated to nitro-TPA and reduced to amino-TPA, and the fluorescence of amino-TPA in 1-butanol is measured in a fluorometer. The method is sensitive to 0.1 p.p.m. as indicated by the recovery of known amounts of TPA added to 10 grams of various tissues from chickens.

Terephthalic acid (benzene-1,4-dicarboxylic acid, TPA) has attracted considerable interest (*3, 7, 8, 10*) as a potentiating agent for a number of well known antibiotic drugs used in the treatment of chronic respiratory disease of domestic fowl. Several reports (*1, 4, 11*) also indicate that the TPA not only does not harm the treated birds but actually increases their growth and production of eggs. The present procedure was developed to provide a sensitive method of determining residues of TPA in chicken tissues for investigational purposes only. There is no recommendation that TPA may be used in conjunction with antibiotics or that TPA can be added to formulations of feed for chickens.

The procedure developed was based on maceration of the chicken tissues with a solution of methanolic ammonium hydroxide, and removal of interferences by precipitation with barium chloride and extraction with chloroform and ethyl ether. After acidification, the TPA is extracted with ether, the ether is evaporated, and the residue is nitrated by heating with a mixture of sulfuric acid and fuming nitric acid (*9*). The nitrated terephthalic acid (nitro-TPA) is then extracted and finally reduced to amino-TPA by titanium trichloride in 1-butanol (*2*), and the resulting fluorescence is measured in a fluorophotometer equipped with suitable filters. The activation peak is at 364 mμ, and the fluorescence peak is at 438 mμ.

Mörner (*6*) obtained nitro-TPA by the reaction of nitric acid on various proteins, but this was not a source of interference in the present method because the authors removed the proteins and separated TPA before nitration. Kauffmann and Weissel (*5*) have made a thorough study of the fluorescence of amino-TPA and related compounds.

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Experimental

Apparatus. Turner Fluorometer (Model 110) or equivalent.

Filters, primary (right hand), 110-811 (7–60); secondary (left hand), 110–816 (2A) plus 110–823 (2nd).

Photometric cells, 12 × 75 mm., matched.

Borosilicate glass test tubes, 19 × 120 mm. with ground-glass stoppers (19/38 joints).

Waring Blendor or equivalent.

Kuderna-Danish evaporative concentrator. The bottom ground-glass joint should be an inner joint to fit the borosilicate test tubes described above.

Reagents and Solvents. All reagents and organic solvents are C.P. or U.S.P. grades unless otherwise specified. The chloroform, benzene, methanol, and 1-butanol should be redistilled before use.

Ethyl ether, U.S.P. grade or better, should be shaken with saturated ferrous chloride solution and redistilled. However, some brands of ether give higher blank results than others. Fisher's anhydrous ethyl ether and Mallinckrodt's U.S.P. ethyl ether were satisfactory.

Nitrating acid. Mix 1 volume of fuming nitric acid (sp. gr. 1.49 to 1.50) and 1 volume of concentrated sulfuric acid (sp. gr. 1.84).

Sodium chloride solution. Saturate distilled water with C.P. sodium chloride. Technical salt is unsatisfactory because of colored impurities extracted by ethyl ether.

Barium chloride ($BaCl_2 \cdot 2H_20$).

Potassium hydroxide solution, 6*N*. Prepare by weighing the potassium hydroxide accurately, correcting for its purity. Avoid contact of the solution with rubber, cork, or metal.

Titanium trichloride (titanous chloride), 20% solution.

Celite, No. 545.

Cotton. Extract cotton in a Soxhlet extractor with acetone for 6 or more hours, dry on a tray in the hood and then overnight in a 100° C. oven, and store in a tightly covered jar.

Terephthalic acid, 99% purity or better. For making a standard solution transfer 50 mg. of TPA, accurately weighed, to a 500-ml. volumetric flask, dissolve in 50 ml. of concentrated ammonium hydroxide, and dilute to volume with methanol. Dilute this solution further with ammonium hydroxide–methanol (1 to 10) to give 1 μg. of TPA per milliliter of solution in the working standard.

