



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
European Union Reference Laboratory for Feed Additives

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

Subject: Addendum to the EURL evaluation reports

References:

- FAD-2010-0342 – Tartrazine (JRC.D.5/CvH/ZE/mds/ Ares(2016)2938957)
- FAD-2010-0348 – Carmoisine (JRC.DG.D.6/CvH/RM/ag/ARES(2011)991812)
- FAD-2010-0349 – Ponceau 4R (JRC.D.5/SFB/CvH/JK/ag/Ares(2012)1483410)
- FAD-2010-0382 – Erythrosine (JRC.DG.D.6/CvH/RM/ag/ARES(2011)861655)
- FAD-2010-0347 – Allura Red AC (JRC.DG.D.6/CvH/RM/ag/ARES(2011)991812)
- FAD-2010-0346 – Indigo Carmine (JRC.DG.D.6/CvH/RM/ag/ARES(2011)861655)
- FAD-2010-0351 – Brilliant Blue FCF (JRC.D.5/SFB/CvH/ZE/ag/Ares(2012)1483410)

Upon the requests from DG SANTE [1, 2], the EURL evaluated the supplementary information provided [3-11] in the frame of the following feed additive dossiers: 2010-0342, 2010-0348, 2010-0349, 2010-0382, 2010-0347, 2010-0346 and 2010-0351 for the analysis of *Tartrazine*, *Carmoisine*, *Ponceau 4R*, *Erythrosine*, *Allura Red AC*, *Indigo Carmine* and *Brilliant Blue FCF* in *feedingstuffs*, respectively. For the quantification of the above mentioned colourants the Applicant submitted a multi-analyte single-laboratory validated and further verified method based on high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [3].

The sample (5 g) is sonicated with mixture of methanol, aqueous bicarbonate solution and acetonitrile, shaken and centrifuged. The supernatant is separated and the extraction is repeated for the second time. The supernatants from the two extractions are combined, diluted and an aliquot after the dilution is evaporated until dryness. The residue is dissolved in the mobile phase for further LC-MS/MS analysis. The analytes of interest are detected by mass spectrometry and the quantification is performed in the multiple reaction monitoring (MRM) mode by using calibration with external standards [3].

The performance characteristics reported by the Applicant and recalculated by the EURL in the frame of the validation [4] and the verification studies [5-11] for the quantification of the above mentioned seven colourants in spiked samples of *feedingstuffs* are presented in Tables 1 and 2. In addition, the Applicant reported a limit of quantification (LOQ) of 1 mg for each individual colourant/kg *feedingstuffs*.

Table 1. The performance characteristics reported by the Applicant and recalculated by the EURL in the frame of the validation and the verification studies for the quantification of *Tartrazine*, *Carmoisine*, *Ponceau 4R* and *Erythrosine* in spiked samples of *feedingstuffs*.

	Tartrazine		Carmoisine		Ponceau 4R		Erythrosine	
	Val.	Ver.	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, mg/kg	1 - 100	10	1 - 100	10	1 - 100	10	1 - 100	10
RSD_r, %	2.8 - 8.3	3.9	1.0 - 6.6	2.5	1.4 - 8.2	2.4	1.8 - 6.2	4.5
RSD_{ip}, %	2.9 - 8.3	3.9	2.6 - 6.6	3.5	2.0 - 10.5	2.4	3.5 - 12.2	6.3
R_{Recr}, %	86 - 94	95	92 - 111	98	81 - 96	101	88 - 100	91
Reference	[4]	[5]	[4]	[6]	[4]	[7]	[4]	[8]

Val. – Validation; Ver. – Verification; RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery rate*.

Table 2. The performance characteristics reported by the Applicant and recalculated by the EURL in the frame of the validation and the verification studies for the quantification of *Allura Red AC*, *Indigo Carmine* and *Brilliant Blue FCF* in spiked samples of *feedingstuffs*.

