

activity as provitamin A. Of lesser importance are neo- β -carotene B, neo- β -carotene U (in canned samples), γ -carotene, and α -carotene. Lycopene and its isomers are inactive and β -carotene has recently been found inactive (9). Results of several biological assays (rat-growth tests), recently conducted on canned tomato samples of widely different β -carotene contents, indicate that the content of β -carotene is at present the best index of provitamin A activity, but that other carotenoids may increase this activity of *Lycopersicon* fruits.

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Colorimetric Determination of DDT in Milk and Fatty Materials

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A procedure has been developed for the determination of DDT as such in food-stuffs containing considerable amounts of fatty matter such as milk, butter, and animal fat. Although the method is not rapid, it permits the detection and determination of DDT in milk in quantities as low as 1 p.p.m.

RECENT articles (1, 13, 16, 17, 21) have focused attention on the possible danger to the public health from contamination of such products as milk, butter, eggs, meat, and fats when farm animals consume DDT-treated feed. Pharmacological investigations (21) have shown that ingested DDT accumulates as such in the fatty tissues of experimental animals and can be excreted in milk. Some of the DDT is metabolized to bis(p-chlorophenyl) acetic acid (3, 20), which is excreted in urine (5, 7, 20). Telford's (16) and Telford and Guthrie's (17) DDT-feeding experiments on goats and rats, using rather high dosages, indicate that such milk may become toxic enough to kill other animals drinking it. In order to evaluate some of these factors, the Bureau of Entomology and Plant Quarantine started an investigation of the contamination of these foods as a result of feeding DDT-treated crops to farm animals.

The problem of analyzing these foodstuffs is complicated considerably by the presence of large amounts of fatty matter which accompany the DDT in organic solvent extracts—for example, about 4 grams of butterfat will be extracted with the DDT from each 100 grams of milk. Any attempt to detect 0.1 mg. of DDT in 100 grams of milk (equivalent to 1 p.p.m.) by any of the published methods of analysis (1, 4, 6, 12, 13, 15, 18) is likely to encounter difficulty because of the large amount of interfering fatty matter. The Schechter-Haller colorimetric method (10, 12), although having the advantage of oxidizing a considerable amount of extraneous matter during the nitration, cannot be used on samples containing more than a few tenths of a gram of fatty matter because of the danger of an uncontrollably violent nitration.

Attempts to nitrate 0.5 gram or more of butterfat containing DDT led to violent nitrations, and even when the nitration was carefully carried out to prevent a violent reaction, the results obtained were low or negative. Neither Woodard *et al.* (21) nor Olier and Calvery (7) mentioned this difficulty when they used the method in pharmacological investigations, probably because there was enough DDT present so that very small samples could be used.

In order to detect very small amounts of DDT, it is necessary to find some method of removing extraneous material and concentrating the DDT. Attempts to concentrate the DDT by chromatography were not successful. Low-temperature precipitation of the fat by cooling a solution with solid carbon dioxide gave rise to difficulties in filtration. Saponification of the fat and use of the unsaponifiable portion would eliminate most of the fatty matter but would, at the same time, convert DDT to its dehydrochlorinated derivative. Such a procedure might be useful if one were not interested in whether the DDT was present as such or had decomposed to its dehydrochlorinated derivative. The method of Stiff and Castillo (14, 15) has this disadvantage.

In the authors' experiments it was highly desirable to establish incontrovertibly the presence of DDT as such and to estimate its concentration. The observation of the solubility of fats and the insolubility of DDT in concentrated sulfuric acid led to the development of the procedure described in this article for eliminating all but a small residue from fatty materials. This residue probably consists mostly of hydrocarbons. A search of the literature disclosed similar methods for the determination of oil deposits on

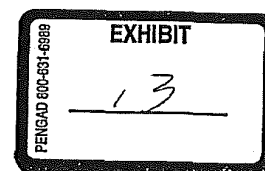


Table 1. Recovery of DDT Added to Milk

DDT Added P.p.m.	DDT Found		Recovery Corrected for Blank %
	Uncorrected for blank P.p.m.	Corrected for blank P.p.m.	
0 (blank)	0.35 ^a	—	—
0 (blank)	0.35 ^a	—	—
1.00	1.27	0.90	80
1.00	1.30	0.93	93
5.00	5.50	5.23	105
5.00	5.40	5.03	101

^a Calculated as DDT; the characteristic blue color was not produced.

leaves (9) and of diphenyl in oranges (2, 16). The relationship to the refining of petroleum with sulfuric acid is also interesting to note.

