European Food Safety Authority

The EFSA Journal (2009) 1097, 1-20

SCIENTIFIC OPINION

Safety and efficacy of the product Ronozyme® NP (6-phytase) for use as feed additive for poultry, weaned piglets and pigs for fattening[1]

Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed

(Question No EFSA-Q-2008-430)

Adopted on 14 May 2009

PANEL MEMBERS*

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SUMMARY

Following a request from European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the safety and efficacy of the product Ronozyme® NP when used as a feed additive for poultry, weaned piglets and pigs for fattening.

The additive Ronozyme® NP is a preparation of 6-phytase produced by the genetically modified micro-organism *Aspergillus oryzae*. Ronozyme® NP is intended to be used in feed for laying hens at a dose range of 600–1500 FYT kg^{-1}, and for the other poultry species/categories, weaned piglets and pigs for fattening at a dose range of 1500–3000 FYT kg^{-1}. In a previous opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), the safety and efficacy of this product when used as a feed additive for chickens for fattening, and the safety for the consumer, user and the environment, as well as the safety aspects of the genetic modification, were established. The FEEDAP Panel considers that the safety aspects other than those related to the new target species are covered in the previous opinion and would not be affected by this extension of use. Therefore, the present opinion focuses on the efficacy in chickens for fattening and the safety for the user of the new formulation (Ronozyme® NP (M)), and on the safety and efficacy of the product for the new target species.

The FEEDAP Panel concludes that Ronozyme® NP improves the utilisation of phytate-bound P in chickens and turkeys for fattening, piglets and pigs for fattening at 1500 FYT kg^{-1} feed, and

[1] For citation purposes: Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on a request from European Commission on the safety and efficacy of the product Ronozyme® NP (6-phytase) used as feed additive for poultry, weaned piglets and pigs for fattening. *The EFSA Journal* (2009) 1097, 1-20

* One member of the Panel did not participate in the discussion on the subject referred to above.

in laying hens at 600 FYT kg^{-1} feed. The efficacy can be extrapolated to all poultry species. The minimum recommended dose for all poultry should be 1500 FYT kg^{-1} feed and 600 FYT kg^{-1} feed for fattening and laying poultry, respectively.

The use of Ronozyme® NP allows the use of diets with a lower level of inorganic P, which may in turn reduce the excretion of P.

Based on the tolerance studies performed and considering the well established mode of action of phytases, it is concluded that Ronozyme® NP is safe at the maximum recommended dose for chickens and turkeys for fattening, laying hens, piglets and pigs for fattening. The FEEDAP Panel considers that these results can be extrapolated to all poultry, considering that the maximum dose does not exceed 3000 FYT kg^{-1} feed for growing birds and 1500 FYT kg^{-1} feed for laying birds.

All solid and liquid forms of the product are considered to be essentially equivalent in terms of safety for the target species and efficacy when used at the same dose.

Ronozyme® NP (M) is assumed to be a dermal and eye irritant as well as a respiratory sensitiser, thus appropriate user safety precautions are required.

No risk for the environment is expected from the use of this product as a feed additive under the conditions specified.

Key words: zootechnical additive, digestibility enhancer, substances which favourably affect the environment, poultry, weaned piglets, pigs for fattening, 6-phytase, efficacy, safety, *Aspergillus oryzae*

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BACKGROUND

Regulation (EC) No 1831/2003[2] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company DSM Nutritional Products[3] for authorisation of the product Ronozyme® NP to be used as a feed additive for poultry, piglets (weaned) and pigs for fattening (category: zootechnical additives; functional groups: digestibility enhancer, substances which favourably affects the environment) under the conditions mentioned under Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[4] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 8 December 2008.

The additive Ronozyme® NP is a preparation of 6-phytase (IUB No. 3.1.3.26) produced by the genetically modified micro-organism *Aspergillus oryzae* (DSM 17594). EFSA adopted an opinion on the safety and efficacy of two formulations of this product (CT and L) when used as a feed additive for chickens for fattening. This included the assessment of the safety for the consumer, the user and the environment as well as the safety aspects of the genetic modification (EFSA, 2008).

This product is authorised in the European Community for use as feed additive for chickens for fattening (until 22 April 2019).[5]

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the efficacy and the safety for the target animal(s), user and consumer and the environment of the product Ronozyme® NP, which is a preparation of 6-phytase produced by the genetically modified micro-organism *Aspergillus oryzae* (DSM 17594), when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on Enzymes as well as Friedrich Schöne for the preparation of this opinion.

[2] OJ L 268, 18.10.2003, p.29
[3] DSM Nutritional Products, represented in the EU by DSM Nutritional Products Sp. z o.o. Poland, Tarczynska 113, 96-320 Mszczonow, Poland
[4] Dossier reference: FAD-2008-0008
[5] OJ L 091, 03.04.2009, p.3

Table 1. **Register entry as proposed by the applicant**

Additive	RONOZYME® NP
Registration number/EC No/No (if appropriate)	To be established
Category of additive	Zootechnical
Functional group of additive	Digestibility enhancer Substances which favourably affect the environment

Description			
Composition, description	Chemical formula	Purity criteria	Method of analysis (if appropriate)
Coated Thermotolerant granulate (CT): 6-phytase 10000 FYT g^{-1}	6-phytase (IUB No. 3.1.3.26) produced by *Aspergillus oryzae* (DSM 17594)	10000 FYT g^{-1}	Colorimetric method measuring the inorganic phosphorus released by the enzyme from phytate.
Aqueous Liquid (L): 6-phytase 20000 FYT g^{-1}		20000 FYT g^{-1}	
Granulates (M): 6-phytase 50000 FYT g^{-1}		50000 FYT g^{-1}	

Trade name	Solid forms: RONOZYME NP (CT) RONOZYME NP (M) Liquid form: RONOZYME NP (L)
Name of the holder of authorisation (if appropriate)	DSM Nutritional Products Ltd.

Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period
		Units of activity kg^{-1} of complete feedingstuffs		
Poultry (excl. laying hens)	-	1500	-	-
Laying hens	-	600	-	-
Piglets (weaned)	-	1500	-	-
Pigs for fattening	-	1500	-	-

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use	Recommended dose per kg of complete feedingstuff: Poultry excl. laying hens 1500-3000 FYT Laying hens 600 – 1500 FYT Piglets (weaned) and pigs for fattening 1500 – 3000 FYT Recommended for feed rich in phytin-bound phosphorus.
Specific conditions or restrictions for handling	-
Post-market monitoring	No additional requirements further to the need for traceability and recall procedures established by Regulation No 178/2002.
Specific conditions or restrictions for use in complementary feedingstuffs	-

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues
-	-	-	-

ASSESSMENT

1. Introduction

The active component of Ronozyme® NP is a 6-phytase derived from a genetically modified strain of *Aspergillus oryzae* (DSM 17594). It is intended to be used in plant-based diets rich in phytate for poultry, piglets and pigs for fattening during the whole feeding period to reduce the need for added inorganic P. The applicant is seeking the authorisation of the product as zootechnical additive, under the functional groups digestibility enhancers and substances which favourably affects the environment.

The safety aspects of the use of this product have been previously established (EFSA, 2008); thus, the FEEDAP Panel considers that the safety aspects other than those related to the new target species are covered in the previous assessment. Therefore, the present opinion focuses on the safety for the user and the efficacy in chickens for fattening of the new formulation (Ronozyme® NP (M)) and on the safety and efficacy of the product for the new target species.

2. Characterisation

The product is produced in three forms: Ronozyme® NP (M), a granular form with an activity of 50000 FYT g^{-1}, Ronozyme® NP (CT), a coated granular form with an activity of 10000 FYT g^{-1}, and Ronozyme® NP (L), an aqueous liquid form with an activity of 20000 FYT g^{-1}. The characterisation of Ronozyme® NP (CT) and (L) has previously been described (EFSA, 2008).

2.1. Characterisation of the product

The manufacturing of Ronozyme® NP (M) differs only from that of Ronozyme® NP (CT) by the omission of a coating phase in the production process, resulting in a higher enzyme activity per unit of weight in comparison with the CT form. This formulation is designed to be used in mash feed.

Ronozyme® NP (M) is a granular form and the particle size analysis (three batches)[6] demonstrated an average particle size of 250 μm, with 3 % particles between 50 and 150 μm and no particles found below 50 μm. The dusting potential was found to be almost zero in the Heubach test (1 mg per 25 g). The density of this preparation is 0.86 g mL^{-1}. The batch to batch variation in terms of activity of the three batches was < 1%, with a mean value 52388 FYT g^{-1}.

The enzyme concentrate complies with the purity criteria recommended for enzyme preparations in Food Chemicals Codex (FCC), in terms of chemical (heavy metals, Pb, Cd, Hg and As) and microbial contaminants (total viable counts, coliforms, *E. coli*, *Bacillus cereus*, Clostridia, *Staphylococcus aureus, Salmonella*). It also conforms to the 'General specifications and considerations for enzyme preparations used in food processing' as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). This was confirmed by the analysis of three batches of the enzyme concentrate.[7] Absence of antimicrobial activity was confirmed by the analysis of three batches of the enzyme concentrate. All the rest of the ingredients used in the enzyme preparation are food or feed grade.[8]

[6] Technical Dossier/Section II/Appendix 2.44
[7] Technical Dossier/Section II/Appendix 2.7
[8] Technical Dossier/Section II/Appendix 2.1

2.2. Stability and homogeneity

The shelf life of Ronozyme® NP (M) was measured[9] in three batches kept in sealed containers at different temperatures (10, 25, 35 and 40 °C) for 12 months, at 50 °C for one month and in open containers at 40 °C/60 % RH for one month. Recoveries after six months were 99, 90, 77 and 67 % when kept at 10, 25, 35 and 40 °C, respectively. Recoveries after twelve months were 98, 89, 72 and 63 % when kept at 10, 25, 35 and 40 °C, respectively. After one month, when kept at 50 °C and 40 °C/60 % RH, recoveries were 67 and 50 %, repectively.

The stability of Ronozyme® NP (M) in premixtures (containing trace elements) was studied in three batches stored at 25 ºC. After three months of storage at 25 ºC, 89 % of the initial activity was retained.

The stability of Ronozyme® NP (M) was investigated with three batches of Ronozyme® NP (M) in swine mash feed.[10] After three months of storage at 25 ºC, 96 % of the initial activity was retained.

The stability of Ronozyme® NP (M) during pelleting was investigated at processing temperatures of 75, 90 and 95 °C in the production of a commercial chicken and layer compound feed.[11] The phytase activity of Ronozyme® NP (M) was slightly affected at processing temperature of 75 °C (recovery 91 %). Significant losses of enzyme activity occurred when the feed was processed at 90 and 95 °C (67 and 48 % recovery, respectively). However, the FEEDAP Panel notes that the product is intended for use in mash feed.

The capacity of Ronozyme® NP (M) to homogeneously distribute in compound feed was studied in two batches[12] when mixed in mash feed; the mean coefficient of variation was 8.9 %.

2.3. Conditions of use

Ronozyme® NP is intended to be used in feed for laying hens at a dose range of 600–1500 FYT kg^{-1}, and for the other poultry species/categories, weaned piglets and pigs for fattening at a dose range of 1500–3000 FYT kg^{-1}. The formulation Ronozyme® NP (M) is designed to be used in mash feed.

