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# Comparison of Gravimetry and Hydrolysis/Derivatization/Gas Chromatography−Mass Spectrometry for Quantitative Analysis of Fat from Standard Reference Infant Formula Powder Using Supercritical Fluid Extraction

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This paper describes a comparative study of the gravimetric versus hydrolysis/derivatization/gas chromatography−mass spectrometry determination of fat in infant formula. Fat was extracted using supercritical carbon dioxide modified with a small amount of ethanol, the extract was weighed, and the total fat was determined gravimetrically. Subsequently, another sample of the supercritical fluid fat extract was hydrolyzed to yield free fatty acids, which were converted to their methyl ester derivatives (FAMEs). Quantification was performed by GC-MS. NIST Standard Reference Material (SRM-1846) was used to validate both fat determination methods. Results showed that the gravimetric average percent fat was 26.86%, whereas the GC-MS method yielded 24.64%. Some peaks were detected in the ion chromatogram from the GC-MS that were identified as nonfatty acids such as aldehydes, which may account for the higher percentage fat measured as weight of extract rather than measured as FAMEs expressed as triglycerides.

**KEYWORDS: Infant formula; fat content; supercritical fluid extraction; gravimetry; gas chromatography; mass spectrometric detection**

## INTRODUCTION

There currently are more than 15 methods for the determination of total fat from food matrices. Methods include solvent extractions such as Soxhlet and those that require sample pretreatment (i.e., acid or base hydrolysis) such as the Roese−Gottlieb and Mojonnier methods. The Roese−Gottlieb method (AOAC 905.02) is internationally accepted as a gravimetric method for the determination of fat in dairy products. Contract and organizational laboratories use additional methods such as modified Mojonnier (AOAC 989.05) and Babcock (AOAC 920.11 B-C) for total fat determination (*1*). Recent studies have introduced supercritical fluid extraction (SFE) using carbon dioxide as an alternative to traditional extraction methods for the measurement of total fat in food products (*2*−*4*). The results of these studies suggest that SFE is a replacement method for traditional gravimetric techniques.

Fat in infant formula improves nutrient composition and promotes good health; therefore, the level and quality of fat in infant formula are required for nutritional labeling information. The definition of fat content, according to the Nutritional Labeling and Education Act (NLEA) (*5*), includes the sum of fatty acids from mono-, di-, and triglycerides, free fatty acids, phospholipid fatty acids, and sterol fatty acids, stoichiometrically expressed as triglycerides.

For infant formula to act as a substitute for human milk, it is important that the formula and human milk contain fairly equivalent amounts of fat. It is known that ∼98% of lipids in human milk fat are triglycerides, with <1% each of diglycerides, free fatty acids, and sterols. Infant formula, if used to replace human breast milk, should have the necessary lipids to help form cellular membrane layers. Some of the long-chain polyunsaturated fatty acids that contribute to membrane synthesis of the brain and nervous system are linoleic and linolenic acids. It has also been observed that skin lesions have been found to develop in infants who are fed milk-based formula of low linoleic acid content. These fatty acids cannot be synthesized in the human body. A breast-feeding mother obtains them from her diet and then passes them on to the newborn. Although data vary among different researchers, human milk fat appears to be made up of about 3.1% palmitoleic acid ($C_{16:1}$), 35−36% oleic acid ($C_{18:1}$), 8−10% linoleic acid ($C_{18:2}$), and 1.2% linolenic acids ($C_{18:3}$). Only small to trace amounts of $C_{20}$−$C_{22}$ unsaturated fatty acids are present. Chief among saturated fatty acids in human milk is palmitic acid ($C_{16:0}$), which is present at levels of 20−25%. The $C_4$−$C_8$ fatty acids make up scarcely 1.5% of human milk fat. A mother who chooses not to breast-

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feed must therefore use some type of infant formula that has the aforementioned essential fatty aids in them (*6*, *7*).

To determine fat content, the NLEA protocol involves a hydrolysis treatment, followed by solvent extraction of lipids, derivatization, and then individual separation of fatty acid methyl esters (FAMEs) by gas chromatography (GC). The percent saturated, monounsaturated, and total fat are then calculated from the resulting FAME profile, and the fat is expressed as triglycerides (*8*, *9*). Other methods for determining fat content have been based on simple gravimetric measurements after the removal of fat from the sample matrix with a suitable solvent. In some cases the lipid material is subjected to acid/base hydrolysis prior to solvent extraction. The most widely used procedure for the removal of fat from a fatty sample is a conventional Soxhlet extraction method; however, in recent years, alternative extraction methods such as supercritical fluid extraction, pressurized solvent extraction, and microwave-assisted extraction have become more attractive because hydrolysis pretreatment is not always performed on the sample. Conventional gravimetric methods are costly, require large volumes of organic solvents, and can potentially alter lipid integrity during extraction (*10*, *11*).

