Separation and Determination of Trace Amounts of Bacteriostats Using a Continuous Distillation Extraction Apparatus

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ABSTRACT

Methodology is described for the determination of very low levels ($\leq$ 100 ng/ml) of triclocarban and triclosan in blood. The technique involves the use of a special continuous distillation extraction apparatus for separating these bacteriostats from blood in a relatively selective and automatic manner. In this procedure the triclosan distills intact, while the triclocarban is hydrolyzed, and the hydrolysis products are distilled and extracted. In addition to describing the apparatus, data are presented on the completeness of the extraction procedure and recoveries from fortified blood samples. Final measurements of the bacteriostats are accomplished by electron capture gas chromatography following appropriate derivative formation to enhance detection sensitivity.

INTRODUCTION

Bacteriostats such as triclocarban and triclosan are used in soaps and other personal care products. For this reason it is important to have very sensitive methods for analyzing trace quantities of these materials in blood and other body tissues. Most trace methods reported in the literature for hexachlorophene (1-5) and various pesticide residues in plant and animal tissue (6-10) suffer from inherent difficulties involving long and tedious multiple extraction and clean-up procedures prior to final measurement. The formation of emulsions during the process often causes additional complications (1,11,12). In this paper we report on the use of a continuous steam distillation/extraction apparatus for the separation of triclocarban (hydrolysis products) and triclosan (intact) from blood in a relatively clean, selective, and automatic manner.

The distillation/extraction head used (Fig. 1) was very similar to that described by Bleidner et al. (13,14) and later modified by Baunok and Geissbuehler (15,16) for their work on determining various urea herbicide residues. In general, the sample to be analyzed is placed in a digestion flask containing aqueous base (or acid) and attached to the lower arm of the apparatus. A nonaqueous solvent, immiscible with and less dense than water (e.g., hexane), is placed in another flask and attached to the upper arm of the apparatus. The U-tube of the unit is filled with enough distilled water to prevent organic solvent from returning to the digestion flask. With a water-cooled condenser in position on top of the apparatus, heat is applied to both flasks at such a rate that approximately equal amounts of condensed vapors of solvent and water pass through the capillary. After the prescribed digestion, distillation/extraction period (3 hr in our procedure), the bacteriostatic components of interest are entirely concentrated in the hexane-containing flask and are ready for subsequent derivative formation and final gas chromatography (GLC) measurement using electron capture detection.

EXPERIMENTAL PROCEDURE

Apparatus and Equipment

The distillation/extraction head used was constructed as shown in Figure 1.

A Hewlett-Packard 5750 gas chromatograph was equipped with Ni^{63} (2 μCi) electron capture detector and operated at pulse interval of 150 μsec; a 5 ft x 1/8 in. stainless steel column containing 10% SE-52 on Chromosorb W AW HMDS, 60/80 mesh, was used with a carrier gas of argon with 5% methane.

Other equipment included round bottom flasks, 250 ml and 100 ml, ℨ 24/40; Liebig condenser, 20 in., ℨ 24/40; heating mantles, 250 ml and 100 ml; and variable transformers. Teflon sleeves, ℨ 24/40 (Fisher Scientific, Fairlawn, NJ), were used between all ground glass connections.

Reagents and Solutions

The chemicals were reagent grade, and the solutions were prepared in distilled water on a weight per volume basis. The following were used: sodium hydroxide, 20%, 50%; hydrochloric acid, 1 *N*; sulfuric acid, 10%; n-hexane (Baker analyzed); Sag 10 antifoam (Union Carbide, New York, NY); potassium sulfite; nitrogen (Linde, H.P. dry); sodium nitrite, 1% (prepared fresh daily); ammonium sulfamate, 10% (replaced every second day); potassium iodide/iodine solution, prepared by dissolving 2.5 g of iodine in 50 ml of 10% aqueous potassium iodide (replaced

