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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

Ethoxyquin

(FAD-2010-0141; CRL/100078)

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-2010-0141 - CRL/100078**
Name of Feed Additive:	***Ethoxyquin***
Active Agent (s):	**Ethoxyquin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium**
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Date:	**26/11/2014**
Report approved by:	**Christoph von Holst**
Date:	**26/11/2014**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *ethoxyquin,* under Article 10(2) for the category/functional group 1(b) 'technological additives'/'antioxidants', according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all animal species and categories. The *feed additive* is intended to be mixed in *premixtures* or added directly to complete *feedingstuffs.* The Applicant proposed a maximum level of 150 mg/kg for *ethoxyquin* alone or for the sum of *ethoxyquin* with butyl hydroxy anisole (BHA, E320) and/or butylated hydroxytoluene (BHT, E321); for dogs, *ethoxyquin* shall not exceed 100 mg/kg. Furthermore the Applicant suggests Maximum Residue Limits (MRLs) in target tissues (muscle, liver, kidney, fat, milk, eggs). As these MRLs are not set up by Commission Regulation (EC) No 37/2010, the correspondent methods of analysis have to be evaluated by the EURL.

For the determination of *ethoxyquin* in the *feed additive*, the Applicant submitted the titrimetric method described in the *"Ethoxyquin* monograph*"* of the Food Chemical Codex (FCC). Even though no performance characteristics are provided, the EURL recommends for official control the internationally recognised FCC method based on titration to determine *ethoxyquin* in the *feed additive.* For the determination of *ethoxyquin* in *premixtures,* the Applicant submitted a single laboratory validated and further verified multi-analyte method based on Reversed Phase High Performance Liquid Chromatography coupled with UltraViolet (RP-HPLC-UV) or Diode-Array Detector (DAD). For the determination of *ethoxyquin* in *feedingstuffs*, the Applicant submitted the ring trial validated RP-HPLC method coupled with Fluorescence Detection (RP-HPLC-FD) published by the Association of Official Analytical Chemists (AOAC 996.13 – "*Ethoxyquin* in feeds"). Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified RP-HPLC-UV/DAD method to determine *ethoxyquin* in *premixtures* and the ring trial validated RP-HPLC-FD method, AOAC 996.13, for the determination of *ethoxyquin* in *feedingstuffs*. For the determination of *ethoxyquin* in target *tissues* the Applicant proposed a single laboratory and further verified RP-HPLC-FD method based on a procedure published in the peer-reviewed journal AOAC international. Based on the performance characteristics presented, the EURL recommends for official control the RP-HPLC-FD method or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC to enforce the MRLs for *ethoxyquin* in the target tissues.

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

Ethoxyquin, technological additives, antioxidants, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought for *ethoxyquin*, under Article 10(2) (re-evaluation of an authorised additive) for the category/functional group 1(b) 'technological additives'/'antioxidants', according to the classification system of article 6 of Regulation (EC) No 1831/2003 [1-2]. *Ethoxyquin* is already authorised as *feed additive* under Commission Directive 70/524/EEC [3].

According to the Applicant, *ethoxyquin* is a yellow-brown chemically synthesised liquid with a minimum purity of 91% [4-6]. The *feed additive* is intended to be mixed in *premixtures* or added directly to complete *feedingstuffs*. Specifically, authorisation is sought for the use of the *feed additive* for all animal species and categories [7]. The Applicant proposed a maximum level of 150 mg/kg for *ethoxyquin* alone or for the sum of *ethoxyquin* with butyl hydroxy anisole (BHA, E320) and/or butylated hydroxytoluene (BHT, E321); for dogs, *ethoxyquin* shall not exceed 100 mg/kg [1,7]. Furthermore the Applicant suggests Maximum Residue Limits (MRLs) in target tissues [1]. As the abovementioned MRLs are not set up by Commission Regulation (EC) No 37/2010, the correspondent methods of analysis have to be evaluated by the EURL [8].The proposed MRLs are listed in Table 1.

Table 1. Maximum Residue Limits (MRLs) in the relevant foodstuffs of animal origin expressed as µg/kg of fresh material [1].

MRLs	Muscle	Liver	Kidney	Fat	Milk	Eggs
Mammals	280	4830	2650	770	190	-
Birds	50	63	-	240	-	52
Fish	2610	-	-	-	-	-

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 *ethoxyquin* 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 [9]

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

For the determination of *ethoxyquin* in the *feed additive*, the Applicant submitted the *"Ethoxyquin* monograph*"* of the Food Chemical Codex (FCC) based on titration with perchloric acid (0.1N) [10-11].

