RAPID METHOD FOR THE DETERMINATION OF FAT IN FECES*

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(Received for publication, August 10, 1948)

Study of fat absorption in a patient requires the determination of the fat content of feces. Quantitative chemical methods available for such determinations are too laborious to be practical for routine analyses. Estimation of the fat content of feces by microscopic examination, practised by many clinicians, is unreliable; this can be demonstrated by comparing the results of microscopic determinations with those of chemical analysis. The results of chemical methods expressing the fat content of a random sample of feces as percentage of dry matter are equivocal. Goiffon (7) and Frazer *et al.* (2) pointed out that the percentage of dry matter in feces is not necessarily constant. It may be quite variable in feces from patients with metabolic disorders or faulty absorption. The observations of Frazer and Goiffon were also confirmed in this laboratory.

Balance studies are the conventional means of evaluating certain aspects of metabolism of nitrogen, calcium, etc. Only a few publications report such studies of fat absorption; yet it seems the only exact method of investigation. Estimation of fat balance is carried out as follows: The patient is kept on a diet containing a known amount of fat. The feces excreted in 24 hours are collected accurately. In a mixed sample of these feces, fat, if necessary differentiated into split and unsplit fat, is determined quantitatively. The total amount of fat consumed in 24 hours, less the amount of excreted fat, represents the amount of retained fat. This may be expressed as per cent of the amount of fat consumed. Since intestinal motility is variable, it is advisable to determine the fat balance on several consecutive days. The frequency of such determinations depends upon the magnitude of the daily fluctuations.

The determination of the fat content of feces may be carried out before or after drying of the feces. There are various objections to the latter method: (1) During drying, conversions may take place by which the ratio of split fat to unsplit fat could be changed; (2) during drying, volatile fatty acids may evaporate; (3) drying takes a long time. These objections are eliminated by the determination of fat in wet feces. Therefore, we

*The data in this paper are taken from the first part of a thesis submitted by J. H. van de Kamer in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of Utrecht, The Netherlands.

have sought a simple method for the determination of fat in wet feces which also meets the requirements of clinical investigation.

Von Liebermann and Székely (13), Folin (3), Polenske (16), Saxon (18), Sonntag (19), Holt, Courtney, and Fales (9), Fowweather (4, 5), Rosenfeld (17), Muller (14), Tidwell and Holt (20), Gephart and Csonka (6), Nielsen (15), Kumagawa and Suto (12), Inaba (10) (method modified by Goiffon (7)), and others have published methods for the determination of fat in wet feces. They depend upon various principles and generally give correct results. Although these methods require less time than those in which feces are first dried, they are still quite time-consuming. Applying the principles published by von Liebermann and Székely (13) and by Saxon (18), we elaborated two methods by which the fat content of feces can be determined simply and rapidly.

Principle of Methods

Feces are saponified according to the procedure of von Liebermann and Székely (13) with concentrated potassium hydroxide in ethanol, giving a solution which contains the soaps derived from the neutral fats and the fatty acids and also the soaps which were originally present in the stool. By adding HCl to the alkaline solution, the fatty acids are liberated. Ethanol is then added and the fatty acids are extracted with petroleum ether. The concentration of ethanol is so chosen that, after shaking the mixture, the petroleum ether and the acid ethanol layers separate quickly; this is expedited by adding NaCl and a small amount of amyl alcohol. Separation is complete after 5 to 10 minutes. In an aliquot sample of the petroleum ether layer the fatty acids are titrated with alkali, with thymol blue as indicator. This method of determining the total fat content requires 35 minutes (Method A).

To determine split and unsplit fat separately, the sample of feces is not treated with alkali, but is boiled for 1 minute with diluted hydrochloric acid in order to convert the soaps into free fatty acids, according to the method of Saxon (18). After addition of ethanol, NaCl, and amyl alcohol, the solution is extracted with petroleum ether. An aliquot sample of the petroleum ether layer is evaporated to dryness and the free fatty acids are titrated with 0.1 N isobutyl alcoholic KOH. Thereupon an excess of the same solution is added and the unsplit fat is saponified by boiling. The excess of alkali is titrated with 0.1 N HCl and thymol blue as indicator (Method B).

