Animal feeding stuffs and per se

Determination of Ronozyme® RumiStar alpha-amylase activity

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Introduction

This Method has been developed to quantify Ronozyme® RumiStar alpha-amylase in concentrated feeding stuffs and per se samples. However, the method cannot be used to evaluate the in vivo efficacy of Ronozyme® RumiStar.

Animal feeding stuffs and per se

Determination of Ronozyme® RumiStar alpha-amylase activity

1. Scope

This Method describes the determination of Ronozyme® RumiStar alpha-amylase activity in concentrated feeding stuffs and per se samples.
The method does not distinguish between Ronozyme® RumiStar alpha-amylase added as a feed additive and other alpha-amylases (e.g. endogenous alpha-amylase) already present in the feed materials.
The method cannot be used to evaluate or compare the *in vivo* efficacy of Ronozyme® RumiStar product. It is not a predictive method of the *in vivo* efficacy of amylases present on the market as they can develop different *in vivo* efficacy per unit of activity obtained with the method described.

The method is suitable and validated exclusively for the determination of Ronozyme® RumiStar alpha-amylase and exclusively in concentrate feeding stuffs and per se.

NOTE 1 The method was developed on the basis of Ronozyme® RumiStar alpha-amylase. Therefore, it might not necessarily be suitable as such for alpha-amylase products presently on the market or which might be developed in the future. The method is thus a tool which is useful only to evaluate the total Ronozyme® RumiStar alpha-amylase in concentrated feeding stuffs or per se samples.

2. Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1. alpha-Amylase unit (KNU)

One KNU is the amount of enzyme that releases in a two step reaction, Ronozyme® RumiStar / α-glucosidase, 6 µmol p-nitrophenol per minute from 1.86 mM ethylidene-G_7-p-nitrophenyl-maltoheptaoside at pH 7.0 and 37 °C.

3. Principle

Incubation of the substrate Red Starch with Ronozyme® RumiStar alpha-amylase depolymerizes the substrate by an *endo*-mechanism to produce low-molecular weight dyed fragments. The low-molecular weight dyed fragments remain in solution after addition of ethanol to the reaction mixture, whereas the high molecular weight material is precipitated. The high molecular weight material is removed by centrifugation and the clear colored supernatant is measured at 510 nm.
The alpha-amylase in the assay solution is quantified with an alpha-amylase standard curve made with a certified alpha-amylase standard for Ronozyme® RumiStar.

4. Chemicals and Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

WARNING This method requires the handling of hazardous substances. It is recommended to use various regulations for potentially hazardous chemicals. Organizational, technical and personal safety has to be observed.

4.1. Ronozyme® RumiStar standard (lot 97-12102; 103.9 KNU / g)

4.2. Hydrochloric acid 25%, p.a.; HCl

4.3. Sodium dihydrogen phosphate dihydrate, p.a.; $NaH_2PO_4 \cdot 2\ H_2O$

4.4. RED-Starch, (Megazyme S-RSTAR)

4.5. Imidazole, p.a.; $C_3H_4N_2$

4.6. Tween 20

4.7. Sodium hydroxide, p.a.; NaOH

4.8. Ethanol absolute p.a.; C_2H_5OH

4.9. RED-Starch substrate solution, 1%, pH 5.0

Dissolve completely 2.0 g of RED-starch in approximately 160 ml distilled water (60°C to 65°C) and cool to room temperature. Add 20ml sodium phosphate buffer (4.14), adjust the pH to 5.0 with 25% hydrochloric acid and fill up to 200 ml with distilled water. Store in the refrigerator. The maximum storage time is 3 months.

4.10. Sodium hydroxide, 5M

Dissolve 100.0 g sodium hydroxide [NaOH] in approximately 400 ml distilled water and cool to room temperature. Fill up to 500 ml with distilled water. Store at room temperature. The maximum storage time is 6 months.

4.11. Tween 20, 10%

Dissolve 10.0 g of Tween 20 with distilled water and fill up to 100 ml. Store at room temperature. The maximum storage time is 6 months.

4.12. Imidazole buffer with 0.02% Tween 20, pH 8.0; 0.1 mol/l

Dissolve 34.0 g imidazole [$C_3H_4N_2$] in approximately 4,8 l distilled water, adjust the pH to 8.00 ± 0.02 with hydrochloric acid (25%) and add 10 ml 10 % Tween 20 (4.11) Fill up to 5 l with distilled water. Store at room temperature. The maximum storage time is 2 weeks.

4.13. Sodium phosphate buffer with 0.02% Tween 20, pH 5.0; 0.1 mol/l

Dissolve 78.0 g sodium dihydrogen phosphate dihydrate [$NaH_2PO_4 \cdot 2\ H_2O$] in approximately 4.8 l distilled water. Adjust the pH with 5 M sodium hydroxide (4.10) to 5.00 ± 0.02 and add 10 ml 10 % Tween 20 (4.11) Fill up to 5 l with distilled water. Store at room temperature. The maximum storage time is 2 weeks.

