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

Dossier related to:	**FAD-2010-0372** **CRL/100140**
Feed Additive:	**Lutein (E161b)**
Active Substance(s):	**Lutein**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis (EURL-FA)**
Report revised by: Date:	**Piotr Robouch (EURL-FA)** **11/10/2012**
Report approved by: Date:	**Christoph von Holst** **11/10/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *lutein (E161b)*, under the category/functional group 2(a) 'sensory additives'/'colourants', for the following subgroups: - (i) substances that add or restore colour in *feedingstuffs*; - (ii) substances which, when fed to animals, add colours to food of animal origin; and - (iii) substances which favourably affect the colour of ornamental fish or birds, according to the classification system of Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the *feed additive* (*lutein, E161b*) is a deep brownish-orange viscous paste with a characteristic odour - obtained by saponification of the extract of *Tagetes* dried petals - containing levels of *total carotenoids & xanthophylls* ranging from 40 to 160 g/kg, which corresponds to a minimum *lutein* (*all-trans-lutein* isomer) content in the *feed additive* ranging from 24 to 96 g/kg and a maximum *zeaxanthin* content in the *feed additive* ranging from 12 to 48 g/kg.

As indicated by the Applicant, the typical formulations of the *feed additive* to be marketed are in liquid or solid forms with a minimum *total carotenoids & xanthophylls* concentrations ranging from 0.5 % to 7 %.

Specifically, authorisation is sought for the use of the *feed additive* for all poultry species, cats and dogs, crustaceans, fish/tilapias, ornamental fish and birds. The *feed additive* is intended to be incorporated in *premixtures*, *feedingstuffs* and *water*.

While no maximum and minimum levels were proposed for cats, dogs, ornamental fish and birds, the Applicant proposed for all poultry species, crustaceans and fish/tilapias a maximum concentration of *lutein* (*all-trans-lutein* isomer only) or *total carotenoids & xanthophylls* of 80 mg/kg in *feedingstuffs* and 40 mg/L in *water* (except for crustaceans and fish/tilapias).

For the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive* the Applicant proposed the internationally recognised FAO JECFA monograph for food additives, as recommended by Commission Directive 2008/128/EC, where identification is based on several tests, including: - solubility; - spectrophotometry; - test for carotenoids, while quantification is achieved by spectrophotometry with or without separation by High Performance Liquid Chromatography (HPLC). Even though no performance characteristics are provided, the EURL recommends for official control the methods recommended by Commission Directive 2008/128/EC and described in the JECFA monograph mentioned above for the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive*.

For the determination of *total carotenoids & xanthophylls* (including all forms of *lutein* and other relevant colouring matters) in *feedingstuffs* the Applicant submitted the official method of the Association of Analytical Communities (AOAC, 970.64) based on saponification, chromatographic separation of carotenes & xanthophylls and further detection of the different fractions by spectrophometry at 436 nm and 474 nm. This method can also be applied to *premixtures* samples after dilution with blank feed. This AOAC method does not distinguish between added and endogenous *carotenoids & xanthophylls*. Even though no performance characteristics are provided, the EURL recommends for official control the above mentioned AOAC official method for the determination of *total carotenoids & xanthophylls* in *premixtures* and *feedingstuffs*.

For the determination of *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs* the Applicant submitted a single laboratory validated and further verified method, based on normal phase High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis). This HPLC-Vis method does not distinguish between added and endogenous *lutein*. The following performance characteristics were reported for *lutein* (*all-trans-lutein* isomer):

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.1 to 11.2%;
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.9 to 11.2%;
- a recovery rate ranging from 78 to 101%; and
- a limit of quantification of 0.7 mg/kg *feedingstuffs*

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis) to determine *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs*.

As the Applicant did not provide any analytical method or experimental data for the determination of *lutein* (*all-trans-lutein* isomer) and *total carotenoids & xanthophylls* in *water*, the EURL cannot evaluate nor recommend any method for official control for their determination in *water*.