Procedure

Cut 10 grams of the chicken tissue to be analyzed into small pieces, and extract in a glass-stoppered 125-ml. Erlenmeyer flask by shaking with 10 ml. of concentrated ammonium hydroxide for 10 minutes. Then add 50 ml. of hot methanol to the extract and macerate in a blender for 2 minutes. Filter the extract with a Büchner funnel through a 6-cm. diameter filter paper precoated by filtration of a slurry of 10 grams of Celite 545 in methanol. After the extract has been filtered completely, transfer the chicken tissue with a spatula from the funnel into the same glass-stoppered, 125-ml. Erlenmeyer flask, leaving as much of the Celite layer in the funnel as possible. Extract the tissue again with another 50 ml. of hot ammonium hydroxide–methanol (1 to 10) with vigorous shaking, and filter the extract through the same Celite 545-packed Büchner funnel. Transfer the chicken tissue again into the Erlenmeyer flask and repeat the extraction and filtration a third time. Rinse the blender and the Erlenmeyer flask thoroughly with ammonium hydroxide–methanol (1 to 10) and filter through the Büchner funnel. Collect all the extracts in a standard-joint Erlenmeyer flask, and evaporate the solution carefully in a steam bath; use a glass tube attached to a vacuum line toward the end of the evaporation.

Add 25 ml. of concentrated ammonium hydroxide, 25 ml. of water, and 20 grams of barium chloride, and shake occasionally for 30 minutes. Filter with a Büchner funnel through filter paper precoated by filtration of a water slurry of Celite 545. Rinse the flask with 25 ml. of 1% ammonium hydroxide solution and filter through the funnel. Add 2 ml. of 6*N* potassium hydroxide solution to the filtrate, and mix thoroughly. Warm the solution on a steam bath, and then let it stand until it cools to room temperature. Filter the solution through a 12.5-cm. diameter, folded, No. 42 Whitman filter paper (or equivalent) into a 500-ml. separatory funnel until the extract has been completely filtered. Rinse the flask and filter paper with 20 ml. of 0.05*N* potassium hydroxide solution.

Extract the filtrate in the separatory funnel with three 100-ml. portions of chloroform and then with two 100-ml. portions of ethyl ether. Discard the organic solvent extracts. Acidify the aqueous layer with concentrated hydrochloric acid to Congo red paper, add 10 ml. excess, and cool the funnel under running water. Extract the acidified solution with 100 ml. of benzene–ether mixture (1 to 9) by shaking vigorously for 1 minute. When the two layers have completely separated, transfer the aqueous layer into a second separatory funnel, and extract with another 75 ml. of benzene–ether. After separation, transfer the aqueous layer into a third separatory funnel, and extract again with another 75 ml. of benzene–ether. After separation, discard the aqueous layer.

Wash the extracts in the three funnels successively with three separate 25-ml. portions of a saturated sodium chloride solution containing 1% of concentrated sulfuric acid. Place a plug of cotton and about 10 grams of anhydrous sodium sulfate in a Gooch crucible holder, wash with 30 ml. of fresh benzene–ether, and discard the filtrate. Filter the organic solvent extract into a Kuderna-Danish evaporative concentrator, evaporate the solvent carefully and completely on the steam bath, and heat the test tube containing the dried residue in a 100° C. oven for 10 minutes.

Nitration of Samples. Cool the test tubes in a beaker of cold water and add 10 ml. of nitrating acid to each. Immerse the test tubes one third to one half their length in a steam bath through holes cut to accommodate the test tubes in a metal holder, and heat for one hour. Cool the test tubes in a beaker of cold water, and add about 20 ml. of cold water to each tube to terminate the nitration.

Extraction of Nitrated Terephthalic Acid. Transfer and rinse the contents of each test tube quantitatively into a 250-ml. separatory funnel with about 60 ml. of water (in addition to the water already in the test tube) and 100 ml. of a benzene–ether mixture (1 to 9). Shake vigorously for at least 1 minute. After the layers have separated clearly, transfer the aqueous layer into a second separatory funnel, and extract with another 75 ml. of the benzene–ether mixture. Then transfer the aqueous layer into a third separatory funnel, and extract with another 75 ml. of the solvent mixture. Draw off and discard the aqueous layer. (The aqueous layers in each funnel should be drained as completely as possible.) Wash the benzene–ether extract in each funnel successively with a 25-ml. portion of saturated salt solution, and repeat with two more 25-ml. washes, discarding the aqueous salt layers. Place a small plug of cotton and about 10 grams of anhydrous sodium sulfate in a Gooch crucible holder, rinse with about 30 ml. of the benzene–ether mixture (1 to 9), and discard the rinse. Starting with the first funnel, filter the benzene–ether layer from each separatory funnel through the plug of cotton into a clean Erlenmeyer flask, and then wash the cotton with benzene–ether. Add a glass bead, evaporate the solvent carefully and completely on the steam bath, and then heat the dried residue in a 100° C. oven for 10 minutes.

