JRC.F.5/CvH/SB/AS/Ares

Subject: Addendum to the EURL evaluation report

Reference: FAD-2010-0248 - Omega-6-fatty acid as octadecadienoic acid (conjugated linoleic acid-methylester) (JRC.D.5/SFB/CvH/SB/mds/Ares (2015) 2247707)

In the corresponding report related to this dossier, the EURL could not recommend a method of analysis for the determination of the *active substance conjugated linoleic acid (CLA) (t10,c12)-methylester*, hereafter indicated as *t10,c12-CLA-Me*, in *premixtures* and *feedingstuffs*. The main reason was that the limited results of the validation and verification studies did not allow for an estimation of the performance characteristics of the method. Subsequently, DG SANTE [1] requested from the Applicant to conduct the missing experiments. The EURL evaluated the supplementary information [2,3] provided by the Applicant in response to this request.

As specified in the corresponding report, the method foresees after the extraction of the sample two separate high performance liquid chromatography (HPLC) determination, namely (1) the determination of total CLA by HPLC-UV (C-18 column) and (2) the percentage fraction of *t10,c12-CLA-Me* in total CLA by applying silver-ion impregnated columns "ChromSpher 5 Lipids". The mass fraction *t10,c12-CLA-Me* in the sample is subsequently calculated by multiplying the content of total CLA in the sample obtained in the first step with the percentage fraction of *t10,c12-CLA-Me* in total CLA obtained in the second step.

The Applicant reported to the EURL the results from the validation and verification studies [2,3] of the methods for the determination of total CLA and *t10,c12-CLA-Me* in *premixtures* and *feedingstuffs*. The raw data for the determination of *t10,c12-CLA-Me* in these matrices were subsequently subjected to statistical assessment by the EURL to calculate the mass fraction of this analyte and the corresponding repeatability relative standard deviation RSD_r (%) and intermediate precision RSD_{ip} (%) of the method. Moreover, the Applicant reported values for the recovery rate (%) for total CLA in the samples.

The results from the validation and verification studies are summarised in Table 1, confirming acceptable results for the precision and recovery rate. The small differences observed for the mean values of the target analyte in the validation and verification study respectively are considered negligible and therefore sufficient transferability could be demonstrated.

Table 1. The performance characteristics of the method for the quantification of *t10,c12-CLA-Me* in *premixture* and two *feedingstuff* samples, obtained within the frame of the validation and verification studies. The values for the recovery rates have been calculated from the results for CLA

	Premixture		**Feedingstuffs**			
			pellet pig finisher		lactating cow	
	Validation	Verification	Validation	Verification	Validation	Verification
Mass fraction (mg/kg)	22099	22325	615	638	4054	3919
RSD_r (%)	4.4	4.9	1.4	2.9	1.7	3.1
RSD_{ip} (%)	5.3	4.9	1.4	2.9	1.8	3.1
R_{rec} (%)	101	102	74	77	92	90
Reference	[2,3]					

RSD_r and RSD_{ip}: relative standard deviations for *repeatability and intermediate precision*; R_{rec} = Recovery rate

For the quantification of t10,c12-CLA-Me in *premixtures* and *feedingstuffs*, based on the performance characteristics available, the EURL recommends for official control the single laboratory validated and further verified method based on liquid chromatography coupled to spectrophotometric detection (HPLC-UV).

Recommended text for the registry entry (analytical method)

For the quantification of *CLA (t10,c12)-methylester* in premixtures and feedingstuffs:

- high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV)

References

[1] Supplementary Information – DG SANTE request cf. Validation and verification of method of analysis for determination of the feed additive. Ares(2020)2289441 - 29/04/2020

[2] Supplementary Information - FAD-2016_0248 and 2016-0033_Response_additional data for method verification.pdf

[3] Supplementary Information. Excel file with raw data. - FAD-2016_0248 and 2016-0033_Raw data for method verification.xlsx

Addendum
- Christoph von Holst (EURL-FA), Geel, 16/02/2022

EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate D: Institute for Reference Materials and Measurements
European Union Reference Laboratory for Feed Additives