APPARATUS AND REAGENTS

Some of the apparatus and reagents are described by Schechter *et al.* (12); it is advisable to use test tubes 25 X 200 mm. or larger for the titrations.

Separatory funnels, 500-ml. capacity, should be prepared in the same manner as the 125-ml. separatory funnels mentioned by Schechter *et al.* (12) and should be dry when used.

Centrifuge and centrifuge bottles with rubber caps.

Sodium sulfate-sulfuric acid. Dissolve 100 grams of *c.p.* anhydrous sodium sulfate (oven-dried) in 1 liter of *c.p.* concentrated sulfuric acid (sp. gr. 1.84) with the aid of heat, and cool to room temperature. Sodium bisulfate is probably formed in the solution.

Fuming sulfuric acid-concentrated sulfuric acid. A mixture of equal volumes of fuming sulfuric acid (20 to 30% sulfur trioxide) and concentrated sulfuric acid (sp. gr. 1.84).

Sodium bicarbonate solution, 5%.

Technical acetone, technical chloroform, and Skellysolve B (a petroleum fraction, boiling at 60° to 70° C.). These solvents should be redistilled before using.

It is advisable to have all apparatus rinsed several times with redistilled technical acetone and dried. The acetone may be saved and recovered.

PROCEDURE

To 100 grams of milk which has been thoroughly mixed before sampling add an equal volume of 95% ethanol. Divide the solution equally between two 200-ml. centrifuge bottles. When the concentration of DDT is higher than 5 p.p.m., it is advantageous to take a correspondingly smaller milk sample. Where larger bottles are available, the sample need not be divided. Add 50 ml. of Skellysolve B to each bottle, cover the bottles with rubber caps, shake vigorously, and centrifuge at 2000 r.p.m. for 15 minutes. Pour the contents of both bottles into a 500-ml. separatory funnel.

After the layers have separated, drain the lower layer in equal portions directly into the same centrifuge bottles. Drain the upper Skellysolve B layer through a 5-cm. tightly packed plug of cotton held in a glass Gooch crucible holder into a 500-ml. Erlenmeyer flask with a standard ground-glass joint. Extract the solution in each centrifuge bottle in the same manner as before with two successive 25-ml. portions of Skellysolve B and a final 50-ml. portion centrifuging for about 10 minutes each time; return the lower layers from the separatory funnel to the centrifuge bottles and filter the upper layers through the plug of cotton into the Erlenmeyer flask. After the last extraction, rinse the separatory funnel with 50 ml. of Skellysolve B, which is also run through the plug of cotton into the Erlenmeyer flask. Add a glass bead to the Erlenmeyer flask and recover the Skellysolve B from the milk extract by distillation on the steam bath, using an all-glass apparatus. While the flask is being heated, insert a tube connected to a vacuum line to remove the last traces of solvent.

Quantitatively wash the residue from the distillation into a 500-ml. separatory funnel with 150 ml. of chloroform. For the analysis of butter or fat, substitute for this residue a 5-gram sample or an extract thereof from which the solvent has been removed. Place 100 ml. of chloroform in a second 500-ml. separatory funnel, and extract the chloroform solutions successively with (1) 50 ml. of sodium sulfate-sulfuric acid, (2) 50 ml. of sodium sulfate-sulfuric acid, (3) 50 ml. of fuming sulfuric acid-concentrated sulfuric acid, and (4) 50 ml. of sodium sulfate-sulfuric acid. If this last wash is not light in color, it is advisable to use

still another sodium sulfate-sulfuric acid wash. Drain each acid wash (lower layer) from the first funnel into the second funnel and finally into a 250-ml. cylinder. The extraction in the second funnel is used to minimize the loss of DDT by the slight emulsification of chloroform in the acid washings. The funnels should be shaken vigorously each time and then allowed to stand for 10 to 15 minutes before draining off the acid layer. In the rare case where an emulsion forms and does not separate in 30 minutes, the mixture may be centrifuged and poured gently back into the separatory funnel. It is well to keep a small beaker under each funnel and to have a wet cloth handy to wipe any acid which may drip.

