

**AOAC TDLM Method Verification Workshop**  
**Example 1: Determination of Total Fat in Cereal**



**122<sup>nd</sup> AOAC**  
ANNUAL MEETING & EXPOSITION

## **Example 1**

### **Determination of Total, Saturated, and Monounsaturated Fats In Foodstuffs by Hydrolytic Extraction and Gas Chromatographic Quantitation: Collaborative Study**

Using gas chromatography (GC), 10 collaborating laboratories measured total, saturated, and monounsaturated fats in 8 blind duplicate pairs of foodstuffs. The method involves a hydrolysis/ether extraction of fat followed by quantitative GC analysis versus an internal standard. Calculations were designed to comply with federal regulations as specified in the Nutrition Labeling and Education Act of 1990. The range of fat contents was about 1–50. Collaborators received and analyzed (in triplicate) a pre-collaborative sample of known fat content as a practice sample. After satisfactory results were obtained, participants received the 16-sample set. The repeatability standard deviations ( $RSD_r$ ) for total fat ranged from 2.04 to 10.6; the reproducibility standard deviations ( $RSD_R$ ) for total fat ranged from 3.74 to 15.8. The hydrolytic extraction– GC method for determination of fat (total, saturated, and monounsaturated) in foodstuffs has been adopted first action by AOAC INTERNATIONAL.

#### **Activity:**

The lab is verifying the method for the determination of total fats in cereal. Use the ALACC Method Verification Guide and information from the AOAC collaborative study to plan how the lab should verify the method.

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### AOAC Collaborative Study Information

**Table 996.06A. Method performance for determination of total fat in foodstuffs by hydrolytic extraction–gas chromatography**

Sample <sup>a</sup>	$\bar{x}$ , %	$s_t$	$s_R$	$r^b$	$R^c$	RSD <sub>t</sub> , %	RSD <sub>R</sub> , %	No. of laboratories excluded <sup>d</sup>
Wheat-based cereal	1.96	0.208	0.260	0.582	0.728	10.6	13.3	—
Peanut butter	46.3	0.86	2.37	2.41	6.64	1.85	5.12	2/10
Fish sticks	11.2	0.354	0.541	0.991	1.51	3.14	4.80	2/10
Parmesan cheese	26.5	0.540	4.17	1.51	11.7	2.04	15.8	—
Chocolate cake (baked)	13.3	0.929	1.95	2.60	5.46	7.00	14.7	—
Fruit snack	3.92	0.087	0.146	0.244	0.409	2.22	3.74	1/10
Ground beef	21.9	1.11	1.82	3.11	5.10	5.06	8.32	1/10
Yogurt	1.46	0.131	0.222	0.367	0.622	8.98	15.2	—

<sup>a</sup> Blind duplicates.

<sup>b</sup>  $r = 2.8 \times s_t$ .

<sup>c</sup>  $R = 2.8 \times s_R$ .

<sup>d</sup> Outliers by either Cochran or Grubb's tests.

Calculate amount of individual triglycerides ( $W_{TG_i}$ ) in test sample as follows:

$$W_{FAME_i} = \frac{P_{t_i} \times W_{t_{C_{11:0}}} \times 1.0067}{P_{t_{C_{11:0}}}} \times R_i$$

$$W_{TG_i} = W_{FAME_i} \times f_{TG_i}$$

where  $P_{t_i}$  = peak area of fatty acid  $i$  in test sample;  $W_{t_{C_{11:0}}}$  = weight of  $C_{11:0}$  internal standard added to sample, g; 1.0067 = conversion of internal standard from triglyceride to FAME;  $P_{t_{C_{11:0}}}$  = peak area of  $C_{11:0}$  internal standard in test sample; and  $f_{TG_i}$  = conversion factor for FAMEs to triglycerides for individual fatty acids (see Table 996.06E).