2.4. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the CRL report can be found in the Appendix.

3. Efficacy

3.1. Efficacy for chickens for fattening

The applicant reported five trials, four of which[13] were already included in the previous assessment (EFSA, 2008). In its previous opinion, the FEEDAP Panel concluded regarding the efficacy of the product when used in chickens for fattening that:

[9] Supplementary information January 2009
[10] Technical Dossier/Section II/Appendix 2.44
[11] Technical Dossier/Section II/Appendix 2.45
[12] Technical Dossier/Section II/Appendix 2.44
[13] Technical Dossier/Section III/Appendixes 3.1 to 3.7

'The results of two balance and two growth trials show that Ronozyme® NP improves the utilisation of phytate-bound P in diets for chickens for fattening. The solid and liquid forms of the product are considered to be essentially equivalent in terms of efficacy. The data confirms the minimum recommended dose of 1500 FYT kg^{-1}.'

The fifth study was a balance trial carried out in order to determine the effect of Ronozyme® NP (M) on phosphorus utilisation and bone mineralisation. However, the results from this study showed efficacy only at 2000 FYT kg^{-1}.

3.2. Efficacy for turkeys for fattening

Three trials were carried out in three different locations with male turkeys for fattening, a summary of the design and some of the results are summarised in Table 2. The diets were based on maize and soybean meal, and a solid form (CT or M) of the product was used in all trials. Two of the trials (1 and 2) were carried out to assess the effect of Ronozyme® NP on the P utilisation while the third one also reports the effects on performance and includes a tolerance dose. The enzyme activities were confirmed in all the studies.

Trial 1 was carried out with a total of 240 six-day-old turkeys (BUT T9), kept in cages of two birds per cage and distributed into eight experimental treatments. A basal diet containing 2.0 g non-phytic P kg^{-1} was supplemented with Ronozyme® NP (CT) at 0, 500, 1000, 1500, 2000 and 3000 FYT kg^{-1}. Two more treatments were considered as positive controls, containing 2.5 or 3.0 g kg^{-1} non-phytic P. The birds were fed the experimental diets for 22 days including a four-day collection of excreta. At the end of the trial, one bird per cage was slaughtered in order to collect the tibia.

Trial 2 was carried out with a total of fifty 21-day-old turkeys (BUT Big 6). A basal diet containing 2.0 g available P kg^{-1} was supplemented with Ronozyme® NP (M) at 0, 1000, 1500, 2000 and 3000 FYT kg^{-1} of complete feed, fed for 17 days, including a five-day collection of excreta. At the end of the study, the birds were slaughtered in order to collect the tibia.

Trial 3 was carried out with 120 one-day-old turkeys (BUT T9), which were fed one of the five experimental diets for 42 days. The dietary treatments were obtained from a basal diet containing 2.5 and 2.0 g kg^{-1} of non-phytate P in starter and grower, respectively, supplemented with Ronozyme® NP (CT) at 0, 1000, 1500, 3000, 30000 FYT kg^{-1}. A balance trial was carried out between days 22 and 25 of life. At the end of the experiment, blood was collected from one bird per cage to measure haematological and biochemical parameters. The same bird was then slaughtered and its tibia collected for analysis of ash.

Table 2. **Effect of Ronozyme® NP on P utilisation and tibia ash content in turkeys for fattening**

Trial	No. of animals (replicates x birds/ replicate) Sex	Duration (days) [1]	Ronozyme® NP dosage (FYT kg^{-1})	Total Dietary P (g kg^{-1})	P Utilisation (%)	Tibia Ash (% of DM)
1[14]	240 (15 x 2) ♂	22	0	5.5	44.9^{a}	27.6^{a}
			500-CT	5.6	50.3^{bc}	28.5^{a}
			1000-CT	5.4	52.9^{cd}	31.0^{ab}
			1500-CT	5.5	56.3^{d}	31.1^{ab}
			2000-CT	5.5	57.4^{d}	33.7^{bc}
			3000-CT	5.5	62.2^{e}	35.3^{c}
			+ Control 1	6.0	45.8^{a}	28.9^{a}
			+ Control 2	6.5	46.2^{ab}	33.3^{bc}
2[15]	50 (10 x 1) ♂	17	0	5.1	44.5^{a}	36.1^{a}
			1000-M	5.1	55.5^{b}	41.3^{b}
			1500-M	5.1	58.0^{b}	42.1^{c}
			2000-M	5.1	62.2^{c}	42.1^{c}
			3000-M	5.1	67.0^{d}	43.9^{c}
3[16]	120 (12 x 2) ♂	42	0	5.8 / 4.9*	65.2^{a}	35.6^{a}
			1000-CT	5.3 / 4.7	70.8^{a}	38.3^{ab}
			1500-CT	5.3 / 4.8	71.2^{b}	37.7^{ab}
			3000-CT	5.5 / 4.7	45.5^{c}	40.8^{b}
			30000-CT	5.5 / 4.6	86.2^{d}	49.0^{c}

[1] The balance trials were started at 21 days of live in trial 1, at 27 days of live in trial 2 and at 22 days of live in trial 3.
* Phosphorus content of the starter and grower diets.
a, b, c, d, e: Values in the same column for a given trial without the same superscript are different (P < 0.05).

High mortalities were recorded in trial 1 after the balance period (between days 24 to 28). High mortality and culling were present in trial 3, particularly during the first 21 days of the trial, in the negative control (29 % and 25 %, respectively).

