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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

Citranaxanthin

(FAD-2010-0201; CRL/100131)

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-2010-0201 - CRL/100131**
Name of Feed Additive:	***Citranaxanthin (Lucantin CX® forte)***
Active Agent (s):	**Citranaxanthin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **01/12/2016**
Report approved by: Date:	**Christoph von Holst** **12/12/2016**

EXECUTIVE SUMMARY

Citranaxanthin is currently authorized in laying hens by Commission Directive 85/429/EEC belonging to the group "colouring matters including pigments" listed in Chapter I of Annex B of Directive 70/524/EEC. In the current application authorisation is sought for *citranaxanthin (Lucantin CX® forte),* under article 10 (2), for the "category"/"functional group" 2 (a) (ii) "sensory additives"/"colourants: substances which, when fed to animals, add colours to food of animal origin" according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically authorisation is sought for the use of the *feed additive* for laying hens.

The *feed additive* consists of deep violet crystals containing a mixture of *citranaxanthin* (10 %) in a carrier of organic and inorganic matrix and variable amounts of antioxidants, preservatives, emulsifiers and water. The *feed additive* is intended to be incorporated directly in *feedingstuffs* or through *premixtures* at a recommended maximum *citranaxanthin* content of 80 mg/kg of complete *feedingstuffs* alone or mixed with other carotenoids and xanthophylls.

For the quantification of *citranaxanthin* in the *feed additive* the Applicant submitted a spectrophotometric ring-trial validated method, while for its quantification in *premixtures* and f*eedingstuffs*, the Applicant proposed a single laboratory validated and further verified method, based on High Performance Liquid Chromatography coupled with a spectrophotometric detector (HPLC-VIS). The following performance characteristics were reported: - precisions (relative standard deviations for *repeatability*, *intermediate precision* and *reproducibility*) ranging from 0.5 to 2.4 % (for the *feed additive*) and between 1.5 to 7.7 % (for *premixtures* and *feedingstuffs*); - recovery rates of 99 % (for the *feed additive*) and ranging from 90 to 102 % (for *premixtures* and *feedingstuffs*); and a limit of quantification of 1 mg/kg *feedingstuffs.* Based on the presented performance characteristics, the EURL recommends for official control the methods mentioned above.

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

Citranaxanthin, Lucantin CX® forte, sensory additives, colourants, laying hens

1. BACKGROUND

Citranaxanthin is authorized in laying hens by Commission Directive 85/429/EEC [1] belonging to the group "colouring matters including pigments" listed in Chapter I of Annex B of Directive 70/524/EEC. In the current application authorisation is sought for *citranaxanthin (Lucantin CX® forte)*, under article 10 (2) (authorisation of an existing product), for the "category"/"functional group" 2 (a) (ii) "sensory additives"/"colourants: substances which, when fed to animals, add colours to food of animal origin" according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically authorisation is sought for the use of the *feed additive* for laying hens [2][3].

The *feed additive* consists of deep violet crystals containing a mixture of *citranaxanthin* (10 %) in a carrier of 73 % organic (i.e. carbohydrates, proteins and vegetable oils) and 5 % inorganic (i.e. silica) matrix and variable amounts of antioxidants, preservatives, emulsifiers and water [4].

The *feed additive* is intended to be incorporated directly in laying hens *feedingstuffs* or through *premixtures* [5]. The recommended maximum content of *citranaxanthin* is 80 mg/kg on complete *feedingstuffs*. When mixed with other carotenoids and xanthophylls, the sum of *citranaxanthin* and of these compounds must not exceed this level [3].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *citranaxanthin (Lucantin CX® forte)* 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 are available from the respective European Union Reference Laboratories [6].

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

For the quantification of *citranaxanthin* in the *feed additive* the Applicant submitted a spectrophotometric ring-trial validated method [7], in which 6 laboratories participated.

The *feed additive* is digested in water with protease. After dilution with acetone the mass fraction of *citranaxanthin* is determined spectrophotometrically at a maximum wavelength of 468 nm. The Applicant analysed this *feed additive* using two different methods (spectrophotometric and HPLC) and obtained similar results, indicating that no interferences could be identified. The performance characteristics obtained in the frame of the ring-trial validated method are reported in Table 1.

