Total Fat Analysis in Milk- and Soy-Based Infant Formula Powder by Supercritical Fluid Extraction

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ABSTRACT: A rapid method for the determination of total fat in infant formula powders using a commercially available supercritical fluid extraction (SFE) instrument was evaluated. The matrices examined were Standard Reference Material SRM 1846 Infant Formula (NIST) and commercial milk- and soy-based infant formula powders. Method verification and validation were done by linear regression analysis using the Method of Standard Additions. A Data Quality Objectives (DQO) format was used to define and evaluate the performance characteristic parameters of the instrumental total fat analysis by SFE. A peer validation study showed excellent agreement with the declared labeled percentage fat values and reproducibility among three participating laboratories. The laboratory relative SD (RSD_R, %) is within Horwitz's limits of acceptability and the HORRAT ratio criteria at the level of the analyte analyzed. Linear regression analysis of all infant formula matrices spiked with added fat showed that the SFE instrument response was due only to the added analyte. By integrating the DQO process with a readily available certified reference material, along with reproducibility indicated by two outside collaborating laboratories, we established verification and validation of the accuracy of the data obtained by SFE.

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KEY WORDS: Collaborative study, fat, infant formula powder, peer-validated supercritical fluid extraction.

The commercial availability of simple bench-top supercritical fluid extraction (SFE) systems utilizing CO_2 offers the potential to measure total fat content of food matrices such as dairy products, meats, and seeds (1–6). Advantages of the SFE technique are rapid, accurate, and high sample volume gravimetric fat determinations and inherent elimination of organic solvent waste disposal problems.

A Data Quality Objectives (DQO) (7,8) approach was utilized in this study to evaluate the performance characteristics of the SFE-based method for the determination of total gravimetric fat in infant formula. To validate the described method, the following parameters were evaluated:

(i) Accuracy. Obtaining correct values for a suitable reference material, SRM 1846 Infant Formula, available from the National Institute of Standards and Technology (NIST) (9).

(ii) Ruggedness. Determining variables such as (i) extraction time (35 min optimum); (ii) ratio of sample size to diatomaceous earth support material (1 g sample/2 g support); (iii) ratio of distilled water to isopropyl alcohol (50%) optimum for both milk- and soy-based infant formula samples); and (iv) extraction flow rate (3–3.5 mL/min optimum).

(iii) Precision. Establishing whether, relative to the SD, multiple determinations fall within the Horwitz Limits of Acceptability and HORRAT ratio criteria at the level of analyte determined. The Horwitz limits of acceptability are based on the Horwitz equation, which is a measure of the predicted variability of analysis at the level of the analyte determined by intralaboratory study and is expressed as $PRSD_r$. The value of the percentage RSD obtained by the actual data is expressed by RSD_r. The Horwitz limits of criteria are satisfied as acceptable if the RSD_r is $\leq PRSD_r$. The HORRAT ratio is the mathematical relationship between the $RSD_r/PRSD_r$. If the HORRAT ratio is between 0.5 and 1.5, then the data are acceptable for the level of the analyte obtained (10,11).

(iv) Scope of applicability. Determining use for milk- and soy-based infant formula powder.

Research data to meet these objectives were obtained by using a commercially available fat analyzer. A peer-verified method (PVM) study was conducted by distribution of samples of the SRM 1846, two commercial milk-based and three commercial soy-based infant formula products to two additional laboratories that participated as collaborators for an interlaboratory study (12). Very good agreement was obtained among the submitting and collaborating laboratories for these peer validation study samples. The use of clearly defined DQO to establish the method performance characteristics, along with the use of a commercially available reference material, provided the mechanism for verification and validation of analytical methodology, thereby ensuring that subsequent users can have confidence in the resultant data (8).

MATERIALS AND METHODS

Reference material and test samples of infant formulas. Reference material SRM 1846 Infant Formula was obtained from the NIST (Gaithersburg, MD). SRM 1846 Infant Formula was also used as a blind unknown check Sample B. Commercial milk- and soy-based infant formulas from three different manufacturers were purchased from local supermarkets. HPLC-grade isopropyl alcohol (IPA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Demineralized water was prepared in the laboratory using a water purifying system (MilliQ UV Plus) obtained from Millipore Corp. (Bedford, MA). The wheat germ oil used for recovery studies

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was purchased from General Nutrition Centers (Pittsburgh, PA). Soybean, safflower, and corn oils were purchased from local supermarkets. LECO-DRY® crystalline silica dispersion agent and glass wool filtering fiber were purchased from LECO Corp. (St. Joseph, MI).

