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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-2007-172
	FAD-2007-0034
Product name:	Advastat
Active Substance(s):	Acarbose
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
	Geel, Belgium
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Date:	8/04/2008
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Date:	9/04/2008

EXECUTIVE SUMMARY

The current application authorisation is sought for *Advastat* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Advastat as a digestibility enhancer for cattle for fattening and dairy cows for milk production.

The product *Advastat* contains the active agent acarbose at a concentration of 10 % (w/w %). The intended use of Advastat is to be added to the cereal concentrate part of the ration, obtaining a final concentration of acarbose in the cereal concentrate ranging from 50 to 200 mg/kg. The cereal concentrate containing Advastat may be fed separately or may be incorporated into a total mixed ration. Maximum inclusion levels of acarbose in feed are proposed, which are 70 mg/kg for cattle for fattening and 35 mg/kg for dairy cows for milk production.

For the determination of acarbose in the *feed additive* the applicant proposed a method based on reversed phase high performance liquid chromatography (HPLC) coupled to Ultraviolet (UV) detection. The target analyte is quantified against standard solutions of acarbose diluted into the mobile phase. Validation experiments were carried at 8 %, 10 % and 12 % of acarbose in the feed additive, obtaining an average value for the recovery of 99 % and relative standard deviations for repeatability ranging between 0.4 and 2.5%. For the determination of the intermediate precision, experiments conducted at acarbose concentration of 10 % yielded a value of 1.1 % for the relative standard deviation for reproducibility.

For the determination of acarbose in *cereal concentrate* and in *feedingstuffs* the applicant proposed an identical method based on reversed phase HPLC coupled to a triple quadrupole mass spectrometer (MS/MS). Acarbose is quantified against matrix matched standard obtained from the dilution of a known amount of acarbose into a feed matrix, which does not contain acarbose (blank matrix). The method was validated on various feed matrices containing acarbose at different concentrations ranging from 35 to 1500 mg/kg. Acceptable performance characteristics were obtained, since the percentage recovery rate varied from 95 to 108% and the precision, expressed as percentage relative standard deviation, varied between 2.8 and 5.4%. Sufficient sensitivity of the method was demonstrated, since the

lowest calibration level corresponding to acarbose concentration in the matrix of 10 mg/kg is well below the maximum levels in feedingstuffs.

The proposed method for the determination of acarbose in feedingstuffs is *only* applicable when matrix matched blank feed samples are available. Within the frame of official control this is – however – not always the case. Upon request from the CRL, additional experiments using the "standard addition technique" were conducted by the applicant, since this technique does not require the use of matrix matched blank feed samples. Milled wheat samples containing about 520 mg acarbose/kg were analysed and the results revealed the following method performance characteristics: The precision, expressed as percentage relative standard deviation was 11 % and the percentage rate of recovery was about 125 %.

The CRL considers both methods, i.e. the method based on matrix matched calibration and the method based on the standard addition technique, suitable for official control purposes within the frame of the authorisation. However, when matrix matched blank test samples are not available, only the "standard addition technique" can be applied.

No further testing or validation is required.

KEYWORDS

Advastat, acarbose, zootechnical additives, digestibility enhancer.

1. BACKGROUND

Advastat is a product for which authorisation as feed additive is sought under the category ‘zootechnical additives’, functional group ‘digestibility enhancers’, according to Annex I of Regulation (EC) No 1831/2003 [1]. The target animals are cattle for fattening and dairy cows for milk production [2]. Advastat is comprised of soybean mill, containing the active agent at a concentration of 10 % (w/w % and up to 1% light liquid paraffin [2]. Advastat is added to the cereal concentrate part of the ration, obtaining a final concentration of acarbose ranging from 50 to 200 mg/kg [2]. The cereal concentrate containing Advastat may be fed separately or may be incorporated into a total mixed ration [3], obtaining a concentration of acarbose in

complete feedingstuffs ranging from 35 to 70 mg/kg [4]. This concentration range of acarbose is calculated from the maximum content of advastat in feedingstuffs as specified in the proposed register entry[1].

2. 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 the 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 methods of analysis, submitted in connection with *Advastat* (EFSA-Q-2007-172) and their suitability to be used for official controls in the frame of authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and qualitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of impurities in the *additive* (e.g. arsenic and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [7].

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

For the determination of acarbose in the *feed additive* the applicant proposed a method based on reversed phase high performance liquid chromatography (HPLC) coupled to

[1] The dossier contains two slightly different *"Proposal of Register Entry"*. The document in reference [2] contains under "*maximum content*" just "700 ppm dry matter of total feed" and "300 ppm dry matter of total feed", without specifying , whether the concentration refers to the active substance or to the product. In section II [4] the proposal of register entry contains the information: "ADVASTAT at 700 ppm dry matter" and "ADVASTAT at 300 ppm dry matter".

