A novel, semiautomated method for the estimation of free fatty acid in serum or plasma

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Summary A modification of the semiautomated assay method of Antonis (1965. *J. Lipid Res.* **6:** 307–312) for free fatty acid is presented. Free fatty acids are extracted from serum or plasma into di-*n*-butyl ether–2-methoxyethanol; the extract is almost free from phospholipids. The acids are analyzed in a portion of extract by a copper soap method using diphenylcarbazide. The extractant, being less dense than water, is easily separated from an aqueous phase both in the extraction of samples and in the assay of copper soaps. The assay is comparable in accuracy with well-tried titrimetric methods and is quicker and easier to operate.

Supplementary key words AutoAnalyzer · continuous flow assays · copper soaps · di-*n*-butyl ether · 1,5-diphenylcarbazide · neutral lipid extraction · 2-methoxyethanol

In our studies of the relationship of plasma lipid concentration to arterial lipid metabolism and atherogenesis, we have required a rapid and accurate method for the estimation of free fatty acid (FFA) in serum and plasma. We present here a new technique in which FFA are extracted manually and then can be assayed by a continuous flow method on a Technicon AutoAnalyzer.

With the exception of the method of Kashket (1), published continuous flow analyses for FFA require preliminary extractions from serum or plasma. These have been fully discussed by Meinertz (2) and, in part, by Baird, Black, and Faulkner (3), and Duncombe and Rising (4). The techniques involve denaturation of serum albumin under acid conditions and the transfer of released FFA into nonpolar solvents. Typical examples are heptane–isopropyl alcohol (3, 5–7) or solvents containing chloroform (4, 8–11). The extracted FFA can then be assayed by a continuous flow technique titrimetrically (3), by fluorimetry after reaction with sodium fluorescienate (7), or by complexing the fatty acids with a transition metal such as copper or cobalt and estimating the metal in the organic phase by reaction with, for instance, diethyldithiocarbamate (8–11), 1,5-diphenylcarbazide (12–15), 1-nitroso-2-naphthol (16), 2-(thiozolylazo)-*p*-cresol (17), or tetraethylthiuram disulphide (18).

A potential disadvantage with the copper soap methods is that phospholipids interfere with the assay (9). Furthermore, with chloroform-based solvents, the organic phase of the biphasic reaction mixture is more dense than the aqueous phase that contains the excess copper reagent, and it is common experience that it is difficult to separate either manually or automatically.

We have developed, therefore, a novel extraction solvent containing 2-methoxyethanol and di-*n*-butyl ether which achieves quantitative recovery of FFA without contamination by phospholipids; being less dense than water, it can also be conveniently separated from an aqueous phase during the extraction of samples and in the subsequent assay. The extracted FFA are complexed with copper and estimated with 1,5-diphenylcarbazide.

Materials and Methods

All reagents are general purpose reagents except where otherwise stated. Bovine serum albumin (BSA; Cohn fraction V) was obtained from Armour Pharmaceuticals, Brighton, U.K., and defatted when so required by the method of Chen (19) using activated charcoal. Radioactive fatty acid, [9,10-^{3}H]palmitic acid and [^{3}H]-*n*-hexadecane standard were obtained from the Radiochemical Centre, Amersham, U.K. Lecithin (essential phospholipid, EPL, a polyunsaturated phosphatidylcholine) was obtained from Nattermann, Köln, West Germany. Tripalmitin, oleic acid, cholesterol, and cholesteryl oleate were obtained from Sigma, London, U.K.

Extraction of FFA. The extractant consists of 2-methoxyethanol—di-*n*-butyl ether 1:1 (v/v). The other reagent required for extraction of serum or plasma is a 0.1 M solution of glycine in water, adjusted to pH 2.7 with sulfuric acid.