Apparatus. A bench-top SFE Fat Analyzer Model FA100 and a four-place analytical balance (Sartorius Model AC121S) from LECO Corp. were used for SFE analysis. A conventional household model microwave oven was used at full power level to dry the collection vials after sampling prior to final weighing.

Instrument parameters. The instrument operating parameters for the FA100 are as follows: (i) pump pressure of 9000 psi; (ii) extraction cell temperature equal to 100°C; (iii) hold time of 15 min; (iv) restrictor temperature at 100°C; (v) total extraction time 35 min; (vi) flow rate (g-CO_2/min) 2.9; (vii) fat units (g fat/g sample) 100%. [Note that instrument manufacturers calibrate and report SFE flow rates in different ways. Each instrument operator will need to convert to these units in accordance with specific instructions from individual instrument manufacturers (13,14).]

Method of extraction. One gram of infant formula powder, 2.0 g of LECO-DRY, and organic modifier (1.0 mL of 50:50 IPA/water) were placed in a beaker, mixed thoroughly, and transferred to extraction thimble tubes. Extraction tubes were placed in the SFE Fat Analyzer following the manufacturer's instructions (13), and the appropriate data were entered into the instrument's software program. Fat extracted from the test portion by the supercritical CO_2 was deposited into a weighed vial containing glass wool, which provided a high surface area to trap the aerosol of extracted fat from the depressurized CO_2 stream. After residual CO_2 was allowed to effuse, the sample was dried for 2 min in the microwave oven to remove traces of extracted water. Fat content of the test portion was determined gravimetrically.

Calculations of percentage of fat in sample (Eq. 1).

$$\% \text{ fat in sample} = \frac{\text{weight fat extracted (g)} \times 100}{\text{weight of test portion (g)}} \quad [1]$$

Method of standard additions (MOSA). MOSA is a means to evaluate and compensate for the presence of matrix and sample preparation effects or to verify their absence. The plotted MOSA line is the standard curve representing the analytical response of the added analyte (fat) in the presence of the matrix, offset by the amount of endogenous fat in the sample. Analyte concentration of fat in the sample is obtained from Equation 2:

$$y \text{ (observed weight fat)} = m \times \text{(added weight fat)} + b \quad [2]$$

where $x = -b/m$ at $y = 0$ (15,16).

Recovery study. For each of the samples, recovery studies were carried out using a MOSA protocol with duplicate additions of supplied wheat germ oil at each of three levels, with the sample matrix being the zero-added point. MOSA analyses were done in triplicate. This resulted in 12 data points for each sample. Each test portion of the sample weighed 1 g for each data point in the MOSA protocol. For instruments that can determine three samples per run, the MOSA protocol was followed. Instruments allowing a different number of test portions per run would follow an appropriate protocol to generate at least two data values at zero and at each of three addition levels.

Statistical analysis. Linear regression analysis and ANOVA were used to evaluate analytical response characteristics of the SFE instrument. Where applicable, the data were examined for outliers, using the appropriate operation in the statistical program. Interlaboratory precision was evaluated by examining the data for acceptable variability using the Limits of Acceptability and the HORRAT ratio criteria of Horwitz *et al*. (11). All assumptions of linear regression analysis were met.

Method performance characteristics. Method performance characteristics examined were (i) effect of instrument response as a function of increasing fat concentration and (ii) the ratio of fat found to fat added in spiked samples of SRM 1846 Infant Formula, and commercial milk- and soy-based infant formula powders. Statistical treatment of fat compositional data was analyzed by Excel (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

DQO are a set of quantitative and experimentally determined values for parameters of fundamental importance in assessing the performance characteristics and suitability of an analytical method for any given purpose (7). Thus, performance characteristics refer to the quality of the results obtained (17). These DQO also provide for establishing both qualitative and quantitative method performance characteristics and are used to evaluate decision criteria for data acceptance (7,8). Integration of the DQO process with use of appropriate reference materials also provides a mechanism for establishing the potential of method performance for accuracy of analytical results (18,19). For this PVM study, the required DQO included verification that the percentage fat data were consistent and within the limits of the reference values for the infant formulas examined. The Horwitz Limits of Acceptability and HORRAT ratio (11) criteria were used for evaluation of the method performance, at the level of the analyte determined (10). Best estimates of statistical parameters resulting from intra- and interlaboratory collaborative studies were evaluated by application of standard statistical procedures (20).

