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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-2010-0174 - CRL/100190**  
**FAD-2010-0216 - CRL/100036**  
**FAD-2010-0253 - CRL/100165**

Name of Feed Additive: **Betaine/ Betaine anhydrous**  
**Betaine hydrochloride**  
**TNIbetain®/ Betaine anhydrous**

Active Substance(s): **Betaine anhydrous**  
**Betaine hydrochloride**

Rapporteur Laboratory: **European Union Reference Laboratory  
for Feed Additives (EURL-FA)**  
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Date: **27/10/2011**

Report approved by: **Christoph von Holst**  
Date: **28/10/2011**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Betaine anhydrous*<sup>1-3</sup> and *Betaine hydrochloride*<sup>2</sup> under the category/functional group 3(a) ‘nutritional additives’/‘vitamins, pro-vitamins and chemically well defined substances having similar effect’ according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all animal species and categories. According to the Applicants, *Betaine hydrochloride*<sup>2</sup> is an off-white to white crystalline powder with a minimum purity of 93 %, while *Betaine anhydrous*<sup>1,2</sup> (produced either by chemical synthesis or extracted from non-genetically modified sugar beet) will be marketed in two forms: - as a brown liquid with a minimum content of 47 % *Betaine anhydrous*; or - as a free flowing crystalline powder with a minimum purity of 96 %. Finally, *Betaine anhydrous*<sup>3</sup> (extracted from genetically modified sugar beet) contains a minimum of 97 % *Betaine anhydrous* and maximum 1.5 % of moisture.

The liquid forms of *Betaine anhydrous* are to be used in *feedingstuffs* only, while the powder forms of *Betaine anhydrous* and *Betaine hydrochloride* are intended to be used in *premixtures* or added directly into the *feedingstuffs* and *water*.

Unlike the previous regulation, where the minimum or maximum concentrations of *Betaine anhydrous* and *Betaine hydrochloride* in *feedingstuffs* and *water* were not specified, Applicants<sup>1</sup> proposed the following concentration ranges for the *Betaine anhydrous*:

- for the powder form: from 0.5 to 2 g/kg in *water* and *feedingstuffs*; and
- for the liquid form: from 2 to 4 g/kg in *feedingstuffs*.

For the determination of *Betaine anhydrous* and *Betaine hydrochloride* in the *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicants proposed two similar single-laboratory validated methods, based on High Performance Liquid Chromatography with refractive index detector (HPLC-RI). Both methods are based on determination of *betaine* content with ion-exclusion chromatography, using a strong cation exchange column (sodium or calcium column). These methods do not distinguish two forms of *feed additive*. Applicant<sup>1</sup> submitted a full validation and verification report and provided the following performance characteristics:

- for the *feed additive*: a *precision* (*repeatability* and *intermediate precision*) ranging from 0.1 to 0.8 %; and a *recovery rate* ( $R_{Rec}$ ) ranging from 94 to 101 %;
- for *premixtures* (samples with contents ranging from 6 to 1000 mg/g): a *precision* ranging from 4.3 to 6.9 %; and  $R_{Rec}$  ranging from 94 to 102 %;

<sup>1</sup> FAD-2010-0174; <sup>2</sup> FAD-2010-216; <sup>3</sup> FAD-2010-0253

- for *feedingstuffs* (samples with contents ranging from 0.7 to 100 mg/g): a *precision* ranging from 4.4 to 8.6 %;  $R_{Rec}$  ranging from 94 to 107 %; and a limit of quantification (LOQ) of 70 mg/kg *feedingstuffs*; and
- for *water*: a *precision* ranging from 0.1 to 2.4 %;  $R_{Rec}$  ranging from 94 to 101 %; and LOQ of 80 mg/L *water*.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified method, using HPLC-RI, as submitted by Applicant<sup>1</sup>, to determine *Betaine anhydrous* and *Betaine hydrochloride* (expressed as *total betaine*) in the *feed additives*, *premixtures*, *feedingstuffs* and *water*, within the concentration range covered by the experimental data.

Furthermore, the EURL identified the internationally recognised US Pharmacopeia 31 Betaine Hydrochloride monograph for the determination of *Betaine hydrochloride* in the *feed additive*. The identification is based on IR absorption, while quantification is based on titration with 0.1 N perchloric acid. Even though no performance characteristics are provided, the EURL recommends (in addition) for official control the USP31 monograph based on titration with perchloric acid for the quantification of *Betaine hydrochloride* in the *feed additive*.