To each 0.2-ml sample of serum or plasma in a centrifuge tube is added 3 ml of glycine solution. The mixture is allowed to stand for 5 min and then 4 ml of extractant is added. Each tube is stoppered and shaken briefly after the addition of extractant and every 5 min thereafter. When 20–30 min have elapsed, each tube is vigorously shaken for at least 20 sec and then centrifuged at 1000 *g* for 10 min to separate denatured protein from the other phase (the volume of upper ether phase is 2.0 ml). The supernatant is transferred to a container for storage at −20°C or to an AutoAnalyzer cup for analysis. It is not convenient to process more than 30 samples in each batch.

Copper reagent. The reagent for formation of copper

Abbreviations: FFA, free (nonesterified) fatty acid; TLC, thin-layer chromatography; PTFE, polytetrafluoroethylene; DPC, 1,5-diphenylcarbazide; BSA, bovine serum albumin; CV, coefficient of variation; SD, standard deviation.

soaps is made up from two stock solutions that are stored at 4°C; the first is 9 g of cupric sulfate pentahydrate in one liter of water, the second is made up by dissolving 100 g of sodium chloride in a solvent consisting of 40 ml of glycerol, 600 ml of water, 40 ml of piperidine, and 5 ml of glacial acetic acid. The copper reagent used in the assay is a 1:1 mixture of the stock solutions.

Color reagent. The color reagent is a solution of 3 g of 1,5-diphenylcarbazide and 5 ml of glacial acetic acid in 1 l of propan-2-ol (AnalaR grade), and is stable at 4°C for at least 2 weeks.

Standards. Oleic acid (Sigma, London, U.K.; Sigma grade) was used as a standard. It showed only FFA by TLC on silicic acid in hexane–diethyl ether–acetic acid 90:10:1 (v/v) and was 98% oleic acid by gas–liquid chromatography (GLC). Standards were prepared by taking appropriate volumes of a stock solution containing 0.5 mmol/l oleic acid in extractant (14.125 mg per 100 ml), adding a further appropriate amount of extractant to give a total volume of 4 ml, then adding 3.0 ml of glycine solution and 0.2 ml of saline and treating as for the samples. Standards were prepared to cover the range of plasma FFA from 0 to 2.5 mmol/l (i.e., 0–0.5 μeq from 0.2 ml of plasma) or for samples with low concentrations of FFA from 0 to 1.0 mmol/l.

AutoAnalyzer system. Continuous flow samples are analyzed on a Technicon AutoAnalyzer II system, the flow diagram for which is shown in **Fig. 1.** Polytetrafluoroethylene (PTFE) is used for the separator (type CO) and mixing coils (A and B), because it adsorbs FFA less than other materials, such as glass and Solvaflex. All pump tubes are Solvaflex and perform adequately for at least a month of daily use. The cups, which must be resistant to attack by di-*n*-butyl ether are either glass or rigid polyethylene.

Extracts are sampled at 40 per hr, controlled by a 7:1 cam to give flattened peaks suitable for use with a digital printer. The sample is mixed with copper reagent in a PT 2 fitting, passes through about 15 cm of 0.5 mm ID PTFE tubing connected to another PT 2 fitting through which is added di-*n*-butyl ether to increase volume and thus ensure good separation of samples in the separator. Air is bled into the stream in a D1 fitting and the mixture passes into an equilibration coil (A) made from 0.9 m of 1.9 mm ID PTFE formed into a coil of three turns, 10 cm in diameter. After leaving the coil the mixture enters a CO-type separator made of PTFE, where the copper reagent is removed. The air-segmented ether stream is mixed with color reagent in a D1 fitting and passes through a delay coil (B) made from 4.4 m of 1.9 mm ID PTFE formed into a coil of 19 turns, 10 cm in diameter,

