An Improved Method for Kelthane Residue Analysis with Applications for Determination of Residues in Milk

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A simplification of the Rosenthal Kelthane method is presented. Kelthane is converted to chloroform and the halogen swept free from extraneous plant material for a subsequent Fujiwara-type color determination. The simplified apparatus, the absence of a steam heating operation, and the lack of need for elaborate cleanup techniques made the method adaptable to rapid routine crop analysis. The method is applied to determination of Kelthane residue in milk.

KELTHANE [4,4'-dichloro-α-(trichloromethyl)-benzhydrol] residues on plant material can be colorimetrically determined by the Rosenthal (*6*) or Eiduson (*2*) method, in which chloroform is liberated from Kelthane by base hydrolysis and swept from extraneous plant material to be converted quantitatively to the red Fujiwara-type dye (*4*) with an aqueous pyridine-alkali mixture. These procedures, however, use specialized equipment for steam heating the pyridine dye solution in the final color development step. In the method presented, the special steam system is eliminated by trapping the chloroform in a glass tube containing the pyridine-base mixture, then placing the tube in a boiling water bath to form the red dye complex. The sensitivity of both the Eiduson method and the Rosenthal method has been improved. At the same time, the method is made more adaptable to routine laboratory use.

George *et al.* (*5*) and recently Eiduson (*3*) presented a method in which the liberation of chloroform and its colorimetric determination are carried out in a single test tube. These procedures involve the use of time-consuming initial cleanup methods for removal of substrate materials. To apply these methods for determination of Kelthane in milk it would be necessary to do a complete separation of the interfering butterfat before determination in the test tube. The present procedure is made applicable to the determination of Kelthane in milk fat by insertion of an 85% sulfuric acid, gas-washing trap in the chloroform evolution system to remove volatile interferences. An organic base, tetraethylammonium hydroxide, is used in place of the normally employed sodium hydroxide for hydrolysis of Kelthane. The organic base is added directly to the extracted milk fat. The employment of this base permits a free flowing medium in the hydrolysis tube from which the generated chloroform can be swept completely.

Procedure

Reagents. Pyridine. Repurify by adding sodium hydroxide pellets to a reagent grade and degassing for a 24-hour period at 100° C., using a small stream of compressed air and condenser set up. Redistill the degassed pyridine and store in an amber bottle.

Tetraethylammonium hydroxide solution, 1*N* reagent. The reagent should be freshly supplied. Degradation traces of ammonia present in this reagent will interfere. Degraded 1*N* solutions of this reagent can be repurified by evaporating the solution to the dry hydroxide compound with the aid of a steam bath and a stream of compressed air. Dry the compound until no traces of ammonia are detected. Then redissolve the compound in the appropriate volume of water to make a 1*N* solution.

Kelthane standard solution. Dissolve 0.1 gram of analytical grade Kelthane (Rohm & Haas Co., Philadelphia, Pa.) in 500 ml. of *n*-hexane (reagent grade). One milliliter of this solution contains 200 μg. of Kelthane. Dilute an aliquot of this solution with *n*-hexane so that 1 ml. contains 10 μg. of Kelthane.

Triton X-45, Rohm & Haas Co., 10% v./v. aqueous mixture.

Apparatus. Spectrophotometer, Beckman Model B or any type that performs satisfactorily at 530 mμ.

Kuderna-Danish evaporative concentrator.

Chloroform evolution apparatus (Figure 1). The hydrolysis tube is from a Kuderna-Danish evaporative concentrator.

Flowmeter, any flowmeter registering 80 ml. of nitrogen per minute.

Preparation of Standard Curve. Pipet 1 ml. of the Kelthane standard solution to a hydrolysis tube (Figure 1). Evaporate the solvent to dryness using a gentle air stream. The tube containing the Kelthane is now used as the hydrolysis tube in the chloroform evolution apparatus.

Apparatus Preparation. Into the receiver tube, place exactly 9 ml. of pyridine, 0.6 ml. of water, and 0.4 ml. of 50% aqueous sodium hydroxide solution. Stopper with a glass top and shake the tube for 1/2 minute to allow equilibration. Connect the receiver tube to the chloroform evolution apparatus in series with a gas washing tube containing 5 ml. of 85% sulfuric acid (Figure 1). Adjust the nitrogen flow rate to 80 ml. per minute.