Method A; Determination of Total Fat Content

Reagents—

Ethanol, 96 per cent, containing 0.4 per cent amyl alcohol.

Ethanol, 96 per cent, neutral to thymol blue.

KOH, 33 per cent.

HCl, 25 per cent, specific gravity 1.13.

Petroleum ether, boiling point 60–80°, or 40–60°. When evaporated to dryness, this must leave no residue which can be titrated or saponified with alkali.

NaOH, 0.1 N.

Thymol blue, 2 per cent, in 50 per cent ethanol.

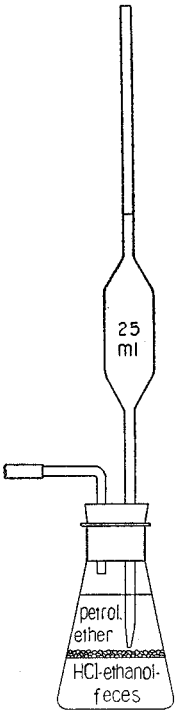

FIG. 1. Erlenmeyer flask with pressure pipette to remove aliquot of petroleum ether.

Apparatus—

150 ml. Erlenmeyer flasks with wide mouths (high model), each provided with a rubber stopper and reflux condenser.

25 ml. pipette, fitted into the flask as illustrated in Fig. 1. With this apparatus the petroleum ether solution is brought into the pipette by blowing and evaporation is prevented.

50 ml. pipette.

5 ml. micro burette.

Procedure—About 5 gm. of feces are weighed in a 150 ml. Erlenmeyer flask. After adding 10 ml. of 33 per cent alkali and 40 ml. of ethanol containing 0.4 per cent amyl alcohol, the mixture is boiled for 20 minutes under a reflux condenser, and then thoroughly cooled. 17 ml. of 25 per cent HCl are added, a graduated cylinder being used, after which the mixture is again cooled. Exactly 50 ml. of petroleum ether are then added, and the flask is closed with a rubber stopper and shaken vigorously for 1 minute. After complete separation, 25 ml. of the petroleum ether layer are transferred into a small Erlenmeyer flask by using the pressure pipette.

After addition of a piece of filter paper, the petroleum ether is evaporated and 10 ml. of neutral ethanol are added. The fatty acids are titrated with 0.1 N NaOH from a micro burette, with thymol blue as indicator, until the yellow color begins to change. If 0.1 N isobutyl alcoholic KOH is available (see Method B), it is possible to titrate the fatty acids directly in the petroleum ether without previous evaporation.

Calculation—For the present, the calculations are carried out according to Goiffon (7), assuming an average molecular weight of 284 for fatty acids.[1] Hence

$$\frac{A \times 284 \times 1.04 \times 2 \times 100}{10{,}000Q} = 5.907\frac{A}{Q} = \text{fatty acids in gm. per 100 gm. feces}$$

in which A = the ml. of 0.1 N alkali used in titration, and Q = the gm. of feces taken for analysis. The factor 1.04 must be used as the petroleum ether layer increases 1 per cent in volume when shaken with alcoholic hydrochloric acid and because 3 per cent of the amount of fatty acids remains in solution in the acid alcoholic layer. Corrections for evaporation of the petroleum ether layer and for volume increase due to fatty acids dissolved in it may be neglected. That the factor used is correct was demonstrated by the fact that the same fat content is found when the solution is extracted quantitatively with petroleum ether and when an aliquot is used as described above.

Method B; Determination of Fatty Acid and Neutral Fat Separately

Reagents—

HCl, 2.5 per cent, specific gravity 1.013, to which 250 gm. of NaCl per liter are added.

Ethanol, 96 per cent, containing 0.4 per cent amyl alcohol.

Ethanol, 96 per cent, neutral to thymol blue.

Petroleum ether, boiling point 60–80°, or 40–60°, as described above.