SFE with $CO_2$ has been performed on a variety of food matrices, especially meat samples. The high efficacy that SFE with $CO_2$ demonstrates toward the removal of oils and fats from such matrices makes it a natural technique for the determination of their fat and oil content. For example, Eller and King have compared gravimetric and GC FAME fat determinations for supercritical fluid extracts of oilseed, ground beef, and bakery samples. They showed that for sunflower oil, cottonseed oil, ground beef, and low-fat bakery products, the gravimetric results were higher than the GC FAME results. The two methods were equivalent for soybeans, canola, and safflower. These observations indicated to these workers that some nonfat material was extracted along with the fatty components by the SFE method (*12*, *13*). Previously, we reported a comparative gravimetric study on quantitative extraction of fat from infant formula using pressurized carbon dioxide and a traditional organic solvent. In this study, supercritical fluid fat extraction conditions were optimized and the methodology was validated against a widely accepted method (i.e., acid hydrolysis). It was concluded that SFE with $CO_2$ was as effective as acid hydrolysis followed by solvent extraction, and it constituted a good choice for fat analysis of infant formula (*11*).

Studies have demonstrated that fat extraction methods do not extract just pure triglycerides. Therefore, it is unlikely that gravimetric measurement of the solvent extract of a sample, with or without prior hydrolysis, will give an accurate determination of fat content (*13*, *14*). Chromatographic techniques performed on the extract are often used to separate the various compounds not only from each other but also from the large amounts of interfering nonfat materials in the sample extract. However, a reliable quantitation method for fat is still not easy, and current methods produce varying fat contents for the same sample. Although fat serves an infant's need for energy and makes a significant contribution to the activity of the central nervous system, at excessive levels in the diet, fat can interfere with calcium absorption and absorption of certain fat-soluble vitamins (*6*). It is important, therefore, to ensure manufacturer's compliance with the presence of regulated fat nutrients at appropriate levels in infant formula.

The National Institute of Science and Technology (NIST; Gaithersburg, MD) released an infant formula standard material (SRM 1846) in 1996 that can be used to validate developing analytical methods. The certified NIST data for fat in infant formula (27.1 ± 0.6%) were obtained from a collaboration of several laboratories where the method of sample preparation ranged from direct liquid−liquid extraction to preliminary acid (or base) hydrolysis followed by extraction. The primary NIST method for determining fat in infant formula utilized an ether-based extraction (*15*). The results from nine different laboratories are shown in **Table 1** to illustrate the data used to establish the certified value. An examination of the Infant Formula Act of 1980 has revealed that there are no standard methods for quantitative determination of fat in infant formula (Infant Formula Act, 1980, Public Law 96-359).

**Table 1.** Methods and Individual Laboratory Results ($n = 6$) Used To Determine Fat Value for NIST Reference Standard 1846[a]

| | av % | SD | % RSD |
|---|---|---|---|
| acid digestion | 25.95 | 0.483 | 1.86 |
| Roese–Gottlieb[b] | 25.43 | 1.092 | 4.29 |
| Roese–Gottlieb | 27.28 | 0.535 | 1.96 |
| Roese–Gottlieb | 26.67 | 0.484 | 1.81 |
| Roese-Gottlieb | 27.83 | 0.052 | 0.19 |
| Roese–Gottlieb | 27.50 | 0.188 | 0.68 |
| Mojonnier[c] | 27.83 | 0.234 | 0.84 |
| Mojonnier | 27.66 | 0.241 | 0.87 |
| Mojonnier | 27.24 | 0.620 | 2.23 |

[a] Ether extraction. [b] Base hydrolysis—nonautomated. [c] Base hydrolysis—automated.

The objective of this paper is to compare the gravimetric and the hydrolysis/derivatization/gas chromatography−mass spectrometric determination of fat on the same supercritical fluid extract in order to understand which method provides a better measurement. The ultimate aim of this study was to develop a methodology for accurate determination of fat that would comply with federal regulations as specified in the NLEA. It would also serve as a quality control procedure to ensure the correct amount and chemical composition of fat in infant formula. In the present study, powdered sample, infant formula standard material (SRM 1846) with a known fat nutrient composition was used as a means to validate fat content measurement of both the gravimetric and hydrolysis/derivatization/GC-MS methods. The resulting methodology was then applied to other infant formulas.