Reaction and Sweeping. Add 3 ml. of xylene (redistilled, reagent grade) to the hydrolysis tube containing the evaporated sample and place without seating under the nitrogen inlet. With a hypodermic syringe, add 2 ml. of 1*N* tetraethylammonium hydroxide solution or 2 ml. of 50% sodium hydroxide solution and immediately seat the tube against the joint. The nitrogen inlet tube must be well below the surface of the aqueous hydroxide layer. Place a vigorously boiling water bath around the reaction tube and allow the nitrogen to flow for 25 minutes. Remove the receiver tube from the assembly, carefully blowing out the contents of the capillary inlet tubing with a gentle nitrogen stream. Stopper the tube and place in a boiling water bath for 5 minutes. At the end of this time, remove the tube and cool in an ice water bath. Adjust the volume of the receiver tube contents to the 10-ml. mark by adding pyridine. Read the absorbance of the red color in a 10-mm. cell at 535 mμ using as a reference solution reagent pyridine.

Milk Analysis

Extraction (Detergent Method). Place a 100-gram sample of whole milk in a 250-ml. centrifuge bottle. Add 3 ml. of 10% potassium oxalate solution (neutralized to pH 7 with a few drops of glacial acetic acid) to the bottle and warm to about 45° C. in a water bath. Then add 10 ml. of a 10% v./v. aqueous mixture of Triton X-45, mix thoroughly,

