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## FOOD COMPOSITION AND ADDITIVES

# WEFTA Interlaboratory Comparison on Total Lipid Determination in Fishery Products Using the Smedes Method

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**Lipid determination by the Smedes method was tested in an interlaboratory trial performed by nine laboratories from seven countries belonging to the West European Fish Technologists Association Analytical Methods Working Group. Five samples of fish and fishery products with different lipid contents, including two blind duplicates, were distributed among the participants. All laboratories applied a slightly modified Smedes method, which included extraction of lipids by cyclohexane and isopropanol, transfer of lipids to the cyclohexane phase by addition of water, phase separation by centrifugation, and gravimetric lipid determination. The results indicate that the RSD for reproducibility ( $RSD_R$ ) was between 4.11 and 6.31% for samples with moderate (7%) and high (14%) lipid content, depending on the sample. Larger SDs among the laboratories were obtained for a cod sample with low lipid content of 0.5%. The method is judged to be suitable as a routine method for lipid determination in fish and fishery products.**

a comparison of those methods revealed that the amount of extracted lipid depends on the matrix (5–7).

The Bligh and Dyer (4) method is routinely applied for total lipid determination in marine biological tissue by many laboratories. This procedure is based on the extraction of lipids, fatty acids, and sterols by chloroform/methanol at different ratios (8). However, chloroform is highly toxic and known to be a human carcinogen. Therefore, methylene chloride ( $CH_2Cl_2$ ) was used as substitute, and was found to be as effective as chloroform (9–11).

Because methylene chloride is currently also suspected to be carcinogenic and has an adverse effect on the environment, Smedes developed a fat extraction procedure using propan-2-ol–cyclohexane–water mixtures, and proved that the method yields results comparable to those of Bligh and Dyer (12). The aim of this study was to compare the results of lipid determination received by experienced laboratories of the Analytical Methods Working Group of the West European Fish Technologists Association (WEFTA) applying the Smedes method under routine conditions on typical fishery products. For practical consideration, in routine analysis, the sample amount was set to 5 g and fixed volumes of solvent mixtures were added, regardless of the lipid content. All laboratories had to use the same protocol.

## Samples and Participants

Five samples (Table 1) were prepared for this exercise, including a cod fillet sample with a low fat content, two blind duplicate raw skinless mackerel fillet samples with medium fat content, and two samples with high fat content (cold smoked salmon fillets and Matjes herring fillets). All samples were homogenized and tested for homogeneity. Mackerel and cod

The composition of fish and fishery products is basically characterized by their moisture, protein, ash, and lipid content, which can be determined by using established standard methods. Indeed, for lipid extraction, several methods have been developed (1–4, among others). However,

**Table 1. Description of test samples sent to participants for analysis**

| Code | Description of sample  |
|------|--|
| G058 | Thoroughly homogenized raw mackerel fillets                                |
| U175 | Thoroughly homogenized raw mackerel fillets, blind duplicate of G058       |
| C499 | Commercially produced Matjes herring fillets, skin off and homogenized     |
| T307 | Commercially produced cold smoked salmon fillets, skin off and homogenized |
| S632 | Thoroughly homogenized skinless cod fillets                                |

were caught during several cruises of the German research vessel "Walther Herwig III." Cod was filleted on board, and both were deep frozen directly after catch. Whole mackerel were thawed, filleted, and skinned. Cod fillets without skin were thawed. A total of 2.5 kg mackerel and 1.5 kg cod fillets were homogenized by means of a vertical cutter-mixer (Feuma Gastromaschinen, Gößnitz, Germany) to yield a fine paste.

A total of 1.5 kg cold smoked salmon fillet slices and 1.5 kg Matjes herring fillets were obtained from local supermarkets. The fillets were passed through a meat grinder and further homogenized by a hand-held mixer (ESGE, Mettlen, Switzerland).

The homogeneity test included 10 water and five fat determinations from randomly taken subsamples of each preparation. All samples were filled in screw-topped plastic tubes, coded by a letter and a three-digit code, and deep-frozen at  $-25^{\circ}\text{C}$ . The samples (~50 g each) were sent deep-frozen to the nine WEFTA laboratories participating in this interlaboratory study together with a detailed protocol for handling the samples. Each participant was asked to carry out a triplicate fat determination according to the common protocol and a duplicate water determination using their home method. All participants gained experience using the method through a first test trial conducted half a year before.