	Allura Red AC		Indigo Carmine		Brilliant Blue FCF	
	Val.	Ver.	Val.	Ver.	Val.	Ver.
Mass fraction, mg/kg	1 - 100	10	1 - 100	10	1 - 100	10
RSD_r, %	1.9 - 9.3	8.7	2.7 - 9.6	2.6	0.8 - 8.2	3.1
RSD_{ip}, %	2.8 - 9.3	8.7	3.0 - 9.6	8.9	2.1 - 11.6	3.1
R_{Recr}, %	92 - 105	105	80 - 88	88	84 - 100	99
Reference	[4]	[9]	[4]	[10]	[4]	[11]

Val. – Validation; Ver. – Verification; RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery rate*.

In addition, samples of a few commercial pet feed products (kibbles) have been analysed using the above mentioned method and acceptable precision was demonstrated. However, lower mass fractions compared to the expected values were measured for *Tartrazine*, *Carmoisine*, *Ponceau 4R* and *Indigo Carmine* [4] in this matrix. No performance

characteristics were presented for *Allura Red AC* and *Erythrosine* when analysing the real samples [4].

The Applicant has attributed the lower mass fractions observed in the samples to the lack of homogeneity of the samples and/or the adverse impact of specific production conditions of the kibbles on these values [4]. It is therefore recommended that additional measures are taken for checking the documentation related to the specific characteristics of the production process of the complete *feedingstuffs* in the case when significantly lower mass fractions of the colourants in comparison to the ones indicated on the labels are obtained during the official control of pet feed samples.

The Applicant did not provide the EURL a method for the determination of the above mentioned colourants in *premixtures* as the *feed additives* (in the form of powder or as the solutions in water) are supposed to be added directly into *feedingstuffs*.

Based on the performance characteristics available the EURL recommends for official control the multi-analyte single laboratory validated and verified LC-MS/MS method for the quantification of *Tartrazine*, *Carmoisine*, *Ponceau 4R*, *Erythrosine*, *Allura Red AC*, *Indigo Carmine* and *Brilliant Blue FCF* in *feedingstuffs*.

Recommended text for the registry entry (analytical method)

For the quantification of *Tartrazine*, *Carmoisine*, *Ponceau 4R*, *Erythrosine*, *Allura Red AC*, *Indigo Carmine* and *Brilliant Blue FCF* in *feedingstuffs*:

- high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

References

- [1] Supplementary Information – DG SANTE request cf. Validation of method of analysis for Sunset yellow, Carmoisine, Ponceau, Allura red, Indigo Carmine, Erythrosine and Brilliant blue, Ares(2019)848759
- [2] Supplementary Information – DG SANTE request cf. Method of analysis for Tartrazine (E102); Ref: FAD-2010-0342, Ares(2019)848653
- [3] Supplementary Information – RV77
- [4] Supplementary Information – Synthetic dyes quantification in pet food (validation file)
- [5] Supplementary Information – eurl-VF_E102
- [6] Supplementary Information – eurl-VF_E122
- [7] Supplementary Information – eurl-VF_E124

- [8] Supplementary Information – eurl-VF_E127
- [9] Supplementary Information – eurl-VF_E129
- [10] Supplementary Information – eurl-VF_E132
- [11] Supplementary Information – eurl-VF_E133

Acknowledgments

The following National Reference Laboratories contributed to this addendum:

- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)
- RIKILT Wageningen UR, Wageningen (NL)

Addendum

- Prepared by Zigmantas Ezerskis
 - Reviewed and approved by María José González de la Huebra and Christoph von Holst (EURL-FA), respectively, Geel, 12/03/2019
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JRC.DG.D.6/CvH/RM/ag/ARES(2011)861655

**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-2010-0346
CRL/100338

Feed additive: Indigo carmine (E 132)

Active Substance(s): Indigo carmine

Rapporteur Laboratory: European Union Reference Laboratory
for Feed Additives (EURL-FA)
Geel, Belgium

Report prepared by: Roberto Molteni (EURL-FA)

Report revised by: Piotr Robouch (EURL-FA)
Date: 08/08/2011

Report approved by: Christoph von Holst
Date: 08/08/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Indigo carmine* under the "sensory additives", functional group 2(a) "colourants", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for cats and dogs, ornamental fish.

