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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-2010-0237 CRL/100352 FAD-2010-0300 CRL/100201
Feed Additive Name:	Butylated hydroxytoluene (BHT)
Active Substance(s):	Butylated hydroxytoluene (BHT)
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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Report approved by: Date:	Christoph von Holst 20/04/2012

EXECUTIVE SUMMARY

In the current applications authorisation is sought for *Butylated hydroxytoluene, E321 (BHT)* under Article 10, category/functional group 1(b) 'technological additives'/'antioxidants', according to the classification system of Annex I of Regulation (EC) No 1831/2003. *BHT* is already authorised as *feed additive* under Commission Directive 70/524/EEC.

According to the two Applicants (FAD 2010-0237 & FAD 2010-0300), *BHT* is a white or colourless solid powder with a minimum purity of 99.5%. Specifically, 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 in complete *feedingstuffs.* Furthermore, the Applicants proposed a maximum level of 150 mg/kg for *BHT* alone or for the sum of *BHT* with Butylated hydroxy anisole (BHA, E321) and/or Ethoxyquin (E324).

For the determination of *BHT* in the *feed additive*, Applicant (FAD-2010-0237) submitted the internationally recognised FAO JECFA method based on Gas Chromatography coupled to Flame Ionization Detection (GC-FID). Even though no performance characteristics of this method are provided, the EURL recommends for official control the internationally recognised FAO JECFA method based on GC-FID to determine *BHT* in the *feed additive.*

For the determination of *BHT* in *premixtures* and *feedingstuffs* Applicant (FAD-2010-0300) submitted a single laboratory validated and further verified multi-analyte method, based on Reversed Phase High Performance Liquid Chromatography coupled with UltraViolet or Diode-Array Detection (RP-HPLC-UV or DAD). The following performance characteristics were reported for concentrations ranging from 5 to 120 g/kg and from 35 to 226 mg/kg, for *premixtures* and *feedingstuffs*, respectively:

- a standard deviation for repeatability (RSD_r) ranging from 1.4 to 6.6%;
- a standard deviation for intermediate precision (RSD_{ip}) ranging from 3.3 to 11.4%;
- a recovery rate (R_{Rec}) ranging from 86.9 to 114%; and
- a limit of quantification (LOQ) below the lowest concentration investigated of 35 mg/kg.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified RP-HPLC-UV (or DAD) method, submitted by the Applicant, to determine *BHT* in *premixtures* and *feedingstuffs*.

According to the Applicant (FAD 2010-0300) the above mentioned multi-analyte technique, submitted for the determination of *BHT* in *premixtures* and *feedingstuffs,* allows the quantification of other synthetic antioxidants such as BHA and Ethoxyquin (in *premixtures*

only). Furthermore, the EURL identified the ring trial validated method by the Association of Official Analytical Chemists (AOAC 996.13 – "Ethoxyquin in feeds") based on isocratic RP-HPLC system coupled with fluorescence detection (RP-HPLC-FD). The following relative precisions (repeatability and reproducibility) were reported: ranging from 0.6 to 6.4% for BHA in *premixtures* and *feedingstuffs*; and ranging from 2.1 to 5.4% and 4.5 to 29% for Ethoxyquin in *premixtures* and *feedingstuffs*, respectively.

Based on the performance characteristics presented, the EURL considers the following methods suitable for official control:

- the single laboratory validated and further verified RP-HPLC-UV (or DAD) method, submitted by the Applicant, for the determination of BHA in *premixtures* and *feedingstuffs* and for the determination of Ethoxyquin in *premixture* (only); and
- the ring trial validated RP-HPLC-FD method characterised by the "Association of Official Analytical Chemists" (AOAC 996.13) for the determination of Ethoxyquin 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
Butylated hydroxytoluene (BHT), *E321*, technological additives, antioxidants, all animal species and categories, Butyl hydroxy anisole (BHA), E320, Ethoxyquin, E324.

1. BACKGROUND

In the current applications authorisation is sought for *Butylated hydroxytoluene, E321 (BHT)* under Article 10, category/functional group 1(b) 'technological additives'/'antioxidants', according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1-4]. *BHT* is already authorised as *feed additive* under Commission Directive 70/524/EEC [5].

According to the two Applicants (FAD 2010-0237 & FAD 2010-0300), *BHT* is a white or colourless solid powder (in flakes or crystalline form) with a minimum purity of 99.5% [6-9]. *BHT* is universally used as antioxidant in feed and food products. Specifically, 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 in complete *feedingstuffs.* Furthermore, the Applicants proposed a maximum level of 150 mg/kg for *BHT* alone or for the sum of *BHT* with Butyl hydroxy anisole (BHA, E320) and/or Ethoxyquin (E324) [3-4, 10-11].

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 *Butylated hydroxytoluene* (*BHT*) 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 [12].

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

For the determination of *BHT* in the *feed additive* both the Applicants proposed to apply analytical methods based on Gas Chromatography coupled to Flame Ionization Detection (GC-FID). Applicant (FAD-2010-0300) presented a single-laboratory validated method without providing the validation and verification reports [13, 14], while Applicant (FAD-2010-0237) submitted the internationally recognised FAO JECFA monographs for food additives [15, 16].

According to the JECFA method, an amount of 10 mg of sample is dissolved and diluted to 50 ml with an “internal standard solution” (2 mg/ml of diphenylamine or 4-tertiary butylphenol in acetone). Under specified conditions, aliquots of the dissolved sample are directly injected in GC. Instrumental determination of *BHT* is performed via FID. The *feed additive* is determined using an external calibration curve built with *BHT* standard solutions containing the abovementioned "internal standard solution".

