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 report

**Reference:**

FAD-2010-0267 – Bixin, Annatto, Norbixin (Norbixin potassium)
(JRC.DG.D.6/CvH/DM/MdS/ARES(2011)769127)

Upon the request from DG SANTE [1], the EURL evaluated the supplementary information provided [2-4] in the frame of the feed additive dossier FAD-2010-0267 for the analysis of *Norbixin* in *feedingstuffs*.

For the quantification of the above mentioned colourant the Applicant submitted a single-laboratory validated and further verified method based on reversed phase high performance liquid chromatography coupled to diode array detection (RP-HPLC-DAD) [2].

The homogenised sample (1 g) is mixed with the aqueous solution of monopotassium phosphate, ascorbic acid and alpha-amylase at pH 7 and the mixture is shaken for 2 h. After an enzymatic reaction, concentrated hydrochloric acid and saturated sodium chloride are added to the reaction mixture. The analyte is extracted with ethyl acetate and centrifuged. The extraction is repeated for another 5 times. The upper organic layer is combined, evaporated, and dissolved in acetone for further solid phase extraction (SPE) clean-up. After the conditioning of the SPE with n-hexane, fat present in the sample is removed by mixture of n-hexane and diethyl ether (1:1, v/v), and acetone. The analyte is eluted with a mixture of methanol and glacial acetic acid (7:3, v/v), and diluted with methanol for further chromatographic analysis. The analyte is detected at 488 nm and the quantification is performed using a matrix-matched calibration curve prepared with *Norbixin* standards at the mass fractions ranging from 0 to 10 mg *Norbixin*/kg starch-based extruded feed [2].

The following performance characteristics of the RP-HPLC-DAD method were obtained in frame of validation and verification studies for a *Norbixin* content ranging from 43 to 76 mg/kg *feedingstuffs* [2-4]: a relative standard deviation for *repeatability* ($RSD_r$) ranging from 1.3 to 5.1 %; a relative standard deviation for *intermediate precision* ($RSD_{ip}$) ranging from 5.2 to 9.5 %; a *recovery* rate ($R_{rec}$) ranging from 88 to 100 % and a limit of quantification of 0.9 mg *Norbixin*/kg *feedingstuffs*, which is below the limits considered as safe for cats and dogs [5]. In addition, few validation data were presented [2] for the

determination of *Norbixin* in *feedingstuffs* at the mass fractions of 2 and 30 mg/kg; the $R_{rec}$ reported were ranging from 95 to 99 % and expanded measurement uncertainty for the mass fraction of 2 mg *Norbixin*/kg *feedingstuffs* was 14 %.

Based on the performance characteristics available the EURL recommends for official control the single laboratory validated and verified reversed phase (RP-HPLC-DAD) method for the quantification of *Norbixin* in *feedingstuffs*.

**Recommended text for the registry entry (analytical method)**

For the quantification of *Norbixin* in *feedingstuffs*:

- Reversed phase high performance liquid chromatography coupled to diode array detection (RP-HPLC-DAD)

**References**


[1] Supplementary Information – DG SANTE request cf. FAD-2010-0267 Norbixin Request validation Method of analysis Ares(2019)2360177

[2] Supplementary Information – Bestimmung des Norbixingehaltes in Tierfutter mit Validierungsdaten

[3] Supplementary Information – Norbixin Validierung

[4] Supplementary Information – Zusammenfassung Verifizierung

[5] EFSA Journal 2017;15(4):4764


**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)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- RIKILT Wageningen UR, Wageningen (NL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)


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Addendum
- Prepared by Zigmas Ezerskis
- Reviewed and approved by María José González de la Huebra and Christoph von Holst (EURL-FA), Geel, 13/05/2019

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
**European Union Reference Laboratory for Feed Additives**

**JRC.DG.D.6/CvH/DM/MdS/ARES(2011)769127**

# 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

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| Dossier related to: | **FAD-2010-0267**<br>**CRL/100269** |
| Feed Additive Name: | **Bixin, Annatto,**<br>**Norbixin (Norbixin potassium)** |
| Active Substance(s): | **Norbixin potassium** |
| Rapporteur Laboratory: | **European Union Reference Laboratory for Feed Additives (EURL-FA)**<br>**Geel, Belgium** |
| Report prepared by: | **Dijana Mitić (EURL-FA)** |
| Report revised by:<br>Date: | **Piotr Robouch (EURL-FA)**<br>**12/07/2011** |
| Report approved by:<br>Date: | **Christoph von Holst**<br>**12/07/2011** |

## EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) under the category/functional group 2(a) "sensory additives"/"colourants", subgroup (i) substances that add or restore colour in feedingstuffs, according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive* was previously authorized under provisions of Council Directive 70/524/EEC for Bixin (E 160b). However, according to the Applicant's request, the subject of this authorization concerns only *norbixin potassium.* Authorisation is sought for the use of the *feed additive* for all species and categories. The *feed additive* is intended to be incorporated in dry, moist and liquid *feedingstuffs*, with no minimum or maximum levels.

For the identification and quantification of *norbixin potassium* in the *feed additive*, the Applicant proposed the internationally recognised FAO JECFA monograph for *Annatto extracts (alkali processed norbixin, acid-precipitated)* in food additives. Identification is based on UV/VIS Absorption and Thin Layer Chromatography, while quantification of the *norbixin potassium* in the *feed additive* is based on spectrophotometry at 482 nm. Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *norbixin potassium* in the *feed additive*.