After the acid extractions are completed, filter the chloroform from the first funnel and then from the second funnel through a 5-cm. tightly packed plug of cotton in a glass Gooch crucible holder into a third 500-ml. separatory funnel. Pipet off any chloroform which has risen to the surface from the combined acid washings in the cylinder and run it through the plug of cotton. Rinse the two funnels and the cotton with chloroform, using about 50 to 100 ml. Add enough 5% sodium bicarbonate solution (about 40 ml.) to the combined chloroform filtrate so that it will remain alkaline when tested with litmus paper after vigorous shaking. After allowing about 10 minutes for a reasonably clear separation, filter only the chloroform layer through a 5-cm. plug of tightly packed cotton in a glass Gooch crucible holder into a 500-ml. Erlenmeyer flask with a standard joint. Wash the sodium bicarbonate solution remaining in the funnel with two successive 30-ml. portions of chloroform, which are also run through the cotton into the Erlenmeyer flask. If the filtrate is not clear, filter again.

Add a glass bead to the Erlenmeyer flask and recover the chloroform on the steam bath, using an all-glass system, until only about 10 ml. of solution are left. Wash this quantitatively into a large test tube (25 X 200 mm. or larger) with acetone, add a glass bead, and cautiously evaporate the solvent on the steam bath, removing the last traces by inserting a tube connected to a vacuum line. Nitrate the residue with 5 ml. of nitrating mixture and complete the analysis as described by Schechter *et al.* (12).

Since there will still be some interference from the small amount of raffinate from the butterfat, it is advisable to make spectrophotometric measurements at 600, 620, and 640 millimicrons (a Beckman quartz spectrophotometer was used) and average the results. Below 600 millimicrons there may be enough interference to cause rather high results, so that it may be impossible to calculate the amounts of *p,p'*-DDT and *o,p'*-DDT and add them to obtain the total DDT. Consequently, it is desirable to use as a standard a sample of the same DDT as was used in the feeding experiment. If it is not available, DDT of the same type and grade may be used.

DISCUSSION

The method of extracting the milk differs from a similar procedure described by Olson *et al.* (8) in that a centrifuge is used to break emulsions rapidly and Skellysolve B is used rather than a mixture of Skellysolve B with ether. In the present procedure it is not necessary to wash the extract with water to remove ethanol, since Skellysolve B alone does not extract ethanol along with the butterfat.

Chloroform is used for the sulfuric acid treatment, because it gives the least trouble with emulsification in the presence of butterfat. The addition of sodium sulfate to the sulfuric acid also seems to aid in preventing emulsification. If solvents other than chloroform are used or if sodium sulfate is not added to the sulfuric acid, emulsions of the consistency of mayonnaise may form. The mixture of fuming sulfuric acid-concentrated sulfuric acid removes material from the chloroform solution of butterfat which is not removed by the sodium sulfate-sulfuric acid washes. The use of straight fuming sulfuric acid containing 20 to 30% of sulfur trioxide has been found to give low results, probably by sulfonation and removal of the DDT. Phosphoric acid (85%) has been tested in this extraction procedure in place of the sulfuric acid but it does not seem to be so satisfactory.

With the amounts of reagents described, persistent emulsions which do not break in 20 minutes will be formed only rarely. If an emulsion is formed, it may be centrifuged or, preferably, the analyses repeated using a smaller sample. If larger samples must be used and emulsions are as a consequence regularly encountered, one or two preliminary extractions of the chloroform solu-

mon with sodium sulfate—concentrated sulfuric acid using gentle shaking is advisable.