Percentage fat composition of milk-based infant formula. For initial validation, the mean data from the submitting and collaborating laboratories were collected for each of known reference material samples (SRM-1846 Infant Formula) and three unspiked unknown matrices (two commercial infant formulas).

The three laboratories were in very good agreement with each other for all of the test samples, as shown in Table 1. Values for mean percentage fat and SD obtained by the three laboratories overlapped the certified value and its assigned uncertainty for the SRM 1846 Infant Formula samples. Mean values for the commercial samples were slightly higher than the

TABLE 1
Mean Percentage Fat Composition of Milk-Based Infant Formulas[a]

	Sample infant formula			
Laboratory	SRM 1846[b]	A	B[c]	C
Submitting				
% Fat	26.1	26.4	26.1	19.0
SD	0.4	1.0	0.4	0.3
RSD_r, %	1.4	3.8	1.5	1.4
CI	0.2	0.6	0.3	0.2
n	9	11	9	11
% Declared value[a]	96.2	105.7	95.9	107.0
MOSA % fat	26.6	27.7	26.4	19.3
Collaborator A				
% Fat	26.6	26.1	26.6	19.0
SD	0.2	0.2	0.1	0.1
RSD_r, %	0.6	0.7	0.4	0.7
CI	0.1	0.2	1.0	0.1
n	3	6	6	6
% Declared value	98.3	104.7	98.1	107.3
MOSA % fat	NA	26.2	26.6	18.7
Collaborator B				
% Fat	26.4	27.1	26.5	19.5
SD	0.3	0.4	0.2	0.3
RSD_r, %	1.1	1.4	0.8	1.7
CI	0.2	0.3	0.1	0.2
n	9	9	9	9
% Declared value	97.5	108.7	97.7	110.2
MOSA % fat	26.3	27.3	26.4	19.1

[a]MOSA, method of standard additions; NA, not available; RSD_r, relative SD for intralaboratory study.
[b]SRM 1846 Infant Formula, certified value 27.1 ± 0.6%.
[c]Sample B was SRM 1846 submitted as an unknown.

TABLE 2
Mean Percentage Fat Composition of Soy-Based Infant Formulas[a]

	Sample infant formula			
Laboratory	Check	A	B	C
Submitting				
% Fat	20.5	26.4	28.1	20.4
SD	0.4	0.2	0.1	0.5
RSDr, %	2.1	0.8	0.5	2.2
CI	0.3	0.2	0.1	0.4
n	6	6	6	6
% Declared value[b]	108.5	109.5	108.0	105.6
MOSA % Fat	20.6	26.5	27.5	20.6
Collaborator A				
% Fat	20.5	26.5	28.1	21.0
SD	0.1	0.1	0.1	0.2
RSDr, %	0.2	0.3	0.4	0.8
CI	0.1	0.1	0.1	0.1
n	3	6	6	6
% Declared value	108.5	110.0	108.0	108.8
MOSA % fat	NA	26.4	27.9	20.7
Collaborator B				
% Fat	20.7	26.6	28.0	20.8
SD	0.2	0.2	0.2	0.1
RSDr, %	0.9	0.8	0.9	0.4
CI	0.1	0.1	0.2	0.1
n	9	9	9	9
% Declared value	109.6	106.6	103.2	109.7
MOSA % Fat	20.7	26.5	26.5	21.0.