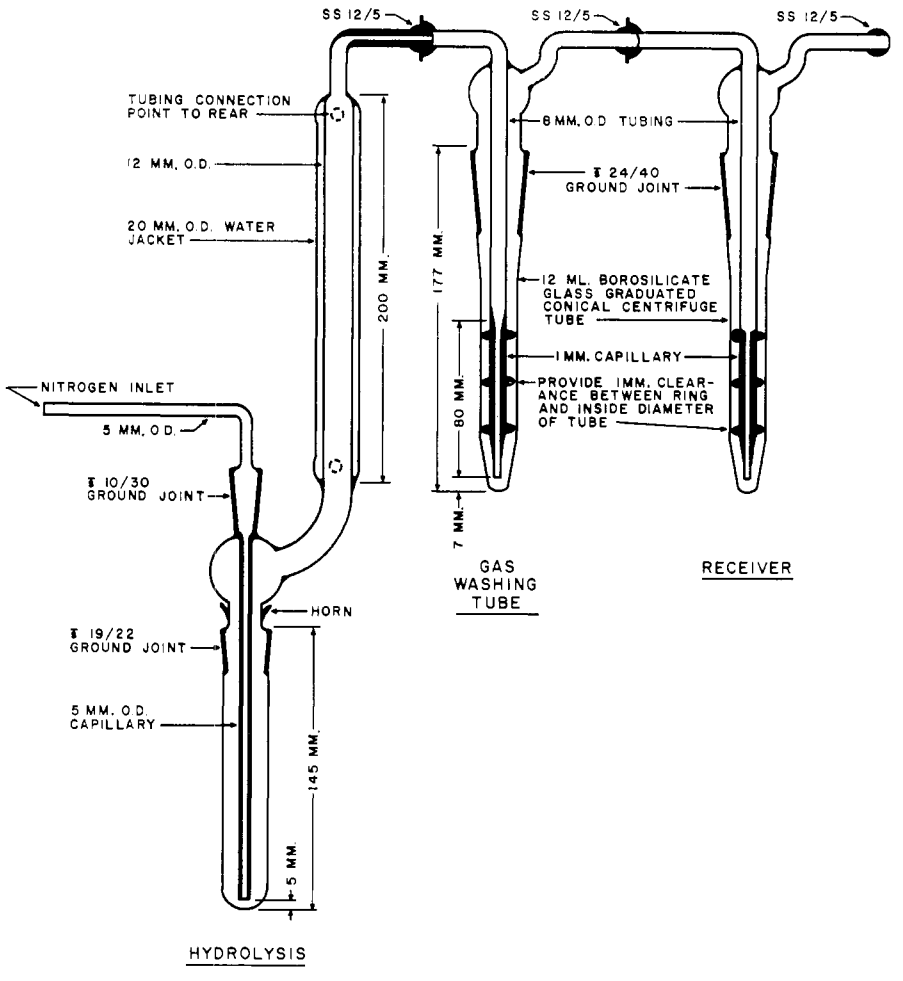

Figure 1. Chloroform evolution apparatus

and maintain at 45° C. for 20 minutes. Allow samples to cool to room temperature. Centrifuge briefly to obtain a clear fat layer, then add 100 ml. of reagent *n*-hexane. Stopper tightly with air-tight (plastic lined) metal lids. Shake gently to gather the fat. Centrifuge until the hexane layer is clear. By means of a siphon arrangement, blow off the hexane layer remaining on the milk. Slowly, so as not to disturb the milk layer, add 50 ml. of hexane and again blow off the hexane into the Kuderna flask containing the hexane from the first extraction. Perform a second and a third extraction by adding hexane, stoppering, shaking, centrifuging, and blowing off the hexane as before. After each extraction, wash with 30 ml. of hexane combining the washings with the previous extracts. Evaporate the hexane down to the fatty residue in the Kuderna apparatus. Remove the last remaining traces of solvent with the aid of a gentle stream of dry air. The tube containing the residue is now used as the hydrolysis tube in the chloroform evolution apparatus.

n-Hexane Extraction of Freeze-Dried Milk. Freeze dry 100 grams of whole milk to obtain the milk solids. Place the solids in a mortar and grind to a fine powder. Transfer the powder to a large beaker using *n*-hexane to wash down the powder sticking to the mortar. Add a total of 300 ml. of *n*-hexane to the beaker and place the beaker on a steam bath for a 10-minute digestion period. Filter the warm hexane through a Büchner funnel provided with Whatman No. 1 filter paper. Transfer the filtrate to a Kuderna-Danish evaporative concentrator. Evaporate the hexane down to the fatty residue in the Kuderna apparatus. Remove the last remaining traces of solvent with the aid of a gentle stream of dry air. The tube containing the residue is now used as the hydrolysis tube in the chloroform evolution apparatus.

Apparatus Preparation for Degassing Sample. Set up the basic chloroform evolution apparatus as illustrated in Figure 1 but without connection of the receiver and gas washing tubes. Do not circulate water through the condenser during this phase of operation.

Degassing Period. Into the reaction tube containing the fatty residue, add 3 ml. of xylene and seat the tube under the nitrogen inlet. Place a boiling water bath around the tube and allow the nitrogen to degas the sample for 30 minutes. At the end of this time, circulate the water in the condenser of the chloroform evolution apparatus and connect it in series with a gas-washing tube containing sulfuric acid and the receiver tube containing the pyridine, base, and water reagents as specified in the standard method.

Reaction and Sweeping. Unseat the reaction tube from the joint, and with a hypodermic syringe add 2 ml. of 1*N* tetraethylammonium hydroxide solution. Then immediately reseat the tube against the joint. Perform the reaction and sweeping operation as described in the standard procedure. Develop the color and read the absorbance of the red dye at 535 mμ.

Calibration. A calibration curve for Kelthane is developed by adding known concentrations of Kelthane to the milk fat extract and then running it through the procedure. The over-all recovery is measured by adding Kelthane to the whole milk prior to the extraction process.

Table I. Reliability of Standard Procedure at Seven Levels of Kelthane Fortification

Kelthane, μg.	Av. Absorbance	Determinations	Std. Dev.
Blank	0.003	5	±0.002
10	0.040	7	±0.004
20	0.082	5	±0.006
30	0.124	8	±0.008
60	0.239	5	±0.010
80	0.320	7	±0.012
90	0.362	7	±0.012
100	0.410	7	±0.015

Discussion

Standard Procedure. The original conditions of the Rosenthal method with respect to concentration of reagents, time of sweeping, and time and temperature of heating for color development have been maintained. Reproducibility of the modified method is shown in Table I with data for seven levels of fortification put through the standard procedure.

Some difficulty was experienced at first in obtaining reproducible results. It was found necessary to equilibrate (by a thorough shaking) the pyridine, water, and 50% sodium hydroxide reagents in the receiver tube before the chloroform sweeping and trapping operation. This degree of mixing and equilibration of reagents is not obtained by merely passing the nitrogen through the reagents, such as occurs in the chloroform trapping operation. At room temperature and with the use of a moderate nitrogen flow rate (80 ml. per minute),

a well mixed trapping solution allows the chloroform to react and complex in the pyridine before any loss from oversweeping has occurred. A complete recovery of the chloroform in the trapping solution is realized.

