Semiautomated method for the colorimetric determination of plasma free fatty acids

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SUMMARY A semiautomated procedure is described for the estimation of plasma free fatty acids. The method requires the preliminary preparation of a phospholipid-free plasma lipid extract which is then analyzed automatically by a colorimetric procedure based on the solubility of copper soaps in chloroform, with subsequent complexing of the copper with diethyldithiocarbamate and measurement of the extinction at 440 mμ.

KEY WORDS semiautomatic · colorimetric · determination · plasma · free fatty acids · lipid extraction · diisopropyl ether · copper soaps · diethyldithiocarbamate

IN THE COURSE of a study of the effects of different adrenergic drugs on the release of free fatty acids (FFA) in plasma, the need arose for a simple, rapid, and reproducible microtechnique for the estimation of plasma FFA. Colorimetric methods for the determination of long-chain fatty acids have been reported by Ayers (1) and Iwayama (2) which are based on the solubility of copper soaps in chloroform with subsequent measurement of the extinction coefficients. Barreto and Mano (3) and Duncombe (4, 5) have recently modified and improved these methods by complexing the copper with sodium diethyldithiocarbamate.

In the present paper the latter method (5) has been adapted to give a semiautomatic determination. The method requires the preliminary extraction of plasma with diisopropyl ether and silicic acid, according to the procedure of Mendelsohn and Antonis (6), giving a phospholipid-free lipid extract which, after being taken to dryness, is redissolved in chloroform and fed into an autoanalyzer (Technicon Ltd., Chertsey, Surrey, England). The chloroform solution of the fatty acids reacts initially with an aqueous copper nitrate–triethanolamine reagent; the excess reagent is separated automatically, and the chloroform phase then meets a solution of sodium diethyldithiocarbamate in butanol before passing through the flow-cell of a colorimeter with subsequent recording of the optical density. In this manner the method has become highly reproducible; it is free from errors caused by incomplete removal of excess copper reagent prior to treatment with thiocarbamate, and 30 specimens of plasma may be analyzed per hour.

REAGENTS AND STANDARDS

All reagents and solvents used were analytical reagent grade. Diisopropyl ether was freed from peroxide by passage through a column of activated alumina just before use. Occasionally $CHCl_3$ may produce high blank values because of the presence of ethanol, but this may be prevented by washing with distilled water and drying over $CaCl_2$ before use. Baker Analyzed reagent grade silicic acid powder was suitable without washing or activation for the preparation of the phospholipid-free lipid extract.

The *copper reagent* consisted of 9 volumes of aqueous 2 M triethanolamine, 1 volume of 2 N acetic acid and 10 volumes of 10% (w/v) $Cu(NO_3)_2$.

The *diethyldithiocarbamate reagent* was a 0.2% (w/v) solution of sodium diethyldithiocarbamate in butanol.

Both reagents were stable for at least a week when stored at 4°.

Fatty Acid Standards

Stock standard solution: 96.15 mg of palmitic acid was dissolved in chloroform and made up to 250 ml (1.5 meq/liter). The working standards were prepared by diluting 1 to 6 ml aliquots of the stock standard to 100 ml with chloroform, contained 15–90 μeq palmitic acid per liter, and corresponded to the range 375–2250 μeq/liter of plasma when carried through the procedure outlined below (1 : 25 plasma extract).

PROCEDURE

Preparation of Phospholipid-Free Extract

Silicic acid (1.2 g) is slurried with 7.5 ml of diisopropyl ether in a stoppered test tube containing a few large glass beads. Plasma (0.3 ml) is added, and the mixture is well shaken until free from lumps. After the silicic acid has settled, a 5 ml aliquot of the supernatant extract is taken off into a test tube, evaporated, and blown to dryness, and the residue is redissolved in 5 ml of chloroform.

The phospholipid-free chloroform extracts of plasma lipids are stable indefinitely if stored at 4°. In the automatic procedure 30 specimens may be analyzed per hour, and therefore a considerable number of extracts (approximately 150) can be prepared, stored, and then analyzed together at a single session.

Automatic Analysis

The Technicon Autoanalyzer sampler II, proportioning pump, colorimeter, and recorder are required, with the arrangement of flow lines shown diagrammatically in Fig. 1.

