Detergent Extraction and Enzymatic Analysis for Fecal Long-Chain Fatty Acids, Triglycerides, and Cholesterol

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We report a procedure for determining fecal long-chain fatty acids (LCFA), triglycerides, and cholesterol after detergent extraction with 10 mL/L Triton X-100, 6 mL/L Brij 30, and 0.1 mol/L HCl in isotonic saline by enzymatic analysis of the extraction supernatant. Mean recoveries of calibrators ranged from 105% to 117%. Assays of fecal extracts were linear with concentration from 0 to 1000 μmol/L for LCFA, from 10 to 90 μmol/L for triglycerides, and from 0 to 300 μmol/L for cholesterol. Within-run CVs were <3% for medium and high concentrations, and 3%, 14%, and 28% for low concentrations of LCFA, cholesterol, and triglycerides, respectively. Correlation with gravimetric determination resulted in coefficients (r²) of 0.72 for total fat, 0.63 for neutral fat, and 0.66 for nonglycerified fat. Assays of extracts and residues prepared for gravimetric determination indicated that ~90% of triglycerides, cholesterol, and LCFA were extracted by organic solvents and that the extracts contain a significant amount of other fats. The proposed method appears accurate, precise, specific, and suitable for routine analysis.

Indexing Terms: feces/fats/lipids/malabsorption/steatorrhea/pancreatic dysfunction

Accurate quantitative determination of steatorrhea is of great importance in the diagnosis of malabsorption. For many years, reference procedures have involved gravimetric or titrimetric measurement of fecal fats extracted with organic solvents (1–3). Despite adequate precision and sensitivity (1, 2, 4–6), several sources of error, e.g., compliance problems, assumption of an average molecular mass for the calculation of fat content (6, 7), and extraction of dietary as well as endogenous lipids (1, 8), have encouraged the search for alternative methods.

Screening methods are designed to minimize cumbersome collection and fecal handling. Serum measurements of, e.g., turbidity (9, 10), triglycerides (11), vitamin A (12), and [14C]triolein/[3H]oleate (13) have produced conflicting results. Some authors report good sensitivity and specificity for fecal microscopy and recommend it as a simple, cost-efficient procedure suitable for office screening (14, 15). Reliability of microscopic results, however, appears to be largely dependent on technician training and thoroughness and on standardization of procedure and interpretation (14, 15). Validation of Sudan stains has been difficult, given that correlation studies with the reference method inherently also compare the use of random specimens with the more traditional 72-h collections. Consequently, reports on correlations with quantitative measurements of fecal fat vary considerably (8, 15).

Isotopic fecal analyses have been developed to eliminate inaccuracies associated with extraction of endogenous fats from feces. Triglycerides or fatty acids labeled with 125I, 14C, or 3H are administered orally in combination with nonabsorbable markers. The measurement of these markers in feces has been moderately correlated with titrimetrically determined fecal fat (4, 16, 17); however, exposure to radioactivity remains a concern with regard to patient safety.

Breath testing for expired CO₂ is in theory a more sensitive indicator of steatorrhea than is fecal fat, and does not require fecal handling. Satisfactory results in the detection of steatorrhea have been reported for breath tests involving 14C and 13C (18, 19). However, additional research is needed to resolve issues such as curvilinear relationships between reference and breath test methods, difficulties relating severity of malabsorption to breath test results, variations in metabolic rates between and within individuals, and delayed expiration peaks (20, 21).

A recently reported technique based on near-infrared reflectance spectroscopy requires little fecal handling and has been claimed to provide precise results and good correlation with titrimetrically determined fecal fat. Issues concerning system calibration and matrix effects are unresolved at this time (7, 22, 23). If confirmed and validated, however, this technique could prove to be a useful clinical tool.

In light of the important role detection of steatorrhea plays in the diagnosis of malabsorption, and given the limitations of existing measures, the need for a method that will provide reliable and precise determination of fecal fats in the clinical lab is evident. Here, we report the development of a procedure that quantitatively extracts triglycerides, long-chain fatty acids (LCFA), and cholesterol from fecal matrix without interfering with subsequent analytical methods. The extracted lipids are analyzed enzymatically by utilizing established methods for assaying serum fats. This technique offers the promise of low-cost, precise, and accurate fecal fat analyses in the routine clinical lab, and should allow more definitive investigation of specimen collection, storage, and transport issues.