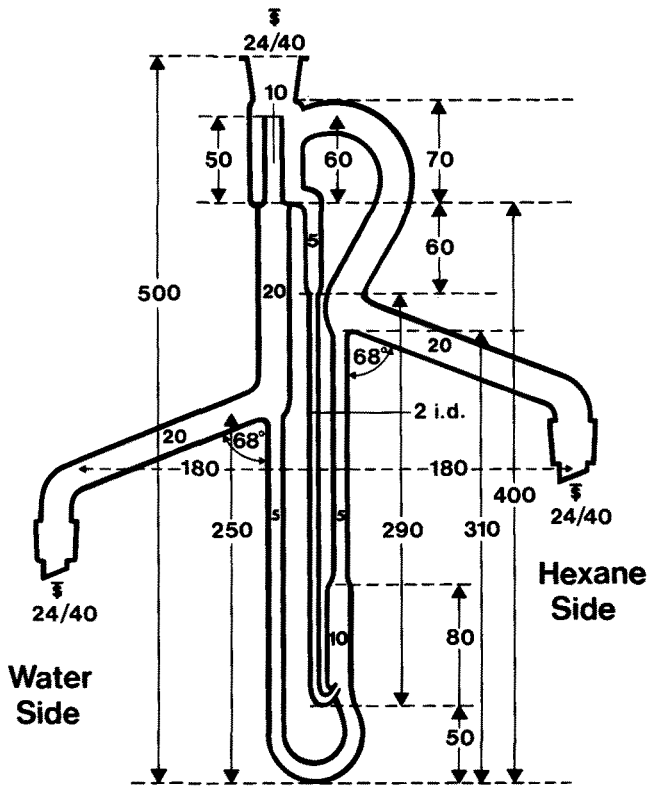

FIG. 1. Distillation/extraction apparatus; dimensions in mm as reported by I. Baunok and H. Geissbuhler. Reprinted from J. Agric. Food Chem. 19:366 (1971). Copyright by the American Chemical Society. Reprinted by permission of copyright owner.

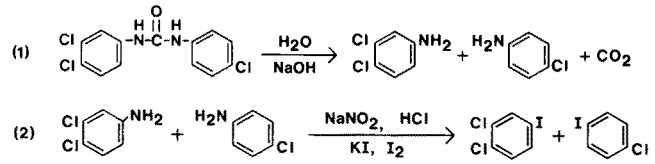

FIG. 2. Summary of chemical reactions involved in triclocarban determination. (1) Hydrolysis of triclocarban to chloroanilines; (2) diazotization and iodination of chloroanilines to chloroiodobenzene derivatives.

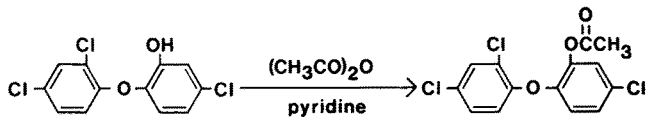

FIG. 3. Acetylation reaction for triclosan determination.

every second day); acetylating solution, prepared fresh daily by mixing 0.8 ml acetic anhydride with 2 ml pyridine; triclocarban solution (a stock solution [1 mg/ml] was prepared by dissolving 100 mg of TCC® [a registered trademark of the Monsanto Co., St. Louis, MO] in 100 ml acetone); and triclosan solution (a stock solution [1 mg/ml] was prepared by dissolving 100 mg of Irgasan DP-300 [Ciba-Geigy, Ardsley, NY] in 100 ml acetone; each stock solution of bacteriostat was further diluted with acetone to a concentration of 1 μg/ml; aliquots [i.e., 25 μl, 50 μl, 100 μl, etc.] of the diluted bacteriostatic solutions were run through the procedure to establish recovery information and determine GLC calibration data).

METHODS

Analytical Procedure for Triclocarban

The analysis scheme for triclocarban (3,4,4′-trichlorocarbanilide) consisted of hydrolysis to chloroanilines (3,4-dichloroaniline and p-chloroaniline), followed by simultaneous distillation and extraction into hexane, diazotization, and reaction with KI/I_2 (Sandmeyer reaction) to form chloroiodobenzene derivatives (3,4-dichloroiodobenzene and p-chloroiodobenzene) and final determination by GLC. The chemical reactions involved in this determination are summarized in Figure 2. The detailed procedure is as follows.

The distillation/extraction (d/e) apparatus was set up by filling the U-tube of the d/e head about 1/3 full with distilled water and attaching a water-cooled condenser.