Improvement of the P utilisation was found at the dose of 500 FYT Ronozyme® NP (CT) kg^{-1} and higher in trial 1, from 1000 FYT Ronozyme® NP (M) kg^{-1} in trial 2 and from 1500 FYT Ronozyme® NP (CT) kg^{-1} in trial 3, as compared to the control. Tibia ash content was significantly higher when the birds were fed diets supplemented from 2000 FYT Ronozyme® NP (CT) kg^{-1} in trial 1, from 1000 FYT Ronozyme® NP (M) kg^{-1} in trial 2 and at 3000 FYT Ronozyme® NP CT kg^{-1} in trial 3, as compared to the control.

3.3. Efficacy for laying hens

Three studies carried out in three different locations were provided by the applicant in order to support the efficacy of Ronozyme® NP in laying hens. A summary of the design and some of the results obtained are shown in Table 3. In general, efficacy was studied by evaluating the performance of the laying hens, the phosphorus digestion and the mineralisation of the tibia.

Trial 1 was carried out with a total of 168 Isa-Brown hens (22 weeks old), which were fed one of the seven experimental diets. A basal diet based on maize and soybean meal, with 3.2 g total P kg^{-1} (1.1 g non-phytic P kg^{-1}) and containing titanium dioxide as external marker, was used as basal diet. The basal diet was supplemented with the two solid formulations of Ronozyme® NP, CT or M, at 0, 600, 900 and 1200 FYT kg^{-1} (confirmed by analysis). After being fed the control diet for 14 days, the hens were fed one of the seven experimental diets for seven weeks (24 to

[14] Technical dossier/Section III/Appendix 3.11
[15] Technical dossier/Section III/Appendix 3.12
[16] Technical dossier/Section III/Appendix 3.13

31 weeks of age). At 31 weeks of age, blood samples were collected from four groups per treatment for blood inorganic phosphorus and calcium determination. At the end of the trial, the hens were slaughtered to sample the contents of the terminal part of the ileum and to collect the right tibia.

Digestibility at the terminal ileum was significantly increased by the supplementation with Ronozyme® NP at 900 FYT kg^{-1} for CT form, and from 600 FYT kg^{-1} for the M form, as compared to the control. At the same time, the supplementation of the basal diet with Ronozyme® NP from 600 FYT kg^{-1} in the case of both formulations increased the ash content of the tibia compared to the control group.

Trial 2, which is also presented as a tolerance study, was carried out with a total of 480 Hy-Line brown hens. A basal diet based on maize and soybean meal, with low P content (3.3 g kg^{-1}; 0.1 g non-phytic P kg^{-1}), was supplemented with Ronozyme® NP (CT) at 0, 600, 900, 1200, 1500 and 15000 FYT kg^{-1} (confirmed by analysis). The experimental period started when the hens were 21 weeks old and lasted for eight weeks. At the end of study, one hen per cage was slaughtered in order to collect ileal contents and the tibia.

Supplementation of the basal diet with Ronozyme® NP (CT) at 1500 FYT kg^{-1} significantly improved ileal phosphorus digestibility as compared to the control diet. Tibia ash content was increased by the supplementation with Ronozyme® NP at 600 FYT kg^{-1}.

The third trial was carried out with 480 ISA white hens (23 weeks of age) and lasted for 15 days. Hens were fed one of the experimental diets resulting from the supplementation of a control diet, with low P content (3.7 g kg^{-1}), with Ronozyme® NP (M) at 0, 600, 900 and 1500 FYT kg^{-1} feed (confirmed by analysis). A positive control diet was also used with a P content of 4.6 g kg^{-1}. At the end of the experimental period, terminal ileum contents were obtained and pooled from all animals per replicate. The right tibia of six hens per replicate was also collected.

Phosphorus digestibility at the terminal ileum was improved by a supplementation with 600 FYT kg^{-1}; however, ash content of the tibia was similar in all groups after 15 days under study.

Improvement of the P digestion was found at the dose of 600 FYT Ronozyme® NP (M) kg^{-1} and higher in trial 1 and trial 3, and from 900 to 1500 FYT Ronozyme® NP (CT) kg^{-1} in trial 1 and 2 (Table 3). Tibia ash content was significantly higher when the birds were fed diets supplemented from 600 FYT Ronozyme® NP (CT and M) kg^{-1} in trial 1 and trial 2 as compared to the control.

Table 3. **Summary of the design and results of the three efficacy trials with Ronozyme® NP in laying hens**

Trial	No of animals (replicates x birds/ replicate) Breed	Duration (age at the start) [1]	Ronozyme® NP dosage (FYT kg^{-1})	Total Dietary P (g kg^{-1})	P ileal Digestibility (%)	Tibia Ash (%)
1[17]	168 (12 x 2) Isa Brown	49 d (24 w)	0	3.2	46.0^{a}	45.0^{a}
			600-CT	3.2	54.0^{ab}	48.0^{b}
			900-CT	3.2	55.6^{bc}	46.9^{ab}
			1200-CT	3.2	62.8^{bc}	47.0^{ab}
			600-M	3.2	61.6^{bc}	49.2^{bc}
			900-M	3.2	65.8^{c}	50.2^{c}
			1200-M	3.2	67.5^{c}	47.8^{b}
2[18]	480 (16 x 5) Hy-Line	56 d (21 w)	0	3.3	44.7^{a}	42.1^{a}
			600-CT	3.3	49.3^{ab}	44.4^{bc}
			900-CT	3.3	46.1^{ab}	44.7^{bc}
			1200-CT	3.4	48.8^{ab}	44.2^{bc}
			1500-CT	3.4	50.5^{b}	42.7^{ab}
			15000-CT	3.4	68.8^{c}	45.6^{c}
3[19]	480 (6 x 16) ISA-white	15 d (23 w)	0	3.7	17.2^{a}	49.9
			600-M	3.7	30.1^{b}	50.2
			900-M	3.7	28.7^{b}	50.8
			1500-M	3.7	36.0^{d}	50.2
			Positive control	4.6	22.4^{c}	48.8

[1] The digestibility was evaluated at 31 weeks of life in trial 1, at 29 weeks of life in trial 2 and at 26 weeks of life in trial 3.
$^{a, b, c, d}$: values in the same column for a given trial without the same superscript are different ($P < 0.05$).