## EXPERIMENTAL PROCEDURES

**Materials.** FAME standards (GLC reference standard, Nestle 37) (>99%) and an internal standard, $C_{11}$ triglyceride (tridecanoin) (T-125), were purchased from Nu-Check Prep, Inc., (Elysian, MN). Methanol and toluene were supplied by Burdick & Jackson Laboratories, Inc. (Muskegon, MI), and acetyl chloride (>99% pure) was purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Reference infant formula sample was purchased from NIST (Gaithersburg, MD). Carbon dioxide (SFE/SFC grade) with helium headspace was supplied by Air Products and Chemicals, Inc. (Allentown, PA).

**Supercritical Fluid Extraction Fat.** An ISCO Suprex (Lincoln, NE) automated Prepmaster (AP-44) system with variable flow restrictor was used for all fat extractions. Infant powder (2.0 g) was thoroughly mixed with 4.0 g of deionized water and sonicated for 10 min to create a homogeneous sample. A portion of this viscous solution (1.0 mL) was taken and mixed with ~3.0 g of Hydromatrix (Varian, Harbor City, CA) to immobilize any excess water. The solid sample mass was placed in a 10-mL extraction vessel and equilibrated for 10 min, and then1 mL of $CH_3OH$ was spiked onto the sample to modify the sample matrix. The methanol-laden mixture was equilibrated for an additional 10 min. SFE was performed at 465 atm and 100 °C at a flow rate of 2 mL/min for 20 min after an initial 10 min static hold. The extraction fluid was 85% $CO_2$/15% ethanol. The variable restrictor temperature was set at 80 °C. The extracted fat was collected via a solid trap of $C_{18}$ bonded

silica at 50 °C, which was rinsed with a 50:50 mixture of $CH_3OH$ and $CH_2Cl_2$ (into a 10 mL preweighed collection vial) after completion of the extraction. The rinse volume was 5 mL; the solid phase trap temperature during rinsing was held at 25 °C.

**Gravimetric Quantitation of Fat.** The preweighed vial, which contained the rinse fat solution, was placed on a hot plate and dried using a stream of nitrogen gas. The difference in weight of the vial before/after extraction and drying was assumed to be fat. The gravimetric percentage of fat in the matrix was determined on the basis of the weight of the original sample.

**Hydrolysis and Derivatization.** After the weight of fat had been obtained via gravimetry, the dried extract was subsequently redissolved in $CH_3OH/CH_2Cl_2$(50:50%) and transferred to a 25 mL screw-cap vial. The solvent was evaporated to dryness, and the extract was redissolved in 10 mL of toluene/$CH_3OH$ (50:50%) containing tridecanoin ($C_{11}$) (45 ng/$\mu$L) as an internal standard. Acetyl chloride (0.5 mL) was added to the solution in order to react with methanol to provide a catalytic amount of HCl for in situ acid hydrolysis. The headspace of the solution was purged with nitrogen, and the vial was capped. The vial containing the extract solution was placed in an oven for 1 h at 100 °C. The vial was allowed to cool to room temperature, then 10 mL of 6% sodium carbonate solution was added, and the resulting solution was mixed vigorously. The vial was centrifuged for 5 min to facilitate phase separation. The top layer was removed for subsequent GC-MS FAME analysis.

**GC-MS Analysis and Quantitation.** A Hewlett-Packard 5890 series II GC incorporating a Supelco SP-2250 (60 m × 0.25 mm, 0.25 $\mu$m film thickness) (Supelco, Bellefonte, PA) column was interfaced to a Hewlett-Packard 5972 series mass selective detector. Injections were made using a Hewlett-Packard 7673 injector. The sample injection volume was 1 $\mu$L with a split ratio (1:50). The GC oven temperature was initiated at 40 °C for 1 min. It was ramped to 145 °C at a rate of 3 °C/min and held for 1 min. Finally, it was ramped to 220 °C at a rate of 5 °C/min and held for 30 min. Ultrahigh-purity grade helium was used as the carrier gas at a flow rate of 1.26 mL/min. The mass detector temperature was set at 280 °C, and the detector was turned on after the first 8.5 min of separation (e.g., elution of the solvent). The weights of the individual FAMEs were calculated on the basis of their integrations relative to the tridecanoin ($C_{11}$) internal standard and were corrected using corresponding GC response factors for each fatty acid (*8*). The weights of the individual FAMEs were converted to equivalent weights of triglycerides using appropriate conversion factors (*16*). Total fat was calculated as the sum of all fatty acids expressed as triglycerides. Identification of compounds in the chromatograms was based on a probability-based matching algorithm library using all of the ion fragments.