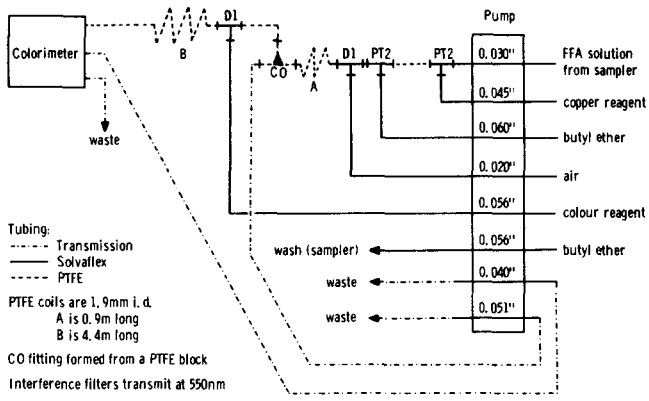

Fig. 1. Flow diagram for the AutoAnalyzer for FFA estimation. All pump tubes are Solvaflex and the remaining ones are transmission tubing. Coils A and B, the separator, type CO, and two short lengths of connection tubing are made from PTFE.

where color develops. It then passes into the colorimeter where air is removed and the absorption is measured at 550 nm.

After use the system is washed with dilute mineral acid, then water, and pumped dry.

Results

Assessment of AutoAnalyzer kinetic parameters. In order to assess the acceptability of the continuous-flow process for copper soap formation and estimation, a series of standards representing plasma concentrations between 0.5 and 2.5 mmol/l (i.e., 0.1–0.5 μeq from 0.2 ml of plasma) were analyzed at the normal rate of 40 samples per hr. The sensitivity of the spectrophotometer was adjusted to give a full scale reading for the highest standard. **Fig. 2** shows a recording of the trace of optical density against time for the various standards. The intensity of color was related linearly to fatty acid concentration. The peak heights at the plateau were approximately the same as the steady state condition.

Analysis of kinetic parameters of the system as described by Thiers, Cole, and Kirsch (20) gave a half-wash time (W½) of 7 sec and a lag phase time (L) of 19 sec. The calculated percentage interaction between samples was 0.7%.

Assessment of efficiency of extraction. In order to assess the efficiency of extraction of FFA two experiments were performed as follows. *1*) FFA of human plasma was extracted by the proposed extractant and by the classical technique of Trout, Estes, and Friedberg (6) and estimated as copper soaps in the AutoAnalyzer.

A sample of human plasma anticoagulated with lithium heparin was diluted with a solution of defatted BSA (6 g/100 ml in 0.9% saline) to give nine control samples containing different concentrations of FFA. Eight aliquots of each control sample were then ex-

