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Validation study of a new procedure for measuring insoluble impurities in animal fat

R. Zasadny[1] and K. Kwiatek

Department of Animal Feedstuffs Hygiene, National Veterinary Research Institute
Al. Partyzantów 57, 24-100 Puławy, Poland

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ABSTRACT

Regulation 1774/2002/EC requires that EU reference laboratories assess the insoluble impurities content in ruminant fat. According to this Regulation, protein-free tallow is defined as fat in which insoluble impurities should not exceed a limit of 0.15%. This article proposes a method that was elaborated, validated and successfully applied on 116 animal fat samples. In the examined contamination range (0.075-0.9%), the values of parameters estimated with this protocol are $y=0.989x-0.009$, $r^2=0.999$ for linearity, 94.7% for mean recovery, 3.3-8.4% for coefficient of variation, 0.012-0.06% for expanded uncertainty, 0.026 and 0.058% for limit of detection (LOD), and limit of quantification (LOQ), respectively. The present study also assesses the robustness of the method and shows that none of the factors can significantly influence the results of the method.

KEY WORDS: insoluble impurities, tallow, insoluble solids, Regulation 1774/2002/EC

INTRODUCTION

The benefits of using animal fat in animal feed are well documented (Krehbiel et al., 1995; Oliver et al., 1997; Richards et al., 1998). For feed and animal producers, the fatty acid composition and low melting point of fat are significant features for the production of a high quality feed pellet and ensure good consistency of meat. For consumers, it additionally has a positive influence on meat attributes and taste (Woodgate and van der Veen, 2004).

Tallow, defined as fat obtained from ruminant tissues, can be removed directly from adipose tissue, skeletal muscles or extracted from other animal wastes, including bones, or fat surrounding specific organs, e.g., the kidney. The residues after tallow rendering are called insoluble impurities or insoluble solids (EC, 1998, 2001). They

[1] Corresponding author: e-mail: rafal.zasadny@piwet.pulawy.pl

are defined as the amount of sediment which usually consists of small proteinaceous particles such as hide, hair, bone, or non- proteinaceous ones such as minerals, metals, soil etc., that are insoluble in organic solvents. Insoluble impurities can decrease fat quality (e.g., smell, colour), its digestibility, promote growth of pathogenic microflora, or cause clogging problems in fat handling screens, nozzles, etc.

Due to the risk of Bovine Spongiform Encephalopathy (BSE), in March 1998 the Scientific Steering Committee (SSC) of the European Commission issued an opinion on the safety of tallow derived from ruminant tissues (revised in 2001). In this opinion, the SSC suggested that at the end of the extraction process tallow should be purified to a maximum insoluble impurities content of 0.15% by weight (EC, 1998, 2001). It was assumed that if insoluble solids contained 100% protein (worst-case scenario), then the infectivity titre would be 0.0015 ID50/g (a dose sufficient to produce a 50% probability of infection in the recipient). This value is 100 times less than the infectivity titre of raw bones (EC, 2000). It was shown, however, that crude, unfiltered tallow did not pose a BSE risk. Moreover, epidemiological studies have also failed to show a connection between the consumption of tallow by cattle and the occurrence of BSE (Taylor and Woodgate, 2003).

There are several analytical procedures for determining the content of insoluble impurities in fat. In the USA, laboratories commonly use the American Oil Chemist Society (AOCS) method Ca 3a-46. However, the Food and Drug Administration (FDA) recommended a method from "Food Chemicals Codes" (2004), while EU specialists suggest using the ISO 663:2001 method (Pearl, 2004). The differences among these methods are fundamental. The FDA method requires 100 g of sample in contrast to the ISO and AOCS methods, which require 20 and 2 g, respectively (Pearl, 2004). The sample weight undoubtedly influences the accuracy of the method but, on the other hand, the larger quantity of sample is associated with greater solvent use.

The aim of this study was to validate a new, less time-consuming method for determining the content of insoluble impurities in fat derived from ruminant and non-ruminant animals, which is applicable to a wide range of contamination levels and requires a small amount of solvent.

MATERIAL AND METHODS

Preparation of fat samples

Blank fat samples (50% ruminant fat, 50% pork fat) were purified by centrifugation (10 min, 3400 × *g*, 2 times), collected in a 500 ml flask and stored at 2-8°C in a freezer. The fat was then spiked by a known weight of insoluble impurities (0.675,

1.35, 2.025, 5.4, and 8.1 g) providing a specified level of contamination (0.075, 0.15, 0.225, 0.6, and 0.9%). Naturally contaminated ruminant fat samples were collected (1800 g) and divided into two equal parts. The first was additionally contaminated by insoluble impurities (1.35 g, corresponding to a level of 0.15%) which were prepared as described below. The second part was not additionally contaminated.

Preparation of insoluble impurities

Insoluble impurities were collected from different fat samples, transferred to a filter and washed in petroleum ether tree times. After the solvent evaporated, the impurities were transferred to a mortar and ground to obtain small particles. They were then dried, weighed and placed in a weighing vessel.