For the quantification of *citranaxanthin* in *premixtures* and *feedingstuffs*, the Applicant proposed a single laboratory validated and further verified method, based on Normal Phase High Performance Liquid Chromatography coupled with a spectrophotometric detector (NP-HPLC-VIS) [8]. The assay comprises an enzymatic digestion of the sample followed by extraction with ethanol and dichloromethane. The extract is injected into an isocratic HPLC system. According to the Applicant the method is able to resolve the main Z-isomers from the all-E-isomers of *citranaxanthin*. These *citranaxanthin* isomers are well separated from the possible carotenoids and other xanthophyllis present in feed [8]. Total *citranaxanthin* (sum of *(all-E)-citranaxanthin* and *(Z)-citranaxanthin* isomers) is quantified by spectrophotometric detection at 466 nm using "one point" external calibration. Upon request of the EURL the Applicant provided experimental evidences proving the equivalence of "one point" external calibration and the commonly accepted "five points" external calibration for the quantification of the total *citranaxanthin* in *feedingstuffs* [9].

The performance characteristics reported in the frame of the validation and verification studies are presented in Table 1. The Applicant also reported a limit of quantification (LOQ) of 1 mg/kg *feedingstuffs* [8].

Table 1. Performance characteristics of analytical methods for the determination of *citranaxanthin* in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS) [7][8] reported in the frame of the validation (Val.), verification (Ver.) and ring-trial (RT)

Method	Matrix	Content	Assay	RSD_r, %	RSD_{ip}, %	R_{Rec} (%)
HPLC-VIS [8]	FS	5-50 (mg/kg)	Val	3.0-7.7	5.5-7.7	98-101
		119 (mg/kg)		3.8	-	-
		4.8-9.3 (mg/kg)	Ver	6.5-7.2[a]	7.2-7.7[a]	90
		115 (mg/kg)		2.3	-	97
	PM	2000 (mg/kg)	Val	1.5	3.0	102
		3640 (mg/kg)	Ver	2.3[a]	2.5[a]	98
Spectrophotometric [7]	FA	9.7-10.6 (g/100g)	Val	1.0[a]	1.0[a]	99
			RT	0.5-1.1	1.7-2.4[b]	-

RSD_r, RSD_{ip} & RSD_R: relative standard deviation for *repeatability*, *intermediate-precision* & *reproducibility,* respectively; R_{Rec}: *recovery* rate (%); a = Recalculated by EURL [10][11]; b = RSD_R%.

Note: A multianalyte method for the determination of carotenoids in *feedingstuffs* and *premixtures* based on reversed-phase HPLC coupled to spectrophotometric detection is being evaluated by the CEN Technical Committee 327 to become a European Standard.

Based on the acceptable performance characteristics available, the EURL recommends for official control, the spectrophotometric method for the quantification of *citranaxanthin* in the *feed additive* and the NP-HPLC-VIS method for the quantification of *citranaxanthin* 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 spectrophotometric method for the quantification of *citranaxanthin* in the *feed additive* and the NP-HPLC-VIS method for the quantification of *citranaxanthin* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *citranaxanthin* in the *feed additive*:

– Spectrophotometric method at 468 nm

For the quantification of *citranaxanthin* in *premixtures* and *feedingstuffs:*

– Normal Phase High Performance Liquid Chromatography with spectrophotometric detection (NP-HPLC-VIS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *citranaxanthin (Lucantin CX® forte)* 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] Commission Directive of 8 July 1985 amending the annexes to the Council Directive 70/524/EEC concerning additives in feedingstuffs (85/429/EEC)

[2] *Application, Reference SANCO/G1: Forw. Appl. 1831/0032-2015

[3] *Application, Proposal for Registry Entry – Annex A

[4] *Technical dossier, Section II, 2.1.3. Qualitative and quantitative composition

[5] *Technical dossier, Section II, 2.5 Condition of use of the additives

[6] 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

[7] *Technical dossier, Section II- Annex_2.6.1.a

[8] *Technical dossier, Section II- Annex_2.6.1.b

[9] *Supplementary Information, Report Calibration CX in Feed VE-01.pdf

[10] *Supplementary Information, eurl_anova_citraxanthin_fa.pdf

[11] *Supplementary Information, eurl_anova_citraxanthin_ver.pdf

*Refers to Dossier no: FAD-2010-0201

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Fødevarestyrelsens Laboratorie Ringsted (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- RIKILT Wageningen UR, Wageningen (NL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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