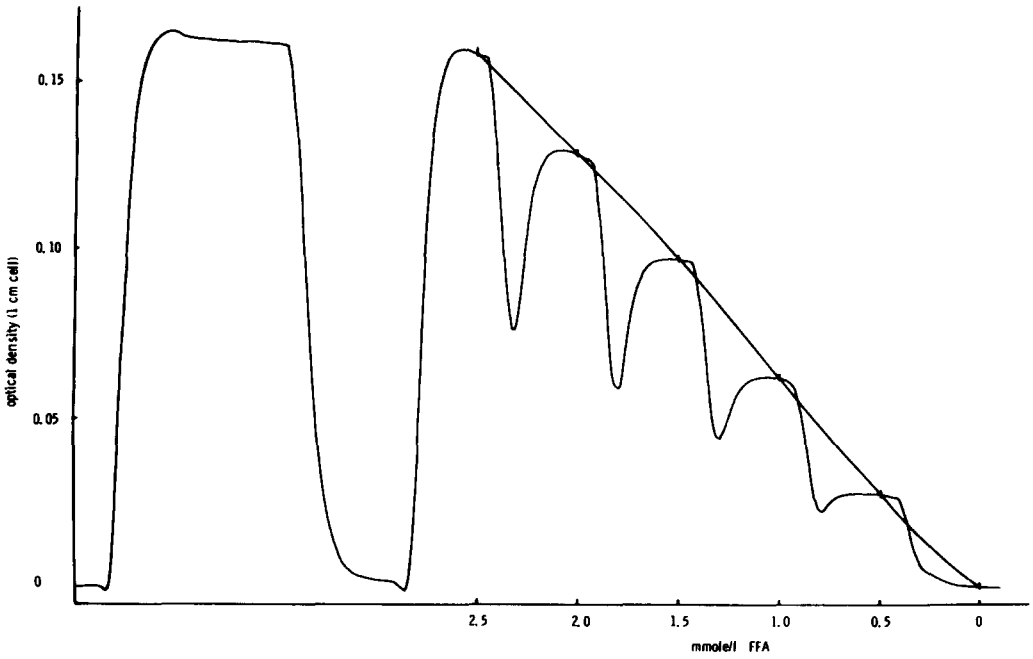

Fig. 2. AutoAnalyzer tracings obtained using the method described for standards showing steady state conditions and peaks equivalent to plasma concentrations in the range 0–2.5 mmol/l.

tracted by the method of Trout et al. (6). The extracts were evaporated at 25°C, dissolved in 2.0 ml of di-*n*-butyl ether, and washed with water. Eight further aliquots of each control sample were extracted by the present method and the FFA of both sets of extracts were estimated by the AutoAnalyzer method as described above. The results are shown in **Table 1.** There were no significant differences between the mean values of each control sample using the two methods of extraction as revealed by Student's unpaired *t* test. The regression line for FFA concentrations measured after the present method of extraction (y) against the concentration in extracts obtained by the method of Trout et al. (x) gave

$$y = 0.045 + 0.94\,x \quad r = 0.996 \quad n = 72$$

The correlation between the two extraction techniques was excellent and the proposed method of extraction gave an acceptable recovery of FFA of 94% of that extracted by the method of Trout et al. (6).

2) The efficiency of extraction was tested by measuring the recovery of ^{3}H-labeled fatty acid from a serum sample into extractant. One μCi of [9,10^3-H]-palmitic acid (sp act 500 Ci/mol) was purified by TLC on silica gel under nitrogen using a solvent of hexane–diethyl ether–acetic acid 90:10:1 (v/v). The FFA spot was eluted with chloroform–methanol 2:1 (v/v) and the extract was washed with saline by the method of Folch, Lees, and Sloane Stanley (21) to remove non-lipid contaminants. The FFA was then dissolved in 0.5 ml of 0.05 M sodium hydroxide and added to 5 ml of fresh rabbit plasma at 40°C. Fifteen 0.2-ml samples of the plasma were extracted by the present method. The radioactivity of 1.0 ml of each extract and also of 10 0.1-ml samples of the plasma was measured in a Nuclear Chicago Mk II scintillation counter using

TABLE 1. Comparison of methods for extracting FFA

	FFA Concentration mmol/l								
Samples	1	2	3	4	5	6	7	8	9
Present method (y)	1.91[a]	1.63	1.29	0.98	0.68	0.36	0.19	0.08	0.02
± SD	0.007	0.010	0.008	0.009	0.004	0.005	0.005	0.005	0.003
Trout et al. (6) (x)	1.99	1.67	1.34	1.00	0.62	0.29	0.14	0.06	0.02
± SD	0.008	0.008	0.010	0.012	0.009	0.008	0.005	0.005	0.002

[a] n = 8 for each sample.

Values obtained for FFA concentrations in nine control solutions of human plasma diluted with a solution of defatted BSA (6 g/100 ml), extracted by the proposed method of Trout et al. (6), and then assayed by the present AutoAnalyzer technique.

10 ml of the Triton X-100–toluene scintillator of Patterson and Greene (22) containing 10% water. The counting efficiency of each sample was measured with an internal standard of [^{3}H]*n*-hexadecane. Assuming that the volume of di-*n*-butyl ether extract obtained from the extraction procedure of plasma is 2.0 ml, the recovery of fatty acid was 106% (SD ± 3% on 15 observations).