A constant-level source of blank solvent (chloroform) was provided in an inverted flask in order to maintain a constant flow of liquid through the system. This replaces the normal reservoir provided with the sampler II, which is not resistant to chloroform. Glass sample cups (0.5 inch internal diameter, 1 inch internal depth, and 3 ml volume) are used instead of the usual polystyrene cups supplied with the autoanalyzer. The cover plate normally supplied, which is also not resistant to chloroform, is fitted with a Teflon disk taped to the under-surface.

Acidflex tubing is used in the pump manifold for all lines in contact with chloroform. Standard Tygon tubing serves for the air line and the aqueous copper reagent, and Solvaflex tubing is used for the butanol solution. The internal diameters of the pump tubing (Technicon Ltd.) are as indicated in Fig. 1. All delivery lines were polythene tubing (Lionel Andrews, Ltd., London, England) of 1.5 mm bore and 0.6 mm wall thickness, except for the

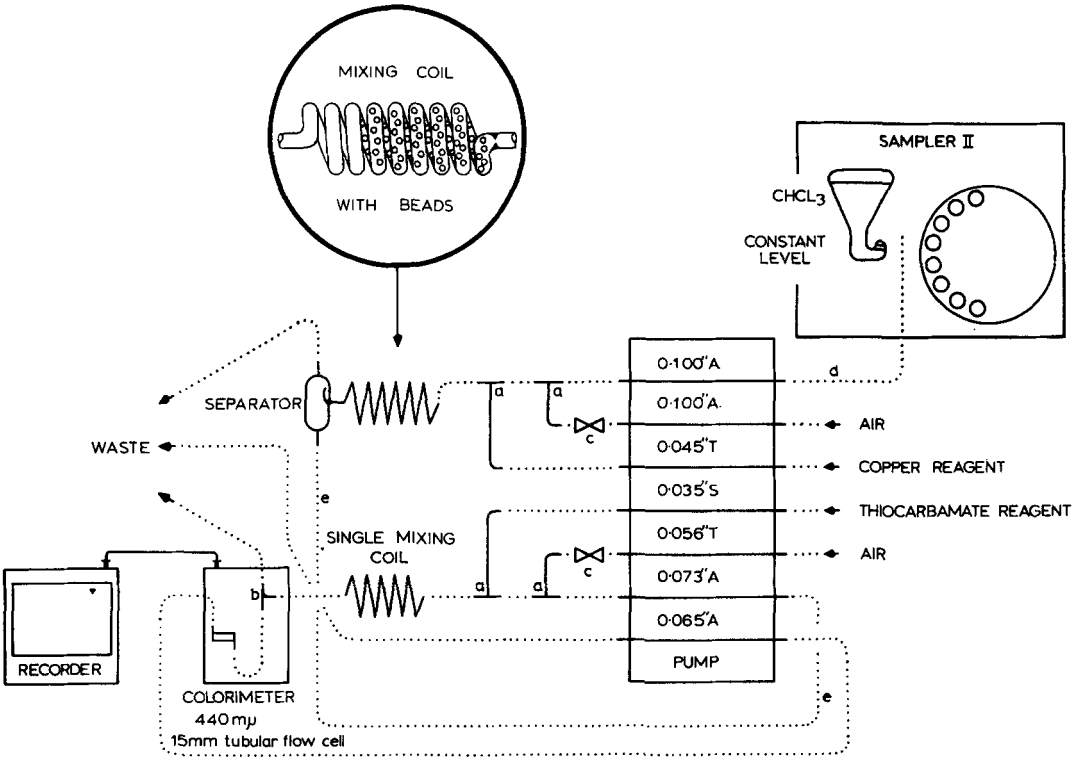

FIG. 1. Flow diagram for FFA estimation.

A, Acidflex
T, Tygon
S, Solvaflex
} pump tubing, bore as indicated (inches).

a, glass h-piece, capillary side arm; *b*, glass debubbler T; *c*, 0.005 inch i.d. standard pump tubing pulse suppressor; *d*, polythene tubing, 1.00 mm i.d., 0.25 mm wall thickness; *e*, polythene tubing, 0.75 mm i.d., 0.60 mm wall thickness.

All other delivery tubing of polythene, 1.5 mm i.d., 0.60 mm wall thickness. All connections with Acidflex tubing and polythene nipples.