Even though no performance characteristics are provided, the EURL recommends for official control the internationally recognised FCC method based on titration to determine *ethoxyquin* in the *feed additive.*

For the determination of *ethoxyquin* in *premixtures*, the Applicant submitted a single laboratory validated and further verified multi-analyte method [12-13]. The method is based on Reversed Phase High Performance Liquid Chromatography system coupled with UltraViolet (RP-HPLC-UV) - UV or Diode-Array Detector (DAD). It consists of an extraction of the *active substance* from the matrix using methanol and ascorbic acid. The extract is further diluted with methanol and mixed thoroughly. The supernatant is filtered and injected into a gradient reversed-phase HPLC system. The *ethoxyquin* content is determined via UV or DAD detector adjusted at 355 nm. The concentration is determined using an external calibration curve. As already mentioned in former reports, several NRLs recommended - based on their experience – to include an additional purification step (i.e. Solid Phase Extraction - SPE) to avoid any potential matrix interferences [14-15]. The

following performance characteristics were reported for *ethoxyquin* concentrations ranging from 5 to 120 g/kg in *premixtures* [12-13]:

- standard deviation for *repeatability* (RSD_r) ranging from 2.1 to 5.2%;
- standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.9 to 5.8%; and
- *recovery* rate (R_{rec}) ranging from 93 to 95.7%.

The multi-analyte method is suitable for the quantification of other synthetic antioxidants such as BHA and BHT. The EURL has already evaluated and recommended this method in the frame of the evaluation of BHA and BHT dossiers (i.e. FAD-2010-0132 [14] and FAD-2010-0237 [15], respectively).

For the determination of *ethoxyquin* in *feedingstuffs* the Applicant submitted the ring trial validated method by the Association of Official Analytical Chemists (AOAC 996.13 – "*Ethoxyquin* in feeds") [10,16]. This method applies for the determination of the *active substance* in a range of 0.5 to 300 mg/kg of pet food and/or meat meal (only). The analytical procedure consists of an extraction with acetonitrile followed by injection in an isocratic RP-HPLC system coupled with Fluorescence Detection (RP-HPLC-FD), adjusted at 360 nm for the excitation and 432 nm for the emission. The following relative precisions (*repeatability* and *reproducibility*) ranging from 4.5 to 29% were reported. Furthermore, on request of the EURL, the Applicant provided supplementary experimental data demonstrating the applicability of the AOAC method to determine *ethoxyquin* in other categories of feeds, such as fish feed, pig feed and poultry feed [17].

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified RP-HPLC-UV method to determine *ethoxyquin* in *premixtures* and the ring trial validated RP-HPLC-FD method, AOAC 996.13, for the determination of *ethoxyquin* in *feedingstuffs*.

Methods of analysis for the determination of the residues of the additive in food.

For the determination of *ethoxyquin* in target *tissues* the Applicant proposed a single laboratory and further verified method based on RP-HPLC-FD. The method is a slight modification of the fit-for-purpose fully validated method published in AOAC international – *"Determination of ethoxyquin by HPLC-FD and its application to the survey of residues in food products of animal origin"* by Aoki et al. [10,18-20].

It consists of a first dilution of the sample (5 g) in a final volume of 20 ml methanol-BHT (25 ml for liquids), followed by homogenisation and filtration (0.2 µm). The sample results in a dense, viscous liquid. Before injection, the extract is further diluted to 100ml with methanol-BHT and mixed thoroughly (step not required for chicken liver, chicken muscle and egg). An

aliquot is finally injected into a gradient RP-HPLC system and *ethoxyquin* is detected with FD adjusted at 370 nm for the excitation and 415 nm for the emission. The concentration is determined using an external calibration curve.

In the AOAC publication, different *ethoxyquin* concentrations, in the range of the proposed MRLs, were investigated for each target *tissue* (except beef kidney) [19-20]. In particular *"…The LOQ* – limit of quantification - *of the foods was 0.01 µg/g, except for pig fat and cow's milk, and the RSD (n = 6) at 0.1 µg/mL of the standard solution was 1.12%. The accuracy of the calculated data of the standard solution was within the range of 94.0 to 101.2%. Recoveries of ethoxyquin from the food products of cattle, pigs, chickens, and salmon were more than 71.0% with an RSD of <9.3%…".* While the validation data provided by the Applicant do not fully comply with the EURL criteria [21], the data provided in the frame of the method verification [22] and recalculated by the EURL [23] are adequate:

- RSD_r ranging from 1.1 to 8.0%;
- RSD_{ip} ranging from 2.5 to 11.3%; and
- R_{rec} ranging from 75.5 to 114%.

