# Validation of the Method for Dosing Linoleic Acid in Food Animal Products

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**Abstract**

One of the big problems of chemical analysis is to ensure its quality and to increase the confidence in the analytical results yielded by a chemical or instrumental analysis. The new internal norms for animal products quality (ANSVSA order 51/2005 regarding the performance of the analytical methods of analysis and data interpretation), aligned to EU requirements, required a unique system of inspection. These requirements stipulate the obligation of the laboratories inspecting animal products to validate each assay method, adapted to the particular type of sample. We therefore aimed to validate the chromatographic method of determining the linoleic acid from meat products. The parameters determined for the validation of this method were in agreement with SR EN ISO/CEI 17025:2005 and refer to: accuracy, reproducibility, sensitivity, precision, limit of detection, and limit of quantification. For validation we used only reference materials and certified standards of analytical purity. The recovery was 95.60% and the incertitude was±0.69 for a sample of 20.673% linoleic acid concentration of total fatty acid. The results prove that the method operates within the admitted limits and is adequate for linoleic acid determination in animal products.

**Keywords**: animal products, chromatography, linoleic acid, method validation, quality

## 1. Introduction

The certification of feeds and animal foods quality requires a continuous effort to optimise the chemical analytical methods and the development of new methods to determine the various food constituents and their level in foods.

Food samples analysis is very important to human consumers, so that the particular products are as competitive as possible on the market.

The polyunsaturated fatty acids are nutrients that have a positive impact on human health [1, 2] and they can be tested to prove the special quality of foods as meat products.

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The method for dosing fatty acids needs first to be validated. The validation of the analytical method is the first step of ensuring the quality in a laboratory [3]. It is the confirmation by examination and supply of objective evidence, that some specific requirements for an intended application are met. Every laboratory has to use the proper measures so as to be capable of delivering confident results proving therefore its qualification and competence.

The specific requirements for the validation of the analytical methods used in the food industry sector are stipulated by law and by national and international regulations.

This paper presents the working steps and results for validation of the gas chromatographic method for determining the linoleic acid from samples which present polyunsaturated fatty acids mixtures. The parameters have been evaluated as indicators of the analytical measurements quality [1, 2, 4, 5].

## 2. Materials and methods

*Principle of the method and field of application*– the fatty acids from the sample are transformed in methyl esters of the fatty acids (FAME); this step is followed by the separation of FAME in a chromatographic column, identification by comparison with the standard chromatograms and the quantitative determination as g FAME/100 g total FAME.

*Reagents and reference material (CRM):*
- $H_2SO_4$ 2% in methanol of analytical purity;
- n-hexane of analytical purity;
- Anhydrous $Na_2SO_4$ of analytical purity;
- Standard solution of methylated fatty acids SUPELCO 37 Component FAME Mix; 10 mg/mL;
- Certified Reference Material (CRM) BCR-163-Beef-Pork Fat Blend EC-JRC-IRMM from Institute for Reference Materials and Measurements *(*IRMM*).*

*Samples for analysis.* The method can be applied on samples of meat products. The fat of the sample is extracted by n-hexane and the fatty acids are esterified to FAME according to [11] using $H_2SO_4$ 2% in methanol and $Na_2SO_4$. A control sample (n-hexane) and a reference sample CRM are analysed in parallel with the samples.

*Chromatographic method*
The method uses gas chromatographer Clarus 500 (Perkin Elmer) fitted with a system for injection in the capillary column (splitting ratio, about 1:100), with oven for heating the column to a programmed temperature; also fitted with flame ionization detector (FID). A high polarity capillary separation column with stationary phase is included.

*Conditions for chromatographic method:*
- Capillary column DB-23 (Agilent), length 60m, diameter 0.250mm; film 0.25µm,
- Temperature 180$^o$C to 220$^o$C, 5$^o$/min
- Carrier gas-hydrogen, 35cm/sec
- FID detector, 260$^o$C; 1 µl injector, 250$^o$C
- Splitting 100:1; vent. 50 mL/min
- Burning gas-air, 420mL/min.

*Calculation*
The relative mass fraction for each component, here for linoleic acid $C_{18:2}$, is calculated with the formula:

$$Wx=A(x)\times f(x)\times 100\%/A(t)$$

where:
Wx-relative mass fraction of component x;
A(x)-peak area corresponding to component x, in area units;
A(t)-sum of the corrected areas of all peaks, excluding the solvent peak, in area units;
f(x)-correction factor for component x.

The validation protocol of the chromatographic method for the determination FAME, respectively for linoleic acid C18:2 were done in agreement with ISO-17025:2005 standard and EURACHEM requirements [6-10]. After equipment standardization it consists in determination of the exactness, accuracy, repeatability and reproducibility, sensitivity, limit of detection and limit of quantification.

## 3. Results and discussion

The standard curve for the linoleic acid $C_{18:2}$ (Fig. 1) was plotted by injection of various concentrations of the standard solution. The curve is linear and the correlation coefficient $R^2$>0.99.