JRC.D.5/SFB/CvH/SB/mds/Ares

Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Omega-6-fatty acid as octadecadienoic acid (conjugated linoleic acid-methylester)

(FAD-2010-0248; CRL/100129)

European Union Reference Laboratory
Feed Additives

Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2010-0248 - CRL/100129**
Name of Product:	***Omega-6-fatty acid as octadecadienoic acid (conjugated linoleic acid-methylester)***
Active Agent (s):	**Conjugated linoleic acid-methylester (trans-10, cis-12-isomer)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA) 28/05/2015**
Report approved by: Date:	**Christoph von Holst 28/05/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 10 for *Omega-6-fatty acid as octadecadienoic acid*, under the category/functional group 3(a) 'nutritional additives'/'vitamins, pro-vitamins and chemically well-defined substances having similar effect', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for pigs and bovines. According to the Applicant, the *active substance* is a *methyl ester of conjugated linoleic acid* (CLA-Me) equivalent to *Omega-6-fatty acid as octadecadienoic acid (trans-10, cis-12-isomer)*, hereafter indicated as *t10,c12-CLA-Me*.

The *feed additive* is to be marketed either as a colourless to yellowish oily liquid or as an off-white to beige solid formulation, to contain a minimum of 9 % of *t10,c12-CLA-Me*. The liquid product is intended to be mixed either in *premixtures* or added directly to *feedingstuffs*, while the solid formulation is specifically intended to be added directly to *feedingstuffs* for bovines only. The Applicant proposed a maximum concentration of *t10,c12-CLA-Me* in *feedingstuffs* of 0.5 %.

For the quantification of *t10,c12-CLA-Me* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on Gas Chromatography coupled to Flame Ionization Detector (GC-FID). Experiments data were provided in the frame of the validation and verification studies. The precision results derived from the validation and verification studies range from 0.14 to 0.65 %. Based on these satisfactory performance characteristics, the EURL recommends for official control this GC-FID method to quantify *t10,c12-CLA-Me* in the *feed additive*.

For the quantification of *t10,c12-CLA-Me* in *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on High Performance Liquid Chromatography coupled to UltraViolet detection (HPLC-UV). The data presented indicates an acceptable method performance profile. However, due to the limited number of available data, a statistical evaluation was not possible. Therefore the EURL cannot comment on the suitability for official control of the HPLC-UV method presented to quantify the total *t10,c12-CLA-Me* content in *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

Conjugated linoleic acid methyl ester (trans-10, cis-12-isomer), Omega-6-fatty acid as octadecadienoic acid, nutritional additives, pigs, bovines

1. BACKGROUND

In the current application authorisation is sought under Article 10 (re-evaluation of the already authorised additive) for *Omega-6-fatty acid as octadecadienoic acid*, under the category/functional group 3(a) 'nutritional additives'/'vitamins, pro-vitamins and chemically well-defined substances having similar effect', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for pigs and bovines [1-3].

According to the Applicant, the *active substance* is a *methyl ester of conjugated linoleic acid* (CLA-Me) equivalent to *Omega-6-fatty acid as octadecadienoic acid (trans-10, cis-12-isomer)*, hereafter indicated as *t10,c12-CLA-Me* [4]. The *feed additive* is to be marketed either as a colourless to yellowish oily liquid (Lutalin®) or as an off-white to beige solid formulation (Lutrell®) [5]. The *feed additive* contains a minimum of 9 % of *t10,c12-CLA-Me* [2]. Lutalin® is intended to be mixed either in *premixtures* or added directly to *feedingstuffs*, while Lutrell® is specifically intended to be added directly to *feedingstuffs* for bovines only [5,6].

The Applicant proposed a maximum concentrations of *t10,c12-CLA-Me* in *feedingstuffs* of 0.5 % [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Omega-6-fatty acid as octadecadienoic acid* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [7].

Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs

For the quantification of *t10,c12-CLA-Me* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on Gas Chromatography coupled to Flame Ionization Detector (GC-FID) [8-10].