Isobutyl alcoholic KOH, 0.1 N. Isobutyl alcohol is boiled for 3 hours

[1] Another paper deals with the determination of the molecular weights.

with 100 gm. of NaOH per 5 liters. It is then distilled and the fraction that comes over between 105–108° is collected. To 5 liters of this solution are added 15 gm. of concentrated 50 per cent KOH solution diluted with 20 ml. of methyl alcohol. The KOH solution is prepared by dissolving solid KOH in an equal amount of water; after standing for several days the clear solution is siphoned off. The diluted isobutyl alcoholic KOH is titrated with 0.1 *N* hydrochloric acid, with thymol blue as indicator, until the yellow color begins to change.

Hydrochloric acid, 0.1 N.

Thymol blue, 2 per cent, in 50 per cent ethanol.

Apparatus—

Cylindrical tubes of 30 cm. length and 4 cm. diameter, provided with 50 cm. reflux condensers with ground joints and glass stoppers.

25 ml. pipette. This is fitted into the cylindrical tubes by means of a stopper, as illustrated in Fig. 1.

100 ml. Erlenmeyer flasks provided with glass bulbs as reflux condensers.

50 ml. pipette.

5 ml. micro burette.

Procedure—About 5 gm. of feces are weighed in a cylindrical tube. After addition of 22 ml. of HCl solution containing NaCl and some grains of pumice, the mixture is boiled for 1 minute under the reflux condenser. The tube is then thoroughly cooled. 40 ml. of 96 per cent ethanol containing 0.4 per cent amyl alcohol are added by means of a graduated cylinder, and exactly 50 ml. of petroleum ether by means of a pipette. After this the tube is closed with the glass stopper and vigorously shaken for 1 minute.

Separation may be facilitated by occasional rotation of the tube. When separation is completed, 25 ml. of the petroleum ether layer are transferred into a 100 ml. Erlenmeyer flask with the help of the pressure pipette (Fig. 1). After addition of a small piece of filter paper, the petroleum ether is evaporated; 2 ml. of neutral ethanol are added and the free fatty acids are titrated with 0.1 N isobutyl alcoholic KOH in the micro burette, with thymol blue as indicator, until the yellow color begins to change.

After addition of 10 ml. of 0.1 N isobutyl alcoholic KOH, the solution is boiled gently for 15 minutes, the flask being provided with a glass bulb as a reflux condenser.

To the hot solution 10 ml. of neutral ethanol are added, after which the excess alkali is titrated immediately with 0.1 N hydrochloric acid until the blue color of the indicator turns yellow.

Calculation—Assuming a molecular weight for fatty acids of 284 and for fat of 297,

$$\frac{A \times 284 \times 1.04 \times 2 \times 100}{10{,}000Q} = 5.907\frac{A}{Q} = \text{fatty acids in gm. per 100 gm. feces}$$

$$\frac{(B - C) \times 297 \times 1.01 \times 2 \times 100}{10{,}000Q} = 5.999\frac{(B - C)}{Q} = \text{neutral fat in gm. per 100 gm. feces}$$

in which A = the ml. of 0.1 N alkali used in titration of the fatty acids, B the ml. of 0.1 N HCl used in the blank titration of 10 ml. of isobutyl alcoholic KOH, C the ml. of 0.1 N HCl used in the titration of fat, and Q the gm. of feces taken for analysis. The factor 1.04 for fatty acid is explained above; for neutral fat the factor is 1.01, since in this case only the increase in volume of the petroleum ether layer has to be corrected.

Remarks

1. After boiling with HCl the solution must be cooled thoroughly; otherwise, after addition of alcohol, esterification of the fatty acids may take place.

2. The use of long cylindrical tubes in Method B is necessary to avoid loss of volatile fatty acids.

3. In order to prevent irregular boiling during evaporation of the petroleum ether, a small piece of filter paper is used. No pumice must be used, as this absorbs fatty acids.

4. It is not necessary to evaporate the petroleum ether quantitatively, as small amounts of this solvent have no influence on the titration nor on the saponification.

5. Saponification of the fatty acids is carried out according to Kolthoff (11) with 0.1 N isobutyl alcoholic KOH. Compared with the usual 0.5 N ethanolic KOH it has two advantages. In the first place, the boiling point of the isobutyl alcoholic solution is about 100° instead of 80° as in the case of ethanol; for this reason the saponification time is reduced considerably. Secondly, the accuracy of the titration is increased because it is possible to use 0.1 N isobutyl alcoholic KOH instead of the usual 0.5 N ethanolic KOH.