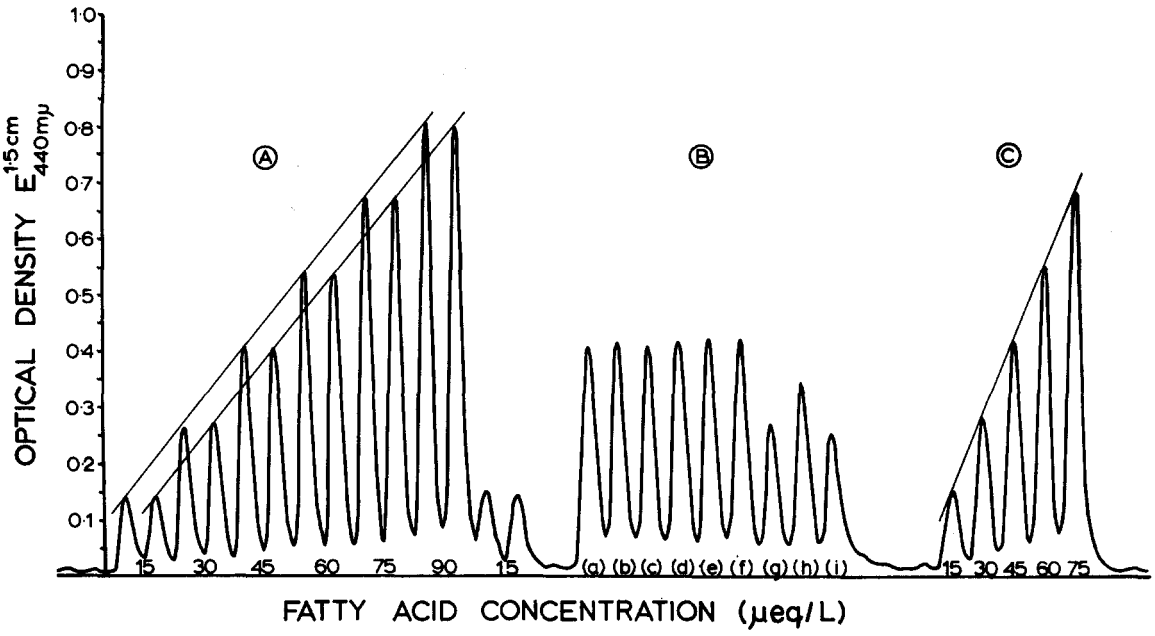

FIG. 2. Relationship between fatty acid concentration and optical density.

A. Palmitic acid standard in chloroform: range 15–90 μeq/liter.
B. Solutions in chloroform of 45 μeq/liter of the following: (*a*) 12:0; (*b*) 14:0; (*c*) 16:0; (*d*) 18:0; (*e*) 18:1; (*f*) 18:2 fatty acids; (*g*) dipalmitoyl lecithin (synth); (*h*) dipalmitoyl cephalin (synth); (*i*) beef brain sphingomyelin.
C. Blank and palmitic acid solutions in diisopropyl ether carried through full extraction procedure: range 15–75 μeq/liter.

line from the sampler to the pump, which was 0.1 mm bore and 0.25 mm wall thickness, and the line from the separator to the pump, which was 0.75 mm bore and 0.6 mm wall thickness. After passing through the pump, the sample line was connected through glass h-pieces fitted with capillary side arms. All connections were made with polythene nipples and acidflex tubing.

The sampler II was loaded with specimens, immediately covered with the cover-plate to prevent evaporation of chloroform from the sample cups, and run at a rate of 30 samples per hr with a 1:2 cam. Operating conditions therefore corresponded to 40-sec sampling of the specimen (approximately 2.26 ml of 1:25 plasma extract) followed by 80 sec of chloroform (blank).

The specimen drawn from the sample plate (or, alternately, chloroform from the reservoir) and air are pumped together with the copper reagent through a special mixing coil filled with glass beads before being separated into two phases. The aqueous copper phase, together with a portion of the chloroform phase, passes to waste, and the remainder of the chloroform phase is redrawn through the pump after which air is drawn in, followed by the dithiocarbamate reagent. The mixture then passes through a single mixing coil before entering the colorimeter, which is fitted with a glass debubbler T and a tubular flow-cell with 15 mm light-path. The extinction is measured at 440 mμ.

Working standards were analyzed at the start of a session, and subsequently as every tenth specimen in order to correct for the baseline drift that may occasionally occur.