4.14. Sodium phosphate buffer, pH 5.0; 1.0 mol/l

Dissolve 78.0 g sodium dihydrogen phosphate dihydrate [$NaH_2PO_4 \cdot 2\ H_2O$] in approximately 450 ml distilled water. Adjust the pH with 5 M sodium hydroxide (4.10) to 5.00 ± 0.02 and fill up to 500 ml with distilled water. Store at room temperature. The maximum storage time is 2 weeks.

4.15. Ethanol STOP and PRECIPITATION reagent, 95%

50 ml distilled water are transferred into a 1 liter volumetric flask and filled up with ethanol absolute. Store at room temperature. The maximum storage time is 6 months.

4.16. Ronozyme® RumiStar stock standard solution

Weigh about 100.0 mg of Ronozyme® RumiStar standard Lot 97-12102 transfer it quantitatively to a 50 ml volumetric flask and dissolve it in 50 ml phosphate buffer with Tween 20 (4.13). Add a magnetic stir bar and stir for 30 – 60 minutes at room temperature. Calculate the exact concentrations of the Ronozyme® RumiStar stock standard solution. The maximum storage time is 1 day.

5. Apparatus

Usual laboratory apparatus, in particular, the following.

5.1 **Water bath**, thermostatically controlled (with inserts for 2 ml tubes).

5.2 **Water bath**, thermostatically controlled (with 4 remote controlled magnetic stirring systems – e.g. TELESYSTEM 06.40, VARIOMAG)

5.3 **pH-meter**, with two decimal digital readout.

5.4 **Magnetic stirrers** (≥ 20 W power)

5.5 **Egg shaped stirring bars** (40 mm x 20 mm)

5.6 **Analytical balance**, sensitivity 0.01 mg.

5.7 **Balance**, sensitivity 0.01 g.

5.8 **Vortex mixer**

5.9 **Centrifuge for 2 ml microcentrifuge tubes**, capable of 11 000-20 000 g.

5.10 **Electronic dispenser**

5.11 **Pipettes (electronic and manual)**, in the range 10 µl to 2 000 µl.

5.12 **Microplate spectrophotometer**

5.13 **Microcentrifuge tubes**, 2 ml.

6. Sampling

A representative sample should be used. It should not have been damaged or changed during transport or storage. Sampling is not part of the method specified. A recommended sampling procedure is given in ISO 6497 [1].

7. Sample preparation

Two weighings are performed for each sample.

7.1 Concentrated feed

Two portions of pellets or mash, of about 50 g each, are weighed into 500 ml conical flasks. 500 ml imidazole buffer with Tween 20 (4.12) are added to the concentrated feed and the mixture is stirred (550 – 650 rpm) on a magnetic stirrer (5.4) for 90 minutes at 50°C with egg shaped stirring bars (5.5). 2 ml of the concentrated feed extract are transferred to a tube (5.13) and centrifuged for 3 minutes in a centrifuge (11'000 to 20'000 g).

NOTE Inhomogeneity in the concentrated feed sample can lead to high CVs. For feed samples showing CVs > 15%: such inhomogeneity can derive from inhomogeneous particle size distribution in products or inhomogeneous feed preparation.

7.2 Solid Ronozyme® RumiStar samples

Two weighings of 0.500 – 1.000 g solid Ronozyme® RumiStar sample are quantitatively transferred to 100 ml conical flasks. 100 ml phosphate buffer with Tween (4.13) are added and the samples are stirred on a magnetic stirrer for 45 – 60 minutes at room temperature with stirring bars. 2 ml sample extract are transferred to a tube (5.13) and centrifuged for 3 minutes in a centrifuge (11 000 to 20 000 g).

7.3 Liquid Ronozyme® RumiStar samples

Two portions of 0.500 – 1.000 g liquid Ronozyme® RumiStar sample are transferred to 100 ml volumetric flasks. The samples are dissolved in phosphate buffer with Tween 20 (4.13) and filled up to 100 ml. The samples are stirred on a magnetic stirrer for 45 – 60 minutes at room temperature with stirring bars.

7.4 Dilution of Ronozyme® RumiStar extracts

Feed extracts have to be at least 10 fold diluted with phosphate buffer (4.13).

The extracts are diluted with phosphate buffer with Tween 20 (4.13) to a final concentration of 0.00025 $KNUml^{-1}$ to 0.00045 $KNUml^{-1}$.

Blank feed extracts are 1:10 diluted.

Dilution example

The expected activity in a concentrated feed sample is 300 $KNUkg^{-1}$ and the final desired concentration is approximately 0.0003 $KNUml^{-1}$.

$$\text{Dilution factor} = \frac{300KNU \times 0.05kg \times ml}{kg \times 500ml \times 0.0003KNU} = 100$$

0.05 kg = weight of the sample; 500 ml = extraction volume

The feed extract should be 1:100 diluted for the enzymatic reaction.

8. Procedure

8.1 Ronozyme® RumiStar standard

The Ronozyme® RumiStar stock standard solution (4.16) is diluted with phosphate buffer with Tween 20 (4.13) according to Table 1.

