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EURL 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-2009-0029** **CRL/090017** **FAD-2010-0044** **CRL/100008**
Name of Feed Additive:	**Sel-Plex 2000 (FAD 2009-29)** **Selenomethionine (FAD 2010-44)**
Active Agent (s):	**Selenomethionine produced by *Saccharomyces cerevisiae***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
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Report approved by: Date:	**Christoph von Holst** **08/03/2011**

EXECUTIVE SUMMARY

In the current applications authorisation is sought for Sel-Plex 2000 (FAD-2009-29) and *selenomethionine* (FAD-2010-44) under Article 4(1),

- under the category of 'zootechnical' functional group 4(d) 'other zootechnical additives' (for FAD-2009-0029); and
- under 'nutritional additives' functional group 3(b), 'compounds of trace elements' (for FAD-2010-0044),

according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *selenomethionine* for all animal species and categories.

The product with the trade name Sel-Plex 2000 (FAD-2009-29) is a selenium enriched inactivated yeast (*Saccharomyces cerevisiae* CNCM I-3060*)* containing 2000 to 2400 mg *total selenium* / kg with a maximum of 3% of residual inorganic selenium. At least 63% of the total organic selenium in Sel-Plex 2000 is *selenomethionine*. The product related to application FAD-2010-44 and with the trade name Selemax 1000 and 2000 is a selenium enriched inactivated yeast (S*accharomyces cerevisiae* YSC 11111-R646*)* containing a minimum of 1000 and 2000 mg *total selenium* / kg, respectively, with a maximum of 2% of residual inorganic selenium. At least 70% of the total organic selenium in Selemax is *selenomethionine*.

Both products are intended to be incorporated in the form of *premixtures* to obtain a maximum dosage of 0.5 mg total selenium /kg in *complete feedingstuffs*, to comply with legal requirements. None of the Applicants proposed minimum doses.

Both Applicants submitted a single laboratory validated and further verified methods developed by the same laboratory, internationally reputed in the field of selenium speciation.

For the determination of *selenomethionine* in the *feed additives* the Applicants proposed a triple proteolytic digestion/extraction followed by anion-exchange high performance chromatography coupled to ICPMS (HPLC-ICPMS). The following performance characteristics were presented:

- a *recovery rate* (R_{rec}) ranging from 94 to 103%;
- a relative standard deviation for *repeatability* (RSD_r) ranging from 1 to 4%; and
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 5 to 8%.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified HPLC-ICPMS method, submitted by the Applicants, to determine *selenomethionine* in the *feed additives*.

For the determination of *total selenium* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicants proposed a microwave digestion using nitric acid and hydrogen peroxide (HNO_3/H_2O_2) followed by inductively coupled plasma mass spectrometry (ICPMS). The following performance characteristics were reported for the *feed additives*: R_{rec} ranging from 94 to 95%; and RSD_{ip} ranging from 2 to 7%.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified ICPMS method, submitted by the Applicants, to determine *total selenium* in the *feed additives*.

However, for the determination of *total selenium* in *feedingstuffs*, the EURL investigated the former ring trial validated VDLUFA method, recently adopted as CEN standard prEN 16159:2010, based on by hydride generation atomic absorption spectrometry (HGAAS). The following performance characteristics are reported:

- a relative standard deviation for *repeatability* (RSDr) ranging from 3.4 to 10%;

- a relative standard deviation for *reproducibility* (RSD_R) ranging from 15 to 23%; and

- a limit of quantification of 0.125 mg/kg, clearly below the legal limit of 0.5 mg Se /kg feed.

For the determination of *total selenium* in *premixtures*, the EURL suggests diluting the *premixtures* samples with ground cereal feed and applying the abovementioned HGAAS method.

Based on the performance characteristics presented, the EURL recommends for official control, the ring trial validated CEN method (prEN 16159:2010) for the determination of total selenium in *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) is not considered necessary.