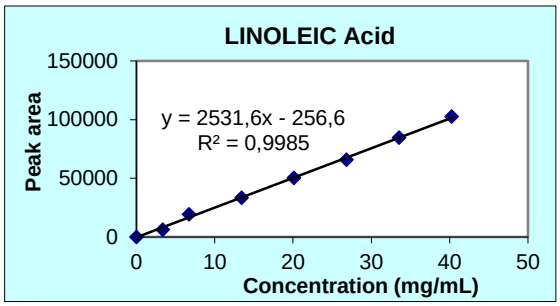


**Figure 1.** Standard curve for linoleic acid

In Table 1 is presented each validation parameter and the abstract of its determination. The reproducibility was evaluated by two analysts who tested the same sample (CRM)–see Table 2.

**Table 1.** Results for method validation parameters

| Definition of the parameter | Results for tested parameter |
|---|---|
| EXACTNESS<br><br>Exactness $\% = \frac{Xmediu}{\mu}100$<br>Where:<br>Xmediu=average of the 6 determinations<br>μ = real value of the reference material<br>Bias $\% = \frac{Xmediu - \mu}{\mu}100$ | A sample with concentration μ=50 μg/mL mixture of fatty acids methyl esters was prepared from the stock solution of 10 mg/mL. (The sample concentration μ=0.225 mg/mL for Linoleic acid $C_{18:2}$)<br>Six repeated determinations were done on this sample and an average of concentrations resulted:<br>X mediu=0.221 mg/mL<br>Exactness=(0.221/0.225)*100=98.22%<br>Bias=1.77% |
| ACCURACY<br><br>CV(RSD) $\% = \frac{s}{Xmediu}100$<br>Where:<br>CV(RSD)=coefficient of variation<br>Xmediu=average of the 6 determinations<br>s=standard deviation | Xmediu=0.221 mg/mL<br>s=0.002835 (see Table 2)<br>CV(RSD)=1.28% (is a RSD calculated)<br>The maximal value of RSD (RSD max) is calculated according to the concentration to be analysed using the Horwitz equation (for the analyte concentration of 1 ppm)=10.72% [7]<br>RSD calculated<RSD max=1.28%<10.72% |
| REPEATABILITY<br>$r=2,8 * s_r$<br>Where:<br>r=limit of repeatability<br>Xmediu=average of the 6 determinations<br>$s_r$ = standard deviation of repeatability | Xmediu=0.221mg/mL<br>$s_r$=0.002835 (see Table 2)<br>r=2.8 x 0.0028=0.00784 |
| INTERNAL REPRODUCIBILITY<br><br>$R=2.8*1.6*S_R$<br>Where:<br>R=limit of reproducibility<br>$S_R$=standard deviation of reproducibility | According to the type of tested method and the performance level of the laboratory the condition R>r must be fulfilled.<br>The testing was repeated by two analysts and the results are presented in Table 2.<br>$s_{R1}$ =0.002835<br>$s_{R2}$=0.002001<br>$s_{R12}$=0.002626<br>$R =2.8*1.6 * S_R$=0.01176<br>R=0.01176>r=0.0079. The condition is fulfilled. |
| SENSITIVITY<br>b=slope of the standard curve<br>or<br>S=ΔY/ΔC<br>Where:<br>S=sensitivity<br>ΔY=variation of the chromatographic peak<br>ΔC=variation of concentration | The value of the slope must be constant for the testing domain.<br>The equation of the linear regression function looks like:<br>Y=bX+a;<br>Y=2531.6X-256.6<br>b=2531.6 |
| LIMIT OF DETECTION<br>LoD=3s<br>s=standard deviation | 5 blank samples were prepared; identified concentration of 0.05 mg/mL<br>Xmean=0.049 mg/mL<br>s=0.000783<br>LoD=3 s=0.002349 mg/mL |
| LIMIT OF QUANTIFICATION<br>LoQ=10 s<br>LoQ>identified concentration<br>s=standard deviation | 5 blank samples were prepared; identified concentration of 0.05 mg/mL<br>Xmean=0.049 mg/mL<br>s=0.000783 mg/mL<br>LoQ=10s+X=0.05683 mg/mL which is>identified concentration 0.05 (0.05683>0.05) |

**Table 1.** Results for method validation parameters

| Definition of the parameter | Results for tested parameter |
|---|---|
| AREA OF APPLICATION<br>LoQ–lower limit of the domain | A standardization curve was plotted for the domain 0–40 mg/mL and the linearity domain was visualised (Figure 1).<br>The lower limit of the domain is LoQ=0.05683 mg/mL<br>The upper limit of the domain is: 1 mg/mL<br>The working domain corresponds to the range in which the concentrations are proportional to the analytical signal |
| RECOVERY<br>Recovery $\% = \frac{(XmediuS - Xmediu)}{Xadaugat}100$<br>XmeanS=average value of the supplemented sample<br>Xmean=average value of the un-supplemented sample<br>Xadded=amount of added analyt = 0.075 mg/ml<br>Recovery must be 80-120% for analyte concentration<100ppm. | Six repeated determinations were done for an un-supplemented sample and average concentration was Xmean=0.221 mg/mL<br>Linoleic acid<br>Six repeated determinations were done for a supplemented sample and average concentration was XmeanS=0.2967 mg/mL<br><br>Recovery %=95.60%<br>80%<95.60%<120% |
| INCERTITUDE<br>u(C0)–Total incertitude<br>u® - Compound incertitude | The sample has 20.673 % linoleic acid of total fatty acids.<br>Incertitude was calculated for multiple sources such as sampling, environmental conditions, used instruments and glassware.<br>u(C0)=0.564<br>u®=0.69 |