3.4. Efficacy for weaned piglets

The applicant has presented three studies done at three different locations in order to provide evidence of the efficacy of Ronozyme® NP in weaned piglets. The main characteristics and results of the trials are summarised in Table 4.

The three trials were designed to study the effect of Ronozyme® NP on the digestibility of P; the second trial also aimed to study the tolerance of the piglets to Ronozyme® NP. In trials 1 and 2, the CT form was used, while the M form was used in trial 3. The analyses for the enzyme activity were provided for all the experiments, showing a good agreement with the expected values.

Trial 1 was a dose-response trial, in which five dietary treatments were obtained by supplementing a basal diet with Ronozyme® NP at 0, 1000, 1500, 2000 and 3000 FYT kg^{-1}. The content of phytic P in the basal diet was 2.99 g kg^{-1}. A total of 40 cross-bred barrows (LW x Landrace, 15.5 kg initial weight) were used in the study. Digestibility was measured in two consecutive periods. Each digestibility trial lasted for 16 days; the piglets were fed the experimental diets ten days before the start of the faeces collection period, which lasted for six days. The total collection of the faeces was done by crating the piglets in metabolic crates; feed was offered restrictively.

Trial 2, which is also presented as a tolerance study, was carried out with a total of 120 weaned piglets (LD x Pi) with an initial body weight of 7.2 kg. The animals were penned in a total of 36 pens in groups of three or four animals. Dietary treatments were obtained by supplementing a basal diet with Ronozyme® NP at 0, 1000, 1500, 3000 and 30000 FYT kg^{-1}; dietary

[17] Technical dossier/Section III/Appendix 3.8
[18] Technical dossier/Section III/Appendix 3.9
[19] Technical dossier/Section III/Appendix 3.10

treatments also included a positive control, obtained by adding 1 g kg^{-1} of dicalcium phosphate to the basal diet. The content of phytic P in the basal diet was 2.72 g kg^{-1}. The animals were fed the experimental diets for six weeks, during which performance was evaluated every two weeks. Diets included an external marker (TiO_2) during the first two weeks. During the second week of the experiment, faeces were obtained in order to study the digestibility of P and Ca.

Trial 3 was carried out with 16 weaned piglets (LW x LD) with an initial body weight of 9.8 kg which were individually crated. The study aimed at evaluating the effect of Ronozyme® NP when included in the diet to a given dose (1500 FYT kg^{-1}). The basal diet presented 2 g of phytic P kg^{-1} and included an external marker (Cr_2O_3). The experimental period lasted for 14 days (Latin Square design), the collection of faeces being carried out during the last three days. The feeding regime was *ad libitum*.

The apparent faecal digestibility of P (Table 4) was significantly increased by the supplementation of the diets with Ronozyme® NP. In two trials (trial 1 and 2), the increases were observed from 1000 FYT kg^{-1}. In trial 3, the lowest dose tested was 1500 FYT Ronozyme® NP kg^{-1}, which also resulted in a higher faecal apparent digestibility of P. The three trials support the efficacy of Ronozyme® NP at the minimum recommended dose of 1500 FYT kg^{-1} feed in weaned piglets.

Table 4. **Summary of the design and results of the three efficacy trials with Ronozyme® NP in weaned piglets**

Trial	No of animals (replicates x pigs/ replicate)	Duration (initial BW)	Diet (form)	Ronozyme® NP dosage (FYT kg^{-1})	Total Dietary P (g kg^{-1})	Faecal Apparent Digestibility (%)	
						P	Ca
1[20]	40 (8 x 1)	16 d (15.5 kg)	Corn/soybean meal/sunflower meal (pelleted)	0	4.6	29.2[a]	57.1[a]
				1000-CT	4.6	53.3[b]	69.6[b]
				1500-CT	4.6	62.4[c]	76.4[c]
				2000-CT	4.6	65.9[c]	77.6[c]
				3000-CT	4.6	66.6[c]	76.1[bc]
2[21]	120 (6 x 3 or 4)	42 d (7.2 kg)	Corn/barley/soybean meal/ whey (pelleted)	0	4.0	43.2[a]	57.8[a]
				1000-CT	3.8	54.8[b]	63.5[ab]
				1500-CT	3.9	65.2[c]	64.3[ab]
				3000-CT	3.8	69.9[c]	67.1[b]
				30000-CT	3.9	70.4[c]	65.0[b]
				Positive control	4.6	43.7[a]	55.5[a]
3[22]	16 (8 x 1)	14 d (9.8 kg)	Corn/soybean meal/rapeseed meal (mash)	0	4.1	18.4[a]	52.6[a]
				1500-M	4.1	35.9[b]	60.3[b]

[a, b, c]: Values in the same column for a given trial without the same superscript are different ($P < 0.05$)

3.5. Efficacy for pigs for fattening

Three experiments carried out at three different locations were provided by the applicant in order to support the efficacy of Ronozyme® NP in growing pigs. The main characteristics of the trials and the results obtained are summarised in Table 5.