KEYWORDS

Selenomethionine, selenium enriched yeast, *Saccharomyces cerevisiae* CNM I-3060; *Saccharomyces cerevisiae* YSC 11111-R646, nutritional additives, zootechnical additive, all species, trace elements

1. BACKGROUND

In the current applications authorisation is sought for Sel-Plex 2000 (FAD-2009-29) and *selenomethionine* (FAD-2010-44) under Articles 4(1),

- under the category of 'zootechnical' functional group 4(d) 'other zootechnical additives', for FAD-2009-0029 [1]; and
- under 'nutritional additives' functional group 3(b), 'compounds of trace elements' for FAD-2010-0044 [2],

according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *selenomethionine* for all animal species and categories [1, 2]. According to the Applicants both products derive from selenium enriched inactivated yeasts (non-GM *Saccharomyces cerevisiae*) [3, 4]. The following characteristics were presented in the two dossiers under investigation:

	Sel-Plex: FAD-2009-0029 [3, 5]	***SeleMax***: FAD-2010-0044 [4, 6]
Saccharomyces cerevisiae strain	CNCM I-3060	YSC 11111-R646
Feed additive containing (in mg *total selenium*/kg)	2000-2400 (in Sel-Plex 2000)	min 1000 and 2000 (in Selemax 1000 and 2000)
Residual inorganic selenium	< 3% w/w	< 2% w/w
Total organic selenium in the form of *Selenomethionine*	ca. 63% w/w	> 70% w/w
Low molecular weight seleno compounds	ca.35%	

Both products are intended to be incorporated in the form of *premixtures* to obtain a maximum dosage of 0.5 mg total selenium /kg in *complete feedingstuffs* [5, 6], to comply with legal requirements. None of the Applicants proposed minimum doses.

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 (EFSA) for each application or group of applications. The methods of analysis submitted in connection with *selenomethionine* and their suitability to be used for official controls in the frame of the authorisation were evaluated for the two dossiers of concern.

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, salmonella, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [7].

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

Both Applicants implemented the experimental methodology developed by the expert research laboratories in Pau [cf. Ultra Trace (UT2A) and Laboratorie de Chimie Analytique et Bio-Organique et Environnement (LCABIE)] – denoted as PAU hereafter - having an international reputation in the field of selenium speciation. PAU developed the methodological approach to investigate selenium speciation in various matrices including yeast [8-11]. Furthermore PAU contributed to the certification campaign of the selenized yeast reference material (SELM-1) for *selenomethionine* and *total selenium* content, organised by the National Research Council of Canada (NRCC) [12]. Finally PAU participated to the pilot key comparison CCQM-P86 organised by the Consultative Committee for Amount of Substances (CCQM in French) to assess the state of the art of measurement capabilities when analysing *total selenium* and *selenomethionine* in yeast tablets [13]. Therefore, the satisfactory results reported by PAU [12, 13] clearly

(i) demonstrate the quality of the measurement capability of the PAU laboratory, comparable to the performance of other reputed National Metrology Institutes, such as NIST (USA), NRCC (CAN), NIM (CHN), LNE (FR), LGC (UK); and

(ii) validate the experimental protocols used for the determination of *total selenium* and s*elenomethionine.*

Experimental

For the determination of *total selenium* (totSe) in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS) the Applicants proposed [14, 15] a microwave digestion using nitric acid and hydrogen peroxide (HNO_3/H_2O_2) followed by inductively coupled plasma mass spectrometry (ICPMS) as developed by PAU [8, 13]. Samples (0.2 to 0.5 g) are mineralised in microwave closed-vessels in the presence of 3+3 mL HNO_3/H_2O_2. The digested solution is

further diluted and the *total selenium* concentration is determined by ICP- MS, using standard addition of inorganic selenium standard together with a ^{103}Rh internal standard. The results were validated using the selenium enriched yeast Certified Reference Material (SELM-1) from NRCC [16] (Table 1).

For the determination of *selenomethionine* (SeMet) in the *feed additives*, *premixtures* and *feedingstuffs* the Applicants proposed [14, 15] high performance liquid chromatography coupled to ICPMS. The sample is digested overnight with protease/lipase solution in neutral buffer then centrifuged. The digestion is repeated three times. After addition of beta-mercaptoethanol the supernatant are pooled together and analysed by anion exchange HPLC-ICPMS, using standard addition of a SeMet standard. The results were validated using the selenium enriched yeast Certified Reference Material (SELM-1) from NRCC [16] (Table 1). A detailed protocol was provided by the Applicant upon request of the EURL [26].

Results

The Applicant (FAD-2010-0044) submitted many results reported by the PAU laboratory for the analysis of his product (cf. Table 1) [18-20]. The methods used are considered to be validated by the SELM-1 certification exercise [12] and the successful participation to the CCQM-P86 inter-laboratory comparison [13].