One hundred milliliters of 20% NaOH, 1 ml of blood sample, three boiling chips, and one drop of Sag 10 antifoam were placed into a 250 ml round bottom flask. This flask was then attached to the lower arm of the d/e apparatus. Forty milliliters of hexane and one boiling chip were placed into a 100 ml round bottom flask. This flask was attached to the upper arm of the d/e apparatus.

With heating mantles and variable transformers in position, heat was applied to both flasks such that approximately equal amounts of water and hexane were vaporized and condensed (this is judged by the size of the solvent globules which are continually formed in the feeding capillary). Alkaline hydrolysis and steam distillation/extraction were continued for 3 hr.

After completion of the distillation/extraction process, the hexane solution was allowed to cool to room temperature. The 100 ml flask containing the hexane distillate was removed from the d/e head, and five drops of 1 *N* HCl were added. Using a stream of nitrogen, but without heat, all the hexane was evaporated (allowing a few drops of aqueous HCl to remain). To this residue was added 10 ml of cold 1 *N* HCl. The flask was stoppered with a ℑ 24/40 glass stopper and the contents mixed by swirling.

The sample was then placed in an ice-water bath containing sodium chloride and cooled to 0 C. For diazotization, 2 ml of 1% freshly prepared sodium nitrite solution were added, and the sample was swirled and allowed to remain in the ice bath for 30 min. Excess nitrite was then destroyed by adding 2 ml of 10% ammonium sulfamate solution. After 5 min, the flask was shaken vigorously until nitrogen generation ceased. To the ice cold sample was added 0.5 ml of KI/I_2 solution. The sample was removed from the ice bath, swirled, and allowed to stand at room temperature for exactly 30 min.

The excess iodine was reduced by adding 200 mg of potassium sulfite powder, whereupon the solution turned colorless. The reaction mixture was made alkaline by adding ca. 1 ml of 50% NaOH and allowed to cool to room temperature.

The sample was then extracted with exactly 10 ml of hexane pipetted into the reaction flask. After vigorous shaking, the layers were allowed to separate and a few milliliters of the upper organic layer were transferred with a pipette to a small vial having a teflon or aluminum lined cap.

Five-microliter portions of sample extract were injected into the gas chromatograph. The peak heights were measured and compared with those obtained from standard triclocarban samples (e.g., 50 ng, 100 ng, etc.) prepared and run through the entire procedure (preferably on the same day that samples were run).

Under the GLC conditions employed (injection temperature 130 C, column temperature 105 C, detector temperature 230 C, flow rate 25 ml/min), the retention times were 2.9 min for p-chloroiodobenzene and 6.5 min for the 3,4-dichloroiodobenzene derivative.

Analytical Procedure for Triclosan

The analysis of triclosan (2-hydroxy-2′,4,4′-trichlorodiphenyloxide) consisted of simultaneous distillation/extraction from acidic solution into hexane, followed by acetyl derivative formation and GLC determination. The chemical reaction involved is shown in Figure 3. The detailed procedure was as follows.

The d/e apparatus was set up by filling the U-tube of the d/e head about 1/3 full with distilled water and attaching a water-cooled condenser.

One hundred milliliters of 10% H_2SO_4, 1 ml of blood sample, three boiling chips, and one drop of Sag 10 antifoam were placed into a 250 ml round bottom flask. This flask was then attached to the lower arm of the d/e apparatus.

Forty milliliters of hexane and one boiling chip were placed into a 100 ml round bottom flask. This flask was attached to the upper arm of the d/e apparatus.

With heating mantles and variable transformers in position, heat was applied to both flasks such that approximately equal amounts of water and hexane were vaporized and condensed and passed down the capillary. Distillation/extraction was continued for 3 hr.

After completion of the distillation/extraction process, the hexane solution was allowed to cool to room temperature. The 100 ml flask containing the hexane extract was removed from the d/e head, and the sample was carefully evaporated to dryness with a stream of nitrogen (*without* heat).

The residue was dissolved in 1.0 ml of hexane. Ten microliters of freshly prepared acetylating reagent was added. The flask was stoppered (ℑ 24/40 glass stopper) and the contents thoroughly mixed by swirling. The sample was then allowed to stand at rom temperature for 90 min (or in a constant temperature bath at 60 C for 15 min).