[20] Technical dossier/Section III/Appendix 3.14
[21] Technical dossier/Section III/Appendix 3.15
[22] Technical dossier/Section III/Appendix 3.16

The three trials were designed to study the effect of Ronozyme® NP in the digestibility of P. The dietary treatments in the three trials were obtained by supplementing a basal diet with Ronozyme® NP at 0, 1000, 1500 and 3000 FYT kg^{-1}, including in all the diets Cr_2O_3 as external marker. In all trials, a solid form of the product was used: M in trial 1 and CT in trials 2 and 3. The enzyme activities were confirmed by analyses in all the trials.

The first trial was carried out with six Large White gilts (31.6 kg initial weight). All animals were fed the four diets following a cross-over design, in four experimental periods. Each experimental period lasted for seven days; faeces were collected on days 6 and 7. The animals were fed in two equal meals at a daily rate of about 80 g $kg^{-0.75}$. Digestible P in the basal diet amounted to 1.10 g kg^{-1}. Apparent digestibility of P and Ca was evaluated in the faeces collected. Also, blood samples were taken from the animals to study the concentration of inorganic P.

In the second trial, a total of twenty-eight crossbred (Pi x (DC x LD)) growing pigs (initial body weight 30 kg; castrated males and females) were individually penned and distributed into the four treatments. For 14 days, the pigs were fed the unsupplemented diet (containing 1.26 g digestible P kg^{-1}), after which the experimental diets were fed to the animals for 14 days, the faeces being collected during the last four days.

In the third trial, a total of thirty-two cross bred (LW x LD) growing pigs with an initial body weight of 47.8 kg were penned in groups of four animals. The pigs were fed for 30 days the unsupplemented diet containing 1.10 g digestible P kg^{-1}. After this period, the animals received one of the four experimental diets for 13 days. During the last three days of administration, faeces were collected individually.

In the three trials, the apparent faecal digestibility of P was improved when the pigs were fed Ronozyme® NP compared to the unsupplemented diets. Significant improvements were found when the basal diet was supplemented from 1000 FYT Ronozyme® NP kg^{-1} in trials 1 and 3, and from 1500 FYT Ronozyme® NP kg^{-1} in trial 2. The three trials support the efficacy of Ronozyme® NP at the minimum recommended dose of 1500 FYT kg^{-1} feed in pigs for fattening.

Table 5. **Summary of the design and results of the three digestibility trials with Ronozyme® NP in pigs for fattening**

Trial	No of animals (replicates x pigs/ replicate)	Duration (days)	Diet (form)	Ronozyme® NP dosage (FYT kg^{-1})	Total Dietary P (g kg^{-1})	Faecal Apparent Digestibility (%)	
						P	Ca
1[23]	6 Cross-over	28	Corn/barley/soy bean meal (Mash)	0	3.2	41.1^{a}	47.9^{a}
				1000-M	3.2	54.4^{b}	51.2^{a}
				1500-M	3.2	55.3^{b}	53.9^{ab}
				3000-M	3.2	65.7^{b}	59.7^{b}
2[24]	28 (7 x 1)	14	Corn/soybean meal (Pelleted)	0	3.3	38.6^{a}	51.8^{a}
				1000-CT	3.4	44.5^{ab}	55.7^{ab}
				1500-CT	3.4	49.7^{b}	59.8^{b}
				3000-CT	3.4	50.9^{b}	60.9^{b}
3[25]	32 (2 x 4)	13	Corn/barley/soy bean meal (Pelleted)	0	4.7	22.8^{a}	48.7^{a}
				1000-CT	4.7	51.0^{b}	58.6^{b}
				1500-CT	4.7	56.7^{b}	58.7^{b}
				3000-CT	4.7	62.1^{b}	60.0^{b}

$^{a, b}$: Values in the same column for a given trial without the same superscript are different ($P < 0.05$)

3.6. Conclusions on efficacy

The solid and liquid forms of the product are considered to be essentially equivalent in terms of efficacy at the same dose.

The FEEDAP Panel does not consider it necessary to modify the conclusions reached in the previous opinion on the use of Ronozyme® NP as feed additive for chickens for fattening (EFSA, 2008):

> 'The results of two balance and two growth trials show that Ronozyme® NP improves the utilisation of phytate-bound P in diets for chickens for fattening. The data confirms the minimum recommended dose of 1500 FYT kg^{-1}.'

The use of Ronozyme® NP in diets for turkeys for fattening showed improvements in the utilisation of dietary P in three balance trials. The data confirms the minimum recommended dose of 1500 FYT kg^{-1}.

Three trials support the efficacy of Ronozyme® NP at the minimum recommended dose of 600 FYT kg^{-1} feed in laying hens.

The mode of action of phytases is well known and therefore the demonstrated efficacy of Ronozyme® NP in the major poultry species can be extrapolated to all poultry with a minimum dose of 1500 FYT kg^{-1} (except for laying poultry, 600 FYT kg^{-1}).

The results obtained in the three digestibility trials provided for weaned piglets and for pigs for fattening show that Ronozyme® NP improves the digestibility of dietary phosphorus. The data confirms the minimum recommended dose of 1500 FYT kg^{-1}.

The use of Ronozyme® NP allows the use of diets with a lower level of inorganic P in all poultry species, weaned piglets and pigs for fattening, which may in turn reduce the excretion of P.

[23] Technical dossier/Section III/Appendix 3.17
[24] Technical dossier/Section III/Appendix 3.18
[25] Technical dossier/Section III/Appendix 3.19

4. Safety

4.1. Safety for the target species

4.1.1. Safety for chickens for fattening

No new data has been provided by the applicant. In its previous opinion (EFSA, 2008), the FEEDAP Panel concluded from the two tolerance trials provided that:

> 'The tolerance trials performed showed that chickens for fattening tolerated a 10X overdose of Ronozyme® NP (CT) without adverse effects on performance or health. Therefore, it is concluded that the use of Ronozyme® NP at the recommended dose is safe for chickens for fattening.'