Upon request from the EURL, the Applicant (FAD-2009-0029) provided three verification studies: - two studies for the determination of *selenomethionine* in the *feed additive* [21, 22] performed by the PAU laboratories using the methods mentioned above, and – a verification study for the determination of *total selenium* in *feedingstuffs* by hydride generation atomic absorption spectrometry (HGAAS) [23]. The corresponding performance characteristics are summarised in Table 2. Selenite and selenate (cf. inorganic selenium) have not been detected in any of the samples analysed (bellow threshold of 2%) [18-20], thus demonstrating that the products contain a minimum of 98% of organic selenium, of which at least 60% consists of *Selenomethionine,* as shown in Table 1.

Table 1: Compilation of results reported by the PAU laboratories, in the frame of the FAD-2010-0044 project – when determining *total selenium* (totSe) and *selenomethionine* (SeMet) in SELM-1 (CRM), *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

The relative standard deviation for *intermediate precision* (RSD_{ip}) is derived from the results reported by PAU for several days of measurements.

Recovery rates are computed when analysing SELM-1.

The ratio of SeMet/totSe are calculated for each individual sample.

Ref	matrix	analyte	Content (mg Se / kg)	std (mg Se / kg)	RSD_{ip}	[SeMet]/[totSe] or (R_{rec})
[20]	CRM	SeMet	1300	100	7.7%	(94%)
[19]	CRM	totSe	1965	98	5.0%	(95%)
[20]	CRM	totSe	1980	40	2.0%	(96%)
[17]	FA	SeMet	2058	102	5.0%	84%
[18]	FA2	SeMet	820	70	8.5%	82%
[19]	FA	SeMet	1578	50	3.2%	62%
[20]	FA	SeMet	1740	150	8.6%	66%
[20]	FA	SeMet	1240	70	5.6%	
[17]	FA	totSe	2445	98	4.0%	
[18]	FA2	totSe	1001	65.0	6.5%	
[19]	FA	totSe	2534	73	2.9%	
[20]	FA	totSe	2650	60	2.3%	
[20]	FA	totSe	2620	70	2.7%	
[19]	PM	SeMet	115	14	11.8%	82%
[20]	PM	SeMet	300	50	16.7%	62%
[20]	PM	SeMet	280	30	10.7%	
[19]	PM	totSe	140	12	8.3%	
[20]	PM	totSe	420	40	9.5%	
[20]	PM	totSe	510	50	9.8%	
[19]	FS	SeMet	0.16	0.02	10.0%	76%
[19]	FS	totSe	0.21	0.01	6.2%	

Table 2: Performance characteristics provided as supplemental information of FAD-2009-0029

	mg Se/kg	method	R_{rec}	RSD_r
SeMet in FA	1230	ICPMS [21,22]	97 - 103 %	1 - 4 %
totSe in FS	1.16	HGAAS [23]	95%	5%

SeMet: *selenomethionine*; totSe: *total selenium*

FA: *feed additive*; FS: *feedingstuffs*

R_{rec}: *recovery rate*; RSD_{ip} = relative standard deviation for *intermediate precision*

The following performance characteristics are derived from the data presented in Tables 1 & 2:

* For the determination of *selenomethionine* in the *feed additive;*
  - a *recovery rate* (R_{rec}) ranging from 94 to 103%;
  - a relative standard deviation for *repeatability* (RSD_r) ranging from 1 to 4%; and
  - a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 5 to 8%.

* For the determination of *selenomethionine* in *premixtures* and *feedingstuffs:*
  - RSD_{ip} ranging from 11 to 17% and 6 to 10%, respectively.

* For the determination of *total selenium*:
  - R_{rec} ranging from 94 to 95% in the *feed additive*;
  - RSD_{ip} ranging from 2 to 7% in the *feed additive*; and
  - RSD_{ip} ranging from 6 to 10% in the *premixtures and feedingstuffs*.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, submitted by the Applicants, based on triple proteolytic digestion followed by HPLC-ICPMS method, to determine *selenomethionine* in the *feed additives.*

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method ICPMS, submitted by the Applicants, to determine *total selenium* in the *feed additive.*

For the determination of *total selenium* in *feedingstuffs*, the EURL investigated the former ring trial validated method developed by the"Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany) [24], recently adopted as CEN standard prEN 16159:2010 [25]. The method for the determination of total selenium by hydride generation atomic absorption spectrometry (HGAAS) after microwave digestion is based on the extraction with 65% nitric acid and 30% hydrogen peroxide. The following performance characteristics are reported:

- a relative standard deviation for repeatability (RSD_r) ranging from 3.4 to 10%;

- a relative standard deviation for reproducibility (RSD_R) ranging from 15 to 23%; and

- a limit of quantification of 0.125 mg/kg, clearly below the legal limit of 0.5 mg Se /kg feed.

