



EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for Feed Additives

 Ref. Ares(2018)2015552 - 16/04/2018

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

**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**

**Mixture of ZnEDTA and Cu(NH₄)₂EDTA chelates on chicory pulp carrier
supplemented with ZnO and CuO
(Stabilflor[®])
(FAD-2017-0044; CRL/170012)**

**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-2017-0044 - CRL/170012**

Name of Product /: ***Mixture of ZnEDTA and Cu(NH₄)₂EDTA
chelates on chicory pulp carrier
supplemented with ZnO and CuO
(Stabilflor®)***

Active Agent (s): ***ZnEDTA and Cu(NH₄)₂EDTA***

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

Report prepared by: **Zigmas Ezerskis**

Report checked by: **Maria Jose Gonzalez de la Huebra
(EURL-FA)
Date: 11/04/2018**

Report approved by: **Christoph von Holst
Date: 11/04/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *mixture of ZnEDTA and Cu(NH₄)₂EDTA chelates on chicory pulp carrier supplemented with ZnO and CuO (Stabilflor[®])* under the category / functional group (4 b) "zootechnical additives"/"gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* is sought to be used for pigs.

Stabilflor[®] is a grey to blue-grey solid mixture, consisting of 60.2 g/kg of ZnEDTA, 10.3 g/kg of Cu(NH₄)₂EDTA, 16.2 g/kg of ZnO, 2.9 g/kg of CuO, and having a respective total zinc and total copper content of 24 g/kg and 4 g/kg *Stabilflor[®]*. According to the Applicant, ZnEDTA and Cu(NH₄)₂EDTA are the active substances. The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* with a proposed minimum and maximum content of *Stabilflor[®]* ranging from 500 mg to 1000 mg /kg *feedingstuffs*, respectively.

For the quantification of ZnEDTA and Cu(NH₄)₂EDTA in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on high performance liquid chromatography coupled to UV detection (HPLC-UV).

The validation and verification studies carried out for the *feed additive*, containing ZnEDTA in the range from 48160 to 72240 mg/kg and Cu(NH₄)₂EDTA in the range from 8240 to 12360 mg/kg, led to precision values expressed in terms of relative standard deviation for *repeatability* (RSD_r) and for *intermediate precision* (RSD_{ip}) between 0.4 to 6.3 %; and *recovery rates* (R_{rec}) from 103 to 117 %.

Based on the acceptable performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified method based on high performance liquid chromatography coupled to UV detection (HPLC-UV) for the quantification of ZnEDTA and Cu(NH₄)₂EDTA in the *feed additive*.

As the accurate quantification of the *Stabilflor[®]* content added to *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine *Stabilflor[®]* in *feedingstuffs*.

For the quantification of ZnEDTA and Cu(NH₄)₂EDTA in *premixtures* and *feedingstuffs* the Applicant applied a modified protocol of the above mentioned HPLC-UV method. The validation and verification studies carried out for *premixtures* and *feedingstuffs*, containing ZnEDTA in the range from 48.2 to 12281 mg/kg and Cu(NH₄)₂EDTA in the range from 8.2 to 2101 mg/kg, led to precision values expressed in terms of RSD_r and RSD_{ip} between 0.4 to 10.4 %; and *recovery rates* from 63 to 107 %. In addition, a limit of quantification (LOQ) for

ZnEDTA ranging from 1.4 to 6.6 mg /kg *feedingstuffs* and a LOQ for *Cu(NH₄)₂EDTA* ranging from 0.4 to 1.4 mg /kg *feedingstuffs* were reported.

Based on the available performance characteristics, the EURL considers the single-laboratory validated and further verified method based on high performance liquid chromatography coupled to UV detection (HPLC-UV) fit-for-purpose for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *premixtures* and *feedingstuffs* in the validated concentration ranges, provided that the measured amounts of the analytes in *feedingstuffs* are corrected for their average *recovery* rates obtained during the validation study.

For the quantification of total zinc and total copper in the *feed additive* the Applicant submitted the international ring-trial validated standard method EN ISO 6869 based on Atomic Absorption Spectrometry (AAS). Furthermore, in the frame of previous dossiers the EURL has evaluated and recommended for the quantification of total zinc and total copper in animal *feedingstuffs* the ring-trial validated methods EN 15510 and EN 15621 based on inductively coupled plasma-atomic emission spectrometry (ICP-AES).