Materials and Methods

Materials. Formaldehyde and HCl were obtained from Baxter Scientific, McGaw Park, IL. NaCl, Triton X-100,
and Brij 30 were purchased from Sigma Chemical Co., St. Louis, MO. Reagent kits for enzymatic determinations of triglyceride and cholesterol were purchased from Roche Diagnostic Systems Div. of Hoffmann-La Roche, Nutley, NJ. The Cobas Calibrator Serum (Roche Diagnostic Systems) served as the calibrator for the triglyceride and cholesterol procedures. The NEFA C kit from Wako Chemicals (Dallas, TX) was used for enzymatic determination of LCFA; the NEFA calibrator (nonesterified fatty acid) contains an aqueous solution of 1.0 mmol/L oleic acid, surfactant, and stabilizers.

Detergent extraction. Aliquots of fecal specimens preserved in 50 mL/L formalin in 0.1 mol/L HCl were diluted 1:15 in stool diluent: 10 mL/L Triton X-100, 6 mL/L Brij 30, and 0.1 mol/L HCl in isotonic saline (NaCl 150 mmol/L). Thoroughly mixed samples (1 mL) were subsequently centrifuged for 15 min at 1050g.

Enzymatic analyses. After extraction, the supernates were analyzed for triglycerides, cholesterol, and LCFA according to enzymatic serum methods. Triglycerides were measured with Roche's modification of the lipoprotein lipase/glycerokinase/glycerophosphate oxidase procedure of Esders et al. (24, 25). Cholesterol was analyzed according to the cholesterol esterase/oxidase/peroxidase method of Allain et al. (26). In both procedures, the absorbance of the final product was measured at 500 nm. LCFA were determined with an acyl-CoA synthetase/acyl-CoA oxidase/peroxidase procedure (Wako Chemicals). Absorbance of the adduct resulting from oxidative condensation was measured at 550 nm. All analyses were performed on a Roche Cobas Fara II analyzer (Roche Diagnostic Systems, Montclair, NJ), and results were expressed as a function of stool wet weight.

Split-sample evaluation. LCFA, triglyceride, and cholesterol contents of 10 fecal specimens were determined by detergent extraction/enzyme analysis as described above. Aliquots of the same samples were further analyzed for total, neutral, and nonesterified fat according to the gravimetric method of Sobel (1). Correlation coefficients were obtained by regression analysis for gravimetrically determined total fat vs total fat determined by detergent extraction/enzyme analysis, neutral fat vs the sum of triglyceride and cholesterol concentrations, and nonesterified fat vs LCFA.

In six randomly selected fecal specimens, split-sample evaluation as described above was followed by enzyme analysis of the extract prepared for gravimetric determination. Triglycerides, cholesterol, and LCFA were further determined enzymatically in the sediment fraction of the extracted stools.

Results

Detergent Extraction

Central to the analysis of fecal LCFA, triglycerides, and cholesterol is the ability to extract and fully solubilize all of the fats in stool. Fatty acid-based surfactants must be avoided, however, because they will interfere with the measurement of fecal fatty acids. We tested the nonionic detergent Triton X-100 at various concentrations for its effectiveness in extraction. LCFA concentrations measured in 50 mL/L formalin-preserved fecal samples diluted 5- to 20-fold with 2, 10, or 20 mL/L Triton X-100 were linear throughout the dilution range. Yields obtained with 10 mL/L Triton X-100 were greater than those obtained with 2 mL/L Triton X-100, and use of 20 mL/L gave little if any increase (Fig. 1). We therefore used 10 mL/L Triton X-100 for further testing.

Because dilution experiments indicated that not all of the LCFA was being extracted with 10 mL/L Triton X-100, we evaluated the inclusion of a second solubilizing agent, the nonionic detergent polyethylene ester Brij 30, in the diluent. The combination of 6 mL/L Brij 30 and 10 mL/L Triton X-100 improved the extraction yields of LCFA. Dilution of at least 2.5-fold was required to fully extract LCFA, but linearity was maintained for greater dilutions (Fig. 2). Yields of LCFA, triglyceride, and cholesterol extracted from fecal specimens having various fat contents showed that a dilution of 1:15 led to consistent results, ensured maximum extraction, and minimized errors associated with greater diluent content. In the final procedure we diluted fecal samples 1:15 with 10 mL/L Triton X-100 and 6 mL/L Brij 30 in isotonic saline before centrifugation.

