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ADAPTATION OF THE QUALITY CONTROL OF THE POLYUNSATURATED FATTY ACIDS AND VITAMIN E-ENRICHED FEEDS TO THE EUROPEAN STANDARDS

ADAPTAREA CONTROLULUI DE CALITATE A NUTRETURILOR IMBOGATITE IN ACIZI GRASI POLINESATURATI SI VITAMINA E, LA CERINTELE NORMATIVELOR EUROPENE

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Considering the new norms in feed quality checking (ANSVSA Order 51/2005 on the performance of the analytical methods and data interpretation), which are aligned to the European norms, the market for feeds implemented a checking system similar to the one for the food industry. These requirements stipulate that the laboratories for feed control must have validated methods for each sample assay. We therefore proposed to validate two chromatographic methods (HPLC and GC) for vitamin E and linolenic acid from the polyunsaturated fatty acids-enriched feeds for layers supplemented with 250 ppm vitamin E. The determined parameters were in agreement with SR EN ISO / CEI 17025:2005 as follows: exactness, reproducibility, sensitivity, accuracy, detection limit, quantification limit. We used for validation only certified reference materials and blanks, of analytical purity. We determined the incertitude for each validated method. We worked on two types of compound feeds for layers enriched in polyunsaturated fatty acids (I); with addition of flax seeds and with enriched linolenic acid (II). While the calculation of incertitude (10,62±0,44µg/g and 15,409±0,6µg/g for linolenic acid and 400±24 µg/g for vitamin E) shows that the methods range within admissible limits. The validated methods are proper for the determination of vitamin E and linolenic acid from feeds enriched in plant fat with a significant supplement of α-tocopherol.
Keywords: validation of methods, fees, chromatography, vitamin E, linolenic acid.

Introduction

The dietary nutrients have a positive effect (increase the feeding value) and a negative effect (ending with the distortion of the human health). The ω:3 fatty acids, the conjugated linolenic acid and the antioxidants (vitamin E, Se) are among the nutrients with positive effects. When feed ingredients rich in double bonds fatty acids are added, antioxidants have to be added, which to remove the unpleasant

effects of autooxidation (rancidity) of the dietary fats. Vitamin E is used most times as dietary antioxidant added to the vitamin premix. (Panaite and all. 2006).

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

The validation of the analytical method is required to ensure the quality and safety of the production processes. It is the confirmation by examination and supply of objective evidence, that some specific requirements for an intended application are met. Therefore, the analytical validation is the first step of ensuring the quality in a laboratory (Tanase and all. 2007).

During the recent years new requirements emerged in feed manufacturing imposing the validation of the analytical methods used for quality checking of the feeds, the specific requirements being stipulated in the legislation.

One of the great problems of the chemical analysis is insuring its quality and increasing the confidence in the results of a chemical or instrumental analysis. This is why the *statistical analysis* of the data is used, which can support the quality of the analytical measurements and of the reason of the analyst in its decisions (Eurachem 2003 ; ICH Q1A(R2) 2003, International Conference 2007).

This paper presents the working algorithm and the data produced by the validation of two methods: gas chromatography (GC) and high performance liquid chromatography (HPLC) used to determine the polyunsaturated fatty acids and vitamin E from the diet formulations enriched in PUFA and supplemented with 250 ppm vitamin E.

Material and Methods

The validation of the chromatographic methods for fatty acids and vitamin E (α tocopherol) determination was done according to a Ordin ANSVSA 51/2005 with a protocol in agreement with ISO-17025:2005 requirements, consisting in equipment standardization, determination of the accuracy, reproducibility, sensitivity, precision, limit of detection and limit of quantification. Only reference materials and certified blanks of analytical purity.

The diet used for validation had the following formulation:

I The diet rich in polyunsaturated fatty acids (PUFA) consisted of corn (32.42 %), wheat (20.00%), peas (10 %), full fat soy (21.98 %), sunflower meal (3.72 %). The diet had 16.21% protein, 2720.9 kcal/ kg metabolisable energy, total polyunsaturated fatty acids (PUFA) 6%, 250 ppm vitamin E.