Ultraviolet (UV) detection [6]. Acarbose is extracted from 1 g of Advastat with 100 ml of methanol. After stirring for 60 minutes, an aliquot of 10 ml is taken from the supernatant and centrifuged. 5 ml of the solvent are taken and diluted by a factor of 10, obtaining a solution with acarbose at a concentration of 0.1 mg/ml. An aliquot of this solution is directly injected into a reversed phase HPLC equipped with a UV detector measuring at 210 nm. The target analyte is quantified against standard solutions of acarbose diluted into the mobile phase. Validation experiments [7] were carried at 8 %, 10 % and 12 % of acarbose in the feed additive, obtaining an average value for the recovery of 99 % and relative standard deviations for repeatability ranging between 0.4 and 2.5. For the determination of the intermediate precision, experiments conducted at acarbose concentration of 10 % yielded a value of 1.1 % for the relative standard deviation for within-laboratory reproducibility.

For the determination of acarbose in *cereal concentrate* and in *feedingstuffs* the applicant proposed an identical method based on reversed phase HPLC coupled to a triple quadrupole mass spectrometer (MS/MS) [8]. Acarbose is extracted from 20 g of the sample with 200 ml of water, shaking for 30 minutes. An aliquot of 15 ml is taken and centrifuged at 3500 rpm. The solution is further diluted by a factor of 10 to 100 depending on the assumed acarbose concentration in the matrix and the diluted extract is subjected to LC-MS/MS analysis without the application of further clean-up procedures. The target analyte is identified by one precursor ion and one transition ion (646.4 >304.4). Acarbose is quantified against matrix matched standard obtained from the dilution of known amount of acarbose into identical feed matrix, which does not contain acarbose (blank matrix). The use of matrix matched standards is most likely required in order to compensate for adverse effect of matrix components on the quantification of acarbose. The method was validated on various feed matrices containing acarbose at different concentrations ranging from 35 to 1500 mg/kg. Acceptable performance characteristics were obtained [9], since the percentage recovery rate varied from 95 to 108% and the precision expressed as percentage relative standard deviation varied between 2.8 and 5.4%. Sufficient sensitivity of the method was demonstrated, since the lowest calibration level corresponding to acarbose concentration in the matrix of 10 mg/kg is well below the maximum levels in feedingstuffs indicated in the proposed registry entry [4].

Since official feed laboratories do not often have matrix matched blank test material available, calibration of the unknown samples against matrix matched standards is not feasible in all cases. Therefore, the CRL requested supplementary information from the applicants to establish, whether the "standard addition technique", which does not require the use of matrix matched standards could alternatively be applied. The difference between these approaches concerns exclusively the mode of preparing the calibration solutions, whereas the extraction conditions and the LC-MS/MS parameters are identical. When applying the "standard addition technique", the unknown sample to be analysed is split into identical sub-samples. One sub-sample is analysed as such, whereas the other sub-samples are fortified with different amounts of the target analyte and afterwards subjected to analysis. Plotting the response of all sub-samples against the added amount of acarbose allows the determination of acarbose in the unknown sample. In order to demonstrate the applicability of the "standard addition technique" to the determination of acarbose in feedingstuffs, the applicant conducted additional experiments [10]. The experimental design included 18 milled wheat samples, fortified with the acarbose at 6 different concentration levels in triplicates. All 21 samples were separately extracted and subjected to LC-MS/MS analysis [11], obtaining the following method performance characteristics: precision expressed as percentage relative standard deviation was 11 % and the percentage rate of recovery was about 125 %.

The CRL considers both methods, i.e. the method based on matrix matched calibration and the method based on the standard addition technique, suitable for official control purposes within the frame of the authorisation. However, when matrix matched blank test samples are not available, only the "standard addition technique" can be applied.

No further testing or validation is required.

4. CONCLUSIONS AND RECOMMENDATIONS

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Reversed phase high performance liquid chromatography (HPLC) coupled to a triple quadrupole mass spectrometer.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Advastat* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Reference SANCO/D/2 Forw. Appl. 1831/23-2007.

[2] Proposed register entry: FAD-2007-0034_Annex_III.pdf.

[3] Proposed register entry: FAD-2007-0034_Annex_III.pdf, "Specific conditions for use in complementary feedingstuffs"

[4] *Section_II.pdf: chapter 2.4 "conditions of use of the additive", page 18

[5] 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 Community reference laboratories, Official Journal of the European Union L 136

[6] *Annex_II-8a.pdf: Analytical Method for Determination of Acarbose in Advastat by HPLC.

[7] * Annex_II-8b.pdf: Validation Documentation for Analytical Method for Determination of Acarbose in ADVASTAT by HPLC*Supplementary information, enclosure 3-extraction test.

[8] * Annex_II-9a.pdf: Analytical method for determination of acarbose in complementary feedingstuffs and premixtures containing minerals by HPLC-MSMS.

[9] * Annex_II-9b.pdf: Validation documentation for analytical method for determination of acarbose in complementary feedingstuffs and premixtures containing minerals by HPLC-MSMS

[10] *Supplementary information: RTQ CRL 14Feb08.pdf

[11] *Supplementary information: RTQ CRL 7March08.pdf

*Refers to Dossier number: FAD-2007-0034

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community 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.

8. ACKNOWLEDGEMENTS

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- Central Institute for Supervising and Testing in Agriculture, Praha, Czech Republic
- Bavarian State institute for Health and Food Safety, Oberschleißheim, Germany
- Plantedirektoratets Laboratorium, Lyngby, Denmark