Based on the available performance characteristics presented, the EURL considers the three standard methods EN ISO 6869, EN 15510 and EN 15621 methods as fit-for-purpose for the quantification of total zinc and total copper in the *feed additive*, *premixtures* and *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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Stabilflor[®], *ZnEDTA*, *Cu(NH₄)₂EDTA*, zootechnical additives, gut flora stabilisers, pigs

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new *feed additive*) for *mixture of ZnEDTA and Cu(NH₄)₂EDTA chelates on chicory pulp carrier supplemented with ZnO and CuO (Stabilflor[®])* under the category / functional group (4 b) "zootechnical additives"/"gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* is sought to be used for pigs [1, 2].

Stabilflor[®] is a grey to blue-grey solid mixture, consisting of 60.2 g of *ZnEDTA*, 10.3 g of *Cu(NH₄)₂EDTA*, 16.2 g of *ZnO*, 2.9 g of *CuO*, and having a respective total zinc and total copper content of 24 g and 4 g/kg *Stabilflor*[®] [3]. According to the Applicant, *ZnEDTA* and *Cu(NH₄)₂EDTA* are the active substances [3]. The *feed additive* is intended to be incorporated

directly into *feedingstuffs* or through *premixtures* with a proposed minimum and maximum content of *Stabilflor*[®] ranging from 500 mg to 1000 mg /kg *feedingstuffs*, respectively [2, 3].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *Stabilflor*[®] 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 [4].

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

ZnEDTA and Cu(NH₄)₂EDTA

For the quantification of the metal EDTA chelates in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on high performance liquid chromatography coupled to UV detection (HPLC-UV) using a hydrophilic interaction chromatography (HILIC) stationary phase [5].

An aliquot of the sample is mixed with water; the mixture is sonicated at 65 °C for 25 min and filtered. The extraction procedure is repeated for the second time using water. The filtrates from the two extractions are combined, filtered, diluted and analysed by hydrophilic interaction HPLC-UV at 210 nm wavelength. The quantification is performed by external matrix-free calibration using the standard solutions of the analytes of interest [5].

The main performance characteristics reported for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in the *feed additive* are summarised in Table 1.

Table 1 Performance characteristics of the single laboratory validated and verified hydrophilic interaction HPLC-UV method for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in the *feed additive* [5]

	ZnEDTA		Cu(NH ₄) ₂ EDTA	
	Validation	Verification	Validation	Verification
Mass fraction, mg/kg	48160 - 72240		8240 - 12360	
RSD _r , %	0.4 - 1.6	1.6 - 4.4	1.0 - 2.2	1.9 - 6.3
RSD _{ip} , %	1.6	4.4	2.2	6.3
R _{rec} , %	115 - 117	112 - 116	105	103 - 111

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery rate*;

The Applicant proposed correcting the measured values for the analytes in the *feed additive* for the average *recovery rates* [5, 6], obtained during the validation study. However, the EURL is considering that the reported range of the *recovery rates* is acceptable and therefore the correction is not necessary.

Based on the acceptable performance characteristics presented the EURL recommends for official control the single-laboratory validated and further verified method based on hydrophilic interaction high performance liquid chromatography coupled to UV detection (HPLC-UV) for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in the *feed additive*. As the accurate quantification of the *Stabilflor[®]* content added to *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine *Stabilflor[®]* in *feedingstuffs*.

For the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *premixtures* and *feedingstuffs* the Applicant applied the modified protocol of the above mentioned HPLC-UV method [5]. The modifications included changes in the sample preparation procedure by using for the extraction a mixture of acetonitrile (60 %, v/v), methanol (20 %, v/v) and 0.04 M sodium acetate (20 %, v/v) (for *premixtures*); and dichloromethane and a mixture of acetonitrile-water for the extraction with further clean-up of the combined extracts using a C18 solid phase (for *feedingstuffs*) [5].

The main performance characteristics reported for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *premixtures* and *feedingstuffs* are summarised in Table 2.