The aperture of the colorimeter was selected, and the sensitivity adjusted, to give 98–100% transmission with the chloroform blank. Under the conditions described, the 90 μeq fatty acid standard gave a reading of approximately 15–20% transmission. With a linearized recorder the corresponding extinctions were 0–0.01 and approximately 0.8, respectively.

At the end of a run the apparatus was washed through with methanol in all lines, and dried by drawing air through the system. With this procedure pump tubing remained satisfactory after months of continuous use.

RESULTS

Fatty Acid Determination

With the above manifold and reagents there is a linear relationship between the extinction and the concentration of FFA (palmitic acid) in the standards, as shown in Fig. 2, where it can be seen that replication of specimens is excellent, the errors introduced by marked changes of concentration are negligible, and the responses given by equivalent solutions of the major fatty acids commonly found in plasma FFA are almost identical. For solutions

containing 45 μeq/liter, the following extinctions were obtained:—lauric acid, 0.396; myristic acid, 0.405; palmitic acid, 0.397; stearic acid, 0.406; oleic acid, 0.411; and linoleic acid, 0.410. That it is essential to remove phospholipids was revealed by the following high extinctions obtained with the same concentrations of these phospholipids: dipalmitoyl lecithin (synthetic), 0.260; dipalmitoyl cephalin (synthetic), 0.330; beef brain sphingomyelin, 0.240.

Preparation of Phospholipid-Free Lipid Extract

The extraction of plasma FFA with silicic acid and diisopropyl ether has been tested by comparison with the microtitration technique of Shafrir and Steinberg (7), and by determination of the recovery of palmitic acid from standards prepared in diisopropyl ether (at the same concentrations as the chloroform working standards), and subjected to the recommended extraction procedure in the presence of saline instead of plasma (i.e., 7.5 ml aliquots of standards in diisopropyl ether, 0.3 ml of saline, and 1.2 g of silicic acid).

For the former comparison the agreement was very good, and the results indicated an average difference between the two methods of 12.2 μeq over the range 360–1400 μeq of fatty acid per liter of plasma, with a standard error of differences between paired items of ±11.6 μeq/liter, indicating that there was no significant difference between the two procedures ($0.4 > P > 0.3$).

In the latter experiment, results were identical with those obtained with the chloroform standards (also shown in Fig. 2), and it was evident that neither the diisopropyl ether nor the silicic acid contributed significantly to a reagent blank, and that fatty acids were not lost through adsorption to silicic acid or incomplete elution by diisopropyl ether.

The complete removal of phospholipids from the diisopropyl ether extracts by adsorption on silicic acid in the recommended procedure has been tested and confirmed by estimation of the phosphorus content using the sensitive microtechnique of Chen et al. (8). With this technique 6.4 μg of P in a final volume of 10 ml gave an extinction of approximately 0.600 at 820 mμ (1 cm light-path). Five-milliter aliquots of the diisopropyl ether extracts derived from specimens of plasma containing approximately 260 mg of phospholipid per 100 ml (which would normally give extinctions of approximately 2.000) showed values of less than 0.005. Removal of phospholipids from the extracts by adsorption on silicic acid was therefore highly efficient (>99.75%). The procedure has been tested at temperatures from 15 to 25°, and in all cases removal of phospholipid was complete. Different batches of silicic acid (Baker) have behaved identically, and the product from another manufacturer (Mallinkrodt) has proved equally suitable provided the particle size was finer than 100 mesh. Zeolite (Taylor & Co., Baltimore, Md.) was equally effective after it had been ground to finer than 100 mesh.

The absence of phospholipids in the extracts has also been confirmed by thin-layer chromatography.

Standard Error of the Method

Free fatty acids have been estimated on duplicate pairs of 20 plasma specimens. The mean difference between duplicate pairs was 12 μeq/liter, with a standard error of a single determination of ±10.5 μeq/liter for the range 220–1300 μeq/liter of plasma FFA. (On duplicate aliquots of the same plasma extracts the standard error was ±7.8 μeq/liter).

The precision of the automated stage of the procedure was tested by loading the sampler with 40 identical extracts (FFA about 45 μeq/liter), and carrying out the procedure as described. The mean extinction obtained was 0.424 with a standard deviation of ±0.006.