The method consists in a direct injection in split mode of the *feed additive* in a fused silica capillary column coated with polyethylene glycol and detected by FID. The *t10,c12-CLA-Me* peak is identified by comparison with the retention times obtained for reference samples. The quantification is done by "corrected area percentage" and adjusted from the water content. This protocol is applicable to the liquid formulation. Furthermore, a slightly modified procedure has been presented, validated and verified for the formulated solid form Lutrell®[11].

Experiments data were provided in the frame of the validation and verification studies. The following precisions - recalculated by the EURL [12] based on the available experimental data - range from 0.14 to 0.18 % (cf. validation) and from 0.2 to 0.65 % (cf. verification). Based on these satisfactory performance characteristics, the EURL recommends for official control the GC-FID method to quantify *t10,c12-CLA-Me* in the *feed additive*.

For the quantification of *t10,c12-CLA-Me* in *premixtures* and *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified method based on High Performance Liquid Chromatography coupled to UltraViolet detection (HPLC-UV) [13-15].

The sample (of maximum 20 g) is weighted into a stoppered flask. BHT, sodium ascorbate, water, ethanol and acetic acid are added. After ultrasonic bath for at least 15 min at 60ºC, the sample is cooled down. 100 ml of extraction solvent (cyclohexane/ethyl acetate 80:20) is added and the flask is stirred for 30 min. A saturated sodium chloride solution is added and the whole is further stirred for 10 min. The solution is finally filtered (0.45 µm) and injected into the HPLC system (C-18 column). The total CLA content (including all conjugated linoleic acid (CLA) and CLA-Me) is quantified at 233 nm via external calibration solutions.

The sample is further processed in order to quantify the specific *t10,c12-CLA-Me* isomer. An aliquot of the undiluted extract is placed in a test tube and evaporated to dryness under nitrogen. An esterification reagent is added to the dried sample and, once the reaction is occurred, water and cyclohexane are added. CLA-Me is extracted into the organic phase during shaking. The supernatant is filtered and injected in the HPLC system. The individual isomers are separated by a series of three silver-ion impregnated columns *"ChromSpher 5 Lipids"*. The individual CLA-Me isomers (including *t10,c12-CLA-Me*) are detected by UV at 233 nm, and the percent peak areas (normalised by the sum of all detected peaks) are

calculated for each individual CLA-ME isomer. Total *t10,c12-CLA-Me* is then quantified by comparison with the previously determined total CLA content.

The data presented indicates an acceptable method performance profile. However, due to the limited number of available data, a statistical evaluation was not possible [12]. Therefore the EURL cannot comment on the suitability for official control of the HPLC-UV method presented to quantify the total *t10,c12-CLA-Me* content in *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified methods based on GC-FID for the quantification of *t10,c12-CLA-Me* in the *feed additive*.

Recommended text for the register entry (analytical method)

For the determination of *Omega-6-fatty acid as octadecadienoic acid (trans-10, cis-12-isomer)* in *feed additive*:

- Gas Chromatography coupled to Flame Ionization Detector (GC-FID)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Omega-6-fatty acid as octadecadienoic acid* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANCO/G1: Forw. Appl. 1831/0021-2012

[2] * Supplementary information, Annex A Register entry_CLA_FAD-2010-0248 mar 2015

[3] * Supplementary information, Addendum_CLA_FAD-2010-0248-13_11_2014, page 2

[4] * Supplementary information, Addendum_CLA_FAD-2010-0248-13_11_2014, page 7

[5] * Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[6] * Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[7] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[8] * Supplementary information, i a Method PM-00685-04e (Lutalin)

[9] * Supplementary information, i b Validation of PM-00685-04e (Lutalin)

[10] * Supplementary information, i c Verification of PM-00685-04e (Lutalin)

[11] * Supplementary information, ii a Method PM-01539-02e (Lutrell)

[12] * Supplementary information, EURL Calculations.xlsx

[13] * Supplementary information, iii a Method AM-00887-02e (Feed)

[14] * Supplementary information, iii b Validation of AM-00887-02 (Feed)

[15] * Supplementary information, iii c Verification of AM-00887-02 (Feed)_Rev01

*Refers to Dossier no: FAD-2010-0248

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Valsts veterinārmedicīnas diagnostikas centrs (VVMDC), Rīga (LV)