II The diet rich in linolenic acid (C18 3n3 and C18 3n6): consisted of corn (60.78 %), soybean meal (23 %), corn gluten (0.5 %), flax seeds (3 %), buckthorn oil (1%). The diet had 15.49% protein, 2767 kcal/ kg metabolisable energy, 6% linolenic acid, 292.6 ppm vitamin E.

Table 1 shows working stages used in both analitycal methods.

Table 1.

Working stages

	GC	HPLC
Reagents and reference materials (CRM)	- H_2SO_4 2% in methanol of acknowledged analytical purity; hexane of acknowledged analytical purity; anhydrous Na_2SO_4 of acknowledged analytical purity; Standard solution of methylated fatty acids; (CRM) Soybean Oil.	- potassium hydroxide 13 % in ethanol; ethanol ; hexane of HPLC purity; petrol ether, boiling point between 400C to 600C; vitamin E standard substance: DL-α- tocopherol, minimal purity 96.0%; anhydrous sodium sulphate; dioxan.
Equipment for measuring/ testing and materials	Gaz cromatograf Perkin Elmer-Clarus 500, fitted with flame ionization detector(FID) and high polarity stationary stage capillary separation column. -carrier gas - hydrogen -combustion gas - air(O_2)	HPLC consisting of: autosampler, 250 mm long column with stationary stage consisting in octadecile groups (C18), detector UV-VIS.
Sample preparation for analysis (chemical processing of the samples)	Preparation of the methylic esters which are thereafter injected in the GC, usually 1 µL. Separation of the methylated fatty acids from the sample by gas chromatography on stationary column. The control sample (n-hexane) and a reference sample CRM are analysed in parallel with the sample for analysis (or with a series of samples for analysis).	The feed sample is saponified with KOH followed by extraction with petrol ether, concentration of the sample in rotavapor and mixing the resiude with hexane. The sample is introduced in a HPLC autosampler. 10 µL is injected in the chromatograph column.
Calculation	Calculation of the relative mass fraction. The relative mass fraction for each component is calculated with the formula: Wx=A(x)xf(x)x100%/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 vitamin E content is expressed in µg/g product and is calculated with the formula: *((Ap/Aet)xCxVx100)/m;* In which: Ap= sample area; Aet= standard area; C=standard concentration; V=volume of the sample; m= sample mass.

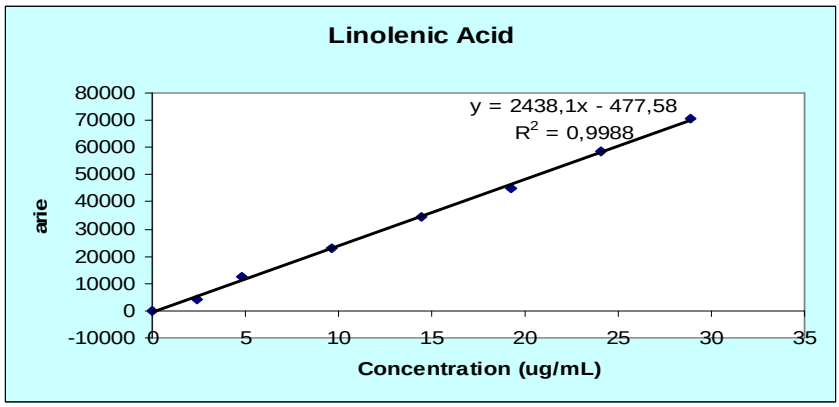

Figure 1 Plotting the standard curve (LINOLENIC ACID)

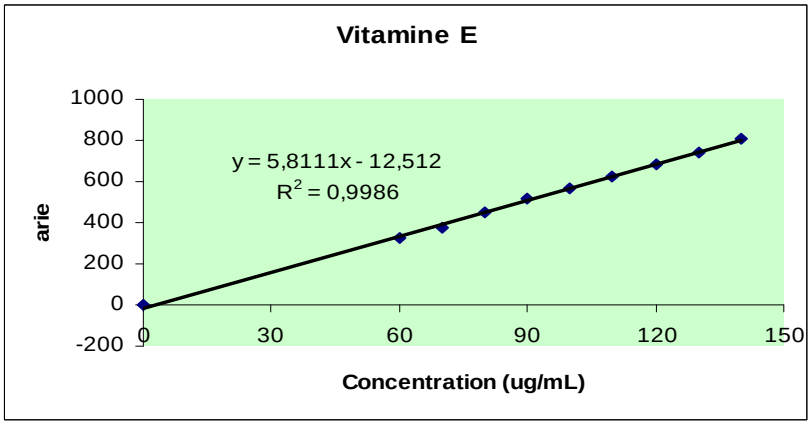

Figure 2. Plotting the standard curve (VITAMIN E)

