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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

Coxipol®

(FAD-2015-0023; CRL/140013)

European Union Reference Laboratory
Feed Additives

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-2015-0023 - CRL/140013**
Name of Product:	***Coxipol®***
Active Agent (s):	**Clopidol**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **20/01/2016**
Report approved by: Date:	**Christoph von Holst** **20/01/2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Coxipol®*, under article 4, for the category "coccidiostats and histomonostats", according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *chickens for fattening*. *Coxipol®* consists of *clopidol* (250 g/kg) as *active substance*, pregelatinised starch, macrogolglycerol ricinoleate as excipients on a rice hulls carrier. *Coxipol®* is intended to be incorporated directly in *feedingstuffs* or through *premixtures*. The Applicant proposed a concentration of *active substance* in *feedingstuffs* of 125 mg/kg and Maximum Residue Limits (MRLs) of 1.5 or 2.0 mg *clopidol/kg* chicken tissues (liver, kidney, muscle or skin/fat). As these MRLs are not set up by Commission Regulation (EC) No 37/2010, the EURL evaluated the correspondent methods of analysis.

For the quantification of *clopidol* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted two single-laboratory validated and further verified methods based on Reversed Phase High Performance Liquid Chromatography coupled with UltraViolet detection (RP-HPLC-UV). The following performance characteristics were recalculated by the EURL based on the experimental data provided: (i) relative standard deviations for *repeatability* (RSD_r) ranging from 0.5 to 5.7 %; (ii) relative standard deviations for *intermediate precision* (RSD_{ip}) ranging from 1.5 to 8.3 %; and (iii) recovery rates (R_{Rec}) ranging from 95 to 109 %. Based on these satisfactory performance characteristics, the EURL recommends for official control the RP-HPLC-UV method for the quantification of *clopidol* in the *feed additive*, *premixtures* and *feedingstuffs*.

For the quantification of *clopidol* in chicken tissues the Applicant submitted a single-laboratory and further verified method based on RP-HPLC coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode (ESI) using matrix matched standards. Based on the performance characteristics presented, the EURL recommends for official control the RP-HPLC-MS/MS method proposed by the Applicant or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs for *clopidol* in the target tissues.

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

Coxipol®, Clopidol, coccidiostat, chickens for fattening

1. BACKGROUND

In the current application authorisation is sought for *Coxipol®*, under article 4(1) (authorisation of a new feed additive), for the category "coccidiostats and histomonostats", according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *chickens for fattening* [1-2].

Coxipol® is a light-brown to brown granulated *feed additive* containing chemically synthesised *clopidol* (250 g/kg) as *active substance*, pregelatinised starch (20 g/kg), macrogolglycerol ricinoleate as excipients (10 g/kg) on a rice hulls carrier [3]; with a minimum guaranteed *clopidol* purity of 98% [4].

Coxipol® is intended to be incorporated directly in *feedingstuffs* or through *premixtures*, to obtain a final concentration of *active substance* in *feedingstuffs* of 125 mg/kg [5]. This product has not to be mixed with other coccidiostats [2].

The Applicant proposed the following Maximum Residue Limits (MRLs) for *clopidol* in chicken for fattening tissues: 1.5 mg/kg for muscle and skin/fat; 2.0 mg/kg for liver and kidney [2]. As these MRLs are not set up by Commission Regulation (EC) No 37/2010 [6], the EURL evaluated the correspondent methods of analysis.

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 *Coxipol®* 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 [7].

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

For the quantification of *clopidol* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted two single-laboratory validated and further verified methods based on Reversed Phase High Performance Liquid Chromatography coupled with UltraViolet detection (RP-HPLC-UV) [8-18].

The *feed additive* sample (~ 0.1g *Coxipol*®) is weighed directly into a 100 ml flask, made up to volume with methanol and homogenised. After 10 min of sonication, the extract is filtered through a filter paper. 1 ml of filtrate is transferred into a 25 ml volumetric flask and made up to volume with the mobile phase (distilled water:acetonitrile:tetrahydrofuran:glacial acetic acid - 83:10:5:2). The obtained solution is filtered and directly injected (20 µl) into the chromatographic system. The *active substance* is then determined by reversed-phase HPLC, detected at 262 nm and quantified by single point external calibration.