LCFA Stability

Initial studies indicated that triglyceride esters were hydrolyzed during storage of fecal specimens, whether formalin-preserved or not. We therefore prepared a fecal preservative consisting of 50 mL/L formalin in 0.1 mol/L HCl to stabilize fecal materials, denature hydrolytic enzymes, and prevent alkaline hydrolysis of triglycerides. Samples were subsequently diluted 1:15 with a diluent of isotonic saline, 6 mL/L Brij 30, 10 mL/L Triton X-100, and 0.1 mol/L HCl. Specimens preserved and stored in acid formalin >2 weeks exhibited minimal loss of LCFA (CV = 4.1%).

Analytical Performance

Recovery. Using the detergent extraction/enzyme procedure, we analyzed the Cobas calibrator for triglyceride, cholesterol, and LCFA.

![Fig. 1. Effectiveness of various concentrations of Triton X-100 in extraction of fecal LCFA.](image-url)

Results for one 72-h fecal specimen, which were found to be consistent with results of other patients' samples. The numbers above each column indicate the concentration of Triton X-100 in mL/L.
ides and cholesterol, and analyzed the oleic acid calibrator provided with the NEFA C kit for LCFA. Each calibrator was then mixed in various volume proportions (from 1:1 to 1:9, standard:fecal specimen) with aliquots of an acid-formalin-preserved fecal specimen of predetermined LCFA, triglyceride, and cholesterol content. The mixtures were subsequently analyzed for triglycerides, cholesterol, and LCFA according to the detergent extraction/enzyme procedure. Mean (±SD) analytical recoveries of the calibrators from the fecal matrix were 106% ± 5% for LCFA, 117% ± 12% for triglycerides, and 107% ± 3% for cholesterol.

**Linearity.** Aliquots of four randomly selected 72-h fecal specimens were diluted 2- to 516-fold with stool diluent and centrifuged for 15 min at 1050g. LCFA, triglycerides, and cholesterol were determined enzymatically. Assays of fecal extracts were linear with concentration for LCFA from 0 to 1000 µmol/L, for triglycerides from 10 to 90 µmol/L, and for cholesterol from 0 to 300 µmol/L.

**Precision.** Within-run precision was determined for low-, intermediate-, and high-concentration specimens. CVs for the latter two samples were <3% for LCFA, triglyceride, and cholesterol procedures. Variability increased as the limit of sensitivity was approached, with CVs for the low-concentration specimens ranging from 3% to 28% (Table 1).

**Split-sample evaluation.** Split-sample comparison of 10 72-h fecal specimens was performed to determine the correlation between the detergent extraction/enzyme method and the gravimetric comparison procedure (1). LCFA, triglyceride, and cholesterol values determined enzymatically were summed to obtain a value for total fat, which correlated by $r^2 = 0.72$ and a slope of 0.28, with gravimetrically determined total fat (Fig. 3A). A coefficient of $r^2 = 0.63$ and a slope of 0.26 were obtained for correlation of gravimetrically determined neutral fat with the sum of triglyceride and cholesterol concentrations (Fig. 3B). Correlation of gravimetrically determined nonesterified fat with LCFA gave a coefficient of $r^2 = 0.66$ and a slope of 0.29 (Fig. 3C). The intercepts were near zero in all three correlation plots.

Gravimetrically determined total fats and enzymatically determined total fat components obtained in fresh stool, the extract, and the sediment resulting from extraction with organic solvents were compared in six randomly selected fecal specimens. Mean fecal fat contents were 4.32 g/100 g stool (wet wt.) according to gravimetric determination, 2.34 g/100 g stool according to enzymatic analysis in detergent-extracted stool, 1.64 g/100 g stool determined in the extract prepared for gravimetric determination, and 0.25 g/100 g stool obtained in the sediment (Fig. 4).

**Distribution of Values in Patients' Samples**

Distributions of LCFA, triglycerides, cholesterol, and total fat (sum of LCFA, triglycerides, and cholesterol) concentrations were determined for 1995 randomly se-

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**Table 1. Within-run precision of LCFA, cholesterol, and triglyceride analyses in fecal extracts.**

<table>
<thead>
<tr>
<th>Range</th>
<th>LCFA</th>
<th>Triglycerides</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>162 ± 5</td>
<td>48 ± 7</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Medium</td>
<td>935 ± 13</td>
<td>224 ± 4</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>High</td>
<td>3449 ± 42</td>
<td>604 ± 4</td>
<td>229 ± 2</td>
</tr>
</tbody>
</table>

$n = 15$ each.
Gravimetric
1.0
'I.
0.8
1.6
0.4
1.2

Extraction,

Total
Fecal
Residue.