## Analyses

### Common Protocol

**Principle.**—Lipids were extracted by cyclohexane and propan-2-ol, and transferred to the cyclohexane phase by the addition of water. Phase separation was performed by centrifugation. Gravimetric fat was determined after separation of the cyclohexane layer and evaporation to dryness.

### Instruments and Chemicals

- (a) *Balance with a precision of 0.1 mg.*
- (b) *High-speed homogenizer.*—Ultra Turrax (Ika Werke, Staufen, Germany).
- (c) *Centrifuge.*—Capable of holding 100 mL tubes; speed approximately 2000 rpm. (If centrifuge is not available, phases also separate over time but the interface is less sharp, which can result in insufficient recovery of the organic phase. At least 80% of the organic phase needs to be recovered; if not, a third extraction is recommended.)

- (d) *Water bath.*
- (e) *Drying oven.*
- (f) *Rotary evaporator.*
- (g) *Pipets.*
- (h) *Deionized water.*
- (i) *Propan-2-ol.*—American Chemical Society (ACS) grade.
- (j) *Cyclohexane.*—ACS grade.
- (k) *Solvent mixture A.*—Propan-2-ol–cyclohexane (w/w) 16–20.
- (l) *Solvent mixture B.*—13% (w/w) of propan-2-ol in cyclohexane.

### Preparation of Sample

The entire sample should be subjected to a systematic cutting and randomization process to ensure a subsample representative of the composition of the whole fish or fishery product. At least 100 g of subsample should be thoroughly homogenized using a high-speed homogenizer. Determination should be performed in duplicate at the least.

### Procedure

Weigh 5 g homogenized fish sample ( $\pm 0.2$  g) in a 100 mL centrifuge tube. Add 36 mL solvent mixture A. Mix using Ultra Turrax homogenizer for 2 min (11 000–13 000 rpm). Add 20 mL water. Mix using Ultra Turrax for 1 min (11 000–13 000 rpm).

Separate the phases by centrifugation (5 min at 2000 rpm). (In case a centrifuge is not available, phases also separate over time, but the interface is less sharp, which can result in insufficient recovery of the organic phase. At least 80% of the organic phase needs to be recovered; if not, a third extraction is recommended.) To prevent some tissue (like liver) from forming an emulsion, NaCl is added.

Transfer the organic phase quantitatively by means of a pipet to a predried and weighed evaporation flask of an appropriate size. Filtration of the organic phase is optional, but makes the method more robust.

Add 20 mL solvent mixture B to the remaining water phase of the centrifuge tube. Mix using Ultra Turrax homogenizer (11 000–13 000 rpm) for 1 min and centrifuge 5 min at 2000 rpm.

Transfer the upper organic phase to the flask containing the first extract, then evaporate the solvent ( $51^{\circ}\text{C}$  at 235 mbar). Dry the residue for 1 h at  $105^{\circ}\text{C}$ . Weigh the residue and calculate the lipid content.

**Table 2. Mean water content, SD, and CV of the samples ( $n = 9$  laboratories)**

| Code | Mean water content, % | SD   | CV, % |
|------|-----------------------|------|-------|
| G058 | 73.03                 | 0.73 | 1.0   |
| U175 | 73.39                 | 0.61 | 0.8   |
| C499 | 67.65                 | 0.64 | 0.9   |
| T307 | 63.32                 | 0.76 | 1.2   |
| S632 | 82.12                 | 0.73 | 0.9   |