Interference by other compounds. During the development of a solvent to minimize the extraction of interfering phospholipids, the extracts from 0.2-ml samples of human plasma and plasma from Cynomolgus monkey and rabbit were monitored by TLC on silica gel with a solvent of hexane–diethyl ether–acetic acid 85:15:2 (v/v), followed by charring in situ (23), and by measurement of phospholipid phosphorus by the method of Itaya and Ui (24, 25). This revealed a concentration of 2.5 μg of phospholipid in extracts of 0.2 ml of plasma compared with 25 μg of phospholipid in extracts of 0.2 ml of similar samples prepared by the method of Duncombe and Rising (4) using chloroform.

As a test for interference by phospholipid, solutions of lecithin (EPL) from 250 to 1000 mg per 100 ml in saline containing BSA (6 g per 100 ml) were prepared. Samples (0.2 ml) were extracted and analyzed by the present method. The lecithin produced results equivalent to 0.010–0.016 mmol/l from 16 observations.

Other lipids, such as cholesterol, cholesteryl esters, and triglycerides, are also extracted by the solvent system described. To test for interference, cholesterol, cholesteryl oleate and tripalmitoylglycerol were purified by TLC and dispersions of each containing 500 mg/100 ml in a solution of BSA (6 g/100 ml saline) were prepared and 0.2 ml of each was analyzed. None produced any interference.

Interference by short chain organic acids was investigated by analyzing extracts of solutions of acids in water (500 mg/100 ml). Acetic, lactic, and hydroxybutyric acids did not give rise to a significant effect but butyric acid solution gave an equivalent FFA concentration of 0.01 mmol/l. It is suggested therefore, that there may be slight interference from unsubstituted medium chain carboxylic acids.

Comparisons of the complete method with other methods. In order to compare the proposed method with other established techniques, two experiments were performed as follows. *1*) Nine control samples of human plasma diluted with BSA solution, as described above, were analyzed by the present method and independently (see acknowledgements) by a modification due to Faulkner, of the semiautomated, titrimetric method of Baird, Black, and Faulkner (3). The results are given in **Table 2** which shows the standard deviation and coefficient of variation. The reproducibility of the proposed method is very satisfactory except at the lowest concentration (mean 0.016 mmol/l) of FFA. The agreement with the titrimetric assay is very good and there was no significant difference, by Student's unpaired *t* test, between the two methods.

2) The proposed method was also compared with the manual extraction and titrimetric method of Trout et al. (6) using the titration mixture of Schnatz (26) on larger series of human plasma, obtained from outdated transfusion supplies, and on Cynomolgus monkey and rabbit plasma. During setting up of the method of Trout et al. (6) it was discovered that washing the heptane extract with sulfuric acid, as described, produced a systematic error as compared with washing with water. The phenomenon was detected even though standards were treated exactly as the samples were. It appears that some sulfuric acid is carried into the heptane phase and that this is greater in samples than in standards. Accordingly, distilled water was used as the wash throughout.

Fig. 3 summarizes the regression of the present method against the modified method of Trout et al.

TABLE 2. Comparison of methods for estimating FFA concentration

	FFA Concentration, mmol/l								
Sample	1	2	3	4	5	6	7	8	9
Present method (y)									
Mean	1.91[a]	1.63	1.29	0.98	0.68	0.36	0.19	0.08	0.02
± SD	0.007	0.010	0.008	0.009	0.004	0.005	0.005	0.005	0.003
CV%	0.4	0.6	0.6	0.9	0.6	1.4	2.7	6.0	18.7
Baird et al. (3) (x)									
Mean	1.90	1.59	1.34	1.10	0.66	0.35	0.20	0.12	
± SD	0.005	0.009	0.007	0.008	0.005	0.005	0.003	0.005	

[a] n = 8 for each sample.

FFA concentrations for nine control solutions of human plasma diluted with a solution of defatted BSA (6 g/100 ml) were estimated by the present method and independently by the method of Baird et al. (3). Regression analysis by the method of least squares gave $y = 0.024 + 1.01\,x$; $r = 0.954$; $n = 64$.