Sample analysis

Sample analyses were performed according to an internal procedure ZHS/PB-02 (2004). Prior to the procedure, glass microfiber filters (Whatman Grade GF/A, ø125 mm) were washed, labeled, dried and weighed. The fat samples were heated (to approximately 80°C) and homogenized using a magnetic stirring hotplate (CAT M17.5). Following this, 100 g of homogenized sample was weighed with a precision of 0.001 g and transferred to a centrifugation tube and centrifuged (Sigma 4K15 laboratory centrifuge) at 3400 × *g* at 40°C for 10 min. The supernatant was removed without disturbing the insoluble impurities settled at the bottom of the tube. The impurities were rinsed thoroughly with 10 ml of petroleum ether (Merck) and mixed using a glass rod. More solvent was added (85 ml) and the samples were centrifuged at 3400 × *g* (at 20°C). After removing the supernatant, the extraction procedure was repeated twice. Next, the defatted insoluble impurities were transferred to the glass microfibre filters. Finally, the preparation was dried for 45 min at 105±2°C and cooled in a vacuum desiccator until the difference between two determined weights did not exceed 0.003 g. The whole procedure was performed under a chemical hood.

Validation

The following parameters of the method were evaluated: linearity, recovery, repeatability, expanded uncertainty, robustness (EURACHEM, 1998; Taverniers et al., 2004). Ten blank samples of animal fat were analysed to verify potential interfering compounds and to estimate such parameters as limit of detection (LOD) and limit of quantification (LOQ).

Robustness test

The aim of this robustness test is to determine if small changes in operating conditions and small modifications influence the test results (Zeaiter et al., 2004). The qualitative results of the test were taken into consideration to calculate robustness. Seven factors: centrifugal force, duration of centrifugation, quantity of solvent used per repeat, duration of solvent centrifugation, drying time, drying temperature, cooling time were examined to see if they could significantly influence the results of the presented method (Table 1).

Table 1. An eight run design template for seven factors

Factors	–1	0	+1
A: Relative centrifugal force, rcf[1]	2753	3400	4112
B: Time of fat centrifugation, min	9	10	11
C: Quantity of used solvent per repeat, ml	85	95	105
D: Time of solvent centrifugation, min	5.5	6	6.5
E: Drying time, min	40	45	50
F: Drying temperature, ºC	95	105	115
G: Cooling time, min	40	45	50

Two-levels design

Results of the robustness test

Factors	Effect	Rankit
A: Relative centrifugal force, rcf	0.0060	0.09
B: Time of fat centrifugation, min	0.0015	0.27
C: Quantity of used solvent per repeat, ml	0.0162	0.46
D: Time of solvent centrifugation, min	0.0062	0.66
E: Drying time, min	0.0127	0.90
F: Drying temperature, ºC	0.0035	1.21
G: Cooling time, min	0.0017	1.71

[1] Sigma 4K15 laboratory centrifuge with 11150 swing-out rotor

In order to decide which of the effects significantly influenced the result, the margin of error (ME) and simultaneous margin of error (SME) values were calculated according to the algorithm of Dong (Vander Heyden et al., 2001; Li et al., 2005). The effect of a particular factor on the result of the method was estimated and plotted in increasing order (Figure 1). It was assumed that if an effect lies below the SME curve but exceeds the ME, it would probably be significant. If an effect is up to the SME, it would be considered significant (Vander Heyden et al., 2001).

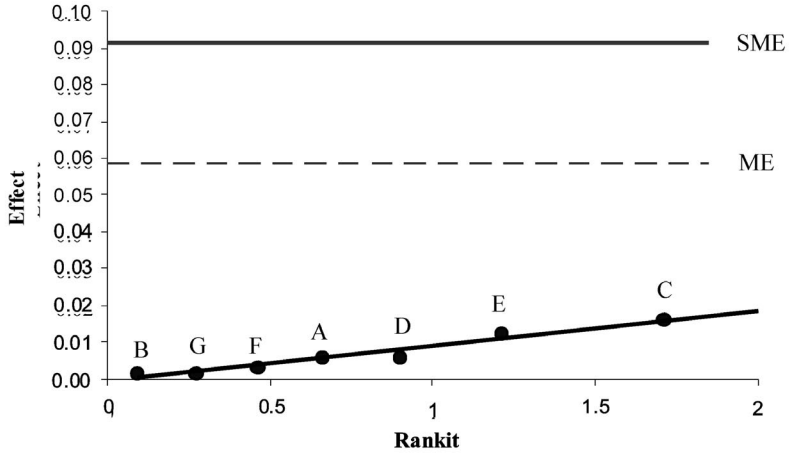

Figure 1. Half-normal probability plot of effects from A to G (values from the Table 1), A - relative centrifugal force (rcf), B - time of fat centrifugation (min), C- quantity of used solvent per repeat (ml), D - time of solvent centrifugation (min), E - drying time (min), F - drying temperature (ºC), G - cooling time (min)

RESULTS

The values of validation parameters from each contamination level are summarized in Table 2. The correlation between coefficient of variation (CV) and concentration of insoluble impurities is significant according to the Horwitz function (Taverniers et al., 2004). Recoveries for artificially contaminated animal fat samples averaged 94.7% and ranged from 92 to 98.4% (Table 2). The obtained intercept, slope value and correlation coefficient (r^2) were 0.989, 0.009 and 0.999, respectively (data not shown). In addition, the "traditional limits" (LOD, LOQ) evaluated with this protocol were 0.026 and 0.058 (Table 2).