**Table 3. Arithmetic means, SDs, and CVs of the lipid determination of various fish samples**

| Laboratory    | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     |
|---------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| G058 Mackerel |       |       |       |       |       |       |       |       |       |
| Mean          | 7.19  | 7.45  | 6.65  | 7.42  | 7.68  | 7.34  | 6.71  | 7.17  | 6.80  |
| SD            | 0.25  | 0.09  | 0.1   | 0.41  | 0.59  | 0.22  | 0.17  | 0.17  | 0.43  |
| CV, %         | 3.43  | 1.23  | 1.48  | 5.47  | 7.66  | 2.93  | 2.51  | 2.38  | 6.40  |
| U175 Mackerel |       |       |       |       |       |       |       |       |       |
| Mean          | 6.98  | 7.81  | 6.62  | 7.61  | 7.62  | 7.22  | 6.94  | 7.16  | 6.73  |
| SD            | 0.08  | 0.16  | 0.20  | 0.05  | 0.51  | 0.04  | 0.14  | 0.27  | 0.15  |
| CV, %         | 1.18  | 2.02  | 2.98  | 0.66  | 6.74  | 0.55  | 2.00  | 3.80  | 2.16  |
| C499 Matjes   |       |       |       |       |       |       |       |       |       |
| Mean          | 14.70 | 14.78 | 13.79 | 14.15 | 14.73 | 14.40 | 13.97 | 12.65 | 13.15 |
| SD            | 0.28  | 0.07  | 0.30  | 0.20  | 1.03  | 0.11  | 0.10  | 0.56  | 0.19  |
| CV, %         | 1.91  | 0.44  | 2.21  | 1.41  | 6.96  | 0.75  | 0.73  | 4.46  | 1.48  |
| T307 Salmon   |       |       |       |       |       |       |       |       |       |
| Mean          | 10.67 | 10.94 | 10.21 | 10.35 | 10.18 | 10.56 | 10.61 | 10.99 | 10.10 |
| SD            | 0.22  | 0.05  | 0.06  | 0.21  | 0.97  | 0.10  | 0.14  | 0.42  | 0.40  |
| CV, %         | 2.02  | 0.41  | 0.54  | 2.07  | 9.48  | 0.92  | 1.34  | 3.86  | 3.91  |
| S632 Cod      |       |       |       |       |       |       |       |       |       |
| Mean          | 0.39  | 0.51  | 0.28  | 0.50  | 0.49  | 0.53  | 0.64  | 0.63  | 0.39  |
| SD            | 0.11  | 0.01  | 0.03  | 0.01  | 0.05  | 0.02  | 0.12  | 0.02  | 0.09  |
| CV, %         | 29.42 | 0.30  | 11.35 | 2.00  | 10.8  | 3.90  | 18.20 | 2.41  | 23.62 |

### Calculations

$$\text{Lipid content (\%)} = \frac{\text{mass of the fat after drying (g)}}{\text{sample weight (g)}} \times 100$$

### Statistical Methods

Statistical processing of the data was carried out according to the collaborative study guidelines of AOAC *Official Methods*<sup>SM</sup> (13) and the International Standard ISO 5725 (14).

## Results and Discussion

### Homogeneity Test

As an additional homogeneity test, each laboratory was asked to analyze the water content of their samples. All laboratories used in principle the AOAC Method **950.46B** (15), by drying 2–5 g material at 105–110°C to a constant weight in an air oven. The mean water contents, the SDs, and the CVs calculated from all results reported by the participating laboratories are listed in Table 2.

The results show very a low variation of the water content between the laboratories, indicating good homogenous samples (Table 2). The CV was <1.2% for all samples.

### Lipid Determination by the Smedes Method

Detailed results on the lipid determination of each participating laboratory are compiled in Table 3. No outliers (Grubbs' test) were detected, and all data were included in further calculations. The results of the statistical analysis are summarized in Table 4. The variability within and between laboratories was relatively low and uniform for all samples with moderate and high lipid content. Larger variations of the lipid content were found for the lean cod sample.

*Samples with moderate and high lipid content.*—The lipid content of the samples ranged between a typical moderate content of 7.2% for the mackerel sample and relatively high 14.1% for the Matjes samples. The lipid content of the cold smoked salmon was in-between, at 10.6%.

The mean repeatability RSD ( $RSD_r$ ), which gives an indication of the within-laboratory variability, ranged between 2.25 and 3.76%, depending on the sample. Only marginal differences were found in the lipid content of the blind duplicates (G058 and U175), showing a high repeatability within the laboratories.

The relative difference between the minimum and maximum lipid content obtained for each series was between 7 and 15%.