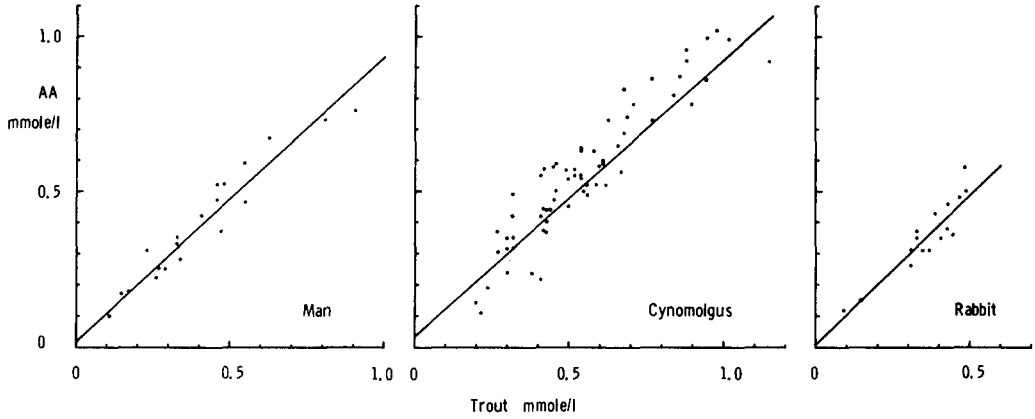

Fig. 3. Comparison between the proposed method, y, and the procedure of Trout, Estes, and Friedberg (6), x, using water instead of acid as a wash for the heptane phase. Regression lines were computed by the method of least squares. *A*. Human plasma; $y = 0.021 + 0.933x$; $r = 0.971$; n = 20. *B*. Cynomolgus monkey plasma; $y = 0.042 + 0.921x$; $r = 0.927$; n = 64. *C*. Rabbit plasma; $y = 0.006 + 0.970x$; $r = 0.916$; n = 16.

(6). The regression and correlation coefficients for human, monkey, and rabbit samples show that the new method provides a reasonable agreement with an established technique for at least three important species. In work with other species it would probably be advisable to examine the correlations with an accepted method, because it is known that plasma FFA in some species, for example cat, are not well extracted even by chloroform.

Discussion

An effective method for the automated analysis of FFA, in addition to being precise, reproducible, and simple to operate, must avoid the extraction of such substances as phospholipids and lactic acid, which may cause erroneous results. Essentially four approaches have been made by various workers. *1*) Extraction was carried out in the AutoAnalyzer followed by titrimetry of the released acid (1). With our equipment, we were unable to prevent precipitated protein from clogging flow lines and unbalancing the separator and so the reproducibility of this method was poor.

2) A manual extraction was performed by the method of Trout et al. (6) and the extract was titrated in the AutoAnalyzer (3). This method is sensitive and accurate, but the extraction procedure is relatively time-consuming and the need for rigid exclusion of carbon dioxide introduces a slight, but unnecessary elaboration. This latter problem has been neatly overcome in the method of Carruthers and Young (7) by estimation with sodium fluoresceinate.

3) Extraction was carried out with an organic solvent, such as di-isopropyl ether (8) or chloroform, in the presence of silicic acid, which removes phospholipids, and then FFA were assayed as copper soaps (4). This method leads to low recovery of FFA (3, 15, 27), probably because some FFA is adsorbed on silicic acid and because extraction of FFA at neutral pH is not efficient (2).

4) An extract was made with chloroform and heptane in the presence of phosphate buffer at pH 6.2 (10) and FFA were assayed as copper soaps (4). In a recent innovation (11) the problem of separation of an organic phase containing the fatty acids from the excess aqueous copper reagent has been overcome by allowing the formation of copper soaps at the surface of a semipermeable membrane made of PTFE lined with Cuprophan. This method gives quantitative extraction of FFA, but there is some contamination by phospholipids.