Over the 80 min period of the analysis, the mean extinctions for successive sets of 10 extracts were 0.421, 0.423, 0.424, and 0.427, respectively. While a trend due to evaporation was indicated, this was slight, and errors introduced in this way were negligible (<1.5%).

DISCUSSION

The procedure recommended consists of two independent stages: (*a*) the preparation of a phospholipid-free plasma lipid extract, and (*b*) the automated analysis of the chloroform extract of fatty acids. Since phospholipids form a complex with copper which is soluble in chloroform and give a response similar to that given by fatty acids when reacted with the copper reagent (5), it is essential that the former are completely removed from the lipid extract before FFA can be estimated. As reported previously (6), and confirmed above, treatment of plasma (or serum) with diisopropyl ether and silicic acid provides a simple and efficient method for the extraction of neutral lipids and FFA, free from other acidic plasma components including phospholipids.

Attempts were made to alter the plasma extraction technique by the use of chloroform instead of diisopropyl ether for the preparation of an extract which would not need reconstitution. Van Handel and Zilversmit (9) and later Cheng and Zilversmit (10) have prepared phospholipid-free serum lipid extracts using chloroform and zeolite (Doucil) for the lysis of the protein-lipid bonds and the adsorption of phospholipids and nonlipid serum components, but made no comment on the recovery of FFA. I found the recoveries using chloroform and either silicic acid or zeolite to be low, variable, and not reproducible. Similar findings have also been reported by Albrink (11). Furthermore, when the plasma chloroform

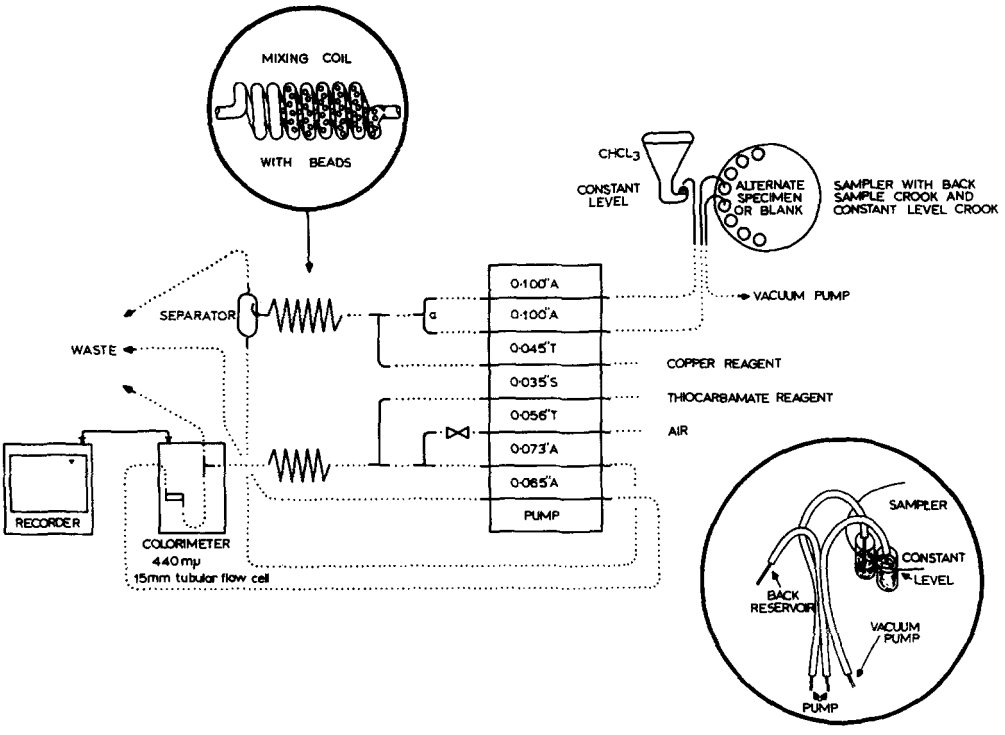

FIG. 3. Flow diagram for FFA estimation (using original sampler).

a, glass Y-piece, all capillary.

extracts were analyzed by the colorimetric technique negative reagent blanks were produced. These could be eliminated by evaporating the extract to dryness and redissolving it in chloroform; but such a procedure would have no advantage over the use of diisopropyl ether.