The reproducibility RSD ( $RSD_R$ ), a good measure for the among-laboratory precision (which includes the within-laboratory repeatability, as well), ranged between 4.11 and 6.31%. The results show that the different laboratories that participated in this collaborative trial were able to determine

**Table 4. Statistical validation of collaborative study on lipid determination<sup>a</sup>**

|                   | Mackerel<br>G058 fat,<br>% | Mackerel<br>U175 fat,<br>% | Matjes<br>C499 fat,<br>% | Salmon<br>T307 fat,<br>% | Cod<br>S632 fat,<br>% |
|-------------------|----------------------------|----------------------------|--------------------------|--------------------------|-----------------------|
| Valid N           | 9                          | 9                          | 9                        | 9                        | 9                     |
| Mean              | 7.16                       | 7.19                       | 14.04                    | 10.51                    | 0.49                  |
| Median            | 7.19                       | 7.16                       | 14.15                    | 10.56                    | 0.50                  |
| Min               | 6.65                       | 6.62                       | 12.65                    | 10.10                    | 0.28                  |
| Max               | 7.68                       | 7.81                       | 14.78                    | 10.99                    | 0.64                  |
| s <sub>r</sub>    | 0.27                       | 0.18                       | 0.32                     | 0.28                     | 0.05                  |
| RSD <sub>r</sub>  | 3.76                       | 2.47                       | 2.25                     | 2.70                     | 10.41                 |
| r                 | 0.75                       | 0.50                       | 0.89                     | 0.79                     | 0.14                  |
| s <sub>L</sub>    | 0.36                       | 0.42                       | 0.74                     | 0.33                     | 0.12                  |
| s <sub>R</sub>    | 0.45                       | 0.45                       | 0.81                     | 0.43                     | 0.13                  |
| RSD <sub>R</sub>  | 6.31                       | 6.30                       | 5.74                     | 4.11                     | 26.05                 |
| RSD <sub>RH</sub> | 2.95                       | 2.97                       | 2.68                     | 2.81                     | 4.44                  |
| R                 | 1.26                       | 1.27                       | 2.26                     | 1.21                     | 0.35                  |

<sup>a</sup> N = Number of valid means; s<sub>r</sub> = repeatability SD (random error); RSD<sub>r</sub> = repeatability relative SD; r = repeatability value; s<sub>L</sub> = among-laboratory variability; s<sub>R</sub> = reproducibility SD; RSD<sub>R</sub> = reproducibility relative SD; RSD<sub>RH</sub> =  $2^{(1-0.5^r \log c)}$  Horwitz equation; R = reproducibility value.

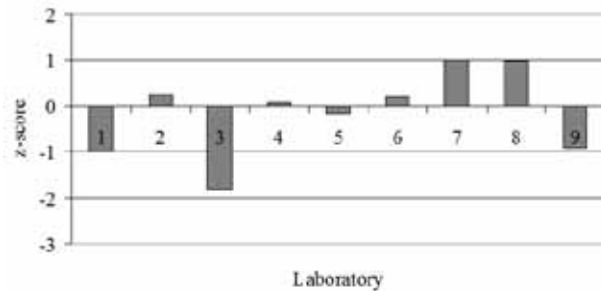
lipid concentrations in fish and fishery products with moderate and high lipid values with acceptable reproducibility when applying the common protocol of the modified Smedes method.

It has been proposed that RSD<sub>R</sub> deviation values found within a range of 0.5–2 times the Horwitz RSD<sub>RH</sub> may be considered an acceptable precision of method performance among laboratories (16). The Horwitz equation ( $RSD_{RH} = 2^{(1-0.5^r \log c)}$ ) is dependent on the analyte concentration (c). This equation has been derived empirically from examination of more than 3000 method performance studies. With the exception of the bad performance data for low fat content (S632), the RSD<sub>R</sub> of the mackerel and Matjes samples (G058, U175, and C499) was approximately twice the Horwitz RSD<sub>RH</sub> (2.14 times the RSD<sub>RH</sub>), or 1.46 times for the salmon samples (T307).

**Lean cod sample.**—The lipid content of the cod sample reported by the laboratories varied considerably at a low level, 0.28–0.64%. The average relative within-laboratory repeatability (RSD<sub>r</sub>) was 10.41%, resulting in a relatively low reproducibility among laboratories with an RSD<sub>R</sub> of approximately 26%. Incomplete homogenization could be excluded as a possible reason, because the determination of the water content showed an excellent agreement.

The variations were likely attributed to inaccuracies during drying of the extracted lipid residue. Considering that the mean lipid content of 0.5% corresponds to an absolute lipid amount of 25 mg, the differences obtained among the laboratories are in the range of a few milligrams.