6. For the extraction of the acid alcoholic solution with petroleum ether, 60 per cent alcohol appears to be most suitable. With lower concentrations, emulsions are easily formed, while at higher concentrations too much fatty acid remains dissolved in the alcoholic layer.

7. The extraction is complete after 1 minute; no more fat is extracted after shaking the mixture for 2, 3, 4, 5, or 10 minutes.

8. Usually phenolphthalein is used as indicator in fatty acid titration. In the yellow-colored petroleum ether solution, however, the change in color of thymol blue from yellow to green is much more evident than the

change of phenolphthalein from yellow to reddish yellow. The two indicators have the same pH range, *viz.* from pH 8 to 10.

9. Addition of NaCl and amyl alcohol to the acid alcoholic solution enhances the separation of the alcoholic and the petroleum ether layer.

10. Titration of the fatty acid was preferred to weighing because it is quicker and because unsaponifiable matter is excluded at the same time.

11. It appeared that fatty acids and fat added to feces could be recovered separately with a relative error of 2 per cent.

Comparison of Various Methods

The results of determinations, carried out according to Methods A and B, were compared with determinations according to other well known methods.

Method of Weibull (21, 22)—About 5 gm. of feces are boiled for 1 hour with 100 ml. of 1.5 N HCl, filtered through a wet filter, and washed with water until all acid has disappeared (neutral reaction to Congo red paper). The filter, after drying overnight at 40°, is extracted with petroleum ether, according to Berntrop (1). The petroleum ether is evaporated and the residue is titrated according to our Method A.

Method of Gorter and de Graaff (8)—About 5 gm. of feces are mixed with 2 ml. of 25 per cent HCl and 10 gm. of sand, evaporated to dryness on a water bath, and extracted according to the procedure of Berntrop (1).

Method of Fowweather (4)—About 3 gm. of feces are mixed in a mortar with 2 ml. of 25 per cent HCl and 20 gm. of desiccated $CaSO_4$, and the mixture is stirred to dryness. After standing for 30 minutes it is stirred again and extracted according to the procedure of Berntrop (1). As it appeared that the results were very irregular, the procedure was modified as follows: After stirring to dryness, the mixture was dried at 40° overnight and afterwards extracted.

Method of Muller (14)—About 5 gm. of feces are mixed with 2 ml. of 25 per cent HCl; then the mass is stirred with some acetone. The acetone is filtered through a Büchner funnel and the residue is washed with acetone until a fine dry powder remains. After evaporation of the acetone, the residue is extracted several times with petroleum ether. The petroleum ether is filtered through a crucible with fritted disk and a layer of cotton wool into a weighed flask. After evaporation the residue is dried at 100° for 1 hour and weighed, or, if possible, titrated. If the fat contains impurities, it may be too dark for titration. The results are given in Table I.

The following conclusions may be drawn from Table I: (*a*) After acid treatment of feces (our Method B) the same amount of fatty acids and fat is found as after alkali treatment (our Method A). (*b*) The first five methods give practically the same results. (*c*) The method of Muller is

not exact, the extracts being too dark for titration; moreover, the values obtained by weighing are irregular and not correct.