4.1.2. Safety for turkeys for fattening

One tolerance trial[26] was carried out with turkeys fed the diet supplemented with the highest recommended dose and tenfold higher dose of Ronozyme® NP. The results of this trial have partly been presented in Section 3.2 (trial 3). Performance, P utilisation and bone mineralisation were evaluated, and haematological and biochemical blood parameters were analysed. The experimental period lasted for 42 days.

A positive response in performance was observed with the supplementation with Ronozyme® NP at increasing doses up to 10x the maximum recommeded dose. Mean final body weights were 1.0, 1.7 and 2.1 kg for the unsupplemented group, the 3000 FYT kg^{-1} group and the tenfold group, respectively. Haematological and biochemical parameters did not show modifications related to the experimental treatments, except for the significantly higher corpuscular haemoglobin in the tenfold dose-fed birds, as compared to the negative control (36 *vs* 39 pg, $P < 0.05$). In the view of the FEEDAP Panel, this may be a consequence of the P-deficient control diet.

4.1.3. Safety for laying hens

One tolerance trial[27] was carried out with laying hens; it has been already presented in Section 3.3 (trial 2). The experimental diets contained 0, 1500 and 15000 FYT Ronozyme® NP kg^{-1}, corresponding to the control, the highest recommended dose and tenfold the highest recommended dose. Performance parameters were evaluated and after eight weeks of experimental period, blood was collected from 16 hens per treatment in order to determine haematological and biochemical blood parameters.

No mortality was observed during the experiment and the results of the trial showed no modifications related to the experimental treatments on the laying performance of the hens (the mean rates of lay were 92 %, 93 % and 94 % for the control, the maximum dose and the tenfold overdose groups, respectively). Blood haematology and clinical chemistry parameters did not show modifications related to the experimental treatments, except in the case of serum inorganic P concentration which was increased linearly with supplementation.

[26] Technical dossier/Section IV/Appendix 4.18
[27] Technical dossier/Section IV/Appendix 4.17

4.1.4. Safety for pigs

The applicant provided a tolerance study[28] in weaned piglets in order to support the safety of Ronozyme® NP for pigs. The study has been previously presented in Section 3.5 (trial 2). Apart from the negative control, Ronozyme NP was included at 3000 (1X) and 30000 FYT kg^{-1} (10X maximum recommended dose). After 42 days of experiment, during which the performance of the animals was evaluated, blood was collected from twelve animals per treatment in order to perform haematological and clinical biochemical analyses.

During the experiment, five animals died and four were culled, with no relation to dietary treatments. The overall results on the performance of the piglets showed that the tenfold dose fed piglets showed no significant differences compared to the negative or the positive control groups.

Haematological and biochemical results fell in all the cases within the physiological values for all the treatments, including the negative control. Thus, there was no difference in those values between positive control and treated groups (1X and 10X).

4.1.5. Conclusions on the safety for the target species

Based on tolerance studies performed in chickens and turkeys for fattening and on laying hens, it is concluded that Ronozyme® NP is safe at the maximum recommended dose of 3000 FYT kg^{-1} for chickens and turkeys for fattening and of 1500 FYT kg^{-1} for laying hens. The FEEDAP Panel considers that these results can be extrapolated to all poultry, considering that the maximum dose should not exceed 3000 FYT kg^{-1} for growing birds and 1500 FYT kg^{-1} for laying birds.

Based on the tolerance trial carried out with weaned piglets and considering the well-established mode of action of phytases, the FEEDAP Panel concludes that Ronozyme® NP is safe for both pig categories (piglets (weaned) and pigs for fattening) at the maximum recommended dose of 3000 FYT kg^{-1}.

4.2. Safety for the user

No new data were provided by the applicant but the safety for the user of the L and CT forms has been assessed previously.

When assessing the safety for the user of the liquid formulation (Ronozyme® NP (L)) and the solid formulation (Ronozyme® NP (CT)), the FEEDAP Panel concluded the following (EFSA, 2008):

> 'The liquid product is used in enclosed systems and the dry product is very low dusting and contains no particles smaller than 150 μm thus the absence of an inhalation study in animals was justified. Since respiratory sensitisation is assumed from the nature of the product the absence of a dermal sensitisation study was justified. A positive result in such a study would not introduce the need for any additional precautions when handling the product.'
>
> 'For skin and eye irritation only the liquid product was tested thus no direct extrapolation to the user safety of the solid form is possible. The eye irritancy and skin irritancy tests showed no evidence of significant irritant potential of the Ronozyme® NP (L). In the absence of specific data it must be assumed that Ronozyme® NP (CT) is both a dermal and eye irritant.'
>
> 'The data suggest no additional precautions beyond those required by the labelling of both Ronozyme® NP products as respiratory sensitisers.'

[28] Technical dossier/Section IV/Appendix 4.19

No inhalation study was provided for Ronozyme® NP (M), which is justified because of the particle size distribution (no particles below 50 µm). However, given the proteinaceous nature of the substance it must be assumed that Ronozyme® NP (M) is a respiratory sensitiser.

Since no data were provided on Ronozyme® NP (M), this form of the product should be treated as the CT form and assumed to be a dermal and eye irritant.

4.3. Safety for the environment

The active ingredient of Ronozyme® NP is a protein and as such will be degraded/inactivated during the passage through the digestive tract of animals. No risk for the environment is expected and no further environmental risk assessment is required.

5. Post-market monitoring

No risks associated with the use of the product are foreseen. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[29] and Good Manufacturing Practice.

