Colorimetric ultramicro method for the determination of free fatty acids

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SUMMARY Previously described colorimetric ultramicro methods for the determination of serum and tissue fatty acids have been improved in sensitivity and selectivity by extracting cobalt rather than copper soaps by means of a solvent lighter than water instead of chloroform. The complex of Co^{++} with α-nitroso-β-naphthol is measured at 500 mμ.

KEY WORDS free fatty acids · extraction · colorimetric determination · cobalt soaps · serum

TITRATION OF FREE FATTY ACIDS (FFA) in lipid extracts, the most frequently used method for their determination, is difficult to perform if only small amounts of biological material are available. The colorimetric methods described in the literature, e.g., that of Carlson and Wadström (1), use relatively complex separation techniques which render it difficult to adapt them for ultramicro determination of FFA. The method of Coleman and Middlebrook (2) is very sensitive, but the intensity of the color that develops depends on the composition of the FFA mixture.

Methods based on the color reactions of copper soaps (3–5) are particularly useful if combined with paper chromatography. When they are applied to biological fluids, however, bilirubin or other colored substances soluble in lipid solvents may interfere with the determination. In addition, when an ultramicro modification of such methods is used, it is difficult to take samples of the lower chloroform extract which are uncontaminated by the upper aqueous phase containing an excess of copper salts. If the volume of the chloroform layer is small the degree of contamination may be great. Hence a method was developed in which interference by bilirubin is eliminated and in which the colored compound is extracted by a solvent less dense than the aqueous phase.

Cobalt nitrate has been used instead of the cuprous nitrate used by Duncombe (4). Cobalt forms soaps with FFA that give a color reaction with α-nitroso-β-naphthol (6).

Apparatus and Reagents. Micropipettes of the type described by Levy (7) and Sanz (8) were made in this laboratory to deliver the following volumes: 50, 250, 300, 400, 500, and 750 μl. The capillary constriction of the pipettes must be smaller than is usual for aqueous solutions.

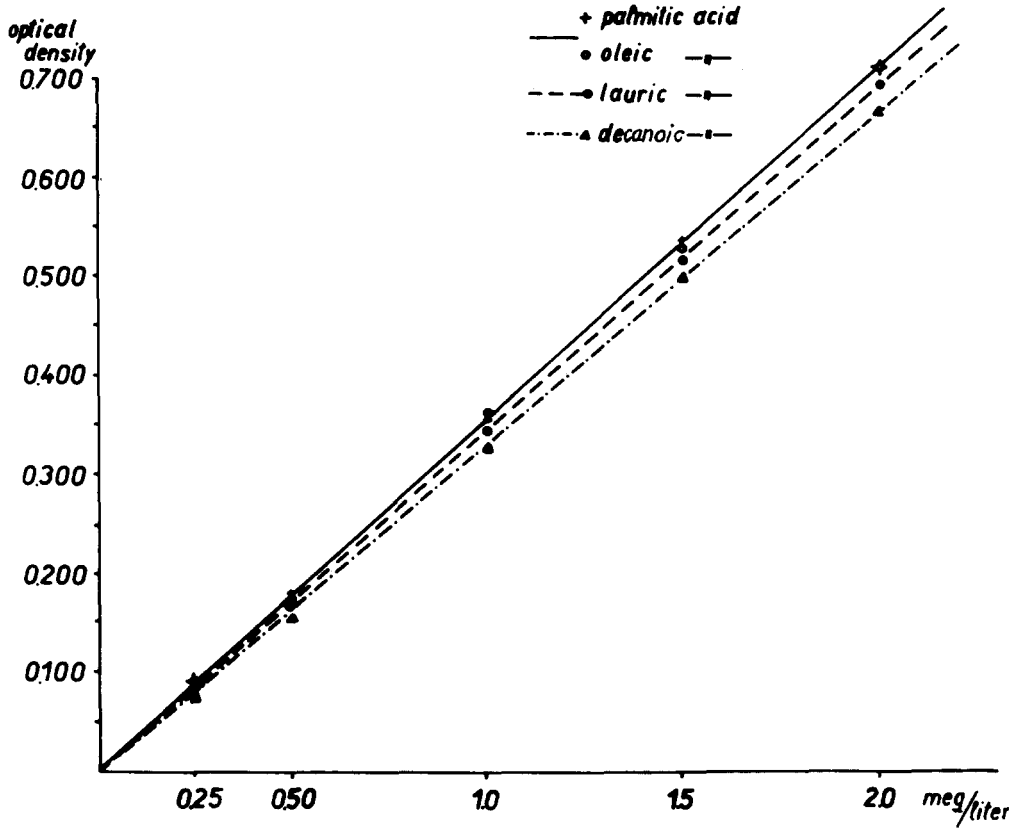

FIG. 1. Determination of several fatty acids by the colorimetric method described.

Test tubes 8 cm × 8–10 mm i.d., with ground glass stoppers, and a vibrating mixer are required.

Solution A: cobalt nitrate–acetic acid–potassium sulfate, prepared by adding to a solution of K_2SO_4 (saturated while boiling, stored in contact with excess crystals, and filtered before use), 6 g of $Co(NO_3)_2 \cdot 6H_2O$ and 0.8 ml of glacial acetic acid to give a total volume of 100 ml at 37°.

Solution B: a saturated Na_2SO_4 solution, prepared by adding Na_2SO_4 to boiling water, kept at 37° overnight.

