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

Dossier related to: FAD-2008-049
CRL/080014

Product name: AviPlus

Active Substance(s): Citric acid, sorbic acid and thymol

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EXECUTIVE SUMMARY

In the current application authorisation is sought for AviPlus according to Article 4 (1) of Regulation (EC) No 1831/2003 under the category "zootechnical additives", group 4(d) "other zootechnical additive", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use AviPlus as additive improving growth and/or feed efficacy of weaned piglets. The additive is intended to be marketed in forms of micro beads, containing 25 % of citric acid, 16.7 % of sorbic acid and 1.7 % of thymol in matrix of saturated vegetable fats.

The active agents of *AviPlus* are citric acid (E 330), sorbic acid (E 200) and thymol (Flavis N° 04.006), all approved additives.

The feed additive is intended to be incorporated into premixtures and/or complete feedingstuffs to obtain a recommended concentration ranging from 1000 to 3000 mg feed additive per kg of complete feedingstuffs for piglets. The corresponding concentration ranges in complete feedingstuffs for piglets are: from 250 to 750 mg/kg for citric acid, from 167 to 501 mg/kg for sorbic acid and from 17 mg/kg to 51 mg for thymol.

For the determination of the **citric acid** (E 330) in the *feed additive* (AviPlus) and *premixtures* a reverse phase high performance liquid chromatography method equipped with ultraviolet/diode array detection (RP-HPLC-UV/DAD) is proposed by the applicant. The following acceptable performance characteristics obtained from the in-house validation study were reported: - a limit of determination (LOD) of 5 mg/kg; - a limit of quantification (LOQ) of 10 mg/kg; - a recovery rate of 100 % determined at different concentration levels; - a repeatability relative standard deviation (RSD_r) of 3.3 % for feed additive and 5.2 % for premixtures.

For the determination of citric acid (E 330) in the *feedingstuffs* the applicant proposes an enzymatic method, based on the CEN standardized method for the determination of citric acid in fruit and vegetable juices (EN 1137). The following acceptable performance characteristics obtained from the in-house validation study were reported: - LOD = 5 mg/kg; - LOQ = 10 mg/kg; - a recovery rate of 100 %; - RSD_r = 1.6 %.

Samples of *feed additive* (AviPlus), *premixtures* and *feedingstuffs* were sent to a second independent laboratory for determination of citric acid. The reported results were in

agreement with those obtained by the applicant, thus demonstrating the transferability of the applicant's method [7].

Based on these acceptable performance characteristics, the applicant in-house validated and verified methods for the determination of citric acid are recommended for official control purposes in the frame of authorisation.

For the determination of the **sorbic acid** (E 200) in the *feed additive* (AviPlus), *premixtures* and *feedingstuffs* the RP-HPLC-UV/DAD method is proposed by the applicant. The following acceptable performance characteristics obtained on the in-house validation were reported: LOD = 10 mg/kg; - LOQ = 25 mg/kg; - a recovery rate of 100 % for *feed additive* (AviPlus) and *premixtures*, 85 % for *feedingstuffs*; - $RSD_r = 2.9$ % for *feed additive*, 4.1 % for *premixtures* and 4.2 % for *feedingstuffs*.

Samples of *feed additive* (AviPlus), *premixtures* and *feedingstuffs* were sent to a second independent laboratory for determination of sorbic acid. The reported results were in agreement with those obtained by the applicant, thus demonstrating the transferability of the applicant's method.

Based on these acceptable performance characteristics, the applicant in-house validated and validated method for the determination of sorbic acid is recommended for official control purposes in the frame of authorisation.

For the determination of the **thymol** in the *feed additive* (AviPlus), *premixtures* and *feedingstuffs* the RP-HPLC-UV/DAD method is proposed by the applicant. The following acceptable performance characteristics obtained on the in-house validation were reported: - LOQ = 2.5 mg/kg; - a recovery rate ranging from 90 to 100 % depending on matrix; - RSD_r ranging from 2.9 to 3.4 %, for different matrixes.

Samples of *feed additive* (AviPlus), *premixtures* and *feedingstuffs* were sent to a second independent laboratory for determination of thymol. The reported results were in agreement with those obtained by the applicant, thus demonstrating the transferability of the applicant's method.

Based on these acceptable performance characteristics, the applicant in-house validated and verified method for the determination of thymol is recommended for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

AviPlus, citric acid, sorbic acid, thymol, zootechnical additives, weaned piglets

1. BACKGROUND

AviPlus is a feed additive for which authorisation is sought according to Article 4 (1) of Regulation (EC) No 1831/2003 under the category "zootechnical additives", functional group 4(d) – "other zootechnical additives", according to Annex I of Regulation (EC) No 1831/2003. This feed additive improves weaned piglet zootechnical performance by stimulation of feed intake and digestive processes (flavour and appetent effect) and favourable bioregulation of the gut microflora [3].