CONCLUSIONS

All solid and liquid forms of the product are considered to be essentially equivalent in terms of safety for the target species and efficacy when used at the same dose. The FEEDAP Panel concludes that Ronozyme® NP improves the utilisation of phytate-bound P in chickens and turkeys for fattening, piglets and pigs for fattening at 1500 FYT kg^{-1} feed, and in laying hens at 600 FYT kg^{-1} feed.

The efficacy of the product can be extrapolated to all poultry species. The minimum recommended dose for all poultry should be 1500 FYT kg^{-1} feed and 600 FYT kg^{-1} feed for fattening and laying poultry, respectively.

The use of Ronozyme® NP allows the use of diets with a lower level of inorganic P, which may in turn reduce the excretion of P.

Based on tolerance studies performed and considering its mode of action, it is concluded that Ronozyme® NP is safe at the maximum recommended dose of 3000 FYT kg^{-1} feed for chickens and turkeys for fattening, piglets and pigs for fattening and of 1500 FYT kg^{-1} feed for laying hens. The FEEDAP Panel considers that these results can be extrapolated to all poultry, considering that the maximum dose should not exceed 3000 FYT kg^{-1} feed for growing birds and 1500 FYT kg^{-1} feed for laying birds.

Ronozyme® NP (M) is assumed to be a dermal and eye irritant as well as a respiratory sensitiser, thus appropriate user safety precautions are required.

No risk for the environment is expected and no further environmental risk assessment is required.

[29] OJ L 35, 8.2.2005, p.1

REFERENCES

EFSA (European Food Safety Authority), 2008. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of the product Ronozyme® NP (6-phytase) for chickens for fattening. http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902199809.htm

DOCUMENTATION PROVIDED TO EFSA

1. Ronozyme® NP as feed additive for poultry, piglets and pigs for fattening. February 2008. Submitted by DSM Nutritional Products.
2. Supplementary information for Ronozyme® NP as feed additive for poultry, piglets and pigs for fattening. Submitted by DSM Nutritional Products on January 2009.
3. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for Ronozyme NP (CT, L, M).
4. Comments from Member States received through the ScienceNet.

APPENDIX

Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for Ronozyme NP (CT, L, M)

In the current application authorisation is sought for Ronozyme NP (CT, L, M) under the category "zootechnical additives", functional groups 4(a) and 4(c), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought to use this product as a digestibility enhancer for *poultry, piglets (weaned) and pigs for fattening*, and as a substance which favourably affects the environment.

The active agent of Ronozyme NP (CT, L, M) is 6-phytase (EC 3.1.3.26), produced by *Aspergillus oryzae* (DSM 17594). The activity of 6-phytase is expressed in FYT (phytase) units. According to the applicant, one FYT unit is the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH = 5.5 and 37^{o}C. The product is intended to be placed on the market as a coated thermo tolerant granulate formulation (Ronozyme NP (CT)) containing 10000 FYT/g of *product*, as a granulate formulation (Ronozyme NP (M)) containing 50000 FYT/g of *product* and as a liquid formulation (Ronozyme NP (L)) containing 20000 FYT/g of *product*. The product is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain a minimum enzyme activity level of 600 FYT/kg of *feedingstuffs* for laying hens, 1000 FYT/kg of *feedingstuffs* for piglets (weaned) and pigs for fattening and 1500 FYT/kg of *feedingstuffs* for poultry excluding laying hens.

For the determination of the activity of 6-phytase in the *feed additive* and *premixtures*, the applicant submitted an in-house validated colorimetric method, based on the release by the 6-phytase of inorganic phosphate during the hydrolysis of sodium phytate at pH = 5.5 and 37^{o}C. The released phosphate forms with molybdate and vanadate ions a coloured complex that is measured at 405 or 415 nm and quantified against a phosphate curve. The content of endogenous phosphate - present in the samples and not related to the phytase activity - is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement.

For the determination of the enzyme activity of 6-phytase in the *feed additive*, the applicant submitted two protocols, which differ in terms of the equipment used - robot versus conventional instruments. Since both methods show comparable performance characteristics, the CRL recommends for official control the use of the method requiring conventional instruments, easily available in official feed laboratories.

The method for the determination of the enzyme activity in *premixtures* is similar to the corresponding method for the analysis of *feedingstuffs*. The method was validated on two different premixtures at the activity range of 80000 to 1700000 FYT/kg of *premixture*. The following performance characteristics were reported: (1) a relative standard deviation for repeatability (RSD_r) ranging from 1.2 to 5.1%, (2) a relative standard deviation for intermediate precision (RSD_R) ranging from 2.4 to 4.2% and (3) a recovery rate ranging from 95 to 99%. Based on these acceptable performance characteristics the method is considered to be suitable for official control at the target activity ranges.

For the determination of the 6-phytase activity in *feedingstuffs*, the applicant submitted the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA). This method is currently under evaluation to become a CEN (European Committee for Standardisation) and ISO (International Organisation Standardization) standard. This method is similar to the one for the determination of the phytase activity in the *feed additive*. The method was ring trial validated covering a phytase

activity from 500 to 1500 FYT/kg of *feedingstuffs* on various feed samples including different phytase products such as Ronozyme P. The performance characteristics obtained were: (1) a RSD_r of 10%, (2) a relative standard deviation for between-laboratory reproducibility of 12% and (3) a limit of detection (LOD) and limit of quantification (LOQ) of 20 and 60 FYT/kg of *feedingstuffs*, respectively. Both limits are well below of the minimum enzyme activity level of 600 FYT/kg proposed by the applicant. These precision data have been calculated from pooled results of all enzyme products including a feed additive that contained the specific enzyme of the present application. Based on the acceptable method performance characteristics the CRL recommends this method for official controls to determine the activity of 6-phytase in *feedingstuffs* at the target activity levels.

Further testing or validation is not considered necessary.