In addition, a limit of quantification (LOQ) for *ZnEDTA* ranging from 1.4 to 6.6 mg /kg *feedingstuffs* and a LOQ for *Cu(NH₄)₂EDTA* ranging from 0.4 to 1.4 mg /kg *feedingstuffs* were reported [5].

Table 2 Performance characteristics of the single laboratory validated and verified hydrophilic interaction HPLC-UV method for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *premixtures* and *feedingstuffs* [5]

	Premixtures				Feedingstuffs			
	ZnEDTA		Cu(NH ₄) ₂ EDTA		ZnEDTA		Cu(NH ₄) ₂ EDTA	
	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.
Mass fraction, mg/kg	8187 - 12281		1401 - 2101		48.2 - 72.2		8.2 - 12.4	
RSD _r , %	0.8 - 1.7	5.4 - 8.4	0.4 - 2.1	2.9 - 4.8	1.1 - 8.5	4.5 - 10.4	2.8 - 8.1	2.0 - 6.1
RSD _{ip} , %	1.7	8.4	2.1	4.8	8.5	10.4	8.1	6.1
R _{rec} , %	105 - 107	107 - 113	101 - 103	99 - 103	71 - 76	66 - 72	65 - 69	63 - 68

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery rate*; Valid.: validation; Verif.: verification

The Applicant proposed correcting the measured mass fractions of the target analytes in *premixtures* and *feedingstuffs* for the average *recovery* rates [5, 6] obtained during the validation study. However, the EURL is considering that the reported range of the *recovery* rates in the case of *premixtures* is acceptable and therefore the correction is not required, while in the case of *feedingstuffs* the correction for recovery is considered necessary by the EURL. Moreover, this is a feasible option, since the measured recovery rates of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *feedingstuffs* showed a low variation in the validation study with RSD_r values of 6.8 % and 4.7 % [5], respectively.

Based on the available performance characteristics presented, the EURL considers the single-laboratory validated and further verified method based on hydrophilic interaction high performance liquid chromatography coupled to UV detection (HPLC-UV) fit-for-purpose for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *premixtures* and *feedingstuffs* at the validated concentration ranges, provided that the measured mass fractions of the analytes in *feedingstuffs* are corrected for the average *recovery* rates, obtained during the validation study.

Total zinc and total copper

For the quantification of total zinc and total copper in the *feed additive* the Applicant submitted the ring-trial validated standard method EN ISO 6869 based on Atomic Absorption Spectrometry (AAS) [7].

A test portion is dissolved in hydrochloric acid, if necessary after ashing in a muffle furnace at 550 °C. Any silica compounds present are removed by precipitation and filtration. The

solution after appropriate dilution is analysed by air-acetylene flame AAS. The quantification is performed using absorbance values of each element in the samples and standard solutions [7].

The Applicant applied the method EN ISO 6869 for analysis of five batches of the *feed additive* and RSD_r values of 1.6 % (for total zinc) and 6.1 % (for total copper) were derived [3]. This is in good agreement with the performance characteristics reported in the frame of the ring-trial for mineral and organic premixes, feed and silage (Table 3).

Furthermore, in the frame of previous dossiers the EURL has evaluated and recommended for the quantification of total zinc [8] and total copper [9] in animal *feedingstuffs* the ring-trial validated standard methods EN 15510 [10] and EN 15621 [11] based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) after wet or pressure digestion. The performance characteristics reported for EN ISO 6869, EN 15510 and EN 15621 are summarised in Table 3.

Based on the acceptable performance characteristics available, the EURL considers the three standard methods EN ISO 6869, EN 15510 and EN 15621 fit-for-purpose for the quantification of total zinc and total copper in the *feed additive*.

The Applicant submitted no method for the quantification of total zinc and total copper in the *premixtures* and *feedingstuffs*. However, the EURL considers the methods EN ISO 6869, EN 15510 and EN 15621 also suitable for the quantification of total zinc and total copper in *premixtures* and *feedingstuffs*.