Calculate amount of total fat in test sample (sum of all fatty acids expressed as triglycerides [including *cis* and *trans* forms of monounsaturated acids]) as follows:

$$\text{Total fat, \%} = \frac{\sum W_{TG_i}}{W_{\text{sample}}} \times 100$$

where  $W_{\text{sample}}$  = weight of test sample, g.

Page 1: Method bias is not relevant to this method because the task force recommended that FDA adopts a chemical definition of fat, to dissuade use of methods that might yield a biased result.

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**Answer**

**Method Verification Category**

The method is Category “Analyte at High Concentration Quantitative 4.”

**Table 5. Category 4: Quantifying an analyte at high concentration and Category 5: Analyte above or below a specified, high concentration (often called a Limit Test)**

Performance Characteristic	Verification	Verification Activity	Reason for Verification
Accuracy	Yes	If the method is a limit test or if the concentration range for which the method is validated is narrow (<1 order of magnitude), analyze one reference material/standard/spike at one concentration. Otherwise, demonstrate accuracy at each concentration level, low middle and high by analyzing one reference material/standard/spike at each level.	Over a narrow concentration range, the accuracy and precision should not vary, therefore, the demonstration at one concentration is sufficient. Over a wide concentration range, the accuracy and precision can vary, thus they need to be verified at the different concentration levels.
Precision	Yes	Perform the repeatability test once. If the method covers a concentration range >1 order of magnitude, then the repeatability test must include low, middle and high concentrations.	Over a narrow concentration range, the accuracy and precision should not vary, therefore, the demonstration at one concentration is sufficient. Over a wide concentration range, the accuracy and precision can , thus they need to be verified at the different concentration levels. Intermediate precision, between analyst, is handled by making sure the analysts are trained and can adequately perform the method.
Specificity	No/Yes	See Specificity in General Requirements	See Specificity in General Requirements

The following Performance Characteristics need verification:

- Accuracy
- Precision
- Possibly Specificity

**Process**

**Identify Uncertainty Components,  $\Sigma c_i x'_i$ .**

Look at equation to identify any source of uncertainty that was not included in the collaborative study and would be a component under  $\Sigma c_i x'_i$ .

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It is often convenient to consider each of the three factors: the sample, the laboratory and the method, when identifying gross uncertainties, as well as any necessary consideration of the individual steps in the procedure.

Calculate amount of individual triglycerides ( $W_{TG_i}$ ) in test sample as follows:

$$W_{FAME_i} = \frac{P_{t_i} \times W_{t_{C_{11:0}}} \times 1.0067}{P_{t_{C_{11:0}}}} \times R_i$$
$$W_{TG_i} = W_{FAME_i} \times f_{TG_i}$$

where  $P_{t_i}$  = peak area of fatty acid  $i$  in test sample;  $W_{t_{C_{11:0}}}$  = weight of  $C_{11:0}$  internal standard added to sample, g; 1.0067 = conversion of internal standard from triglyceride to FAME;  $P_{t_{C_{11:0}}}$  = peak area of  $C_{11:0}$  internal standard in test sample; and  $f_{TG_i}$  = conversion factor for FAMEs to triglycerides for individual fatty acids (*see* Table 996.06E).

Calculate amount of total fat in test sample (sum of all fatty acids expressed as triglycerides [including *cis* and *trans* forms of monounsaturated acids]) as follows:

$$\text{Total fat, \%} = \frac{\sum W_{TG_i}}{W_{\text{sample}}} \times 100$$

where  $W_{\text{sample}}$  = weight of test sample, g.

### Uncertainty from FAMEs

FAMEs is the ether extraction, transesterification of fatty acids to their methyl esters

All factors, except  $f_{FA_i}$  would have been varied during the collaborative study and are included in the uncertainty,  $S_R$ . This  $f_{FA_i}$  uncertainty was not combined in the inter-collaborative study. Hence, it is a  $\Sigma c_i x'_i$  component.

