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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2005-071
Name of Additive:	Sel-Plex® 2000
Active Substance:	Selenium
Rapporteur Laboratory:	National Veterinary Institute (SVA), Uppsala, Sweden
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Date:	06 December 2005
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Date:	08 December 2005

1. EXECUTIVE SUMMARY

In the current application authorisation is sought for Sel-Plex® 2000, a selenised yeast product generated by the strain *Saccharomyces cerevisiae* CNCM I-3060. The applicant proposes to place Sel-Plex® 2000 in the category 'nutritional additives', 'compounds of trace elements', group 3b, and in 'zootechnical additives', 'other zootechnical additives', group 4d, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, for the zootechnical claim, authorisation is sought to use Sel-Plex® 2000 to improve the quality and nutritional value of animal derived food products. Sel-Plex® 2000 is intended for use in dairy and beef cattle, laying hens and chickens for fattening.

The active substance in SelPlex® 2000 is selenium (Se), which predominantly (>97.0 %) is present as selenomethionine. Sel-Plex® 2000 contains at least 2000 mg/kg of selenium, of which 97-99 % is organically bound.

The appearance is a light to dark brown, free flowing powder with a slight yeast aroma.

The feed additive is intended to be mixed into compound feedingstuffs at a concentration of 0.2-0.3 g Sel-Plex®2000 per kg compound feedingstuff, depending on the category of animal. The registry entry proposed by the applicant foresees a maximum concentration of total Se in feed of 0.5 mg/kg.

The active substance is measured as total selenium regardless of its chemical form, i.e. independently of whether it is present as organically bound Se or as inorganic Se.

For the determination of the active substance in the *feed additive* a method which includes mineralisation of Se with nitric acid/hydrogen peroxide in closed vessels in a microwave oven followed by detection by ICP-MS (Inductively Coupled Plasma – Mass Spectroscopy), is proposed. The method is based on well known principles for the analysis of selenium.

For the determination of the active substance in *premixtures* and *feed* the same method as above is proposed. The method's performance characteristics include the following: Relative standard deviation for repeatability (RSD_r) of 10.9 % at 509 mg/kg for mineral feed, 9.8 % at 7.1 mg/kg in milk replacer and 16.5 % at 4.4 mg/kg in "beef concentrate", a premixture intended for cattle. These performance characteristics are considered acceptable. However,

this method has not been validated for its suitability for the determination of the active substance in feedingstuffs where its concentration is lower than in premixtures.

For official control regarding the determination of the active substance in premixtures and in feedingstuffs, the CRL recommends a fully ring trial validated analytical method that has been conducted at relevant concentrations of the active substance in relevant matrices. The method and the results from the related interlaboratory study are presented in the method collection of the "Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten" (VDLUFA, Germany). The obtained method performance characteristics for this method are considered acceptable, since the relative between-laboratory reproducibility standard deviation (RSD_R) for a premixture containing 112 mg/kg of Se was 7.3 % and the between-laboratory RSD_R for a feedingstuff matrix containing 0.48 mg/kg of Se was 7.4 %. However, the validated method includes different options for the mineralisation procedure and also for the type of instrumentation, since either Zeeman graphit furnace Atomic Absorption Spectrometry (AAS) or Hydrid AAS can be used for the final measurement of Se. Therefore, the laboratory has to select a specific analytical procedure based on these options and must demonstrate that the method performance criteria as obtained in the ring trial can be met. The VDLUFA method shows different limits of quantification depending on the specific analytical procedure selected, but they are all sufficiently below the legal limit of 0.5 mg Se /kg feed and therefore acceptable for the purpose of analysis.

