Determination of Fat in Infant Formula by Robotic Automated Method

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A method has been developed for the determination of fat in infant formula using a commercially available robotic system. The procedure and chemistry at large are the same as the manual method, *Official Methods of Analysis* 16.064, by the Association of Official Analytical Chemists (AOAC). Both liquid and powder forms of milk-protein-based and soy-protein-based matrices were analyzed in this study. The robotic operations are described in detail. The evaluation of the accuracy is accomplished by comparing the data obtained by the robotic automated method to those obtained by the official manual method. The analysis of variance does not indicate a statistically significant difference (p-value 0.0620, mean difference 0.0056%) between the mean results of the two methods for the milk-protein-based infant formula. The results of other matrices tested by both methods agreed within 1% relative of each other. The precision of the robotic automated method is slightly better than the manual method as shown by the overall relative standard deviation (RSD) of 0.167% vs 0.269%. The ruggedness of the instrument has been satisfactory. The results of this study suggest that the robotic automated method is suitable for this application.

The determination of total fat content in milk or milk-like foods using the procedure as described in AOAC 16.064 (1) has been widely accepted in the food industry. The Infant Formula Council has adopted this procedure as the reference method in the collaborative studies for milk-protein-based infant formulas (2). The manual method is commonly known as Roese-Gottlieb or Mojonnier Method. Fat is extracted by the ethanol/ethyl ether/petroleum ether solvent system, and the fat content is determined by the weight of the residue after the solvent is removed by evaporation. This assay is one of the most labor-intensive and time-consuming assays in our laboratory. Exposure to the organic solvents also makes it a less-desirable test for the laboratory personnel. The exploration of an automated system was initiated with cost reduction and improved safety as the primary goals.

Other robotic systems have been reported to perform a variety of different assays (3,4). Criteria employed to evaluate the suitability of the robotic automated system to perform this analysis were: a) The accuracy of the method compared to the official manual method; b) the precision of the determinations; c) the applicability to various matrices, i.e., liquid and powder forms of milk-protein- and soy-protein-based formulas; and d) the ruggedness of the instruments which is expected to perform the assay continuously with minimum attention by the analyst.

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EXPERIMENTAL

Chemicals. Ammonium hydroxide, concentrated 28-30%. Ethyl ether, U.S.P., petroleum ether, boiling range 35-60°C, ethyl alcohol, 95% or reagent alcohol absolute S.D.A., formulation 3A. All reagents used are reagent grade or better.

Manual method. The manual method is the same as described in the Official Method of Analysis (1). The exception is that only two, instead of three, extractions were used.

Robotic automated method. The sample size and reagents used are the same as those used in the manual method.

The robotic system is a product of the joint effort of two manufacturers, Zymark Corp. (Hopkinton, MA) and Forcoven Products (Humble, TX). Minor optimizations of the software were added to accommodate the requirements of our laboratory. The description and position of the components of the instrument are shown in Figure 1.

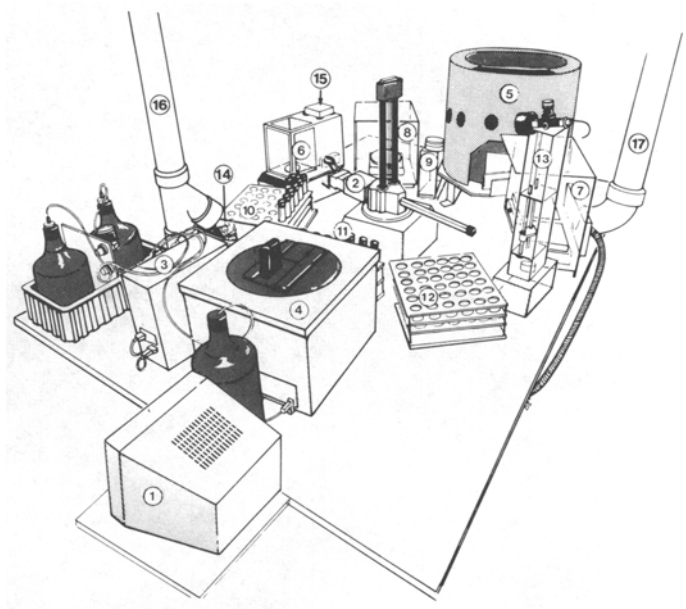

FIG. 1. Illustration of the Robotic Instrument.