Premixtures (PM) and *feedingstuffs* (FS) samples are grinded and homogenised. Samples (0.4 g PM or 10 g FS) are weighed directly into a 300 ml iodine flask and methanol is added. *Clopidol* is extracted by shaking mechanically for 1 hour. After particles sedimentation or centrifugation, 1.0 ml of the supernatant is transferred into a 20 ml volumetric flask (2 ml for FS). The extract is made up to volume with water:methanol for PM (only water for FS). After filtration through a nylon membrane, 20 µl of solution are injected into the chromatographic system. The *active substance* is then determined by RP-HPLC (gradient elution), detected at 262 nm and quantified by external calibration curve. The performance characteristics recalculated by the EURL based on the experimental data provided by the Applicant [10-12,15,16] are presented in Table 1. Furthermore, the Applicant reported a Limit of Detection (LOD) and Quantification (LOQ) of 1 and 5 mg/kg, respectively.

The Applicant investigated the method specificity assessing the technique with three different feeds for birds (i.e. starter, grower and finisher) [17]. Even though no potential interferences were reported in the frame of the single-laboratory validation and verification studies, the EURL remarks that an extraction without purification steps and the use of a wavelength not very specific (i.e. 262 nm) could lead to potential interferences. Whenever the reported values are above the maximum authorised content, further analysis by a more selective method is recommended for confirmation (e.g. HPLC coupled to mass spectrometry and/or additional clean-up).

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the RP-HPLC-UV method for the quantification of *clopidol* in the *feed additive*, *premixtures* and *feedingstuffs*.

Table 1. Performance characteristics of analytical method for the quantification of *clopidol* in the feed additive (FA), premixtures (PM) and feedingstuffs (FS) as calculated by the EURL based on the experimental data reported in the frame of the validation and verification studies [12].

Matrix	Concentration	RSD_r (%)	RSD_{ip} (%)	R_{Rec} (%)
FA	250 g/kg	0.5 - 1.8	1.5 - 1.8	98.4 - 101.8
PM	10 – 75 g/kg	0.9 - 3.5	2.0 - 8.3	94.5 - 109.3
FS	5 – 200 mg/kg	1.4 - 5.7	3.6 - 4.8	95.7 - 102.6

RSDr: relative standard deviation for repeatability; RSDip: relative standard deviation for intermediate precision; RRec: recovery rate.

Methods of analysis for the determination of the residues of the additive in food.

For the quantification of *clopidol* in chicken muscle, the EURL identified a ring-trial validated method based on RP-HPLC-UV described by AOAC [19].

The test sample is extracted with acetonitrile and purified with alumina followed by anion exchange solid phase extraction cartridges. The final extract is injected in the chromatographic system and *clopidol* is quantified at 270 nm. While satisfactory performance characteristics were reported, the method could not be recommended since it did not comply with the requirements set by Commission Decision 2002/657/EC [20].

For the quantification of *clopidol* in chicken tissues (liver, muscle, kidney and skin/fat) the Applicant submitted a single-laboratory and further verified method based on RP-HPLC coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode (ESI) using matrix matched standards [8,21-22].

Minced tissue (1 g) is spiked with an appropriate amount of clopyralid (internal standard) and placed into a centrifuge tube. 5 ml of acetonitrile is added and the tissue sample is shaken for 10 min and further centrifuged for additional 10 min at 4ºC. 2.5 ml of supernatant is then transferred into a clean tube, shaken and centrifuged once again. 100 µl of the acetonitrile layer is transferred, diluted and vortex mixed. An aliquot is further transferred and diluted again in water/acetonitrile. The final extract is filtered and transferred into a vial for injection in the RP-HPLC-MS/MS system. *Clopidol* is detected by MS/MS (positive mode) after ionisation by ESI.