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**

Betaine anhydrous, betaine hydrochloride, TNiBetain, nutritional additive, vitamins, all animal species and categories

## 1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use in water) and 10(2) (re-evaluation of additives already authorised under provisions of Council Directive 70/524/EEC) for *Betaine anhydrous*<sup>1-3</sup> and *Betaine hydrochloride*<sup>2</sup> under the category/functional group 3(a) ‘nutritional additives’/‘vitamins, pro-vitamins and chemically well defined substances having similar effect’ according to Annex I of Regulation (EC) No 1831/2003 [1].

Authorisation is sought for the use of the *feed additive* for all animal species and categories [1]. Furthermore, *Betaine anhydrous* linked to application<sup>3</sup> is produced by genetically modified sugar beet H7-1 (KM-000H71-4) [2, 3].<sup>4</sup>

According to the Applicants, *Betaine hydrochloride* is a white crystalline powder with a minimum purity of 93 % [4], and *Betaine anhydrous* (produced either by chemical synthesis or extracted from non-genetically modified sugar beet molasses or vinasses) will be marketed in two forms:

- as a free flowing light brown<sup>1</sup> or white<sup>2</sup> crystalline powder with a minimum purity of 96 % [4]; and
- as a brown liquid<sup>1</sup> with a minimum content of 47 % *Betaine anhydrous* [5,6].

While *TNIBetain*<sup>3</sup> contains a minimum of 97 % *Betaine anhydrous*, and maximum 1.5 % of moisture [2].

The liquid forms of *Betaine anhydrous* are to be used in *feedingstuffs* only [6], while the powder forms of *Betaine anhydrous* and *Betaine hydrochloride* are intended to be used in *premixtures* or added directly into the *feedingstuffs*, and *water* [3,6,7].

As in the previous regulation [8], the minimum or maximum concentrations of *Betaine anhydrous* and *Betaine hydrochloride* in *feedingstuffs* and *water* are not specified [2,4]. However Applicant<sup>1</sup> proposed the following concentration ranges levels for *Betaine anhydrous* [5]:

- for the powder form: from 0.5 to 2 g/kg in *water* and *feedingstuffs*; and
- for the liquid form: from 2 to 4 g/kg in *feedingstuffs*.

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<sup>1</sup> FAD-2010-0174; <sup>2</sup> FAD-2010-216; <sup>3</sup> FAD-2010-0253; <sup>4</sup> The manufacturing process does not have any impact on the active substance and *Betaine anhydrous* is chemically the same substance in all three applications.

## 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 these particular dossiers, the methods of analysis submitted in connection with *Betaine anhydrous* and *Betaine hydrochloride*, 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 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 *Betaine anhydrous* and *Betaine hydrochloride* in the *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicants proposed two similar single-laboratory validated methods [10-13], based on High Performance Liquid Chromatography with refractive index detector (HPLC-RI). Both methods are based on determination of *betaine* content with ion-exclusion chromatography, using a strong cation exchange column (sodium or calcium column). These methods do not distinguish two forms of *feed additive*. Applicant (FAD-2010-0174) submitted a single laboratory validated method [14-16] and further verified [17-19] by a second independent laboratory. The *feed additive* samples (700 mg – 1 g) are diluted with water, filtered through 0.2 µm membrane filter to HPLC vials and analysis is performed using sodium based cation exchange column [11]. For the determination of *betaine* in *premixtures* and *feedingstuffs* [12,13], the samples (1 g for *premixtures* and 10 g for *feedingstuffs*) are first diluted in water, ultrasonicated for 30 minutes and mixed with magnetic stirrer for 30 minutes. The extracted solution is then centrifuged for additional 10 minutes at 3500 rpm. Proteins and lipids are then removed by C18 solid phase extraction and

eluate is filtered through 0.2 µm membrane filter to HPLC vials and analysis is performed using calcium based cation exchange column. A chromatogram of the separated components is obtained by differential refractometry of the eluent. Calibration is performed using an external standard. The following performance characteristics for the quantification of total *betaine* are derived from validation and verification studies [14-19]:

- for the *feed additive*: a *precision* (*repeatability* and *intermediate precision*) ranging from 0.1 to 0.8 %; and a *recovery* rate ( $R_{Rec}$ ) ranging from 94 to 101 %;
- for *premixtures* (samples with contents ranging from 6 to 1000 mg/g): a *precision* ranging from 4.3 to 6.9 %; and  $R_{Rec}$  ranging from 94 to 102 %;
- for *feedingstuffs* (samples with contents ranging from 0.7 to 100 mg/g): a *precision* ranging from 4.4 to 8.6 %;  $R_{Rec}$  ranging from 94 to 107 %; and a limit of quantification (LOQ) of 70 mg/kg *feedingstuffs*; and
- for *water*: a *precision* ranging from 0.1 to 2.4 %;  $R_{Rec}$  ranging from 94 to 101 %; and LOQ of 80 mg/L *water*.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified method, using High Performance Liquid Chromatography with refractive index detector (HPLC-RI), as submitted by Applicant (FAD-2010-0174), to determine *Betaine anhydrous* and *Betaine hydrochloride* (expressed as *total betaine*) in the *feed additives*, *premixtures*, *feedingstuffs* and *water*, within the concentration range covered by the experimental data.

In addition the EURL identified the internationally recognised US Pharmacopeia 31 Betaine Hydrochloride monograph [20] for the determination of *Betaine hydrochloride* in the *feed additive*. The identification is based on IR absorption, while quantification is based on titration with 0.1 N perchloric acid, where 1 mL of perchloric acid is equivalent to 15.36 mg of *Betaine hydrochloride*. Even though no performance characteristics are provided, the EURL recommends (in addition) for official control the USP31 monograph based on titration with perchloric acid for the quantification of *Betaine hydrochloride* in the *feed additive*.

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 single-laboratory validated and further verified High Performance Liquid Chromatography method with refractive index detector (HPLC-RI) to determine *Betaine anhydrous* and *Betaine hydrochloride* in the *feed additives, premixtures, feedingstuffs* and *water*;

in addition to

- the US Pharmacopoeia method – US Pharmacopeia 31 *Betaine Hydrochloride* monograph, based on titration with perchloric acid to determine *Betaine hydrochloride* in the *feed additive*.

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

For the determination of *Betaine hydrochloride* in the *feed additive*:

- Titration with perchloric acid (US Pharmacopeia 31, *Betaine hydrochloride monograph*) or
- High Performance Liquid Chromatography method with refractive index detector (HPLC-RI)

For the determination of *Betaine anhydrous* in the *feed additive*:

- High Performance Liquid Chromatography method with refractive index detector (HPLC-RI)

For the determination of *Betaine anhydrous* and *Betaine hydrochloride* in *premixtures, feedingstuffs* and *water*.

- High Performance Liquid Chromatography method with refractive index detector (HPLC-RI)

#### 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Betaine anhydrous* and *Betaine hydrochloride* 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] \*+#Reference SANCO/D/2: Group Forward Slip Ares(2011)276755
- [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] +Application, Proposal for Register Entry - Annex A
- [5] \*Application, Proposal for Register Entry - Annex A
- [6] \*Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis
- [7] +Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis
- [8] Official Journal of the European Union, C 50 of 25.2.2004, p. 1, *List of the authorised additives in feedingstuffs (1) published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs*
- [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 – Annex\_II\_13
- [11] \*Technical dossier, Section II – Annex\_II\_B18\_MoA\_HPLC product
- [12] \*Technical dossier, Section II – Annex\_II\_B19\_MoA HPLC premix
- [13] \*Technical dossier, Section II – Annex\_II\_B20\_MoA HPLC feed
- [14] \*Technical dossier, Section II – Annex\_II\_B21\_Validation HPLC\_Product
- [15] \*Technical dossier, Section II – Annex\_II\_B22\_Validation\_HPLC Premix
- [16] \*Technical dossier, Section II – Annex\_II\_B23\_Validation\_HPLC Feed
- [17] \*Technical dossier, Section II – Annex\_II\_B24\_Verification\_Product
- [18] \*Technical dossier, Section II – Annex\_II\_B25\_Verification\_Premix
- [19] \*Technical dossier, Section II – Annex\_II\_B26\_Verification\_feed
- [20] US Pharmacopeia (USP31) Betaine Hydrochloride

\* Refers to Dossier No. FAD-2010-0174

+ Refers to Dossier No. FAD-2010-0216

# Refers to Dossier No. FAD-2010-0253

## 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.



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## 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)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Kmetijski inštitut Slovenije, Ljubljana (SI)