The uncertainty in the factor  $f_{FA_i}$  must be assessed and added if significant. A literature search showed the factors are well known and the uncertainty contribution is not significant.

### Sampling

Since the collaborative study used prepared samples, the uncertainty from the sampling step was not included in the collaborative study. The lab would have to assess the

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uncertainty from sampling in the lab, that is, the uncertainty introduced when the lab takes its sub-sample. This could be done by taking duplicates at the sub-sampling stage and calculating the standard deviation contributed at the sub-sampling stage. If the uncertainty is significant, the lab would have to include the uncertainty from sub-sampling in its estimate of uncertainty for the method.

There are many references on how to estimate the uncertainty from sampling, so this example will not include the procedure.

For this example, it is assumed the lab has done a sub-sampling study and verified the contribution from sampling is not significant.

### Repeatability

From the collaborative study the repeatability is  $s_r = 0.208\%$

The lab conducted a repeatability study by analyzing a reference material 7 times and calculating the standard deviation ( $s_w$ ) to be 0.185%, which is less than that obtained in the collaborative study. Hence, the repeatability in the lab is acceptable.

An F test could be used to demonstrate the lab's repeatability is not significantly different from that from the collaborative study.

### Bias

The same data as used for repeatability is used for bias. The approach is taken from ISO Guide 33.

### Uncertainty of bias estimate

The estimate of lab bias is, in its self, uncertain and this uncertainty should be maintained at an insignificant level, where practical. This is done by choosing  $n$  such that the uncertainty  $\sqrt{s_w^2/n} < 0.2s_R$ . For this example  $n$  is 4 which is less than the 7 repeat analyses included in the repeatability study. The uncertainty from the bias estimate is insignificant.

### Bias Estimate

The certified value,  $\mu$ , is subtracted from the mean of the 7 results used in the repeatability study,  $m$ , to obtain an estimate of the lab bias,  $\Delta$ . The bias is acceptable if  $|\Delta| < 2s_D$ , where  $s_D$  is the standard deviation of results obtained by repeated measurement on a reference material used for checking control of bias.  $s_D$  is calculated from the data in the collaborative study.

$$s_D^2 = s_L^2 + s_w^2/n$$

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Given that the reproducibility standard deviation  $s_R$  is given by  $s_R^2 = s_L^2 + s_r^2$ , the equation becomes

$$s_D^2 = s_R^2 - s_r^2 + s_w^2/n$$

Hence,  $\Delta < 2\sqrt{(s_R^2 - s_r^2 + s_w^2/n)}$  is the acceptability test for  $\Delta$ .

The Certified Reference Value,  $\mu$ , is 2.14% and the mean of the 7 results used in the repeatability study,  $m$ , is 2.08%.

$$\Delta = m - \mu$$

$$\Delta = 2.08 - 2.14 = -0.06 \quad \text{and} \quad |\Delta| = 0.06$$

$$2\sqrt{(s_R^2 - s_r^2 + s_w^2/n)} = 2\sqrt{(0.260^2 - 0.208^2 + 0.185^2/7)} = 0.342$$

$0.06 < 0.342$ , thus the lab bias is acceptable and is confirmation that the laboratory component of bias is within the population of values represented in the collaborative study.

### Specificity

The AOAC food triangle is used to define the matrix for the method. The lab is using this method for wheat based cereals which are in food triangle location 5. Since all the samples the lab is analyzing are in this category there is no impact on specificity and no further assessment of specificity is needed.