For the reduction and development of fluorescence, add 20 ml. of 1-butanol, and swirl to dissolve the residue in the flask. Add 1 drop of titanium trichloride from a medicine dropper, stopper the flask, and shake well. Add 40 ml. of water saturated with 1-butanol, shake well again, centrifuge, and discard the aqueous layer. Fill a fluorometric cuvette with the butanol solution, and measure the amount of fluorescence in a fluorophotometer previously adjusted to zero with 1-butanol. The fluorescence is stable for at least 4 hours at room temperature.

Prepare a standard curve from aliquots of the work-

ing standard TPA solution in ammonium hydroxide–methanol with the same procedure. The standard curve should be a straight line with a slope of 0.11 μg. of TPA per Turner fluorometer unit under the experimental conditions described. The range selector on the fluorometer should be used in the 3X position unless greater sensitivity is desired.

Tests Made with Method

The percentage recovery of TPA from chicken meat was determined as follows: Aliquots of a standard solution of TPA were pipetted into a series of glass-stoppered Erlenmeyer flasks containing 10-gram samples of chicken meat. Each sample was evaporated on a steam bath until the odor of ammonia and alcohol was gone. The samples were then analyzed by the method described (Table I). The average of the recoveries was about 85%.

In a study of the potentiation effect of TPA on aureomycin, four groups of 20 New Hampshire chicks were each fed 3 weeks on a practical broiler diet that contained 100 mg. per kg. of aureomycin plus TPA at levels of 0.0, 0.5, 2.0, and 3.0 %. At the end of 3 weeks, birds in each group were sacrificed, and samples of tissue were collected for analysis of TPA residues (Table II).

Discussion

The authors' fluorometric method is generally suitable for estimating residues of TPA in chicken tissues. Interferences are largely removed by precipitation with barium chloride and filtration and by extractions with solvents. However, certain general precautions should be followed to avoid excessively high chicken tissue control or reagent blank readings: The chemical reagents and solvents used must be highly purified grades, and the solvents should be redistilled; all glassware should be cleaned with cleaning solution and thoroughly rinsed with water; contact of the analytical solutions with stopcock grease, rubber, cork, soap powder, or soap film must be avoided because these materials can introduce undesirable additional fluorescence and cause high readings.

With a Turner spectrophotofluorometer and the filters specified, the practical working range of the method is limited to 1 to 11 μg. of TPA. With an Aminco-Bowman spectrophotofluorometer, the maxima of activation and fluorescence were 364 and 438 mμ, respectively, and as little as 0.01 μg. could be detected. If necessary, the sensitivity of the method could be increased, but a more rigorous cleanup procedure would then be necessary.

The treatment of the extract with 2 ml. of 6*N* potassium hydroxide solution followed by paper filtration

Table I. Recovery of Terephthalic Acid Added to 10 Grams of Chicken Meat

TPA Added, μg.	TPA Recovered, μg.	%
0.0	0.0	
	0.1	
2.7	2.4	85.0
	2.5	88.8
4.5	3.9	84.4
	4.1	88.8
6.3	5.2	82.5
	5.4	84.0
8.1	6.9	83.7
	7.1	86.6
9.9	8.6	85.9
	8.4	83.9
	Av.	85.4

Table II. Residues of Terephthalic Acid in 10 Grams of Tissues from Chickens Treated with TPA

TPA Added to Feed, %	Amount of TPA Found,[a] μg.
Liver	
0.0	0.1
	0.1
	0.0
0.5	2.7
	2.4
	2.6
2.0	8.2
	8.8
	8.3
3.0	16.2
	16.4
	16.1
Fat	
0.0	0.1
	0.1
3.0	21.0
	20.1
Skin	
0.0	0.0
	0.0
0.5	1.2
	1.8
	1.3
3.0	13.6
	13.0
	13.8
Dark Meat	
0.0	0.0
	0.0
3.0	9.8
	10.0
White Meat	
0.0	0.0
	0.0
3.0	7.6
	7.9

[a] Corrected for control sample results.

is an important step in the cleanup procedure which greatly decreases the amount of interference.

Amino-TPA in 1-butanol fluoresces strongly, but the intensity of the fluorescence is diminished by acid and is completely quenched by strong acid. To remove the acid added with the titanium trichloride reagent, the authors washed the butanol solution of amino-TPA with water. To avoid any change in the concentration of the butanol solution of the amino-TPA, water saturated with butanol was used for the washes.

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