[a]See Table 1 for abbreviations.
[b]For SRM 1846, declared = certified value of 27.1 ± 0.6% (9). For commercial samples, declared = label value.

declared label values, for which no uncertainty is known (21). The percentage fat values obtained for SRM 1846 Infant Formula were slightly lower than the declared value of 27.1 ± 0.6%. This reflects the fact that the assigned fat values for SRM 1846 were obtained by acid hydrolysis–organic solvent extraction methods, which extract other lipid materials in addition to fat defined as TG (22). The SFE solventless extraction with supercritical CO_2 does not remove polar materials such as phospholipids from the sample when compared with the classical solvent extraction (23). Method comparison study used to verify the fat content measurement of powdered infant formula is equally effective as obtained either by acid hydrolysis/organic extraction, gravimetric, or SFE methods (24,25).

Percentage fat composition of soy-based infant formula. Table 2 shows the mean data from the submitting and collaborating laboratories for each of three unspiked, unknown soy-based matrices of three different commercial infant formulas. For all samples, the percentage fat composition values slightly exceed the values declared on the labels of the three commercial soy-based infant formulas. These results are not surprising, because within defined limits manufacturers are permitted to add more of the nutrient than the label designates and still remain in compliance (21). The fat values determined by SFE and MOSA were found to be essentially the same. Sample C was used as a blind check sample for soy-based infant formula powder. All three laboratories obtained the same percentage fat values.

The Horwitz Limits of Acceptability at the level of the analyte determined is obtained from an experimentally derived exponential equation, which predicts the relative SD (RSD_r) among laboratories.

$$PRSD_R\,(\%) = 2C^{-0.1505} \quad [3]$$

This predicted relationship is independent of analyte, matrix method, and time of sample analysis of the interlaboratory study (10). Method performance is evaluated by the HORRAT ratio parameter (11), which is used to evaluate the acceptability of laboratory data obtained by interlaboratory study of a proposed method. The HORRAT ratio parameter is the ratio of the experimentally found relative standard deviation among laboratories (RSD_R) divided by the predicted RSD obtained by the Horwitz formula (%RSD/$PRSD_r$). The method is acceptable if the obtained HORRAT ratio is between 0.5 and 1.5.

Tables 3 and 4 show the calculated RSD_R among laboratories and the resultant HORRAT ratio values obtained for both milk- and soy-based infant formula powders. RSD for multiple determinations of both the SRM 1846 Infant Formula and the commercial samples (with exception of one matrix from one laboratory) was well within the defined Horwitz Limits of Acceptability and HORRAT ratios at the level of analyte tested.

Linear response and recovery as a function of increased concentration. Linear regression analysis of typical MOSA curves of SRM 1846 Infant Formula and commercial

TABLE 3
Horwitz Criteria for Interlaboratory Milk-Based Infant Formula Study[a]

Milk-based infant formula	Laboratory		
	Submitting	Collaborating A	Collaborating B
SRM 1846			
% Fat mean	26.1	26.6	26.4
SD	0.4	0.2	0.3
RSD_r, %	1.4	0.6	1.1
$PRSD_r$	1.2		
Limit of acceptability	1.1		
HORRAT ratio	0.8		
A			
% Fat mean	26.4	26.1	27.1
SD	1.0	0.2	0.4
RSD_r, %	3.8	1.5	1.4
$PRSD_r$	1.2		
Limit of acceptability	1.8		
HORRAT ratio	2.2		
B			
% Fat mean	26.1	26.6	26.5
SD	0.4	0.1	0.2
RSD_r, %	1.5	0.4	0.8
$PRSD_r$	1.2		
Limit of acceptability	0.6		
HORRAT ratio	0.5		
C			
% Fat mean	19.0	19.0	19.5
SD	0.3	0.1	0.3
RSD_r, %	1.4	0.7	1.7
$PRSD_r$	1.2		
Limit of acceptability	1.3		
HORRAT ratio	1.2		

[a]$PRSD_r$, predicted RSD_r; for other abbreviation see Table 1.