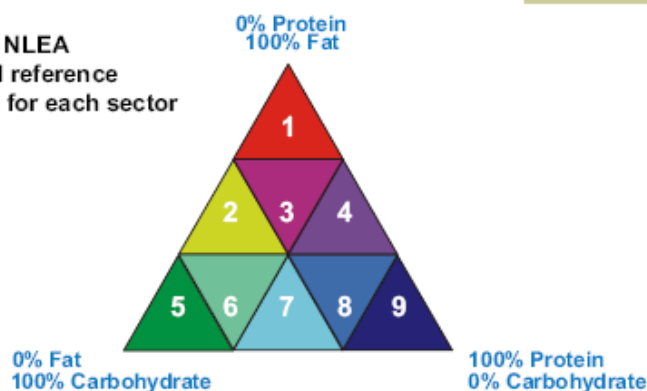
The ALACC method verification guide states:

#### General Requirements—Specificity

For specificity in all categories of methods, if samples are identical to those for which the method is intended and validated, and the method is based on basic principles then no verification is needed. If the samples have the same matrix, the specificity which is based on basic principles, will not be impacted. Basic principles are chemical reactions, e.g. reaction of Ag with Cl to create a precipitate. For some methods, the specificity can be affected by the instrument used. In these cases the lab should assess if the instrument differences could affect the specificity, and if so, include specificity in the verification, e.g. the different resolution and/or detection systems in inductively coupled plasma optical emission spectrophotometers may result in different interferences.

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- Established to address NLEA
- Analytical methods and reference materials must be valid for each sector



"The food matrix organizational scheme can be used to select one or two food matrices representing each sector, for development of a series of reference materials representing all foods. In some sectors, several samples may be necessary to account for differences in all the types of protein, fat, or carbohydrate."

W. R. Wolf and K. W. Andrews, "A System for Defining Reference Materials Applicable to All Food Matrices", *Fresenius' J. Anal. Chem.*, 353:73-76 (1995)

"Careful selection of two foods or food products from each sector will cover the entire range of carbohydrate, protein, and fat, as well as other food attributes."

W. Ikins, et. al., "A Food Matrix Organizational System Applied to Collaborative Studies", *The Referee AOAC International*, 17(7) 1,6,7 (1993).

### Continued Verification of Performance

The lab has a thorough QC program that sufficiently verifies precision and accuracy.

### Uncertainty Estimate

Since the prior studies have established due control of bias and precision within the testing laboratory, and no factors arise from operations not conducted during the collaborative study, the reproducibility standard deviation is used for estimating the uncertainty standard deviation, leading to an expanded uncertainty of  $U = 0.520\%$  where  $k = 2$ .

### Conclusion

This study demonstrated that the bias and precision are within those values generated in the collaborative study. The samples are sufficiently similar to those in the collaborative study, such that there is no impact on specificity.

The method for the **Determination of Total, Saturated, and Monounsaturated Fats In Foodstuffs by Hydrolytic Extraction and Gas Chromatographic Quantitation** for AOAC Food Triangle location 5, has been successfully verified.

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	Wheat Based Cereal					
$y$	$\mu$	$\delta$	$B$	$e$		
Total Fat	= ideal result	+ method bias	+ lab bias	+ repeatability	+	$\sum c_i x'_i$ sum of effects NOT included in collaborative study
“Target Values”		<p>Page 1 Method bias is not relevant to this method because the task force recommended that FDA adopts a chemical definition of fat, to dissuade use of methods that might yield a biased result.</p>	<p>Collaborative Study</p> $s_L^2$ $s_R^2 = s_L^2 + s_r^2$ <p>Thus</p> $s_L^2 = s_R^2 - s_r^2$	<p>Collaborative Study</p> $s_r^2$		<p>Examine the equation and procedure identified two effects</p> <p>FAMES &amp; Sampling</p>
Source of Lab Data			<p>Repeatability study of CRM with CRV <math>\mu</math></p> $m$	<p>Repeatability study of CRM</p> $s_w$		<p>See text Identify Uncertainty Components (C.2.4), <math>\sum c_i x'_i</math></p>
Calculations			$\Delta = m - \mu$ $ \Delta  < 2\sqrt{(s_R^2 - s_r^2 + s_w^2/n)}$	<p>F test</p>		
Additional Items		<p>Specificity</p> <p>Long Term Verification</p>				