Five-microliter portions of the sample solutions were

injected into the gas chromatograph. The peak heights were measured and compared with those obtained from standard triclosan samples (e.g., 25 ng, 50 ng, etc.) prepared and run through the entire procedure (preferably on the same day that samples were run).

Under the GLC conditions employed (injection temperature 215 C, column temperature 205 C, detector temperature 310, flow rate 50 ml/min), the retention time for acetylated triclosan was 3.7 min.

RESULTS AND DISCUSSION

The use of antifoam was required to prevent the blood samples from bubbling excessively during reflux, especially in the early (digestion) stages of the distillation/extraction process. Union Carbide's Sag 10 was found to be suitable; some other antifoam agents were found to impede the distillation/extraction process (resulting in low recoveries) or to contain volatile matter which subsequently interferred with the GLC determination.

To reduce possible contamination and residue build-up (from previous samples) in the flasks, condensers, and distillation/extraction apparatus, reagent blanks consisting of 20% NaOH (or 10% H_2SO_4 for triclosan determinations) and hexane were distilled and extracted in the equipment between sample runs. The glassware was then successively rinsed with ethanol (3A), distilled water, and acetone and finally oven dried at 130 C before reuse.

Triclocarban Method

Because triclocarban itself is not sufficiently volatile to be determined by GLC, it was necessary to split the compound into volatile components which could be gas chromatographed. The procedure which was developed is based upon the classical alkaline hydrolysis of substituted ureas to the corresponding aromatic amines. The method, however, differs slightly from published procedures (13-16) in that the distillate containing the substituted anilines was evaporated in the presence of a small amount of HCl instead of being extracted with aqueous HCl. This change was made in order to eliminate sample transfers and additional glassware wherever possible.

The iodo derivatives, chosen because of their strong response to electron capture detection, were prepared by reaction with KI/I_2 at room temperature instead of in a boiling water bath (15,16). This modification simplified the procedure without diminishing product yield. Using this procedure, the ratio of the GLC peak heights for the p-chloroiodobenzene and 3,4-dichloroiodobenzene derivatives of the triclocarban hydrolysis products have been quite reproducible even at various triclocarban concentrations (Table I). This constancy allows us to be more confident that the GLC peaks observed are due to the initial presence of triclocarban and not from other sources which may give one or the other of the hydrolysis products.

Calibration curves prepared by plotting peak height of the derivatives of the hydolysis products versus triclocarban concentration are shown in Figure 4. The curves are essentially linear in the concentration range of interest (0-200 ppb).

The validity of the method was shown by intentionally spiking blood samples at various triclocarban levels and measuring the recoveries obtained. Typical gas chromatograms of control blood, standard triclocarban, and blood spiked with triclocarban are shown in Figure 5. Recovery values from blood samples spiked with 100 ppb and 50 ppb of triclocarban have averaged ca. 87% and are summarized in Table II. The calculated recovery values were obtained by comparing spiked samples with triclocarban standards run through the entire method.

One should comment on the absolute recoveries attainable by this method. As might be expected, the Sandmeyer reaction does not give quantitative yields, our absolute recoveries being 40% for p-chloroiodobenzene and ca. 70% for the 3,4-dichlorobenzene derivative. It has been our practice, therefore, to run standards through the entire method along with test samples in an effort to equalize possible contamination or interferences contributed by

TABLE I

Reproducibility of Chloroiodobenzene Formation from Triclocarban

Triclocarban concentration (ppb)	Peak I height / Peak II height[a]
100	0.70
100	0.73
100	0.69
100	0.81
100	0.78
50	0.79
50	0.72
50	0.66
50	0.63
50	0.69
	Average ± standard deviation: 0.72 ± 0.06

[a]Peak I is p-chloroiodobenzene; Peak II is 3,4-dichloroiodobenzene.