Table 3 The performance characteristics of the ring-trial validated EN ISO 6869, EN 15510 and EN 15621 methods for the quantification of total zinc and total copper in animal *feedingstuffs*

	EN ISO 6869		EN 15510		EN 15621	
	<u>total zinc</u>	<u>total copper</u>	<u>total zinc</u>	<u>total copper</u>	<u>total zinc</u>	<u>total copper</u>
Mass fraction, mg/kg	44 – 14600	14 – 39100	27.4 – 3826	6.8 – 775	26.6 – 3618	7.3 – 470
RSD_r, %	1.7 – 7.6	1.2 – 14.5	1.7 – 8.8 ^(*)	2.9 – 12 ^(*)	1.5 – 5.4	2.6 – 6.8
RSD_R, %	3.2 – 15.0	2.9 – 23.8	5.0 – 19 ^(*)	8 – 22 ^(*)	2.7 – 22	3.8 – 12
LOQ, mg/kg	5	5	3	3	1	1
Reference	[7]		[10]		[11]	

RSD_r and RSD_R: relative standard deviations for *repeatability* and *reproducibility*, respectively; (*) the largest precision values were obtained for mineral premixtures.

Zinc oxide and copper oxide

For the quantification of *zinc oxide* and *copper oxide* in the *feed additive* the Applicant suggested subtracting the mass fractions of *zinc* and *copper* in the metal chelates from the corresponding contents of total zinc and total copper, and applying multiplication factors derived from the ratio of molar masses of the metal oxides to the atomic weights of the metal elements [12].

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 as last amended by Regulation (EU) 2015/1761) 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 method based on hydrophilic interaction high performance liquid chromatography coupled to UV detection (HPLC-UV) for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in the *feed additive*

As the accurate quantification of the *Stabilflor[®]* content added to *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control to determine *Stabilflor[®]* in *feedingstuffs*.

Furthermore, the EURL considers fit-for-purpose:

- the single-laboratory validated and further verified method based on hydrophilic interaction high performance liquid chromatography coupled to UV detection (HPLC-UV) for the quantification of *ZnEDTA* and *Cu(NH₄)₂EDTA* in *premixtures* and *feedingstuffs* in the validated concentration ranges, provided that the measured mass fractions of the analytes in *feedingstuffs* are corrected for the average *recovery* rates, obtained during the validation study;
- the standard method EN ISO 6869 based on atomic absorption spectrometry (AAS) and the standard methods based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) after wet or pressure digestion, namely EN 15510 and EN 15621, for the quantification of total zinc and total copper in the *feed additive*, *premixtures* and *feedingstuffs*

Recommended text for the register entry (analytical method)

For the quantification of ZnEDTA and Cu(NH₄)₂EDTA in the *feed additive*:

- hydrophilic interaction high performance liquid chromatography coupled to ultra-violet detection (HPLC-UV)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Stabilflor[®]* 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 SANTE_E5_FWD. APPL. 1831-0033-2017
- [2] *Application, Proposal for Register Entry – Annex A
- [3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed 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] *Technical dossier, Section II – Annex II_12
- [6] *Supplementary information – Reply for the request for supplementary info_2018.02_Dr.BATA Ltd
- [7] EN ISO 6869:2000 – *Animal feeding stuffs – Determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc - Method using atomic absorption spectrometry*
- [8] #Zinc Group – JRC.D.5/CvH/ZE/mds/Ares(2016)99245
- [9] #Copper Group JRC.D.5/CvH/ZE/mds/Ares(2017)2353146
- [10] EN 15510:2007 – *Animal feeding stuffs – Determination of calcium, sodium, phosphorus, magnesium, potassium, iron, zinc, copper, manganese, cobalt, molybdenum, arsenic, lead and cadmium by ICP-AES*
- [11] EN 15621:2012 – *Animal feeding stuffs – Determination of calcium, sodium, phosphorus, magnesium, potassium, sulphur, iron, zinc, copper, manganese and cobalt after pressure digestion by ICP-AES*
- [12] *Technical dossier, Section II – Annex II_7

*Refers to Dossier no: FAD-2017-0044

#http://irmm.jrc.ec.europa.eu/EURLs/EURL_feed_additives/authorisation/evaluation_reports/

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Laboratorio Arbitral Agroalimentario. Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid (ES)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- RIKILT Wageningen UR, Wageningen (NL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabriels (ES)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 – Labore Landwirtschaft, Nossen (DE)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)