## RESULTS AND DISCUSSION

Gravimetric methods are used routinely for the determination of total fat. In some cases organic solvent extraction is accomplished directly, whereas in others acid or base hydrolysis (i.e., digestion) precedes extraction with organic solvent. The goal of this study was to (a) quantitatively extract fat from a standard reference material (SRM) with supercritical carbon dioxide with no prior digestion, (b) weigh the extracted portion, and (c) then subject the extract to, firsr, hydrolysis, thus freeing fatty acids, second, derivatization to FAMEs, and, third, separation/analysis of the FAMEs via GC-MS. The SRM chosen for this study was infant formula. Results from several independent laboratories with the same SRM are also given for comparison.

In our work, the weight of the supercritical fluid (SF) extract was assumed to be a direct measure of fat content (e.g., the only material extracted was fat). Our gravimetric results are shown in **Table 2** where ethanol-modified $CO_2$ was employed. Five replications were employed with an average percent extractables (i.e., fat) equal to 26.86%, RSD = 2.99%. The measured percent fat extracted matched well the certified fat value of 27.1 ± 0.6%. As can be observed, >99% recovery relative to the value provided by NIST was obtained. Independent of our study, Isco, Inc., performed the same SFE (7500 psi of $CO_2$, 100 °C, 15% ethanol, 2 mL/min, 30 min) with similar results. Extractor 1 (Isco Fast Fat HT 1) gave 27.54% ($n$ = 25), RSD = 1.25%, extractor 2 (Isco Fast Fat HT 2) gave 27.84% ($n$ = 4), RSD = 0.25%, and extractor 3 (Isco SFX 3560) gave 27.81% ($n$ = 12), RSD = 1.42%.

**Table 2.** Weight Percent of Fat Extracted by Modified $CO_2$ from NIST Infant Formula Powder As Determined by Gravimetry versus GC-MS of Hydrolyzed/Derivatized Supercritical Fluid Extracted Fat

| extraction | SFE gravimetry (%) | SFE/GC-MS (%) |
|---|---|---|
| 1 | 27.07 | 23.61 |
| 2 | 27.33 | 25.47 |
| 3 | 26.30 | 24.42 |
| 4 | 25.80 | 24.20 |
| 5 | 27.98 | 25.50 |
| av | 26.86 | 24.64 |
| % RSD (SD) | 3.10 (0.83) | 3.35 (0.83) |

Assay of the SF extract by hydrolysis/methylation/GC-MS yielded a lower average value (~2.5%) wherein fat is expressed as the triglyceride equivalent (**Table 2**) than both the NIST reference value (27.1 ± 0.6%) and our gravimetric value (26.86 ± 0.83%). The precision of both gravimetric and GC-MS methods in our laboratory was similar. The discrepancy between the two values may be attributed to a number of factors. The SFE method involves trapping of the extractables on a solid sorbent material followed by rinsing the extractables from the solid sorbent with organic solvent into a vial. Sample loss could occur when the extract is transferred during the derivatization process. Furthermore, during the methylation step the fatty residue is carried to dryness to effect a solvent exchange. Low molecular weight FAMEs could have evaporated during the drying step. The low GC-MS value could also be rationalized in the following way. The solvent delay was set to ~8.5 min; therefore, no GC-MS peak could be detected prior to 8.5 min. It has been observed in our laboratory with GC-FID that some low molecular weight FAMEs (e.g., $C_4$ and $C_5$) can be eluted prior to the solvent peak and thus not be detected by our GC-MS protocol.