For determination of selenium in *animal derived food products* the same ICP-MS method as above is proposed by the applicant. The method's performance characteristics included a limit of detection (LOD) of 3 ng Se/ml for milk and blood; and a LOD of 15 ng Se/g for freeze dried egg. Within laboratory reproducibility standard deviations were 0.4 ng Se/ml at 7.1 ng Se/ml milk, corresponding to a within-laboratory RSD_R of 5.6 %; and 13 ng Se/ml at 165 ng/ml blood, corresponding to a within-laboratory RSD_R of 7.9 %. The performance characteristics for freeze dried eggs were validated with a certified reference material (NIST RM8415) and the within laboratory reproducibility standard deviation was found to be 0.05 mg Se/kg at 1.15 mg Se/kg, corresponding to a within-laboratory RSD_R of 4.3 %. The found method performance characteristics are considered acceptable but since there are no legal

limits for the active substance in animal products fixed by European legislation, the suitability of the proposed method for official control cannot be evaluated.

For characterisation of SelPlex® 2000, two DNA based techniques are proposed by the applicant to determine the presence of *Saccharomyces cerevisiae* CNCM I-3060, a pulsed field gel electrophoresis (PFGE) method and polymerase chain reaction (PCR) method. The proposed PCR method was validated by a collaborative study and is considered appropriate for official control purposes at feed additive level, providing that viable colonies of the *Saccharomyces cerevisiae* can be obtained for the PCR analysis, but the method has not been validated for identification of the feed additive in feed.

Further testing or validation is not considered necessary.

2. KEYWORDS

Sel-Plex® 2000, Se, selenised yeast, nutritional additives, other zootechnical additives, poultry, pigs, bovines.

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4. BACKGROUND

Sel-Plex® 2000 is a feed additive consisting of selenised yeast produced by *Saccharomyces cerevisiae* CNCM I-3060. The applicant proposes to classify the additive as belonging to the category nutritional additives, compounds of trace elements, group 3b, and, zootechnical additives, "other zootechnical additives", group 4d intended to improve the quality of animal products. The applicant states that organically bound selenium (Se), predominantly in the form of selenomethionine, is the dominant fraction (97-99 %) of the total amount of selenium in the additive. Selenium is regarded as the active substance. The intended use (*cf.* EFSA-Q-2005-071) of the current application is to enrich the content of dietary selenium to poultry, pigs and bovines in order to supply for nutritional needs and to enhance the quality and nutritional value of animal products (i.e. meat, milk, eggs), by mixing the feed additive into feed to a maximum concentration (including naturally occurring Se from other sources) of 0.5 mg Se/kg in complete feedingstuffs.

5. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the methods of analysis and validation studies submitted in connection with Sel-Plex® 2000, *cf.* EFSA-Q-2005-071, for official control purposes were evaluated.

6. EVALUATION

The numbering system under this point refers to that of Section II of the Annex of Commission Directive 2001/79/EC (2.5 Control methods). Methods of analysis for determining of the presence of the active substance, Se, in various matrices and corresponding validation data are given in Section II (2.5) of the dossier.

Description of the methods used for the determination of the criteria listed cf. pt. 2.5.1 of the Annex of Commission Directive 2001/79/EC, i.e. composition and purity of the additive

Determination of the active substance in the additive

The active substance in SelPlex 2000® is selenium. The method proposed by the applicant involves mineralisation of Se with nitric acid/hydrogen peroxide (HNO_3/H_2O_2) in closed vessels in a microwave oven followed by detection by ICP-MS (inductively coupled plasma – mass spectroscopy). This is considered acceptable but ICP-MS is an advanced technology requiring expensive instrumentation and alternative methods do exist. In general, the analysis of Se can be separated in two steps, namely mineralisation of Se and final determination. The mineralisation step includes digestion in strong oxidising acids such as nitric and perchloric acids or mixtures of acids and oxidising agents such as hydrogen peroxide. The mineralisation step may be carried out in open vessels with reflux or in closed vessels (bombs). Heating can be achieved through conventional heating (block thermostats, ovens) or by microwaves. The applicant has utilised microwaves and a mixture of nitric acid and hydrogen peroxide. For the

final determination of Se several options are available, for example ICP-Atomic Emission Spectroscopy (ICP-AES), Atomic absorption spectroscopy (AAS) using graphite furnace or hydride generation to enhance sensitivity. Several procedures exist and in the method book of 'Verbandes Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten' (VDLUFA Methodenbuch III, 1993) [1] some variants are described.
(*Cf.* the requirements listed in point 2.1.3 of the Annex).