The active substances in AviPlus are citric acid, sorbic acid and thymol, all currently approved in the EU legislation [1], [2].

AviPlus is intended to be marketed in forms of protected microbeads containing 25 % of citric acid, 16.7 % of sorbic acid, 1.7 % of thymol and 49.8 % of hardened vegetable oils or hydrogenated triglycerides of vegetable origin [3].

The target concentrations of the feed additive in complete feedingstuffs are 1000 mg/kg for the minimum content and 3000 mg/kg for the maximum content [4]. The corresponding concentration ranges in complete feedingstuffs for piglets are: from 250 to 750 mg/kg for citric acid, from 167 to 501 mg/kg for sorbic acid and from 17 mg/kg to 51 mg for thymol.

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, 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 AviPlus, were evaluated for their suitability for official control.

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 undesirable substances in the *additive* (e.g. arsenic, heavy metals, mycotoxins and dioxins) are available at the respective Community Reference Laboratories [5].

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

Citric acid (E 330)

Feed additive and premixture

For the determination of **citric acid** in *feed additive and premixture*, an in-house validated method based on RP-HPLC-UV/DAD method is proposed [6].

Approximately 0.2 g of the *feed additive* are accurately weighed into a 40 ml test tube, extracted by 5 ml of diethyl ether. Then 30 ml of 0.1 M solution of sodium hydroxide is added and the mixture is extracted in an ultrasonic bath at room temperature. After centrifugation the upper etheric phase is collected into a 40 ml test tube and extraction is repeated with further 30 ml of sodium hydroxide solution. The alkali layers from both extractions are combined into 100 ml volumetric flask and filled up to the mark with water. 5 ml of the solution is transferred into 25 ml volumetric flask, then pH is adjusted with hydrochloric acid to the value 3 – 4 and filled up to the mark with distilled water.

Approximately 0.5 to 2 g of *premixture* are accurately weighed into a 40 ml test tube, extracted twice with 25 ml of water in a water bath at 60 °C and then in an ultrasonic bath. Both extracts are combined in 100 ml volumetric flask and filled up to the mark with water.

The extracted solution (from feed additive or premixture) is injected after filtration into the HPLC apparatus. External standard calibration is used for the quantification of citric acid.

The following acceptable performance characteristics obtained from the in-house validation study were reported [6]: - LOD = 5 mg/kg; - LOQ = 10 mg/kg; - a recovery rate of 100 %

determined at different concentration levels; - $RSD_r = 3.3 \%$ for *feed additive* and 5.2% for *premixtures*.

Samples of *feed additive* (AviPlus), *premixtures* and *feedingstuffs* were sent to a second independent laboratory for determination of citric acid. The reported results were in agreement with those obtained by the applicant, thus demonstrating the transferability of the applicant's method [7].

The CRL considers the proposed in-house validated and verified method for determination of citric acid in *feed additive* and *premixture* suitable for official control purposes in the frame of authorisation.

For the determination of citric acid in *feedingstuffs* the applicant proposes an enzymatic method [8], based on the CEN standardized method for the determination of citric acid in fruit and vegetable juices (EN 1137:1994) [10].

Approximately 0.5 g of *feedingstuffs* is accurately weighed into a 20 ml test tube and extracted with 5 ml of water in a water bath at $70 \text{ }^\circ\text{C}$. After centrifugation and filtration the solution is analysed with the enzyme kit and the amount of citric acid is quantified by measured absorbance UV at 340 nm.

The following acceptable performance characteristics obtained from the in-house validation study were reported [8]: - LOD = 5 mg/kg; - LOQ = 10 mg/kg; - a recovery rate of 100 %; - $RSD_r = 1.6 \%$.

Samples of feed additive (AviPlus), premixtures and feedingstuffs were sent to second independent laboratory for determination of citric acid. The reported results were in agreement with those obtained by the applicant, thus demonstrating the transferability of the applicant's method [7].

The CRL considers the proposed in-house validated and verified method for determination of citric acid in *feedingstuffs* suitable for official control purposes in the frame of authorisation.

Sorbic acid (E 200) and thymol

For the determination of the sorbic acid and thymol in the *feed additive* (AviPlus), *premixtures* and *feedingstuffs* the RP-HPLC-UV/DAD method is proposed by the applicant [9].