For the determination of *total selenium* in *premixtures*, the EURL suggests diluting the *premixtures* samples with ground cereal feed and applying the abovementioned HGAAS method.

Based on the performance characteristics presented, the EURL recommends for official control, the ring trial validated CEN method (prEN 16159:2010) for the determination of *total selenium* in *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) 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 presented by the Applicants, using high performance liquid chromatography and inductively coupled plasma mass spectrometry (HPLC-ICPMS) after triple proteolytic digestion, to determine *selenomethionine* in the *feed additives;*

- the single laboratory validated and further verified method presented by the Applicants, using inductively coupled plasma mass spectrometry (ICPMS) after HNO_3/H_2O_2 mineralisation, to determine *total selenium* in the *feed additives;*

- the CEN ring trial validated method (prEN 16159:2010), using hydride generation atomic absorption spectrometry (HGAAS) after microwave digestion to determine *total selenium* in *premixtures* and *feedingstuffs.*

Recommended text for the register entry (analytical method)

For the determination of *selenomethionine* in the *feed additives*:

- high performance liquid chromatography and inductively coupled plasma mass spectrometry (HPLC-ICPMS) after triple proteolytic digestion;

For the determination of *total selenium* in the *feed additives*:

- inductively coupled plasma mass spectrometry (ICPMS) after HNO_3/H_2O_2 mineralisation;

For the determination of *total selenium* in *premixtures* and *feedingstuffs:*

- hydride generation atomic absorption spectrometry (HGAAS) after microwave digestion (prEN 16159:2010).

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Selenomethionine (Sel-Plex2000* and *Selemax 2000 & 1000)* 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/Ref:SANCO/D/2:Forw.Appl.1831/0023-2009

[2] +Application/Ref:SANCO/D/2:Forw.Appl.1831/0040-2010

[3] *Technical dossier, Section II – Vol.1 and Vol.2

[4] +Technical dossier, Section II – Identity

[5] *Application, (Annex A), FAD-2009-0029_RegEntry

[6] +Application, (Annex A), FAD-2010-0044_RegEntry

[7] 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

[8] *Technical dossier, Section II, Annex 2-1-6:
Encinar *et al. Anal Chim Acta 500 (2003) 171*

[9] *Technical dossier, Section II, Annex 2-6-1:
Polatajko *et al*. JAAS 19 (2004) 114

[10] *Technical dossier, Section II, Annex 2-6-3:
Encinar *et al.* Anal Chem 76 (2004) 6635

[11] +Technical dossier, Section II, Annex 2.6.1.b:
Polatajko et al. Anal Bioanal Chem 381 (2005) 844

[12] +Technical dossier, Section II, Annex 2.6.1.a:
Mestrer *et al.* Anal Bioanal Chem 385 (2006) 168

[13] *Supplementary Information, CCQM-P86.pdf
Goenaga-Infante *et al*. Anal Bioanal Chem 390 (2008) 629

[14] *Technical dossier, Section II (Volume 2)

[15] +Technical dossier, Section II_Identity

[16] CRM SELM-1 certificate, issued by NRCC
www.nrc-cnrc.gc.ca/obj/inms-ienm/doc/crm-mrc/eng/SELM-1_e.pdf
(last visited on 17/02/2010)

[17] +Technical dossier, Section II, Annex 2.1.3.a

[18] +Technical dossier, Section II, Annex 2.1.3.b

[19] +Technical dossier, Section II, Annex 2.4.1.e (21/11/2008)

[20] +Technical dossier, Section II, Annex 2.4.1.e (23/06/2009)

[21] *Supplementary Information, SeMet Validation (UT2A).pdf

[22] *Supplementary Information, SeMet verification Pau 2010.pdf

[23] *Supplementary Information, 2-6-6 Validation active subst in feeds.pdf

[24] VDLUFA Methodenbuch III (2003), 11.6.1 - Selen

[25] prEN 16159:2010 - "Animal feeding stuffs: - Determination of selenium by hydride generation atomic absorption spectrometry (HGAAS) after microwave digestion (extraction with 65% nitric acid and 30% hydrogen peroxide)"

[26] * Supplementary Information, SeMet ISO 1-28-11_final.pdf

*Refers to Dossier no: FAD-2009-0029
+Refers to Dossier no: FAD-2010-0044

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:

- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava – Slovakia.
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien – Austria.
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino - Italy.
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes – France.