Indigo carmine is a synthesized dark-blue powder or granules, soluble in water, consisting of a minimum of 85 % total colouring matters calculated as sodium salt. *Indigo carmine* is intended to be incorporated directly in *feedingstuffs* as a solution in *water* (either added directly as a solid to the feedingstuffs in the presence of water or by addition of an aqueous solution), with no recommended minimum or maximum levels.

For the determination of *Indigo carmine* in the *feed additive*, the Applicant proposed the internationally recognised FAO JECFA monograph in food additives. Identification is based on spectrophotometry with a maximum in water at 610 nm and Thin Layer Chromatography (TLC), while quantification of *Indigo carmine* in the *feed additive* is based on spectrophotometry at 610 nm in aqueous solution, as recommended by Commission Directive 2008/128/EC laying down specific purity criteria concerning colours for use in foodstuffs. Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *Indigo carmine* in the *feed additive*.

The Applicant did not provide any experimental method or data for the determination of *Indigo carmine* in *premixtures*, *feedingstuffs* and *water*. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *Indigo carmine* in *premixtures*, *feedingstuffs* and *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

Indigo carmine, sensory additive, colourants, cats and dogs, ornamental fish

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use in water and new species) and 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) for *Indigo carmine* under the "sensory additives", functional group 2(a) "colourants" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for cats and dogs, ornamental fish [2].

Indigo carmine is a synthesized dark-blue powder or granules, soluble in water (16 g/L at 20°C) [3], consisting of a minimum of 85 % total colouring matters calculated as sodium salt [2]. The Applicant states that the purity criteria set in the Commission Directive 2008/128/EC for the food additive apply to the requirement for the *feed additive* [3]. *Indigo carmine* is intended to be incorporated directly in *feedingstuffs* as a solution in *water* (either added directly as a solid to the feedingstuffs in the presence of water or by addition of an aqueous solution), with no recommended minimum or maximum levels [2]. However, a typical maximum concentration of 500 mg/kg *feedingstuffs* is suggested by the Applicant [3].

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 *Indigo carmine*, 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 and feedingstuffs

For the determination of *Indigo carmine* in the *feed additive*, the Applicant proposed the internationally recognised FAO JECFA monograph for food additives [5]:

- Identification of *Indigo carmine* in the *feed additive* is based on - spectrophotometry at 610 nm in water and - Thin Layer Chromatography (TLC), with Retention factors (R_f) determined using several chromatographic conditions for confirmation.
- Quantification of *Indigo carmine* in the *feed additive* is based on spectrophotometry at 610 nm in aqueous solution, as recommended by Commission Directive 2008/128/EC. Total colouring matters are quantified using Procedure 1 described in the JECFA monographs n. 1 (Vol. 4) [5].

Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *Indigo carmine* in the *feed additive*.

The Applicant did not provide any experimental method or data for the determination of *Indigo carmine* in *premixtures*, *feedingstuffs* and *water*. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *Indigo carmine* in *premixtures*, *feedingstuffs* and *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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control of the *feed additive* the identification tests and the quantification assay recommended by Commission Directive 2008/128/EC and described in the JECFA monographs n. 1 (Vol. 4), Combined Compendium for Food Additive Specifications.

The Applicant did not provide any experimental method or data for the determination of *Indigo carmine* in *premixtures*, *feedingstuffs* and *water*. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *Indigo carmine* in *premixtures*, *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the quantification of *Indigo carmine* in the *feed additive*:

- spectrophotometry at 610 nm (Commission Directive 2008/128/EC referring to FAO JECFA monographs n. 1 (Vol. 4))

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Indigo carmine* 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. 1831/00185/(10337)-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 monographs n. 1 (Vol. 4), Combined Compendium for Food Additive Specifications

* Refers to Dossier No. FAD-2010-0346

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

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- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer (DE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
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