Preparation of cobalt reagent. Triethanolamine, 1.35 volumes, is made up to 10 volumes with solution A. Solution B, 7 volumes, is added and the mixture is shaken. This reagent is not stable and must be prepared fresh for every series of analyses. Solution A and B are kept at 37°.

Indicator. Stock solution: 0.4% α-nitroso-β-naphthol in 96% ethanol. This is diluted with ethanol by a factor of 12.5 before use.

Extraction mixture was prepared according to Dole (9).

Chloroform–heptane 5:1 (v/v) was made up using redistilled chloroform and heptane.

Procedure. To 250 μl of Dole's extraction mixture in one of the narrow test tubes, 50 μl of serum is added. The liquids are mixed by vibration, care being taken not to allow them to reach the stopper. The test tubes are cooled for 10 min in a bath of melting ice. Heptane (300 μl) is added, then 500 μl of water, and the contents of the tube are thoroughly mixed. After the phases have separated, 300 μl is drawn from the upper heptane phase

TABLE 1 LACK OF EFFECT OF WASHING THE DOLE EXTRACT OF SERUM BY PROCEDURE OF TROUT ET AL. ON FFA COLORIMETRIC DETERMINATION

Sample	FFA in Extract	
	Unwashed*	Washed†
1	0.26, 0.33	0.30, 0.34
2	0.39, 0.43	0.39, 0.37
3	1.59, 1.65	1.63, 1.73
4	0.42, 0.41	0.43, 0.43
5	0.27, 0.25	0.26, 0.28

* Dole extract of serum.
† Extract washed with 0.05% H_2SO_4 (10).

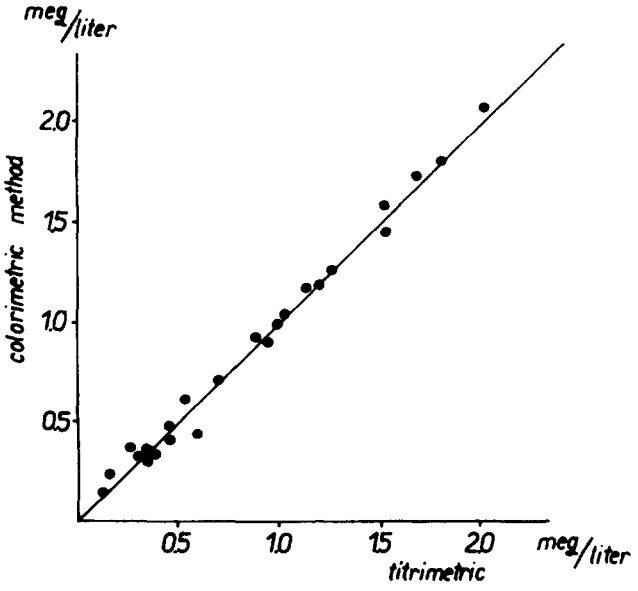

FIG. 2. Comparison between the colorimetric method and the titrimetric method of Trout et al. (10), as applied to human serum (24 subjects).

and transferred to another test tube. Chloroform–heptane (400 μl) is added, followed by 500 μl of the cobalt reagent, and the solution is thoroughly mixed for 3 min. The mixture is centrifuged for at least 15 min at 2500 rpm, and 600 μl of the upper chloroform–heptane phase is transferred to a test tube containing 750 μl of the indicator solution.

It is important to treat samples and the standard solutions simultaneously. For blank and standards, 50 μl of water instead of serum is carried through the operation. Only two standards are necessary: palmitic acid in Dole's extraction mixture, 0.2 and 0.4 meq/liter. It is necessary that the 250 μl pipette used for the standard solutions (or extraction mixture) has exactly 5 times the volume of that used for serum. The other pipettes need not be calibrated so exactly, provided the same pipettes are used for all determinations.

Values are read 30 min later at 500 mμ in 1-ml cuvettes (1 cm path length). The color is stable for several hours. For concentrations greater than 2 meq/liter it is advisable to dilute the sample with water 1:1 (v:v).

Results. Several fatty acids were tested (Fig. 1). The method was compared with the titration method of Trout, Estes, and Friedberg (10), which eliminates some acidic interfering substances. Human serum was analyzed by both methods (Fig. 2). Table 1 shows that FFA determinations in extracts from sera were not affected by washing the extract according to Trout et al. The standard deviation of the method calculated from 30 parallel determinations was ±0.021. Table 2 shows that lactic acid and bilirubin do not interfere with the determination.

TABLE 2 LACK OF INTERFERENCE BY LACTIC ACID AND BILIRUBIN

Lactic Acid Concn*	"FFA" Determined	Bilirubin Concn†	"FFA" Determined
mg/100 ml	*meq/liter*	*mg/100 ml*	*meq/liter*
10	0.006	10	0.0015
20	0.005	20	0.007
30	0.004	30	0.014
40	0.003		
50	0.015		
60	0.006		

* Sodium lactate in water. Solution then treated like serum.
† Bilirubin was dissolved in water; a few drops of 0.02 N NaOH were then added. This solution then treated like serum.

Discussion. The advantages of the method reported are (*a*) the small volume of serum required, 50 μl instead of 300 μl (11), (*b*) the use of the upper layer for FFA determination, and (*c*) the lack of interference by bilirubin and other substances. It is valuable particularly in work with newborn infants and small laboratory animals. The method can be used for FFA determination in any fluid or tissue which can be extracted with Dole's mixture and is being routinely used for analyses of adipose tissue and incubation media in this laboratory. It is, however, absolutely necessary to have at least two standard solutions analyzed for every series of estimations.

Manuscript received May 8, 1964; accepted January 26, 1965.

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