Even though no performance characteristics of this method are provided, the EURL recommends for official control the internationally recognised FAO JECFA method based on GC-FID to determine *BHT* in the *feed additive.*

For the determination of *BHT* in *premixtures* and *feedingstuffs* Applicant (FAD-2010-0237) proposed to apply the above described internationally recognised FAO JECFA method based on GC-FID [15-16]. This method applies for the determination of the *active substance* in the *additive* and in food matrices. Alternatively, Applicant (FAD-2010-300) submitted a single laboratory validated and further verified multi-analyte method specifically designed for *premixtures* and *feedingstuffs* [13, 17-20] and based on Reversed Phase High Performance Liquid Chromatography coupled with UltraViolet or Diode-Array Detection (RP-HPLC-UV or DAD). The method consists of an extraction of the *active substance* from the matrix using methanol (together with ascorbic acid for *premixtures*). The extract is further diluted with methanol and mixed thoroughly. The supernatant is filtered and injected into a gradient reversed-phase HPLC system. The *BHT* content is determined via UV (or DAD) adjusted at 285 nm. The concentration of *BHT* is determined using an external calibration curve. Based on their previous experience, the NRLs recommended including an additional purification step, using Solid Phase Extraction (SPE), to avoid any potential matrix interferences (cf. FAD-2010-0132).

The following performance characteristics were reported for BHT concentrations ranging from 5 to 120 g/kg [17, 18] and from 35 to 226 mg/kg [19, 20] in *premixtures* and *feedingstuffs*, respectively:

- a standard deviation for *repeatability* (RSD_r) ranging from 1.4 to 6.6%;
- a standard deviation for *intermediate precision* (RSD_{ip}) ranging from 3.3 to 11.4%;
- a *recovery* rate (R_{Rec}) ranging from 86.9 to 114%; and
- a limit of quantification (LOQ) below the lowest concentration investigated of 35 mg/kg [19].

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified RP-HPLC-UV (or DAD) method, submitted by one Applicant, to determine *BHT* in *premixtures* and *feedingstuffs*.

The maximum level of 150 mg/kg *feedingstuffs* proposed by the Applicants applies for *BHT* alone or for the sum of *BHT*, BHA (E320) and/or Ethoxyquin (E324) [3-4, 10-11]. According to Applicant (FAD-2010-0300) the above mentioned RP-HPLC-UV (or DAD) method, submitted for the determination of *BHT* in *premixtures* and *feedingstuffs,* is suitable also for the quantification of other synthetic antioxidants such as BHA and Ethoxyquin (in *premixtures* only). Furthermore, the EURL identified the ring trial validated method by the Association of Official Analytical Chemists (AOAC 996.13 – "Ethoxyquin in feeds") [21]. The AOAC method 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*) were reported:

- for BHA in *premixtures* and *feedingstuffs*: ranging from 0.6 to 6.4%;
- for Ethoxyquin in *premixtures* [17-20]: ranging from 2.1 to 5.4%; and
- for Ethoxyquin in *feedingstuffs* [21]: ranging from 4.5 to 29%.

Based on the performance characteristics presented, the EURL considers the following methods suitable for official control:

- the single laboratory validated and further verified RP-HPLC-UV (or DAD) method, submitted by the Applicant, for the determination of BHA in *premixtures* and *feedingstuffs* and for the determination of Ethoxyquin in *premixture* only, and
- the ring trial validated RP-HPLC-FD method characterised by the "Association of Official Analytical Chemists" (AOAC 996.13) for the determination of Ethoxyquin 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:

- the FAO JECFA method based on GC-FID to determine *BHT* in the *feed additive*;
- the single laboratory validated and further verified method using RP-HPLC with UV (or DAD) detection to determine *BHT* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- Gas Chromatography coupled to Flame Ionization Detection (GC-FID) (FAO JECFA)

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

- Reversed Phase High Performance Liquid Chromatography coupled to UltraViolet or Diode-Array Detection (RP-HPLC-UV or DAD, 285 nm)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *BHT* 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. 1831/0069-2011
[2] +Application, Reference SANCO/D/2 Forw. Appl. 1831/0068-2011
[3] *Application, Proposal of Register Entry – Annex A
[4] +Application, Proposal of Register Entry – Annex A
[5] Council Directive 70/524/EEC concerning additives in feedingstuffs - List of authorised additives in feedingstuffs (2004/C50/01)
[6] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[7] +Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[8] * Technical dossier, Section I; 2.2 Characterisation of the active substance(s)/agent(s)
[9] + Technical dossier, Section I; 2.2 Characterisation of the active substance(s)/agent(s)
[10] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[11] +Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[12] 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
[13] +Technical dossier, Section II: 2.6.1 Method of analysis for the active substance
[14] +Technical dossier, Section II;Annex_II_2.6.1_01
[15] *Technical dossier, Section II: 2.6.1 Method of analysis for the active substance
[16] *Technical dossier, Section II; Annex_II_44 Method of Analysis
[17] +Technical dossier, Section II;Annex_II_2.6.1_02
[18] +Technical dossier, Section II;Annex_II_2.6.1_03
[19] +Technical dossier, Section II;Annex_II_2.6.1_04
[20] +Technical dossier, Section II;Annex_II_2.6.1_05
[21] AOAC Official Method 996:13 – Etoxyquin in Feeds

* Refers to Dossier No. FAD-2010-0237

+ Refers to Dossier No. FAD-2010-0300

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:

- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin, PL
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, IT
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby, DK
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim, DE
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena, DE
- RIKILT-Instituut voor Voedselveiligheid, Wageningen, NL
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien, AT
- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren, BE
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana, SI
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, CZ
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes, FR