Results and Discussions

The most important aspects of the methods used in determination were investigated. The parameters determined by the two methods, according to the obtained standard curves are shown in Table 2.

Table 2.

Validation parameters

<table>
<tr><th>Determination parameters</th><th>GC Method
Reference material :Soybean Oil 100µg/mL</th><th>HPLC Method
Reference material: α- tocophoferol</th></tr>
<tr><td>Exactness</td><td>A sample was prepared with concentration
µ=50 µg/mL mixture of fatty acids methyled esters from the stock solution of 100 µg/mL (19.25 µg/mL for the linolenic acid).
6 repeated determinations were done on the sample with concentration µ and the Yi values of the peak area were measured.
The standardization curve was used to calculate the values Xi of concentration obtained experimentally.
meanX = 19.003 µg / mL
Exactness=(19.003/19.25)*100 = 98.72 %
Bias = 1.28 %</td><td>A sample was prepared with concentration µ = 90 µg/ml de α-tocopherol from the stock solution of 1000 ppm. 10 repeated determinations were done on the sample with concentration µ=90 µg/ml and the Yi values of the peak area were measured.
The standardization curve was used to calculate the values Xi of concentration obtained experimentally.
meanX = 90.57 µg / mL
Exactness = 100.64 %
Bias = 0.64%</td></tr>
<tr><td>Accuracy (precision)%</td><td>meanX = 19.003 µg / mL
s = 0.147845
CV(RSD) = 0.78 %
The maximal value of RSD is determined according to the concentration to be analysed using the Horwitz equation (for the concentration of 1 ppm, max RSD = 10.72%)
RSD calc < RSD max = 0.78% < 10.72%</td><td>meanX = 90.57
S = 0.757
CV(RSD) = 0.836
The maximal value of RSD is determined according to the concentration to be analysed using the Horwitz equation (for the concentration of 1 ppm, max RSD = 10,72%)
RSD calc < RSD max = 0,836%<10,72%</td></tr>
<tr><td>Repeatability</td><td>meanX = 19.003 µg / mL
s = 0.147845
r=2.8 x 0.147845= 0.414</td><td>meanX = 90.488 µg / mL
s = 0,8132
r=2,8 x 0.8132 = 2.27</td></tr>
<tr><td>Internal Reproducibility</td><td>According to the type of tested method, of the performance level of the laboratory; R>r
The analysts were done by two analysts using the same working method.
s_{r1}= 0.14785
s_{r2} = 0.1164
s_{R12} = 0.13213
R_L=2.8*1.6 * S_R= 0.5919
R = 0.5919 > 0.4140</td><td>According to the type of tested method, of the performance level of the laboratory; R>r
The analysts were done by two analysts using the same working method.
s_{r1}= 0,8132
sr2 = 0,3379
s_{R12} = 1.1511
R_L=2,8*1,6 * S_R= 5.157
R = 5.157 > 2.27</td></tr>
<tr><td>Sensitivity</td><td>The value of the slope must be constant for the working domain
The equation of the linear regression function looks like:
Y = bX+a;
Y =2438.1X -477.58
b=2438.1</td><td>The value of the slope must be constant for the working domain
The equation of the linear regression function looks like:
Y = bX+a;
Y =5.8111X – 12.512
b= 5.8111</td></tr>
<tr><td>Limit of detection</td><td>5 blank samples were prepared, with concentration of 0.05µg/ mL
meanX = 0.049 µg / mL
s= 0.000783
LoD = 3s = 0.002349 µg / mL</td><td></td></tr>
<tr><td>Limit of Quantification</td><td>5 blank samples were prepared, with concentration of 0.05µg/ mL
meanX = 0.049 µg / mL
s= 0.000783 µg / mL
Tested concentration: 0.05µg/ mL
LoQ =10s + X =0.05683 µg / mL
LoQ > tested concentration 0.05683 > 0.05</td><td></td></tr>
<tr><td>Area of Application</td><td>A standardization curve was plotted for the domain 0–30 µg / mL and the linearity domain was visualised.
The lower limit of the domain is
LoQ = 0.05683 µg / mL
The uppper limit of the domain is: 30µg/mL
The working domain corresponds to the range in which the concentrations are proportional to the analytical signal</td><td></td></tr>
<tr><td>Tracing</td><td>6 repeated determinations were done on a sample which contained 19.25µg/mL linolenic acid
6 measurements were done on the same sample with analyt supplement (5µg/mL) and final concentration of 24,25 µg / mL.
meanX =19.003 µg / mL
<table>
<tr><th>blank+0.05µg Arie</th><th>Concentration traced on the detection limit</th></tr>
<tr><td>372</td><td>0.049</td></tr>
<tr><td>348</td><td>0.049</td></tr>
<tr><td>290</td><td>0.048</td></tr>
<tr><td>397</td><td>0.050</td></tr>
<tr><td>410</td><td>0.050</td></tr>
<tr><td>mead</td><td>0.049</td></tr>
<tr><td>STDEV</td><td>0.000783</td></tr>
</table>
meanX S = 23.945 µg / mL
Tracing % = 98.84%
80 % < 98.84% < 120%</td><td>6 repeated determinations were done on a sample which contained 80 µg/mL α-tocoferol.
6 measurements were done on the same sample with analyt supplement (20 µg/mL and final concentration of 100 µg/ mL.
<table>
<tr><th>Conc. vit E traced on the conc 80 µg/ml</th><th>Conc. vit E traced on the conc 100 µg/ml</th></tr>
<tr><td>80,05</td><td>98,5</td></tr>
<tr><td>79,8</td><td>99</td></tr>
<tr><td>78,05</td><td>101,02</td></tr>
<tr><td>78,9</td><td>99,8</td></tr>
<tr><td>80,01</td><td>99,08</td></tr>
<tr><td>Mead 79,362</td><td>99,48</td></tr>
<tr><td colspan="2">Tracing = 100,59</td></tr>
</table></td></tr>
</table>