The EURL could therefore confirm that the performances characteristics presented by the Applicant are in line with those published in the study of *Aoki et al*. [19-20]. Moreover, the proposed technique, belonging to the *feed additive* group B of Annex I of the Council Directive 96/23/EC [24], fully complies with the confirmatory requirements set by Commission Decision 2002/657/EC [25].

Based on the performance characteristics presented, the EURL recommends for official control the RP-HPLC-FD method proposed by the Applicant or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC to enforce the MRLs for *ethoxyquin* in the target tissues.

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 FCC method based on titration to determine *ethoxyquin* in the *feed additive*;
- the single-laboratory validated and further verified method based on RP-HPLC-UV/DAD detection for the determination of *ethoxyquin* in *premixtures*;
- the ring-trial validated AOAC 996:13 method, based on RP-HPLC-FD detection for the determination of *ethoxyquin* in *feedingstuffs*;
- the proposed RP-HPLC-FD method - or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC - for the quantification of *ethoxyquin* in *tissues*.

Recommended text for the register entry (analytical method)

For the determination of *ethoxyquin* in *feed additive*:

- Food Chemical Codex "Ethoxyquin monograph"

For the determination of *ethoxyquin* in *premixtures*:

- High Performance Liquid Chromatography coupled to UltraViolet or Diode-Array detection (HPLC-UV/DAD)

For the determination of *ethoxyquin* in *feedingstuffs*:

- High Performance Liquid Chromatography coupled to Fluorescence Detection (HPLC-FD) - AOAC 996:13

For the determination of *ethoxyquin* in *tissues*:

- High Performance Liquid Chromatography coupled to fluorescence detection (HPLC-FD) or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *ethoxyquin* 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, Proposal for Registry Entry – Annex A

[2] *Application, Reference SANCO/D2/WP/ng/724693

[3] Council Directive 70/254/EEC concerning additives in feedingstuffs – List of authorised additives in feedingstuffs (2004/C50/01)

[4] *Technical dossier, section II: 2.1.3 Qualitative and quantitative composition

[5] *Technical dossier, section II: 2.1.5 Physical state of each form of the product

[6] *Technical dossier, section II: 2.3 Manufacturing process, including any specific processing procedure

[7] *Technical dossier, section II: 2.5 Conditions of use of the additive

[8] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

[9] 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

[10] *Technical dossier, section II: 2.6 Methods of analysis and reference samples

[11] Food Chemical Codex monograph "Ethoxyquin", FCC 7 (2010), p.333-34

[12] *Supplementary information, Annex_Q2_ETX_validation_premix.pdf

[13] *Supplementary information, Annex_Q2_ETX_verification_premix.pdf

[14] [1] FAD-2010-0132, BHA, Ref. Ares(2012)40826 – 13/01/2012 https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2010-0132.pdf

[15] [2] FAD-2010-0237&FAD-2010-0300, BHT, Ref. Ares(2012)490799 – 20/04/2012 https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2010-0237%2B0300.pdf

[16] AOAC Official Method 996:13 – Ethoxyquin in Feeds

[17] *Supplementary information, Annex_Q1_ETX_analytics_Feed_AOAC.pdf

[18] *Supplementary information: RM125-0001-P-VAL EQ in Tissues by HPLC-FL.pdf

[19] Aoki Y, Kotani A, Miyazawa N, Uchida K, Igarashi Y, Hirayama N, Hakamata H, Kusu F. "Determination of ethoxyquin by high-performance liquid chromatography with fluorescence detection and its application to the survey of residues in food products of animal origin", J AOAC Int. 2010 Jan-Feb; 93(1):277-83.

[20] *Supplementary information, Ethoxyquin_Tissue_method_Aoki_2010_AOAC.pdf

[21] *Supplementary information, Method of analysis, 2013_08_27_Validation report ethoxyquin analysis in tissues.pdf

[22] *Supplementary information, Annex_table_2.1_Ethoxyquin.pdf

[23] *Supplementary information, Precision-recovery verification Tissues.xlsx

[24] Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directive 85/358/EEC and 86/469/EEC and Decision 89/187/EEC and 91/664/EEC

[25] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

*Refers to Dossier no: FAD-2010-0141

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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- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Cabrils (ES)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby, Fødevarestyrelsen, Ringsted (DK)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren (BE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- RIKILT-Instituut voor Voedselveiligheid, Wageningen (NL)