Biological tissues may be treated with sodium sulfate as described by Smith and Stahlman (13) or Ofner and Calvery (7). After evaporation of the solvent used for extraction, the residue may be dissolved in chloroform and submitted to the sulfuric acid extraction procedure described above.

p,p'-DDA, or bis(*p*-chlorophenyl)acetic acid, when put through the sulfuric acid treatment, is removed and does not interfere in the analysis for DDT. If *p,p'*-DDA is to be determined in biological tissues, it should first be separated from the sample by utilizing its acidic properties. It can be extracted from an ether solution of the sample (or extract thereof) with sodium bicarbonate solution, which should be separated, acidified, and extracted with fresh ether. Evaporation of the ether will give a residue containing the *p,p'*-DDA. This can usually be nitrated directly and determined by comparison with *p,p'*-DDA standards run by the Schechter-Haller colorimetric procedure (12). If traces are to be determined and interferences are encountered, the residue instead of being nitrated directly can be submitted to a modified sulfuric acid treatment. The modification consists in omitting the fuming sulfuric acid—concentrated sulfuric acid and the sodium bicarbonate washes and using only our sodium sulfate—sulfuric acid washes and one wash with 40 ml. of water. Duplicate determinations on 1.00 mg. of *p,p'*-DDA using this modification have given recoveries of 95 and 97%.

Table II. Analyses of Milk, Butter, and Fat for DDT

Sample, ID No.	DDT P.p.m.	Sample, ID No.	DDT P.p.m.
Milk 10,054	3.3	Milk 10,167	21.21
10,056	4.4	10,168	20.20
10,109	15, 15, 15	10,169	21.24
10,110	15, 15, 16	10,169	25.26
10,141	19, 20	10,169 (blank) ^a	0.47, 6.47
10,142	20, 20	Butter 10,200	456, 456
10,181	22, 23	10,201	530, 534
10,182	20, 20	Fat from steak, 10,350	178, 179
		Lean meat from steak, 10,350	4.4

^a Calculated as DDT; the characteristic blue color characteristic was not produced.

The application of the sulfuric acid treatment prior to the nitration of the sample may eliminate interfering substances if they are soluble in sulfuric acid or easily sulfonatable. For example, a 5.0% solution of DDT in Velsicol NR-70 (chiefly tetramethyl-naphthalene) when analyzed by direct nitration gave values of 10.5 and 10.7%, whereas when the sample was put through the sulfuric acid treatment described under Procedure, values of 5.2 and 5.1% were obtained.

RESULTS

The procedure was first tested on technical DDT and dehydrochlorinated *p,p'*-DDT [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene] to determine the percentage recovery of these materials in the absence of butterfat. When 1.00 mg. of technical DDT was dissolved in 150 ml. of chloroform and carried through the described treatment, 100% recovery was obtained in duplicate runs. When 1.00 mg. of dehydrochlorinated *p,p'*-DDT was treated in the same manner, it was recovered to the extent of 97 and 99% in duplicate runs. Since dehydrochlorinated *p,p'*-DDT is evidently not removed by the sulfuric acid treatment, its presence to any appreciable extent in a sample may be detected by its effect on the absorption spectrum of the developed color. Dehydrochlorinated *p,p'*-DDT gives a red color in the Schechter-Haller colorimetric test (12), with an absorption spectrum different from that of the blue color of DDT. *o,p'*-DDT and its

dehydrochlorinated derivative likewise would be expected to be unaffected by the sulfuric acid treatment. Any *p,p'*-DDA [bis(*p*-chlorophenyl)acetic acid] which is not removed by the sulfuric acid washes will be removed in the sodium bicarbonate wash of the chloroform solution and hence will not interfere in this determination.

The complete procedure, starting with the extraction of the milk sample, was tested by adding known amounts of technical DDT to the milk. To 100 grams of milk 0.1 mg. of technical DDT in acetone was added to give 1 p.p.m., and to 100 grams of milk 0.50 mg. of technical DDT in acetone was added to give 5 p.p.m. The results of these analyses together with the analyses of the original milk without any DDT added (blank analyses) are given in Table I. The procedure when applied to milk containing as low as 1 p.p.m. of DDT gave an easily discernible characteristic blue color. The blank milk, to which no DDT was added, gave only a yellow color.

Table II shows the results of analyses of milk, butter, and fat samples for DDT content. These samples came from cows which were given DDT-treated feed, except ID 10,107, which came from a control cow that did not receive any DDT. The results are in duplicate or triplicate and have been rounded off to the nearest whole number except for the blanks. Some of the deviations may have been due to inadequate mixing and sampling of the milk, which in many instances had separated on standing. The blue color, characteristic of DDT, was developed in all cases except for the blank milk, ID 10,197.

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