Characterisation of producing microorganism

For characterisation of the authorised strain of the microorganism used in SelPlex® 2000, *Saccharomyces cerevisiae* CNCM I-3060, two DNA based techniques are proposed by the applicant which are pulsed field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). The applicant uses the PCR method for routine control. The PFGE method would be used additionally, if necessary. The PFGE method is provided in form of three literature references. The proposed PCR method [2] was validated by a collaborative study [3] and is considered appropriate for official control purposes, providing that viable colonies of *Saccharomyces cerevisiae* CNCM I-3060 are present in the matrix under investigation to be used for the PCR analysis.
(*Cf.* the requirements listed in point 2.2.3 of the Annex).

Stability of the additive

The same method as described above is proposed for stability studies. This is considered suitable.
(*Cf.* the requirements listed in point 2.3.2 of the Annex).

Description of the qualitative and quantitative analytical methods for routine control of the active substance (selenium) in premixtures and feed.

The same ICP-MS method as above is proposed by the applicant. Quantitation is based on standard addition *(spiking*), i.e. samples are analysed as such and spiked with a known amount of Se. From the relation between the spiked and the unaltered sample the amount of Se in the material can be calculated. Validation data for the method can be found in the applicant's study of homogeneity of premixtures and feedingstuffs. Repeatability studies of

the method have been performed for “dairy cows mineral feed”, “milk replacer”, and “beef concentrate”, a premixture intended for cattle. Calculations from this within laboratory study were done according to ISO 5725. Results include a RSD_r of 10.9 % at 509 mg/kg for mineral feed, a RSD_r of 9.8 % at 7.1 mg/kg in milk replacer and a RSD_r of 16.5 % at 4.4 mg/kg in beef concentrate. The obtained method performance characteristics are considered acceptable, provided that the method is applied within a quality assurance system using control charts and relevant reference materials. However, the method relies on expensive high-tech instrumentation, ICP-MS, and validation is not carried out on feedingstuffs. Another method by VDLUFA [1] has been validated at relevant levels through a ring test. The obtained method performance characteristics for this method are considered acceptable, since the relative between-laboratory RSD_R for a premixture containing 112 mg/kg of Se was 7.3 % and the between-laboratory RSD_R for a feedingstuff matrix containing 0.48 mg/kg of Se was 7.4 %. This method allows for several options for the mineralisation procedure and also for the type of instrumentation, since either Zeeman graphit furnace Atomic Absorption Spectrometry (AAS) or Hydrid AAS can be used for the final measurement of Se. Both instruments are frequently used in routine laboratories. Therefore, the results from the ring test provide a benchmark for laboratories, against which the specific protocol selected from the options offered by the VDLUFA method need to be tested. This is a valuable tool in the quality management, which can be used to demonstrate proficiency in the analysis of Se in feed. Provided that the method is applied within a quality assurance system with control charts, reference materials etc., the VDLUFA method is considered as fit for official control.
(*Cf.* pt. 2.5.2 of the Annex of the Commission Directive 2001/79/EC).

Description of the qualitative and quantitative analytical methods for determining the active substance in target tissues and animal products.

For the determination of selenium in animal products, the methods proposed for determining Se in feed is identical with the method proposed for use with feedingstuffs, premixtures and feed additives.

The proposed method was in-house validated on several samples. Quantitation was based on ^{77}Se and ^{78}Se for milk and blood; and ^{77}Se, ^{78}Se and ^{82}Se for eggs (white and yolk), respectively. Validation was in all cases based on standard addition.