In the method described here, we have attempted to overcome certain of the difficulties inherent in the above approaches. The novel extraction solvent was chosen because of the known low solubility of phospholipids in ethers such as di-*n*-butyl ether (2). In order to promote the efficient transfer of fatty acid from the aqueous phase, 2-methoxyethanol was incorporated into the solvent and the extraction was performed at pH 2.7. As now developed, the extraction system ensures adequate transfer of FFA and has the considerable advantage over two-phase systems containing chloroform that the organic phase is on top and can thus be sampled easily without contamination by denatured protein.

In initial attempts to form copper soaps of FFA dissolved in butyl ether, the reagent proposed by Antonis (8) containing triethanolamine was tried. It was unsuccessful, apparently because the amine is insoluble in di-*n*-butyl ether; the use of piperidine or pyrrolidine overcomes this problem.

In the assay of the copper complexes in di-*n*-butyl ether, it was observed that diphenylcarbazide was 4

times more sensitive than diethyldithiocarbamate (see also reference 13). The stability of the color was much improved by the addition of acetic acid, which probably hinders side reactions. It was also observed that the rate of color development and fading was dependent upon the ratio of the di-*n*-butyl ether phase to color reagent; in the system described the time to reach maximum color intensity is about 5 min and the required delay is conveniently achieved by a coil of PTFE (coil B).

In any new method for continuous flow analysis it is important to establish conditions such that samples are separated cleanly and do not interfere with one another. In this method this was achieved by the use of PTFE for the mixing coils and the separator. Analysis of the kinetic parameters showed that the half-wash (W½) of 7 sec and the lag phase time (L) of 19 sec compare very favorably with values of other methods on an SMA-12 hospital analyser (Technicon Instruments Corporation), where W½ lies between 9 sec for calcium and 21 sec for alkaline phosphatase assays and L between 6 sec for albumin and 32 sec for calcium assays (20). The percentage interaction between samples of 0.7% is very acceptable.

It is also important in any technique involving a biphasic system for FFA extraction and copper soap formation to prevent contamination of the organic phase with the aqueous phase. In biphasic systems using chloroform alone, this is difficult because the organic phase is denser than water and the lower phase must be sampled. This problem was overcome in the present method by the use of di-*n*-butyl ether as organic solvent, which forms an upper phase. A similar approach, but employing the addition of heptane to a chloroform solvent, has been used by others (15, 16). In the method of Elphick (11), the separation of copper reagent from organic phase by a semipermeable membrane gave stable operation and low baseline noise and drift, thereby improving the sensitivity down to 3×10^{-4} μmol. The only disadvantage in that approach would seem to be the need to replace semipermeable membranes and the fact that the organic phase is still a lower phase in the extraction procedure. In our technique, phase separation in analysis of copper soaps was extremely satisfactory and stable without special apparatus and the baseline stability allowed detection of 1×10^{-3} μmol.

The proposed extraction solvent gives a very satisfactory recovery of FFA, while discriminating against phospholipids. Of the medium chain acids only butyric acid gave a significant interference.

The proposed method gives fairly good agreement with the titrimetric method of Trout et al. (6), modified to use a water wash instead of acid, to remove contaminating short chain acids. For plasmas of man, Cynomolgus monkey, and rabbit, the recoveries of the present method compared with the modified Trout method were 93.3%, 92.1%, and 97.0%, respectively. The correlation coefficients, r, for the two methods were also reasonable being 0.971 (n = 20), 0.927 (n = 64) and 0.916 (n = 16). In our hands, the variability of the method of Trout et al. (6) (CV = 12.5%) was greater than the AutoAnalyzer technique (CV = 6.9%) and this almost certainly explains a lower value of r than might have been desirable. In a comparison by Elphick (11) of his technique with other methods, a lower correlation coefficient, i.e., greater scatter, was found against the method of Trout et al. (6), $r = 0.975$, n = 27 than against the method of Itaya and Ui (9), $r = 0.987$, n = 21.

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