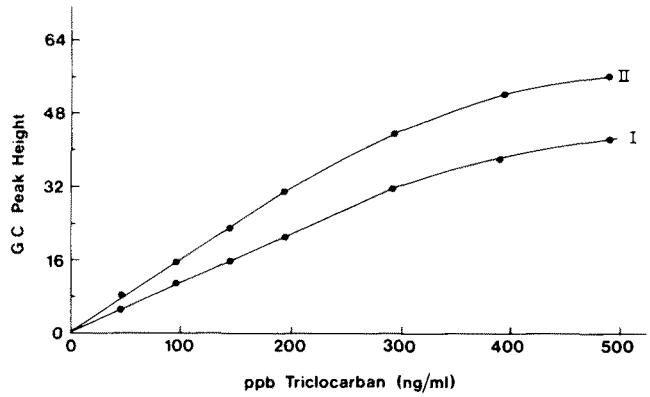

FIG. 4. Gas liquid chromatography calibration curves for iodo derivatives of triclocarban hydrolysis products, peak height versus triclocarban concentration (ng/ml). I is p-chloroiodobenzene and II is 3,4-dichloroiodobenzene.

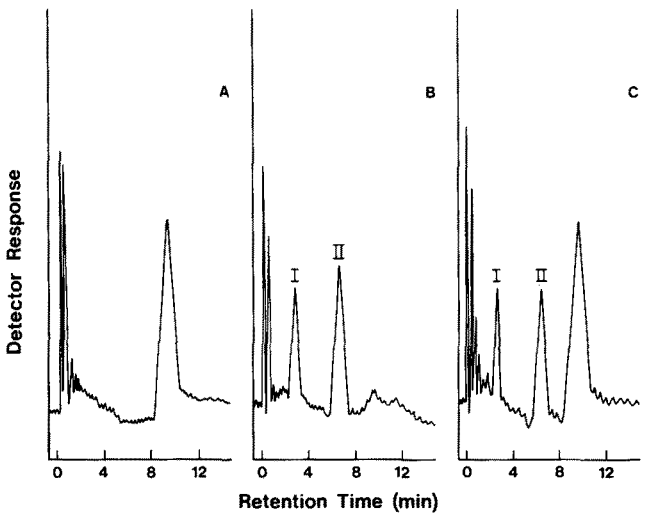

FIG. 5. Gas liquid chromatograms from A, 1 ml whole blood; B, standard triclocarban, 100 ng/ml; C, 1 ml whole blood spiked with 100 ng triclocarban. All samples were hydrolyzed and derivatized as described in the triclocarban procedure. I is p-chloroiodobenzene and II is the 3,4-dichloroiodobenzene derivative.

TABLE II

Recoveries of Triclocarban from Fortified Blood Samples[a]

Triclocarban added (ppb)	Recovery (%)
100	86
100	79
100	95
50	83
50	88
50	92
	Average: 87.2 ± 5.8%

[a]Samples were fortified by adding aliquots of a standard solution of triclocarban in acetone (1 ng/μl) to the digestion flask.

TABLE III

Efficiency of Distillation/Extraction Procedure for Triclosan

Triclosan added (ng)	Recovery (%)
25	98
25	96
50	91
50	97
100	93
	Average: 95.0 ± 2.9%

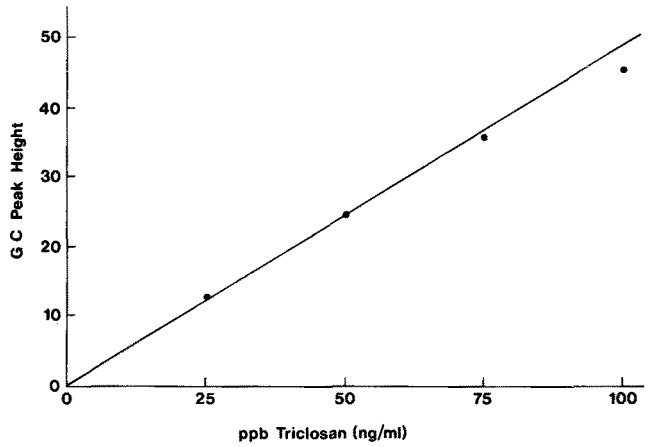

FIG. 6. Gas liquid chromatography calibration curve for acetylated triclosan, peak height versus concentration (ng/ml).

TABLE IV

Reproducibility of Triclosan Method at the 100 ppb Level

Sample number	Peak height (arbitrary units)
1	19.3
2	18.7
3	19.9
4	18.6
5	19.6
6	17.5
Average ± standard deviation: 18.9 ± 0.9	
Percent relative standard deviation: 4.6%	

reagents and also to discount any method variables including nonquantitative absolute yields (1,7,14,17).