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
lutein, E161b, zeaxanthin, carotenoids, xanthophylls, sensory additives, colourants, all poultry species, cats and dogs, crustaceans, fish/tilapias, ornamental fish and birds

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use of a feed additive) and 10(2) (re-evaluation of additives already authorised under provisions of Council Directive 70/524/EEC) for *lutein (E161b)*, under the category/functional group 2(a) 'sensory additives'/'colourants', for the following subgroups: - (i) substances that add or restore colour in *feedingstuffs*; - (ii) substances which, when fed to animals, add colours to food of animal origin; and - (iii) substances which favourably affect the colour of ornamental fish or birds [1, 2], according to the classification system of Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the *feed additive* (*lutein, E161b*) is a deep brownish-orange viscous paste with a characteristic odour - obtained by saponification of the extract of *Tagetes* dried petals - containing levels of *total carotenoids & xanthophylls* ranging from 40 to 160 g/kg, which corresponds to a minimum *lutein* (*all-trans-lutein* isomer) content in the *feed additive* ranging from 24 to 96 g/kg and a maximum *zeaxanthin* content in the *feed additive* ranging from 12 to 48 g/kg [2, 3].

As indicated by the Applicant, the typical formulations of the *feed additive* to be marketed are in liquid or solid forms with a minimum *total carotenoids & xanthophylls* concentrations ranging from 0.5 % to 7 % [3].

Specifically, authorisation is sought for the use of the *feed additive* for all poultry species, cats and dogs, crustaceans, fish/tilapias, ornamental fish and birds [2]. The *feed additive* is intended to be incorporated in *premixtures*, *feedingstuffs* and *water* [2, 3].

While no maximum and minimum levels were proposed for cats, dogs, ornamental fish and birds [2], the Applicant proposed for all poultry species, crustaceans and fish/tilapias a maximum concentration of *lutein* (*all-trans-lutein* isomer only) or total *carotenoids & xanthophylls* of 80 mg/kg in *feedingstuffs* and 40 mg/L in *water* (except for crustaceans and fish/tilapias) [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 *lutein (E161b)* 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, mycotoxins, and dioxins) are available from the respective European Union Reference Laboratories [4].

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

For the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive* the Applicant proposed the internationally recognised FAO JECFA monograph for food additives [5], as recommended by Commission Directive 2008/128/EC, where identification is based on several tests, including: - solubility; - spectrophotometry; - test for carotenoids. For the quantification of *total carotenoids & xanthophylls* spectrophotometry at 446 nm is applied, while High Performance Liquid Chromatography (HPLC) coupled with spectrophotometry at 446 nm is used for the quantification of *lutein* (*all-trans-lutein* isomer only) and *zeaxanthin*.

Even though no performance characteristics are provided, the EURL recommends for official control the methods recommended by Commission Directive 2008/128/EC and described in the above mentioned JECFA monograph for the determination *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive*.

For the determination of *total carotenoids & xanthophylls* (including all forms of *lutein* and other relevant colouring matters) in *feedingstuffs* the Applicant submitted the official method of the Association of Analytical Communities (AOAC, 970.64) based on saponification, chromatographic separation of carotenes & xanthophylls, and further detection of the different fractions by spectrophometry at 436 nm and 474 nm [6]. This method can also be applied to *premixtures* samples after dilution with blank feed. This AOAC method does not distinguish between added and endogenous *carotenoids & xanthophylls*.

Even though no performance characteristics are provided, the EURL recommends for official control the above mentioned AOAC official method for the determination of *total carotenoids & xanthophylls* in *premixtures* and *feedingstuffs*.

For the determination of *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method, based on normal phase High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis) [7]. This HPLC-Vis method does not distinguish between added and endogenous *lutein*.