TABLE 4
Horwitz Criteria for Interlaboratory Soy-Based Infant Formula Study[a]

Soy-based infant formula	Laboratory		
	Submitting	Collaborating A	Collaborating B
A			
% Fat mean	26.4	26.6	26.6
SD	0.2	0.1	0.2
RSD_r, %	0.8	0.3	0.8
$PRSD_r$	1.24		
Limit of acceptability	0.6		
HORRAT ratio	0.5		
B			
% Fat mean	28.1	28.1	28.0
SD	0.1	0.1	0.2
RSD_r, %	0.5	0.4	0.9
$PRSD_r$	1.2		
Limit of acceptability	0.6		
HORRAT ratio	0.5		
C			
% Fat mean	20.4	20.6	20.8
SD	0.5	0.2	0.1
RSD_r, %	2.2	0.8	0.4
$PRSD_r$	1.3		
Limit of acceptability	1.1		
HORRAT ratio	0.9		

[a]See Tables 1 and 3 for abbreviations.

soybased infant formula powders is shown in Figures 1 and 2. The MOSA curves obtained by all three laboratories have slopes near unity, indicating complete recovery of added fat for the spiked samples over the range tested. All three laboratories also obtained R^2 values near unity ($R^2 \geq 0.99$) for all samples included in this study, indicating little variation in this complete recovery over the range investigated. From the linear regression equation, the coefficient of determination (R^2) shows that the increase in instrument response is linear and accounted for by only the increased analyte (fat) being measured (8,15,16).

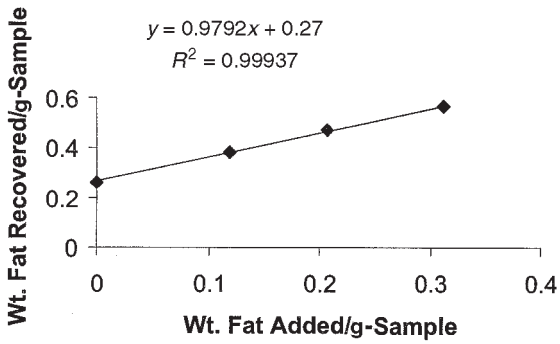

FIG. 2. MOSA recovery curve of commercial soy-based infant formula. See Figure 1 for abbreviation.

Recovery of different oils. The recovery values reported in this study were done using an available sample of wheat germ oil as a representative fat. Because infant formulas are formulated with a variety of different oils, it is necessary to ensure

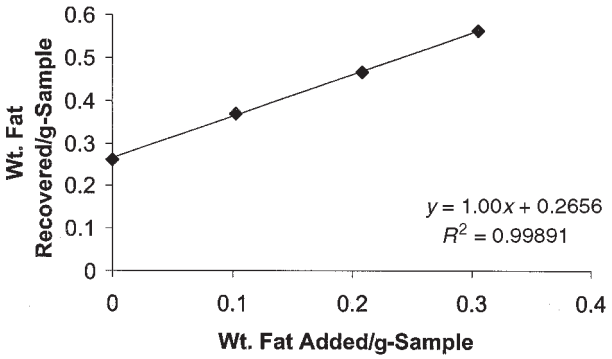

FIG. 1. MOSA recovery curve of SRM 1848 Infant Formula. MOSA, method of standard additions.

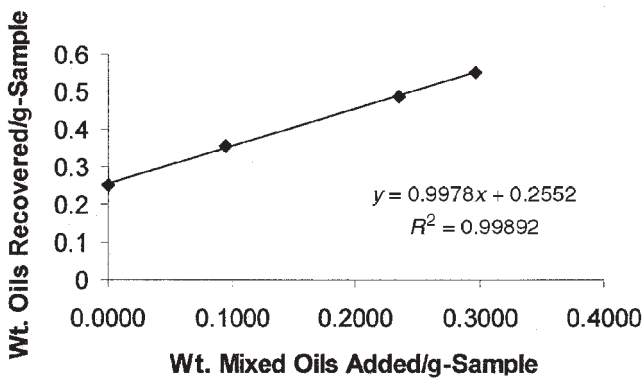

FIG. 3. Recovery of added mixed oil from SRM 1846 Infant Formula.

that the wheat germ oil is representative of other oils potentially used in infant formula. We obtained commercial samples of safflower, corn, and vegetable oils, which are commonly used to formulate infant formula. An equal-part mixture was made of the three different types of oil. The MOSA study using the SRM 1846 Infant Formula sample and this oil mixture was done. Figure 3 shows complete fat recovery and linearity as obtained in the previous MOSA studies. Thus, this SFE method is valid for all types of oils used in infant formulas.

This method has been accepted by AOAC International as PVM 2:2002 (26).

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