Conclusively,
by

The

Discussion

The proposed method of detergent extraction and subsequent enzymatic analysis of LCFA, triglycerides, and cholesterol appears to fully solubilize and recover fecal fats and allows for accurate and precise determination of analyte concentrations. Further, the use of potentially harmful and flammable organic solvents required by the gravimetric and titrimetric comparison procedures (1, 2) is avoided.

Fecal microscopy, while cost-effective and attractive as a screening method, has to date not been validated conclusively, largely because of its semiquantitative nature. Differences between the microscopic and the other comparison methods with respect to the collection period of specimens and the classes of lipids identified by either method (8, 15) confound validation studies. In fecal microscopy, triglycerides are identified by the "neutral stain," whereas the "split fat stain" provides semiquantitative information on triglyceride and fatty acid content (8, 14), excluding cholesterol, cholesteryl esters, and phospholipids (8).

The titrimetric method of van de Kamer et al. (2) similarly allows the differential determination of neutral fats and nonesterified fats. However, lipids identified by the neutral fat procedure include not only triglycerides but also cholesteryl esters derived from biliary secretion and phospholipids originating from sloughed epithelial cells (8, 27); thus, the presence of these endogenous lipids leads to overestimation of neutral lipids. Overestimation of nonesterified fat may result from concomitant titration of acids other than fattyested, acid-formalin-preserved samples from patients. Positively skewed distributions were obtained for all four analytes (Fig. 5), with skewness and coefficient of kurtosis of 2.1 and 9.1, respectively, for total fats; 4.2 and 32 for triglycerides; 2.7 and 22 for cholesterol; and 1.9 and 6.6 for LCFA. We used the 80th percentiles of the distributions to generate the following expected reference intervals for fecal fat (g/100 g stool, wet wt.): total fat, <2.5; LCFA, <1.6; triglycerides, <0.4; and cholesterol <0.4.

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acids. Gravimetric determination of fecal fat further overestimates fat content through the inclusion of non-saponifiable matter (1). It remains controversial whether the more specific microscopic determination or the more inclusive petroleum ether extraction followed by gravimetric or titrimetric determination provides a more accurate indicator of steatorrhea (8, 15, 27).

Enzymatic analysis after detergent extraction eliminates inaccuracies associated with the presence of endogenous lipids and non-saponifiable matter or with the titration of acids other than fatty acids. According to the slopes of the correlation plots, the proposed method measures about one-third of the fats identified by the gravimetric comparison method. Enzymatic analysis of the extracts prepared for gravimetric determination indicates that organic solvents extract a substantial amount of fats other than triglycerides, cholesterol, and LCFA. Further, the amount of total fats measured enzymatically in the sediment suggests that not all triglycerides, cholesterol, and LCFA are extracted by organic solvents.

For the titrimetric method, the upper normal limit of fecal fat is frequently set at 5% of stool weight (5 g/100 g). However, an upper normal limit for fat of 1 g/100 g was established for microscopically obtained results. At the same time, stool containing fat at 5 g/100 g by the method of van de Kamer et al. (2) has been found negative for steatorrhea by the Sudan stain (27). These results have been interpreted as suggesting a greater specificity of fecal microscopy.

Projected reference intervals for total fat according to the proposed detergent extraction/enzyme procedure are above those suggested for fecal microscopy. This may be partially explained by the inability of Sudan stain to assess cholesterol content (8); further, it suggests greater sensitivity and accuracy of the proposed method. In addition, the reference intervals projected for the detergent extraction/enzyme analysis method are considerably lower than the upper normal limit established by van de Kamer et al., which supports the claim of overestimation of fecal lipids by titrimetric measurement (27). Comparatively low estimated reference intervals and the moderate slopes of the correlation plots indicate greater specificity of the detergent extraction/enzyme analysis procedure than of gravimetric fecal fat determination.

In conclusion, detergent extraction followed by enzymatic analysis makes accurate and precise fecal fat determination available to the clinical laboratory. We hope that the introduction of easily performed assays will expedite the study of outstanding issues, such as the sensitivity and selectivity tradeoffs of random stools vs 1-, 2-, or 3-day collections.

References