**Figures 1** and **2** show the total ion current chromatograms (TIC) for a commercial FAME standard mixture and one of our infant formula SRM SF extracts after hydrolysis/derivatization. The TIC of the infant formula SF extract showed several peaks that could be assigned to nonfatty acids. This observation could account for the higher gravimetric values as all extractables are assumed to be fat. Quantification of each identifiable FAME in the supercritical extract was carried out (**Table 3**). The major components were observed to be $C_{12:0}$ (13.74%), $C_{16:0}$ (12.01%), $C_{18:0}$ (11.58%), $C_{18:1}$ (39.98%), and $C_{18:2}$ (14.37%). Results for a fat sample subjected to only organic solvent extraction as far as FAME composition is concerned were strikingly similar to the SFE results. The base prehydrolysis Mojonnier (i.e., not automated) and Roese−Gottlieb (i.e., automated) methods likewise gave similar FAME results to SFE and LSE (**Table 3**).

In a separate study, Mojonnier (fat in milk AOAC Method 989.05), which employs base ($NH_4OH$) hydrolysis, was performed (Mid-West Laboratory, Omaha, NE) on the standard infant formula prior to solvent extraction with ether. The dried solvent extract gave 27.41% (i.e., 101% recovery) by weight (e.g., assumed to be fat). When this extract was derivatized and fat determined by GC-MS of FAMEs, the fat percentage was

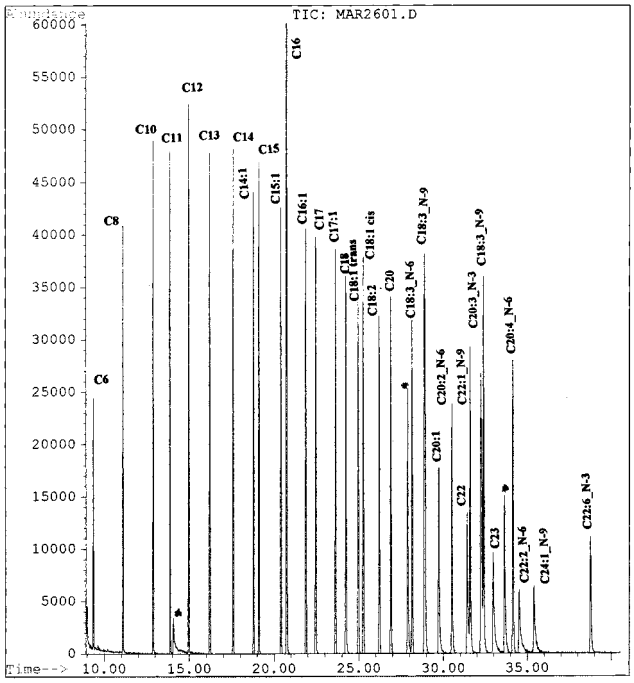


**Figure 1.** GC-MS of FAME standard (*, impurity).

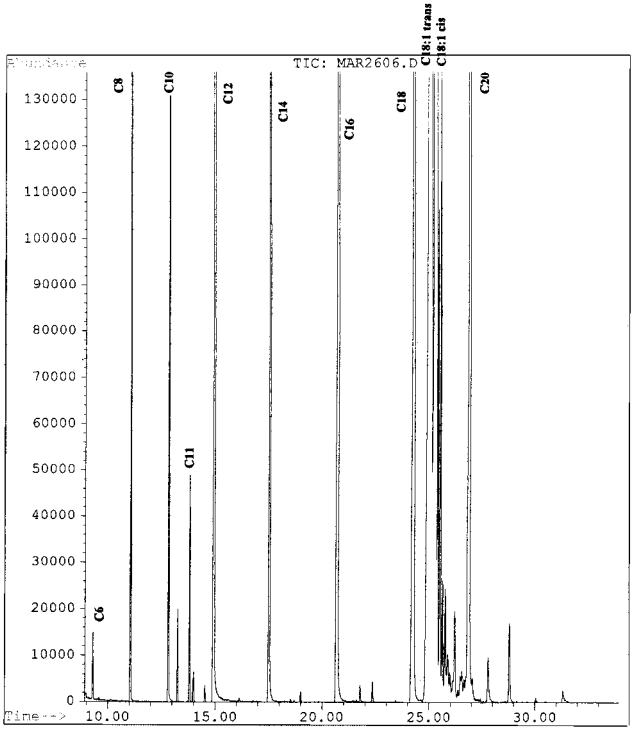


**Figure 2.** GC-MS of standard reference material infant formula after SFE and derivatization.

found to be 26.17% (i.e., 96% recovery). Solvent extraction of the same infant formula as received without any prehydrolysis but followed by GC-FID of FAMEs yielded 25.29% fat (i.e., 92% recovery).