Appropriate amount of *premixture* or *feed additive* containing at least 5 mg of **thymol** is accurately weighed into a 100 ml volumetric flask, extracted with 3 ml of diethyl ether for 5 minutes and diluted up to mark with ethanol. The solution is injected after filtration into the HPLC system.

Approximately 2.0 g of *feedingstuffs* are accurately weighed into a 40 ml test tube, extracted by 5 ml of diethyl ether. Then 10 ml of ethanol is added and the solution is sonicated for 10 minutes. After centrifugation, the upper layer is collected into 25 ml volumetric flask and re-extracted by another 10 ml of ethanol. Both extracts are combined in another 25 ml volumetric flask and filled up to the mark with ethanol. The solution is injected after filtration into the HPLC system. External standard calibration is used for the quantification of thymol.

Appropriate amount of *premixture* or *feed additive* containing at least 50 mg of **sorbic acid** is accurately weighed into a 100 ml volumetric flask, extracted with 3 ml of diethyl ether for 5 minutes and diluted up to mark with ethanol. The extract is injected into HPLC system after dilution with equal mixture ethanol-water and filtrated.

Approximately 50 g of *feedingstuffs* is accurately weighed into a 500 ml flask test tube and 450 ml of potassium hydroxide solution (18 g/l) is added. The solution is shaken vigorously to disperse the sample homogenously and placed into water bath of 70 °C for one hour with occasional shaking. The solution is then sonicated for 15 minutes. The centrifuged extract is diluted with water and the pH is set to 4 - 5. Finally the solution is injected into the HPLC system after filtration. External standard calibration is used for the quantification of sorbic acid.

The following acceptable performance characteristics obtained from the in-house validation study for sorbic acid were reported: - LOD = 10 mg/kg; - LOQ = 25 mg/kg; - a recovery rate 100 % for *feed additive* (AviPlus) and *premixture*, 85 % for *feedingstuffs*; - $RSD_r = 2.9\%$, for *feed additive*, 4.1 % for *premixtures* and 4.2 % for *feedingstuffs*.

The following acceptable performance characteristics obtained in the in-house validation for thymol were reported: - LOQ = 2.5 mg/kg; - a recovery rate ranging from 90 to 100 % depending on the matrix; - RSD_r ranging from 2.9 to 3.4 %, depending on the matrix.

Samples of feed additive (AviPlus), premixtures and feedingstuffs were sent to a second independent laboratory for determination of sorbic acid and thymol. The reported results were in agreement with those obtained by the applicant, thus demonstrating the transferability of the applicant's method. [7].

The CRL considers the proposed in-house validated and verified method for determination of sorbic acid and thymol in *feed additive*, *premixtures* and *feedingstuffs* suitable for official control purposes in the frame of authorisation [7].

Further testing or validation is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of the AviPlus authorisation the CRL recommends the analytical methods proposed by the applicant for official control of *feed additives*, *premixtures* and *feedingstuffs* at the target concentration levels of citric acid, sorbic acid and thymol.

Further testing or validation is not considered necessary.

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

Characterisation of the active substances in the *feed additives*: citric acid, sorbic acid, thymol

Determination of **citric acid** in *feed additives*, *premixtures* - Reverse phase high performance liquid chromatography method equipped with ultraviolet/diode array detection (RP-HPLC-UV/DAD).

Determination of **citric acid** in *feedingstuffs* - Enzymatic determination of citric acid content - NADH (reduced form of nicotinamide adenine dinucleotide) spectrometric method.

Determination of **sorbic acid** and **thymol** in *feed additives*, *premixtures* and *feedingstuffs* - RP-HPLC-UV/DAD.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

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

6. REFERENCES

- [1] European Parliament And Council Directive No 95/2/EC Of 20 February 1995 On Food Additives Other Than Colours And Sweeteners
- [2] 1999/217/EC: Commission Decision Of 23 February 1999 Adopting A Register Of Flavouring Substances Used In Or On Foodstuffs Drawn Up In Application Of Regulation (EC) No 2232/96 Of The European Parliament And Of The Council Of 28 October 1996
- [3] * Section II, 2.1 Identity Of Additive
- [4] * Annex III – Proposal of Registry Entry
- [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. 24.5.2006.
- [6] * Section II. Annex II 2.6.1q
- [7] * Section II. Annex II 2.6.1.3
- [8] * Section II. Annex II 2.6.1.r
- [9] * Section II. Annex II 2.6.1u
- [10] EN 1137:1994 – Fruit and Vegetable Juices - Enzymatic determination of citric acid content - NADH (reduced form of nicotinamide adenine dinucleotide) spectrometric method

*Refers to Dossier No: FAD-2008-0049.

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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