The detection limit of the method is ca. 50 ppb (ng/ml) based upon a 1 ml blood sample. According to the procedure, this is equivalent to injecting 25 picograms of triclocarban into the gas chromatograph. Because the samples are quite clean in the GLC region of interest, it appears that lower levels of triclocarban in blood could probably be observed by starting with a larger sample size.

TABLE V

Recoveries of Triclosan from Fortified Blood Samples[a]

Triclosan added (ppb)	Recovery (%)
100	97
100	82
100	101
100	86
100	97
50	92
50	86
50	99
25	93
	Average: 92.6 ± 6.6%

[a]Samples were fortified by adding aliquots of a standard solution of triclosan in acetone (1 ng/μl) to the digestion flask.

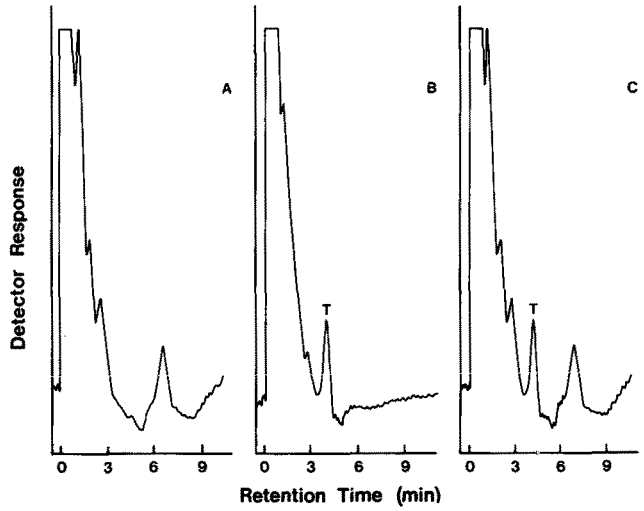

FIG. 7. Gas liquid chromatograms of acetylated extracts from A, 1 ml whole blood; B, standard triclosan, 50 ng/ml (T); C, 1 ml whole blood spiked with 50 ng triclosan (T).

Triclosan Method

Although previous reports of distillation/extraction procedures (for various herbicide residues, etc.) have involved hydrolysis from basic solution, triclosan because of its phenolic group, hydrolytic stability, and steam volatility (18) was distilled/extracted from acidic solution. The acetyl derivative was formed in order to sharpen the triclosan GLC peak, which has a tendency to tail.

To check the efficiency of the overall method, known amounts of triclosan were distilled/extracted and analyzed according to the procedure and compared (in the gas chromatograph) with standard solutions of triclosan acetate. The results (Table III) show an average yield of 95% in the 25-100 ng range.

A linear calibration curve relating GLC peak height with triclosan concentration is shown in Figure 6. An indication of the precision of the method can be obtained from the data in Table IV. This series of six (6) individual triclosan samples (at the 100 ng level) were run through the entire procedure and had a relative standard deviation of only 4.6%.

The validity of the method was demonstrated by running blood samples with different amounts of intentionally added triclosan. The recovery results, which averaged 93%, are summarized in Table V. Typical GLC traces of control blood, standard triclosan, and blood spiked with triclosan are shown in Figure 7. It is apparent that the method can detect 25 ppb of triclosan in a 1 ml blood sample. (This is equivalent to an injection of 125

picograms of triclosan into the gas chromatograph). In addition, we have been able to extend the detection to ca. 8 ppb by increasing the sample size from 1-3 ml.

The described distillation/extraction apparatus can be an effective means of digesting and separating steam distillable materials or their hydrolysis products from whole blood or serum in a quantitative and essentially automatic manner. Although the d/e head has to be custom made, the analytical technique requires a minimum of operator attention and the extracts obtained generally require no additional clean-up.

In the procedures described above for triclocarban and triclosan, the samples required no manual transfer once the process began. In fact, aliquots of final product (after derivatization) were frequently injected into the gas chromatograph directly from the 100 ml flask which was attached to the d/e head in the intial steps of the procedure.

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