TABLE I

Comparison of Results of Various Methods

Sample No.	Various methods	Duplicate analyses, per cent of fresh feces		
		Fatty acids	Fat	Fatty acids + fat
1	Our Method A			6.2, 6.2
	" " B	5.8, 5.5	0.51, 0.51	6.3, 6.0
	Weibull	5.5, 5.6	0.64, 0.64	6.1, 6.2
	Gorter and de Graaff	5.6, 5.6	0.68, 0.58	6.3, 6.2
	Fowweather (modified)	5.6, 5.5	0.62, 0.58	6.2, 6.1
	Muller (titrated)	5.2, 5.3	0.70, 1.10	5.9, 6.4
	" (weighed)			6.5, 7.0
2	Our Method A			5.6, 5.6
	" " B	5.0, 5.0	0.70, 0.60	5.7, 5.6
	Weibull	5.0, 5.0	0.24, 0.36	5.3, 5.4
	Gorter and de Graaff	4.9, 4.9	0.51, 0.53	5.4, 5.4
	Fowweather (modified)	5.0, 4.9	0.48, 0.53	5.5, 5.4
	Muller (titrated)	5.1, 5.2	1.30, 1.50	6.4, 6.7
	" (weighed)			6.0, 6.2
3	Our Method A			10.5, 10.5
	" " B	9.1, 9.1	1.4, 1.5	10.5, 10.6
	Weibull	9.2, 9.1	0.9, 0.9	10.1, 10.0
	Gorter and de Graaff	9.2, 9.2	1.1, 1.0	10.3, 10.2
	Fowweather (modified)	8.9, 9.1	1.2, 1.3	10.1, 10.4
	Muller (end-point not visible)			
	" (weighed)			11.7, 10.3
4	Our Method A			5.6, 5.6
	" " B	4.9, 4.9	0.8, 0.8	5.7, 5.7
	Weibull	5.0, 4.9	0.5	5.4
	Gorter and de Graaff	4.8, 5.0	0.6, 0.6	5.4, 5.6
	Fowweather (modified)	4.8, 4.8	0.5, 0.6	5.3, 5.4
	Muller (end-point not visible)			
	" (weighed)			6.6, 6.7

SUMMARY

Methods for the determination of the total amount of fat in feces and of the relative proportions of free and combined fatty acids are described.

They are based on the principle that fatty acids and fat can be extracted almost quantitatively with petroleum ether from an acidic, alcoholic solution of about 60 per cent ethanol, saturated with NaCl and containing a small amount of amyl alcohol.

By these methods the fat in feces can be measured within 35 to 45 minutes with an error not exceeding 2 per cent.

BIBLIOGRAPHY

1. Berntrop, J. C., *Z. angew. Chem.*, **15,** 121 (1902).
2. Cooke, W. T., Elkes, J. J., Frazer, A. C., Barkes, J., Peeney, A. L. P., Sammons, H. G., and Thomas, G., *Quart. J. Med.*, **15,** 141 (1946).
3. Folin, O., and Wentworth, A. H., *J. Biol. Chem.*, **7,** 421 (1909–10).
4. Fowweather, F. S., *Brit. J. Exp. Path.*, **7,** 7 (1926).
5. Fowweather, F. S., and Anderson, W. N., *Biochem. J.*, **40,** 350 (1946).
6. Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, **19,** 521 (1914).
7. Goiffon, R., Manuel de coprologie chimique, Paris (1942).
8. Gorter, E., and de Graaff, W. C., Klinische Diagnostiek, 5th edition, Leiden, 383 (1941).
9. Holt, L. E., Courtney, A. M., and Fales, H. L., *Am. J. Dis. Child.*, **17,** 38 (1919).
10. Inaba, R., *Biochem. Z.*, **8,** 348 (1908).
11. Kolthoff, I. M., cited in de Graaff, W. C., Schoorl, N., and van der Wielen, P., Commentaar op de Nederlandsche Pharmacopee, 5th edition, Utrecht, **3,** 304 (1927–31).
12. Kumagawa, M., and Suto, K., *Biochem. Z.*, **8,** 212 (1908).
13. von Liebermann, L., and Székely, S., *Arch. ges. Physiol.*, **72,** 360 (1898).
14. Muller, P., *Nederl. Tijdschr. Geneesk.*, **79,** 3960 (1935).
15. Nielsen, G., *Acta paediat.*, **31,** 225 (1943).
16. Polenske, E., *Arb. k. Gsndhtsamte.*, **33,** 563 (1910).
17. Rosenfeld, G., *Biochem. Z.*, **200,** 280 (1928).
18. Saxon, G. J., *J. Biol. Chem.*, **17,** 99 (1914).
19. Sonntag, G., *Arb. k. Gsndhtsamte.*, **51,** 25 (1919).
20. Tidwell, H. C., and Holt, L. E., Jr., *J. Biol. Chem.*, **112,** 605 (1935–36).
21. Weibull, M., *Z. angew. Chem.*, 450 (1892).
22. Weibull, M., *Z. angew. Chem.*, 199 (1894).

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J. Biol. Chem. 1949, 177:347-355.