For highly lipemic plasma (or serum) it is preferable to use aliquots smaller than 0.3 ml in the extraction procedure, and to add water or saline to render conditions identical with those recommended, so as to ensure quantitative extraction of all the neutral lipids. In post-heparin plasma, in which neutral lipid concentrations may be normal but higher concentrations of FFA are found, the recommended extraction procedure is used and the chloroform extracts are diluted before analysis.

In the automated procedure with the recommended sampling rate, the extinction coefficients are approximately two-thirds of those obtained in the manual method (5). Identical values may be obtained by increasing the time of sampling of the specimens until plateaus, instead of peaks, are recorded. This is not essential in practice, however, since the heights of the peaks have been found to be directly proportional to the heights of the plateaus; and in either case the relationship between the height attained and the concentration of the sample is linear.

In the automated analysis the results were more reproducible when twice the strength of the reagents recommended by Iwayama (2) and Duncombe (5) were used. Proportions of reagents were also slightly different. These changes have been introduced mainly to suit the requirements of the sample cups and tubing available with the autoanalyzer, and do not appear to have affected the specificity or the sensitivity of the method.

The separation of the chloroform and aqueous phases in the automated technique has proved to be far more efficient than in the manual method where extreme care must be taken to prevent contamination of the chloroform phase by traces of the copper reagent, and duplication and even triplication have been found essential for the production of reliable results.

Errors due to evaporation of chloroform have been found to be negligible, provided that the cover plate fits directly on top of the sample cups, as shown by the results when the sampler was fully loaded with 40 identical specimens, covered, and run for a period of 80 min (room temperature 23°). Nevertheless in an attempt to find a relatively nonvolatile solvent other than chloroform to redissolve the samples, a number of chlorinated hydrocarbons were tested. In all cases, as reported by Duncombe (5), the sensitivity was lower than with chloroform. 1,1,2-Trichloroethane (bp 113°) gave approximately two-thirds of the extinction obtained with chloroform, but the relationship between concentration and extinction for a series of palmitic acid standards was

linear. This solvent could therefore be used as an alternative to chloroform in laboratory conditions of extreme humidity and temperatures higher than 25°, where the possibility of evaporation and consequent concentration of specimens while in the sampler is greatly increased.

ADDENDUM

The procedure described above may be carried out using the original sampler (I) supplied by Technicon, but the following modifications to the sampler are essential for successful operation.

The sampler unit was modified by soldering to the original sampling crook two additional crooks made of suitably shaped metal tubing, the first in the same direction as the original crook and the second in the opposite direction (see inset, Fig. 3).

The additional sampling crooks on the sampler unit were required (*a*) to provide a constant fluid level in the sample cups and (*b*) to maintain a constant flow and ratio of fluid to air through the system. A capillary line connected to vacuum (water pump) was adjusted so that its tip extended about ⅛ inch below the rim of the glass sample cup preceding the one being sampled for analysis. All sample cups should be filled above this level so that excess fluid may be aspirated and run to waste. By thus ensuring a constant level for subsequent sampling a source of variability in the air:fluid ratio was eliminated.

The second additional sampling line was adjusted to pick up blank solvent (chloroform) from the constant level reservoir set behind the sampler unit at the precise moment that sampling from the sample cup had stopped. The lengths of the two delivery lines from the sampler to the pump unit were carefully adjusted so that the alternate flow of air and fluid through these tubes produced a uniform pattern at the point of mixing in the glass Y-joint, prior to meeting the copper reagent. It is advisable to check the accuracy of this balancing before the copper reagent is sampled by using a series of sample cups containing chloroform. Imprecise balancing, with large air gaps between specimens and blanks, may lead to an insufficient excess of chloroform entering the separator over that being redrawn through the pump. This would lead to copper reagent being redrawn through the pump and subsequently reacting with the diethyldithiocarbamate. It is important to prevent this since even slight contamination by traces of copper reagent will materially affect the results. If contamination should occur, then the whole system may be thoroughly cleaned by drawing methanol through all the lines.

The sample plate was loaded *alternately* with specimens and chloroform blanks, and run at a rate of 60 samples per hour. Operating concitions therefore corresponded to 40-sec sampling of specimen followed by 80 sec of chloroform (blank), and were the same as in the procedure with sampler II.

In order to minimize evaporation of chloroform from the sample cups leading to concentration of specimens, the sampler was covered with a polystyrene plate immediately after the table was loaded.

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