**Table 2.** Testing for internal reproducibility by 2 analysts (linoleic acid C 18:2)

| Analyst 1 | | Analyst 2 | |
|---|---|---|---|
| Peak Area (6 tests) | mg/mL | Peak Area (6 tests) | mg/mL |
| 10789 | 0.211 | 10576 | 0.218 |
| 10443 | 0.215 | 10658 | 0.219 |
| 10854 | 0.222 | 10823 | 0.222 |
| 10919 | 0.223 | 10772 | 0.221 |
| 10875 | 0.223 | 10645 | 0.219 |
| 10776 | 0.221 | 10894 | 0.223 |
| mean | 0.221 | mean | 0.220 |
| standard deviation | 0.002835 | standard deviation | 0.002001 |

The reproducibility (R) of the results was 0.01176, higher than the repeatability (r) of 0.0079. The limit of quantification (LoQ) was 0.002349 mg/mL, higher than the limit of detection (LoD) of 0.05683 mg/mL. Recovery was 95.60% which is between 80%-120% according to the analytical methods requirements for analytes higher than 10 µg/kg [6-10]. Method exactness was very good, by a coefficient of variation 98.22%.

In order to determine the interval in which the value of linoleic acid lies, associated with confidence interval of 95%, the incertitude was calculated. For a sample concentration of 20.673% linoleic acid of total fatty acids and the compound incertitude U(r) was±0.69.

All these results indicate that the validation parameters were within the admitted limits for this method, which can be successfully used to determine the linolenic acid C18:2 concentrations in food products.

Several meat products have been tested by this validated method and the results for fatty acids concentrations are presented in Table 3 and Table 4. Further dilutions of the samples were necessary in order that the sample concentration fits with the standard curve. Linoleic acid is presented also in Tables 3 and 4; the samples had concentrations (% of total fatty acids) between 9.59 and 19.65 for fillet and between 12.88 and 36.55 for ham. These values are similar as concentrations of Romero

(2013) [12] which tested Argentinian traditional sausages and identified 0.02–0.19% linoleic acid.

**Table 3.** Concentration of fatty acids in meat products (fillet)

| Meat products (fillet) | Linoleic Acid C18:2n6 | SFA | MUFA | PUFA | UFA |
|---|---|---|---|---|---|
| | | (% of total fatty acids) | | | |
| Fillet | 14.14 | 39.33 | 42.23 | 18.34 | 60.57 |
| Fillet MM | 12.09 | 35.52 | 49.37 | 15.11 | 64.48 |
| Fillet Vitality | 12.68 | 36.48 | 47.88 | 15.48 | 63.35 |
| Fillet MM | 9.59 | 37.64 | 50.94 | 11.42 | 62.36 |
| Smoked fillet | 11.97 | 35.92 | 48.69 | 15.07 | 63.76 |
| Traditional fillet | 19.65 | 34.88 | 42.34 | 22.78 | 65.12 |
| Fillet AZUGA | 11.73 | 36.82 | 49.17 | 13.99 | 63.16 |
| Traditional fillet | 11.55 | 37.89 | 45.69 | 16.20 | 61.88 |

Saturated Fatty Acid (SFA); Monounsaturated Fatty Acid (MUFA); Polyunsaturated Fatty Acid (PUFA); Total Unsaturated Fatty Acid (UFA).

**Table 4.** Concentration of fatty acids in meat products (ham)

| Meat products (ham) | Linoleic Acid C18:2n6 | SFA | MUFA | PUFA | UFA |
|---|---|---|---|---|---|
| | | (% of total fatty acids) | | | |
| Praga ham | 12.88 | 38.14 | 44.98 | 16.79 | 61.77 |
| Turkey ham Vitality | 36.21 | 32.53 | 28.46 | 39.02 | 67.47 |
| Presed ham extra | 21.24 | 32.16 | 43.25 | 24.29 | 67.54 |
| House ham | 14,.80 | 35.35 | 47.38 | 17.26 | 64.65 |
| Praga Turkey ham | 25.14 | 30.34 | 40.51 | 28.31 | 68.82 |
| Mediteranean ham | 36.55 | 28.79 | 30.63 | 40.24 | 70.87 |
| Turkey roll | 16.67 | 30.18 | 50.23 | 19.03 | 69.26 |

Saturated Fatty Acid (SFA); Monounsaturated Fatty Acid (MUFA); Polyunsaturated Fatty Acid (PUFA); Total Unsaturated Fatty Acid (UFA).

## 4. Conclusions

This analytical method is suitable for the determination of the linoleic acid and also for other polyunsaturated fatty acids in animal foods samples. It can be applied in the food industry because of its good results.

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