The Applicant did not submit any experimental data for the quantification of *norbixin potassium* in *feedingstuffs.* Therefore, the EURL could not evaluate nor recommend any method for official control to determine *norbixin potassium* in *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**
*bixin, annatto, norbixin, norbixin potassium*, E 160b, sensory additives, colourants, all animal species and categories.

## 1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use) and 10(2) (re-evaluation of additives already authorised) under the category/functional group 2(a) "sensory additives"/"colourants", subgroup (i) substances that add or restore colour in feedingstuffs [1, 2], according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive* was previously authorized under provisions of Council Directive 70/524/EEC for Bixin (E 160b). However, according to the Applicant's request, the subject of this authorization concerns only *norbixin potassium* [2].

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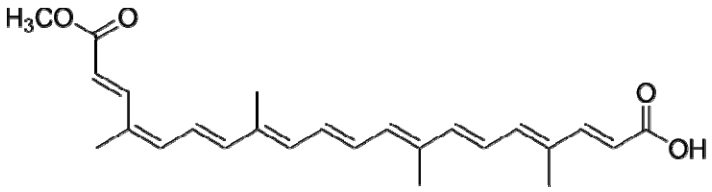
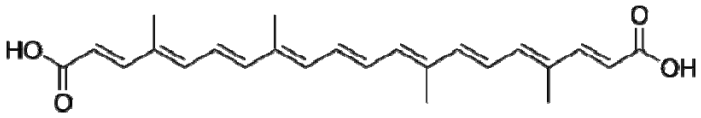
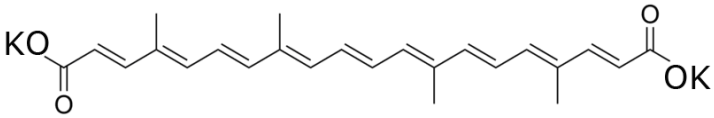
|  | Bixin |
|  | Norbixin |
|  | Norbixin Potassium |

The active substance is prepared by removal of the outer coating of the seeds of the annatto tree (*Bixa orellana L.*) with aqueous alkali (potassium or sodium hydroxide). Bixin is hydrolysed to norbixin in hot alkaline solution (60 °C) and acidified to precipitate the norbixin, with a minimum content of 35% [3, 4]. One of the possible preparations, described by the Applicant [2, 3], is an orange coloured aqueous liquid containing 2.3 to 2.7 % of *norbixin potassium*, to which 1.9 % potassium hydroxide is added to stabilise the compound.

Authorisation is sought for the use of the *feed additive* for all species and categories [2]. The *feed additive* is intended to be incorporated in dry, moist and liquid *feedingstuffs*, with no minimum or maximum levels [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 *Norbixin potassium*, 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 [5].

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

For the identification and quantification of *norbixin potassium* in the *feed additive*, the Applicant proposed the internationally recognised FAO JECFA monograph for *Annatto extracts (alkali processed norbixin, acid-precipitated)* in food additives [4].

Identification of the *norbixin potassium* in the *feed additive* is based on UV/VIS spectrophotometry, where the sample in 0.5% potassium hydroxide solution shows absorbance maxima at about 453 and 482 nm, and Thin Layer Chromatography (TLC). For the TLC a 5% solution of sample in 95% ethanol is prepared and applied to the activated TLC plate. The plate is allowed to dry and develop using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia until solvent front ascends about 10 cm. After drying, *bixin* and *norbixin* appear as yellow spots. After spraying with 5% sodium nitrite solution and then with 0.5 mol/L sulfuric acid the spots immediately decolourise.

Quantification of the *norbixin potassium* in the *feed additive* is based on spectrophotometry at 482 nm. It follows the experimental protocol described in the Food Colours, Colouring Matters Content by Spectrophotometry (Vol.4), procedure 1, using 0.5% potassium hydroxide as solvent.

Upon request by the EURL, the Applicant provided spectroscopic data [6], without validation or verification data. Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *norbixin potassium* in the *feed additive*.

For the quantification of *norbixin potassium* in *feedingstuffs* the Applicant submitted an overview of suitable HPLC-based methods [7]. However, as no validation nor verification data were provided, the EURL could not evaluate nor recommend any method for official control to determine *norbixin potassium* in *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 of the *feed additive* the identification tests and the quantification assay described in the JECFA monograph *Annatto extracts (alkali processed norbixin, acid-precipitated)*.

The Applicant provided no experimental data for *feedingstuffs*, therefore the EURL could not evaluate nor recommend any method for the quantification of *norbixin potassium* in *feedingstuffs*.

***Recommended text for the register entry (analytical method)***

For the quantification of *norbixin potassium* in the *feed additive:*

- spectrophotometry at 482 nm (JECFA monograph on *Annatto extracts (alkali processed norbixin, acid-precipitated))*

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Norbixin potassium* 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. 1831/00161/(10339)/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] *Technical dossier, Section II – Annex_II_8_Norbixin_JECFA

[5] 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

[6] *Supplementary information, 2011-06-22 Reply to EURL

[7] *Supplementary information, Scotter 2011

* Refers to Dossier No. FAD-2010-0267

## 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)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUFA) Speyer, Speyer (DE)