The sensitivity of the Rosenthal method has been improved. One hundred micrograms of Kelthane give an absorbance reading of 0.410 as compared to the Rosenthal absorbance value of 0.330. The increased sensitivity of the modified method is brought about by the elimination of loss of color intensity occurring in the Rosenthal method due to a volume dilution step which was necessary to rinse down residual colored solution on the glass beads of the chloroform trap. Also, the improved trapping quality of the pyridine, base, and water reagents, brought about by a thorough mixing of these reagents, allows the chloroform to react and complex before any loss from oversweeping has occurred.

The acid trap may or may not be employed for preparation of the standard curve depending upon its required cleanup usage for the particular substrate encountered. For most crops, it will not be necessary. Standard values are the same with or without the acid trap.

Both sodium hydroxide and tetraethylammonium hydroxide have been specified in preparation of the standard curve. Absorbance values obtained from use of either of these two reagents should coincide unless, as noted under reagents, the organic base solution is impure. Sodium hydroxide is most normally used for routine crop analysis since no purification of this reagent is ever needed. A special procedure for purification of the pyridine is described. Experience has shown that the normally supplied reagent will produce slightly colored reagent blanks. Various cleanup methods were tried on the solvent, but only a degassing technique followed by distillation alleviated this problem.

Milk Procedure. Two methods of milk extraction are given. Both the detergent method (*7*) and the hexane extraction of the freeze-dried solids give an efficiency of 95%. Up to 200 grams of whole milk may be processed by either of these procedures providing approximately 8 grams of extracted milk fat. This quantity of fat containing the Kelthane residue is directly treated with base for hydrolysis of the Kelthane. Sodium hydroxide, the normally used hydrolytic reagent, could not be employed because of the formation of soapy resins from which the generated chloroform could not be efficiently swept. The use of tetraethylammonium hydroxide permits a free flowing medium in the hydrolysis tube and allows a complete evolution of the chloroform.

The amount of base used is not quantitatively related to the amount of milk fat. Under the conditions of the experiment, quantitative hydrolysis of the fat does not occur. Only a relatively small amount of base need be employed to maintain the condition of basicity necessary for hydrolysis of the Kelthane.

A degassing operation is carried out to rid the fatty sample of volatile interferences which could distill into the pyridine trapping solution causing color distortion in the final solution for absorption measurement. The degassing should be performed without water cooling the condenser to allow maximum efficiency in removal of the volatiles. A 30-minute degassing time was found sufficient for the cleanup of 8 grams of sample. However, as a measure of safety, the sulfuric acid, gas-washing tube will remove any traces of interference not completely eliminated within this time period.

It should not be necessary to use the degassing technique for preparation of a standard curve, as long as the hydrolysis medium, xylene, is free of volatile interferences. There is no loss of Kelthane involved in this cleanup step. For most crops, the degassing step may be omitted especially when the amount of interfering volatiles is small and can be completely absorbed in the sulfuric acid trap.

Table II. Recovery of Kelthane Added to Various Crops

Crop	Sample, Grams	Av. Control Absorbance	P.P.M. Added	Av. Recovery, %	Std. Dev., %	Determinations
Apple leaves	15.5	0.010 ± 0.005	1.0	90	12	6
Alfalfa	16.7	0.030 ± 0.008	1.0	63	13	7
Almond husk	150.0	0.013 ± 0.004	0.1	81	10	4
Almond nut	12.5	0.015 ± 0.005	1.0	66	8	4
Bean plant	15.0	0.015 ± 0.004	1 0	85	8	6

Recovery and Sensitivity. The overall recovery obtained by adding 1 to 100 μg. of Kelthane to 200-gram whole milk samples is 91 to 95%. With the use of 5-cm. path length absorption cell, a sensitivity of 0.01 p.p.m. was obtained.

Interferences. In general, any volatile chlorinated hydrocarbon which forms the Fujiwara complex will interfere as will any compound which liberates these chlorinated hydrocarbons upon base hydrolysis. In very sensitive experiments where 1 or 2 μg. have to be determined, special precautions should be taken to ensure that the room atmosphere is free of chloroform and its analogs.

Other Applications. The modified method may be used for analysis on all plant extractives previously reported by Rosenthal *et al.* (*6*). In addition, Table II shows recovery values obtained when the procedure was applied to other extractives.

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