1. Zymate II PC
2. Robotic Arm
3. Multi-Lab-Dispenser
4. Centrifuge
5. Fat Pan Dispenser
6. Balance
7. Evaporation Station
8. Drying Station
9. Pan Disposal
10. Sample Tube Rack
11. Rinse Tube Rack
12. Discard Tube Rack
13. Scanner and Aspiration Station
14. Linear Shaker Station
15. Heat Sink
16. Exhaust Vent
17. Exhaust Vent

The samples are weighed into the sample tubes, and water and ammonia are added manually. They are then placed in the sample tube rack station (Fig. 1, no. 10). Through the command of the Zymate II computer, (Fig. 1, no. 1), a fat pan is transferred from the pan-dispenser

(Fig. 1, no. 5), weighed at the balance (Fig. 1, no. 6), and placed on the solvent aspiration station (Fig. 1, no. 13) by the robot arm (Fig. 1, no. 2). Then a sample tube is transferred to the shake table (Fig. 1, no. 14), and solvents are added to the sample through the multilab-dispenser (Fig. 1, no. 3). The extraction is accomplished by shaking of the sample and the solvent on the shake table. At the end of the extraction, the sample is removed and placed into the centrifuge (Fig. 1, no. 4). After the centrifugation, the sample is transferred to the scanner (Fig. 1, no. 13). The detection of the interface between the organic and aqueous layers is accomplished by using a light beam scanned through the sample, from top to bottom. The difference of the light transmitted between the two phases is detected as the interface. The transfer needle is then positioned at ca 0.5 mm above the interface. The upper layer (organic) is transferred into the fat pan by aspiration with positive pressure of nitrogen gas. Then the sample tube, with the lower layer (aqueous), is returned to the shake table for the second extraction. A rinse tube, containing 1:1 mixture of ethyl ether and petroleum ether, is transferred from the rinse tube rack (Fig. 1, no. 11) to the aspiration station (Fig. 1, no. 13). An aliquot of ca 10 ml of the solvent mixture is used to rinse off the residue in the tubings and needles, and the rinse is combined with the ether extract in the sample pan.

The sample pan with the first ether extract is placed onto the hot plates at the evaporation station (Fig. 1, no. 7) for removal of the solvent. When the second extraction is completed, the ether extract is removed in the same manner and combined with the first extract. After the ether is completely evaporated (ca 22 min), the fat pan is transferred onto the third hot plate (Fig. 1, no. 8) to dry off any residual moisture in the extracted fat. The sample is then cooled to room temperature on a heat sink (Fig. 1, no. 15) before being weighed back. The "fat plus pan weight" for each sample from the balance (Fig. 1, no. 6) will be recorded and tracked by the computer. The percentage fat results are then calculated by the computer and printed out in a report.

In order to exclude the weights contributed by the reagents and moisture on the fat pans, duplicates of reagent blanks are included at the beginning of each run, and the average result of the reagents blanks is subtracted from the sample results in the calculation by the computer.

Exhaust vents (Fig. 1, nos. 16 and 17) are placed in appropriate positions, effectively removing ether vapor from the work area. The monitor screen of the Zymate II PC continuously displays the process so that the operator can keep track of the operation. The operator can also interrupt or stop the operation if needed.

Statistical analysis. The Statistical Analysis System (SAS, SAS Institute Inc., Cary, NC), General Linear Models Procedure was used to generate the Variance Component Analysis. Additionally, an estimate of each method mean and its standard error were obtained. The means were compared via a t-test assuming unequal variances (Satterthwaite's approximation). Because the numbers of the individual determinations of the Robotic method were different on days 5 and 11, a weighted least squares analysis as described by Johnson and Milliken (5) was used to estimate the mean of the Robotic method and its standard error. The SAS IML matrix algebra language was used in comparing the means of the results obtained by the two methods. These analyses are detailed in the following Results and Discussion section.

RESULTS AND DISCUSSION

Verification of the execution of the assay procedure by the robot. This is done by visual observation by the analyst while the robot is carrying out the assay. The sample identification tracking and the calculation of results are verified by comparing the results from the automated program to those obtained by manual tracking and calculation.