The detection limit was 3 ng Se/ml for milk and blood, and 15 ng Se/g for freeze dried egg. Within laboratory reproducibility standard deviations were 0.4 ng Se/ml at 7.1 ng Se/ml milk , corresponding to a within laboratory RSD_R of 5.6 %; and 13 ng Se/ml at 165 ng/ml blood, corresponding to a within laboratory RSD_R of 7.9 %. The performance characteristics for freeze dried eggs were validated with a certified reference material (NIST RM8415) and the within laboratory reproducibility standard deviation was found to be 0.05 mg Se/kg at 1.15 mg Se/kg, corresponding to a within laboratory RSD_R of 4.3 %. The found method performance characteristics are considered acceptable.

Since there are no legal limits for the active substance in animal products fixed by European legislation, the suitability of the proposed for official control cannot be evaluated.

(*Cf*. pt. 2.5.3 of the Annex of the Commission Directive 2001/79/EC).

CHECKLIST FOR METHODS SUBMITTED BY THE APPLICANT

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned on Premixtures accompanied by information on:				
	- Sampling Method used		**X**		
	- Percentage Recovery		**X**		
	- Specificity	**X**			
	- Accuracy	**X**			
	- Precision	**X**			
	- Limit of detection		**X**		
	- Limit of quantification		**X**		
	- Validation procedure used	**X**			
1.2	Is/Are the method(s) mentioned on Feedingstuffs accompanied by information on:				
	- Sampling Method used		**X**		
	- Percentage Recovery		**X**		
	- Specificity		**X**		
	- Accuracy		**X**		
	- Precision		**X**		
	- Limit of detection		**X**		
	- Limit of quantification		**X**		
	- Validation procedure used		**X**		
2.1	Is/Are the method(s) mentioned on Target tissues accompanied by information on:				
	- Sampling Method used		**X**		
	- Percentage Recovery		**X**		
	- Specificity		**X**		

		Y	N	N/A	Comments
	- Accuracy		**X**		
	- Precision		**X**		
	- Limit of detection		**X**		
	- Limit of quantification		**X**		
	- Validation procedure used		**X**		
2.2	Is/Are the method(s) mentioned on Animal products accompanied by information on:				
	- Sampling Method used	**X**			
	- Percentage Recovery		**X**		
	- Specificity	**X**			
	- Accuracy	**X**			
	- Precision	**X**			
	- Limit of detection	**X**			
	- Limit of quantification	**X**			
	- Validation procedure used	**X**			
3.	If the method(s) has/have been devised, consideration has been given to the fact that their limits of quantification must be below the MRLs.			**X**	

7. CONCLUSIONS AND RECOMMENDATIONS

Methods of analysis for determining the active substance of SelPlex 2000® in the additive, in premixtures, in feed and in animal products and tissues are proposed, and validation data are provided. Although not in full compliance with international standards, the validation data are considered sufficient to conclude that further testing or validation is not considered necessary. The recommended methods do, however, rely on highly specialised and expensive equipment (ICP-MS), not readily available in control laboratories. Therefore, the CRL proposes the procedures recommended in VDLUFA Methodenbuch III, 1993[1] for official control purposes. The VDLUFA method has been validated through a ring test.

8. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of Sel-Plex® 2000 have been sent to the CRL.

The dossier has been made available to the CRL by EFSA.

9. REFERENCES

The dossier provided by the applicant is divided into various documents structured according to the Annex of Commission Directive 2001/79/EC.

[1] VDLUFA Methodenbuch III, 1993, Selen 11.6.1

[2] Nes, F., Lavallée F., Dubourdieu D., Aigle M., Dulau L. 1993. Identification of yeast strains using the polymerase chain reaction. J. Sci. Food Agric., 62, 89-94

[3] Leuschner R.G.K., Bew J., Fourcassier P., Bertin G. 2004. Validation of the Official Control Methods based on polymerase chain reaction (PCR) for identification of authorised probiotic yeast in animal feedingstuff. System. Appl. Microbiol. 27,492-500

10. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the National Veterinary Institute (SVA) in Uppsala, Sweden.