The reported variation of the lipid content of the cod sample is typical for lipid values of cod (17). Our results show that in addition to the biological variation in the cod lipid content, an analytical uncertainty has to be considered when analyzing the lipid content of lean fish species. Consequently, the protocol



**Figure 1. Assessment of laboratory performance for fat content of cod sample (S632); assigned value: 0.50% fat.**

should be improved for the analysis of lean fish species (lipid content  $\leq 1\%$ ).

Although there were large variations between the results of the laboratories when applying the method on the cod sample, all performances still meet the requirements for proficiency testing of analytical chemistry laboratories, as shown in Figure 1. The performance of laboratories is often assessed by the differences between their results ( $\bar{x}$ ) and the assigned value ( $X_a$ ) and converted into a z score (18). For the fat determination in cod, the z score was calculated as follows:  $z = (\bar{x} - X_a)/s_L$  ( $s_L$  = among-laboratory variability). The median was taken as the assigned value. Z scores in the range of  $-2$  to  $2$  are commonly designated as acceptable.

## Conclusions

The modified Smedes method yielded comparable lipid content in fish and fishery products with moderate and high lipid values with acceptable among-laboratory reproducibility when applied by different laboratories having experience in the analysis of fish and fishery products. For the lean cod sample, larger variations were obtained but all results were still in a range required for a successful performance in a proficiency test. The z scores of all laboratories were between  $-2$  and  $2$ . Nevertheless, the protocol needs to be improved for the determination of the lipid content of lean fish species.

As the method avoids the use of toxic solvents, most of the WEFTA laboratories participating in this interlaboratory trial have switched to the Smedes method, and have judged the method suitable for routine analysis of the total lipid content of fish and fishery products.

## References

- (1) Soxhlet, F. (1879) *Dingler's Polytechnisches J.* **232**, 461–465
- (2) Stoldt, W. (1952) *Fette und Seifen* **54**, 206–207. <http://dx.doi.org/10.1002/lipi.19520540406>
- (3) Folch, J., Lees, M., & Stanley, G.H.S.A. (1957) *J. Biol. Chem.* **226**, 497–509
- (4) Bligh, E.G., & Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917. <http://dx.doi.org/10.1139/o59-099>
- (5) Hubbard, W.D., Sheppard, A.J., Newkirk, D.R., Prosser, A.R., & Osgood, T. (1977) *J. Am. Oil Chem. Soc.* **54**, 81–83. <http://dx.doi.org/10.1007/BF02912395>
- (6) Kolar, K., Faure, U., Torelm, I., & Finglas, P. (1993) *Fresenius*

- J. Anal. Chem.* **347**, 393–395. <http://dx.doi.org/10.1007/BF00635463>
- (7) Manirakiza, P., Covaci, A., & Schepens, P. (2001) *J. Food Comp. Anal.* **14**, 93–100. <http://dx.doi.org/10.1006/jfca.2000.0972>
- (8) Lee, C.M., Trevino, B., & Chaiyawat, M. (1996) *J. AOAC Int.* **79**, 487–492
- (9) Chen, I.S., Shen, C.-S.J., & Sheppard, A.J. (1981) *J. Am. Oil Chem. Soc.* **58**, 599–601. <http://dx.doi.org/10.1007/BF02672373>
- (10) Oehlenschläger, J. (1986) *Infn. Fischw.* **33**, 188–190
- (11) Cequier-Sanchez, E., Rodriguez, C., Ravelo, A., & Zarate, R. (2008) *J. Agric. Food Chem.* **56**, 4297–4303. <http://dx.doi.org/10.1021/jf073471e>
- (12) Smedes, F. (1999) *Analyst* **124**, 1711–1718. <http://dx.doi.org/10.1039/a905904k>
- (13) Method Validation Guidelines (1995) *J. Assoc. Off. Anal. Chem.* **78**, 143A–160A
- (14) International Organization for Standardization (1986) *Precision of test methods—Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests, ISO 5725*, Geneva, Switzerland
- (15) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **950.46B**
- (16) IUPAC (1990) *Pure Appl. Chem.* **62**, 149–162. <http://dx.doi.org/10.1351/pac199062010149>
- (17) Love, R.M. (1975) *J. Fish. Res. Board Can.* **32**, 2333–2342. <http://dx.doi.org/10.1139/f75-269>
- (18) IUPAC (2006) *Pure Appl. Chem.* **78**, 145–196. <http://dx.doi.org/10.1351/pac200678010145>