In both analytical methods the samples had to be diluted further to achieve a better chromatographic separation and to plot Gaussian-form peaks and to frame the sample concentration on the used standardising curve. The analytical methods are suitable for the determination of the polyunsaturated fatty acids and vitamin E from compound feeds with good results, being used as analytical methods in the food and pharmaceutical industries.

Calculation of the Incertitude

GC	Concentration µg/g			Concentration µg/g + Incertitude	
u(C0)	NC enriched in fatty acid PUFA	NC enriched in linolenic acid and flax seeds	u®	NC enriched in fatty acid PUFA	NC enriched in linolenic acid and flax seeds
	0.5326	0.5123		10.619 ± 0.435	15.409 ± 0.61
HPLC	**Concentration µg/g**			**Concentration µg/g + Incertitude**	
u(C0)	**NC enriched in fatty acid PUFA**	**NC enriched in linolenic acid and flax seeds**	**u®**	**NC enriched in fatty acid PUFA**	**NC enriched in linolenic acid and flax seeds**
	5.06	**4.962**		**398.65±23.71**	**460±26.9**

u(C0) – Composed Incertitude
u® - Total Incertitude

The reproducibility test of the results by the two methods was within normal limits (0.42-0.6%), the experiments being replicated one week later; the accuracy (0.8-2%).The exactness of the methods is very good, ranging between 98-99%. The calculated interval of confidence is 94% for the determined concentrations.

Conclusions

- The analytical methods were used successfully to determine the concentration of linolenic acid and vitamin E from the compound feeds given to layers.
- The analysed samples (used compound feeds) are very important to poultry feeding so that poultry products are as competitive as possible on the market.
- The calculation of incertitude (10.62±0.44µg/g and 15.409±0.6µg/g for the determination of the CF linolenic acid, 400±24 µg/g for the determination of the vitamin E) show that the method ranges within admissible limits.

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