Comparison of the determination of total fat in milk-protein-based infant formula. A milk-protein-based infant formula from the same lot was analyzed by both methods simultaneously through an 11 day period. The results are shown in Figure 2. A variance component analysis was performed to compare the precision of the two methods (Table 1). The between-day components of variance were almost identical. The within-day precision of the Robotic method was statistically significantly better than that of the manual method (p-value 0.0027, F-statistic 5.909). This difference accounts for the overall RSD of the Robotic method being lower than that of the manual method. A t-test comparing the two method means with a standard error of the difference of 0.002822 and 18 degrees of freedom (Satterthwaite's approximation) did not show the mean difference of 0.0056% to be significant at the 95% confidence level, p-value = 0.0620 (Table 2). The difference required for significance was 0.0059%.

TABLE 1

Variance Component Analysis: Determination of Fat in Milk-Protein-Based Infant Formula, Manual vs Robot Automated Method

	% Fat	
	Manual	Robotic
Mean[a]	3.4955	3.4899
Variance:		
Between days	0.000024 (10)	0.000023 (09)
Within day[b]	0.000065 (11)	0.000011 (14)
Overall	0.000089 (10)	0.000034 (09)
Standard dev.	0.0094	0.0058
RSD	0.269%	0.167%

Numbers in parentheses are degrees of freedom of the variance estimates. Estimates were determined using the SAS formulas for the Expected Mean Squares for each method.

[a]Not significantly different, T-statistic = 1.9844, p-value = 0.0620, 18 degrees of freedom (Satterthwaite).
[b]Significantly different, F-statistic = 5.909, p-value = 0.0027.

Comparison of the determination of total fat in liquid and powder forms of milk-protein-based and soy-protein-based infant formula. The two methods, manual and robotic-automated, were used to analyze five samples which represent the major matrices of infant for-

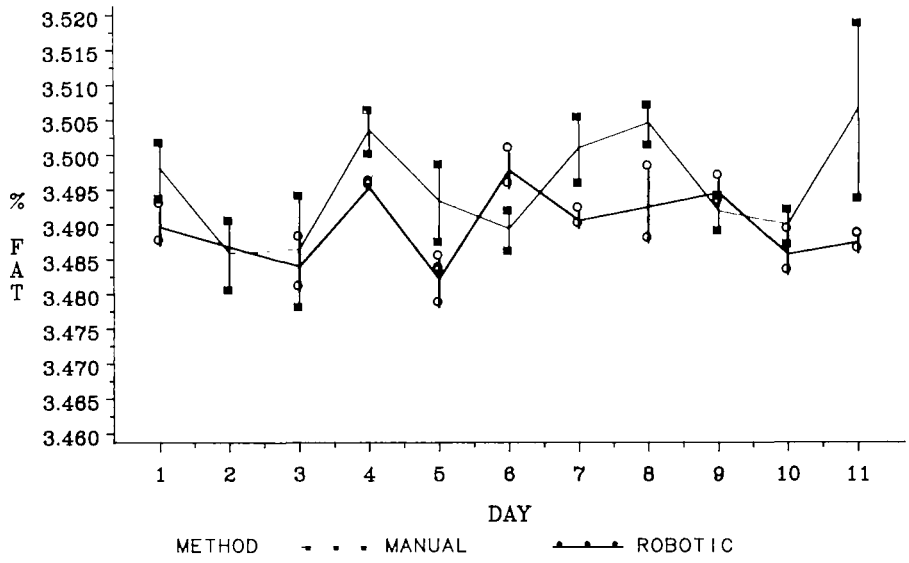

FIG. 2. Determination of Fat in Milk-Protein-Based Infant Formula Manual vs Robotic

TABLE 2

Mean Comparison by Day Determination of Fat in Milk Protein Based Infant Formula Manual vs Robotic Automated Method

	Method			
	Manual	Robotic		
Day	% Fat[a]	% Fat[a]	% Difference	Significance[b]
1	3.4980	3.4896	.0084	NS
2	3.4859	—	—	—
3	3.4864	3.4839	.0025	NS
4	3.5035	3.4952	.0083	NS
5	3.4933	3.4822	.0111	NS
6	3.4894	3.4977	-.0083	NS
7	3.5010	3.4906	.0104	NS
8	3.5046	3.4925	.0121	NS
9	3.4920	3.4946	-.0026	NS
10	3.4900	3.4857	.0043	NS
11	3.5066	3.4875	.0191	NS

[a]All means are based on two determinations except for the Robotic method Day 5 and Day 11 means, for which there were four results.
[b]The difference required for significance is .0205, except for days 5 and 11, for which it is .0198. The comparisonwise error rate is .005, providing a maximum experimentwise error rate of .05.