The difference in the amount of insoluble impurities in naturally contaminated samples and additionally contaminated sample showed that the obtained recovery was statistically similar to the mean recovery of the spiked blank samples. However, the results of naturally contaminated samples were characterized by a high spread and expanded uncertainty of 0.11% (vs 0.046% at the spiked 0.6 level). There were also differences in the coefficient of variation, 11.9 vs 3.8% at the spiked 0.6 level (Table 2).

The robustness of the test results suggest that this method of measuring insoluble impurities is relatively insensitive to small (10%) changes (Table 1). None of the seven factors exceeded the ME curve and this are considered non-significant for the results of the method (Vander Heyden et al., 2001).

Table 2. Validation data of artificially contaminated samples

Level of contamination, %	Average weigh % ± SD[1]	CV, %	Expanded uncertainty[2] %	R/ RA[3]		LOD/ LOQ, %
0.075	0.069 ± 0.006	8.4	0.120	92.0		
0.15	0.140 ± 0.010	7.0	0.021	93.3		
0.225	0.211 ± 0.011	5.0	0.022	93.8	94.7	0.026/ 0.058
0.6	0.577 ± 0.022	3.8	0.046	96.2		
0.9	0.886 ± 0.029	3.3	0.060	98.4		

Validation data of naturally contaminated samples

Level of additional contamination, %	Average weight, % ± SD [1]	CV %	Recovery %	Expanded uncertainty[2] %
0.15	0.460 ± 0.054	11.9	97.9	0.11

[1] SD -standard deviation (n=6 replicates), [2]k=2, [3]R - recovery for each level of contamination, %; RA - the average recovery for all levels of contamination, %

DISCUSSION

The present study is based on the SSC opinion stating that insoluble solids have a proteinaceous nature (EC, 2001). In 2000 the SSC confirmed that "Since BSE infectivity has a tendency to fractionate with the proteinaceous rather than the fatty fraction during the production of tallow, the effect of protein contamination of tallow must be separately evaluated" (EC, 2000).

Our previous studies (data not shown) with the ISO method revealed practical problems with preparation of blank samples. Centrifugation or filtration does not give fat cleared from substances soluble in fat but insoluble in petroleum ether. This may lead to overestimated results in the determination of insoluble impurities. The Polish standard method (PN-88/C-04288/05) provides the possibility of rinsing filters in the Soxhlet apparatus (PKNMiJ, 1988). However, small changes in methodology gave different results (Żelazowska, 2005).

Lack of validation made it difficult to compare the methods for determining insoluble impurities. Additionally, it is difficult to evaluate a validation study when there are no available reference materials. On the other hand, it is hard to obtain strictly equivalent Home Reference Material (HRM). The insoluble impurities used in this study were extracted from approximately 50 samples, but this was not enough to carry out a full validation study. For example, determinations at the highest expected level (approximately 3.5%) were not done because this would require an additional 21 g of insoluble impurities for six replicates. Naturally extracted insoluble impurities were partly (25%) replaced by prepared meat-and-

bone meal (MBM), which is a very similar material but not an equivalent. From our experience, recovery for fat contaminated by MBM and insoluble impurities is the same (data not published). The differences are in repeatability because, in general, MBM is more homogenous and gives a narrower spread of results. The main influence on CV factor is the appropriate homogenization of the sample and it is better to use a magnetic stirrer (data not published).

Furthermore, a prevalidation study revealed that were no statistically significant differences in results between examining 100% ruminant fat and 50% ruminant/ 50% pork fat. However, an important part of the procedure is a good choice of filter. In the present study we used a Whatman GF/A filter, which has low weight and water absorption, although it is very fragile. Recovery results showed that they increased according to the concentration of insoluble impurities (Table 2). This may have been caused by insufficient particle retention by the filter. Therefore, at a low level of contamination this correlation was probably more evident.

The SSC opinion (1998) also presented two alternative ways of examining tallow based on determining the nitrogen level or molecular weight of residue peptides or polypeptides (EC, 1998). Assaying total nitrogen in the sample should be considered. On the other hand, the idea of fat being examined for the presence of protein or polypeptides with a mol. wt. of up to 10,000 Daltons is unrealistic. The processing conditions of tallow are not sufficient to hydrolyse proteins that are part of insoluble impurities.

CONCLUSIONS

In conclusion, the proposed method is fitting for purposes of routine quantitative measurement of insoluble impurities in ruminant fat and tallow mixed with pork fat. However, without corresponding validation data, it is difficult to determine if statistically equivalent results are obtained by two different methods. Furthermore, implementing another safety criterion for tallow, such as a limit on total nitrogen in the sample, should be considered. This assay is based on the well-known Kjeldahl or Dumas methods.

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