The grinded sample (0.2 - 20 g) is extracted with a n-hexane:ethanol:acetone:toluene (10:6:7:7, v/v). The extract is injected into an isocratic normal phase HPLC system to resolve the geometrical isomers of capsanthin, *zeaxanthin* and *lutein*. The xanthophylls are quantified at 450 nm using β-carotene as the calibration standard. Experimentally determined relative response factors of different xanthophylls in relation to β-carotene are used for the correction of specific absorbances of the different xanthophylls. The performance characteristics determined for *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs* [8] are presented in Table 1. Furthermore, the Applicant reported for *lutein* (*all-trans-lutein* isomer) a limit of quantification (LOQ) of 0.7 mg/kg *feedingstuffs*.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis) to determine *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs*.

The Applicant did not provide any analytical method or experimental data for the determination of *lutein* (*all-trans-lutein* isomer) and *total carotenoids & xanthophylls* in *water*. Therefore, the EURL cannot evaluate nor recommend any method for official control to determine *lutein* (*all-trans-lutein* isomer) and *total carotenoids & xanthophylls* in *water*.

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.

Table 1: Method performance characteristics for the determination of *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs* [8]

	Premixtures		Feedingstuffs	
	Validation	**Verification**	**Validation**	**Verification**
Content [mg/kg]	11.3 - 18.0		16.4 - 42.0	
RSD_r (%)	4.6	4.5 - 11.2	0.1 - 0.9	0.5 - 5.2
RSD_{ip} (%)	-	11.2	-	0.9 - 5.2
R_{rec} (%)	84	101	78 - 94	82 - 90

RSD_r, RSD_{ip} - relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{rec} - *recovery* rate;

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- the methods described in FAO JECFA monograph *'lutein from tagetes erecta'* monograph No. 3 (2006), Combined Compendium for Food Additive Specifications - as recommended by Commission Directive 2008/128/EC - for the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive,* where identification is based on: - solubility; - spectrophotometry; - test for carotenoids, while quantification is achieved by spectrophotometry with or without separation by High Performance Liquid Chromatography (HPLC).
- the AOAC official method 970.64, based on chromatographic separation after saponification and further detection by spectrophometry at 436 nm and 474 nm, for the determination *of total carotenoids & xanthophylls* (including all forms of *lutein* and other relevant colouring matters) in *premixtures* and *feedingstuffs*.
- the single-laboratory validated and further verified method using High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis) for the determination of *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs*.

The Applicant did not provide any analytical method or experimental data for the determination of *lutein* (*all-trans-lutein* isomer) and total *carotenoids & xanthophylls* in *water*. Therefore, the EURL cannot evaluate nor recommend any method for official control to determine *lutein* (*all-trans-lutein* isomer) and *total carotenoids & xanthophylls* in *water*.

Recommended text for the register entry (analytical method)

For the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive*:

- High Performance Liquid Chromatography (HPLC) with spectrophotometry - Commission Directive 2008/128/EC referring to FAO JECFA monograph *'lutein from tagetes erecta'* monograph No. 3 (2006), Combined Compendium for Food Additive Specifications

For the determination of *total carotenoids & xanthophylls* in *premixtures* and *feedingstuffs*:

- Liquid chromatography with visible detection (LC-Vis) - AOAC official method 970.64

For the determination of *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs*:

- High Performance Liquid Chromatography with visible detection (HPLC-Vis)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *lutein* 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/D/2 Forw. Appl. 1831/00160 (10173)-2010

[2] *Application, Proposal for Register Entry – Annex A

[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis

[4] 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

[5] FAO JECFA Combined Compendium of Food Additive Specifications, *'lutein from tagetes erecta'*, Monograph No. 11 (2011)
http://www.fao.org/ag/agn/jecfa-additives/specs/monograph11/additive-255-m11.pdf
(last visited on 11/10/2012)

[6] AOAC Official Method 970.64 Carotenes and Xanthophylls in dried plant materials and mixed feeds
http://www.aoac.org/omarev1/970_64.pdf
(last visited on 11/10/2012)

[7] *Technical dossier, Section II, Annex_II_28

[8] *Technical dossier, Section II, Annex_II_32

* Refers to Dossier No. FAD-2010-0372

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:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 - Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)