Relevant chicken tissues were investigated at different *clopidol* concentrations [21]. Three levels of the *active substance* (i.e. MRL/2; MRL and 2MRL) were examined for each target *tissue*. Four identification points were set for *clopidol* using one parent and two daughter ions. Quantification is based on the transition m/z 192.07 > 101.00 while confirmation is based on the transition m/z 192.07 > 87.00 to comply with the confirmatory requirements set by Commission Decision 2002/657/EC [20].

Table 2. Performance characteristics of analytical method for the quantification of *clopidol* in tissues.

Tissues		µg/kg (#)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
			Valid	Verif	Valid	Verif	Valid	Verif
Chicken [21-22]	Liver	1000	1.6	---	10.4	---	109	---
		2000	3.0	0.9-5.7	9.0	4.9	103.2	98.4
		4000	1.9	---	6.0	---	95.2	---
	Muscle	750	6.8	---	15.3	---	92.9	---
		1500	5.8	5.4-9.4	5.5	9.4	106	96.6
		3000	6.9	---	6.6	---	99.1	---
	Kidney	1000	8.3	---	12.2	---	101	---
		2000	3.6	4.5-7.0	11.1	5.6	100.1	95.8
		4000	2.2	---	6.3	---	99.8	---
	Skin/Fat	750	6.1	---	1.6	---	104.4	---
		1500	4.3	4.5-6.3	3.6	6.2	100.6	99.9
		3000	5.4	---	10.9	---	95.4	---

RSD_r: relative standard deviation for *repeatability*; RSD_{ip}: relative standard deviation for *intermediate precision*; R_{Rec}: *recovery* rate; # Fortified level.

The performance characteristics derived from the validation and verification studies are presented in Table 2. Furthermore the Applicant determined an LOQ of 0.5 mg/kg for all the target tissues.

Based on the performance characteristics available, the EURL recommends for official control the RP-HPLC-MS/MS method proposed by the Applicant or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs for *clopidol* in the target tissues.

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:

− High Performance Liquid Chromatography coupled with UltraViolet detection (HPLC-UV) for the quantification of *clopidol* in the *feed additive*, *premixtures* and *feedingstuffs*; and

− Reversed-Phase HPLC coupled to triple quadrupole mass spectrometer (RP-HPLC-MS/MS) - or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC - for the quantification of *clopidol* in chicken *tissues.*

Recommended text for the register entry (analytical method)

For the quantification of *clopidol* in the *feed additive*, *premixtures* and *feedingstuff*:

- High Performance Liquid Chromatography coupled with spectrophotometric detection (HPLC-UV)

For the quantification of *clopidol* in *tissues*:

- Reversed-Phase High Performance Liquid Chromatography coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Coxipol®* 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 SANCO/G1: Forw. Appl. 1831/0018-2015

[2] *Application, Proposal of Registry Entry – Annex A

[3] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[4] *Technical dossier, Section II: 2.1.4 Purity

[5] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[6] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

[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, 2.6 Methods of analysis and reference samples

[9] *Technical dossier, Section II, Annexes, Reference II.24

[10] *Technical dossier, Section II, Annexes, Reference II.25

[11] *Technical dossier, Section II, Annexes, Reference II.26

[12] *Supplementary Information, PerfEURL.xlsx

[13] *Technical dossier, Section II, Annexes, Reference II.27

[14] *Technical dossier, Section II, Annexes, Reference II.30

[15] *Technical dossier, Section II, Annexes, Reference II.28

[16] *Technical dossier, Section II, Annexes, Reference II.29

[17] *Technical dossier, Section II, Annexes, Reference II.31

[18] *Technical dossier, Section II, Annexes, Reference II.32

[19] Determination of clopidol residues in chicken tissues by liquid chromatography: collaborative study. Pang GF et All, J AOAC Int. 2003 Jul-Aug; 86(4):685-93

[20] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

[21] *Technical dossier, Section II, Annexes, Reference II.33

[22] *Technical dossier, Section II, Annexes, Reference II.36

*Refers to Dossier no: FAD-2015-0023

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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- RIKILT Wageningen UR, Wageningen (NL)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)