TABLE 3

Mean Comparison by Product Matrix Determination of Fat in Various Infant Formulas Manual vs Robotic Automated Method

	% Fat			
	Method			
Matrix type	Manual	Robotic	Diff.	Signifi-cance[c]
1. Soy-protein conc. liq.	7.0087[a]	6.9796[b]	0.0291	NS
2. Soy-protein ready-to-feed	3.6500[a]	3.6498[b]	0.0002	NS
3. Soy-protein powder	28.5162[a]	28.2698[a]	0.2464	S
4. Milk-protein conc. liq.	6.7919[a]	6.8079[b]	-0.0160	NS
5. Milk-protein powder	28.5200[a]	28.3758[a]	0.1442	NS

[a]Mean of duplicate determinations.
[b]Mean of quadruplicate determinations.
[c]The mean difference required for significance by matrix is: 1. 0.0419, 2. 0.0236, 3. 0.1703, 4. 0.0406, and 5. 0.1704. The per comparison (product) error rate is 0.01, providing a maximum experimentwise error rate of 0.05.

mula currently available in the market, i.e., ready to feed liquid, concentrated liquid, powder forms of the milk and soy-protein-based infant formulas. The comparisons of the method means are based on a relative standard deviation of 0.27% (10 degrees of freedom) for the manual method, and 0.17% (9 degrees of freedom) for the Robotic method. The method difference required for significance was calculated (95% maximum experimentwise error rate) for each of the matrices tested. The difference and an indication of significance are included in Table 3. The analysis indicated that the means of the soy-protein-based powder obtained by the two methods are statistically different. The actual magnitude of the difference is less than 1% relative to the mean value of either method. For each of the other matrices, the difference between the two methods is not statistically significant.

Performance and limitation of the robotic instrument. The initial installation and optimization of the robotic instrument took approximately five weeks. It has been in operation in our laboratory for over 10 months. Seven technicians have been trained to operate the instrument with no difficulty. The down-time for the instrument is estimated to be ca 15%, which was caused mostly by the peripheral devices, e.g., bent needles, leaking solenoids, and electrical power interruptions. The reliability of the robotic arm and the computer have been satisfactory. We

are confident that with experience and a stable power supply, most of the down-time can be avoided.

A technician typically can perform 36 single determinations in six hours. With the robotic automation, the actual manual labor time has been reduced to approximately three hours, a savings of 50% in labor cost. However, the robotic instrument processes the samples sequentially, one at a time, as compared to the manual procedure which is capable of processing 4-8 samples simultaneously. Therefore, the overall assay time per sample is longer for the automated method. This is a disadvantage if quick turnaround time is needed.

Safety is a very important issue as the assay involved ethyl ether, petroleum ether, and ethyl alcohol, all of which are extremely flammable. All hot-plates should be explosion-proof and all pneumatic devices should use compressed nitrogen gas for their operations. Ventilation of the work area should be adequate to eliminate any accumulation of ether vapor, and an alarm system should be available in case of an emergency.

Although the variances of each method were quite small and there was no overall mean difference, it was noted that the majority of the differences were in the same direction (Tables 2 and 3), i.e., the Robotic method tended to yield slightly lower results. However, from a practical standpoint, the results obtained in this study agreed within 1% relative of each other, supporting the conclusion that the robot-automated instrument is applicable for the determination of total fat in infant formulas. The procedure at large is the same as the manual method with only minor changes to accommodate the instrument requirements. The method is satisfactory and may be used for the majority of the infant formula matrices. A savings in labor cost and a reduction of potential health hazards are realized by the implementation of this method.

Due to the interface detection mechanism, this method is not applicable to samples with particulates that adhere to the sidewall of the sample tube.

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