The optimized gravimetric SFE method was next applied to several commercially available infant formulas (**Table 4**). Without a prehydrolysis step, the gravimetrically measured fat agreed quite well with the percent fat stated on the labels of the four vendors listed. Base hydrolysis followed by ether extraction gave similarly good results, but an additional experimental step and organic solvent usage was required.

**Table 3.** Comparison of GC FAMEs Profiles for NIST (SRM-1846)

| fatty acid profile | solvent extraction (%) | base hydrolysis (%) Mojonnier | base hydrolysis (%) Roese–Gottlieb | SFE (%) |
|---|---|---|---|---|
| $C_{4:0}$ | 0.08 | 0.11 | 0 | 0 |
| $C_{6:0}$ | 0.16 | 0.31 | 0 | 0.04 |
| $C_{8:0}$ | 3.53 | 3.78 | 1.96 | 0.39 |
| $C_{10:0}$ | 2.67 | 2.94 | 1.66 | 0.30 |
| $C_{12:0}$ | 14.74 | 14.26 | 13.42 | 13.74 |
| $C_{14:0}$ | 5.95 | 5.80 | 5.80 | 5.98 |
| $C_{14:1}$ | 0.03 | 0 | 0 | 0 |
| $C_{15:0}$ | 0.06 | 0.06 | 0 | 0.06 |
| $C_{16:0}$ | 11.29 | 11.56 | 11.41 | 12.01 |
| $C_{16:1}$ | 0 | 0 | 0.09 | 0.10 |
| $C_{17:0}$ | 0.16 | 0.10 | 0.09 | 0.11 |
| $C_{17:1}$ | 0 | 0 | 0.05 | 0 |
| $C_{18:0}$ | 10.98 | 10.88 | 11.05 | 11.58 |
| $C_{18:1}$ | 36.18 | 36.12 | 38.22 | 39.98 |
| $C_{18:2}$ | 12.89 | 12.88 | 14.29 | 14.37 |
| $C_{20:0}$ | 0.32 | 0.32 | 0.32 | 0.42 |
| $C_{20:1}$ | 0.13 | 0.13 | 0.26 | 0.11 |
| $C_{18:3}$ | 0.36 | 0.36 | 0.39 | 0.37 |
| $C_{21:0}$ | 0.05 | 0 | 0 | 0 |
| $C_{20:2n-6}$ | 0.03 | 0 | 0 | 0 |
| $C_{22:0}$ | 0.21 | 0.23 | 0 | 0.23 |
| $C_{24:0}$ | 0.13 | 0.16 | 0 | 0.13 |
| $C_{24:1n-9}$ | 0.04 | 0 | 0 | 0 |
| $C_{22:1}$ | 0 | 0 | 0.15 | 0 |
| $C_{20:4}$ | 0 | 0 | 0 | 0.07 |
| other fatty acids | 0 | 0 | 0.62 | 0 |
| total | 99.99 | 100 | 99.78 | 99.99 |

**Table 4.** Gravimetric Percent Fat Collected from Various Brands of Concentrated Liquid Infant Formula

| brand | manufacturer | % fat via SFE | % fat via base hydrolysis/ether extraction[a] | % fat on label |
|---|---|---|---|---|
| Follow-Up, concentrate | Nestle | 4.99 (1.8)[b] | 5.20 | 5.21 |
| Good Start, concentrate | Nestle | 6.66 | 6.65 | |
| Good Start, ready to feed | Nestle | 3.51 | 3.50 | |
| Good Start, powder | Nestle | 26.71 | 26.47 | |
| Next Step Enfamil, concentrate | Mead Johnson | 6.65 (1.8) | 6.40 | 6.73 |
| Enfamil, concentrate | Mead Johnson | 6.49 (1.8) | 6.80 | 6.73 |
| Similac, concentrate | Ross Lab | 3.46 (1.9) | NA[c] | NA |
| Isomile, concentrate | Ross Lab | 6.92 (2.4) | 7.00 | 6.94 |
| soy baby formula, concentrate | Gerber Food | 6.80 (1.7) | 6.70 | 6.73 |

[a] Two replicate extractions, analyzed by outside laboratory. [b] Number in parentheses is % RSD ($n \geq 3$). [c] Not available.

In summary, traditional hydrolysis/solvent extraction and direct SFE gravimetric methods yield similar accuracy and precision. Both of these extracts produce similar fatty acid profiles, and they may contain more than triglycerides; nevertheless, the measured weight gain is assumed to be fat. SFE appears to be a reliable, replacement technique for fat determination in liquid and powder infant formula.

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