Table 1 — Dilution steps Ronozyme® RumiStar standard curve

Standard	Parts Stock Solution (4.16)	Parts Buffer (4.13)	Dilution Factor	KNU / ml[a]
A	1	249	250	0.00083
B	1	499	500	0.00042
C	1	749	750	0.00028
D	1	999	1000	0.00021

[a] The exact concentrations have to be calculated (4.16)

8.2 Ronozyme® RumiStar standard curve

For each sample blanks are included. For calculation of Ronozyme® RumiStar alpha-amylase activity, the blank values are subtracted from the sample values.
Triple determinations and two blanks are performed for each dilution. The procedure is described in Table 2.

Description of the assay: 200 µl diluted standard sample (8.1) are pipetted into a 2 ml tube (5.13). The sample is pre-incubated for 5 min at 40°C. 0.4 ml pre-heated (40°C) Red-Starch substrate (4.9) is added. The sample is mixed and incubated for exactly 60 min at 40°C. After 60 min 1 ml STOP and PRECIPITATION reagent (4.15) is added. The sample is mixed and kept for 15 min at room temperature. The sample is mixed again and then centrifuged for 3 min at 11 000 - 20 000 g. The clear supernatant is measured at 510 nm.
Blanks are NOT incubated at 40°C and the STOP and PRECIPITATION reagent (4.15) –STEP 1- is added prior to the Red-Starch substrate (4.9) –STEP 2.

8.3 Concentrated feed samples

Triple determinations and two blanks are performed for each extraction (7.1). The procedure is described in Table 2.
Description of the assay: 200 µl properly diluted feed extract (7.1 and 7.4) are pipetted into a 2 ml tube (5.13). The sample is pre-incubated for 5 min at 40°C. 0.4 ml pre-heated (40°C) Red-Starch substrate (4.9) is added. The sample is mixed and incubated for exactly 60 min at 40°C. After 60 min 1 ml STOP and PRECIPITATION reagent (4.15) is added. The sample is mixed and kept for 15 min at room temperature. The sample is mixed again and then centrifuged for 3 min at 11 000 - 20 000 g. The clear supernatant is measured at 510 nm.
Blanks are NOT incubated at 40°C and the STOP and PRECIPITATION reagent (4.15) –STEP 1- is added prior to the Red-Starch substrate (4.9) –STEP 2.

8.4 Solid Ronozyme® RumiStar samples

Triple determinations and two blanks are performed for each extraction (7.2). The procedure is described in Table 2.
Description of the assay: 200 µl properly diluted extract from solid Ronozyme® RumiStar (7.2 and 7.4) are pipetted into a 2 ml tube (5.13). The sample is pre-incubated for 5 min at 40°C. 0.4 ml pre-heated (40°C) Red-Starch substrate (4.9) is added. The sample is mixed and incubated for exactly 60 min at 40°C. After 60 min 1 ml STOP and PRECIPITATION reagent (4.15) is added. The sample is mixed and kept for 15 min at room temperature. The sample is mixed again and then centrifuged for 3 min at 11 000 - 20 000 g. The clear supernatant is measured at 510 nm.
Blanks are NOT incubated at 40°C and the STOP and PRECIPITATION reagent (4.15) –STEP 1- is added prior to the Red-Starch substrate (4.9) –STEP 2.

8.5 Liquid Ronozyme® RumiStar samples

Triple determinations and two blanks are performed for each extraction (7.3). The procedure is described in Table 2.

Description of the assay: 200 µl properly diluted extract from liquid Ronozyme® RumiStar (7.3 and 7.4) are pipetted into a 2 ml tube (5.13). The sample is pre-incubated for 5 min at 40°C. 0.4 ml pre-heated (40°C) Red-Starch substrate (4.9) is added. The sample is mixed and incubated for exactly 60 min at 40°C. After 60 min 1 ml STOP and PRECIPITATION reagent (4.15) is added. The sample is mixed and kept for 15 min at room temperature. The sample is mixed again and then centrifuged for 3 min at 11 000 - 20 000 g. The clear supernatant is measured at 510 nm.

Blanks are NOT incubated at 40°C and the STOP and PRECIPITATION reagent (4.15) –STEP 1- is added prior to the Red-Starch substrate (4.9) –STEP 2.

Table 2 — Procedure Ronozyme® RumiStar samples

Assay steps	Standard and samples	Blank
Diluted extract	200 µl	200 µl
Pre-incubation at 40 °C	5 min	NO
RED-Starch substrate, 40 °C (4.9)	0.4 ml	0.4 ml (STEP 2)
Mix	YES	YES
Incubation at 40 °C	60 min	NO
STOP and PRECIPITATION reagent (4.15)	1 ml	1 ml (STEP 1)
Mix	YES	YES
Room temperature	15 min	15 min
Mix	YES	YES
Centrifugation	3 min 11 000 – 20 000 g	3 min 11 000 – 20 000 g

9. Calculations

9.1 Formulation standard curve

A standard curve is made with the ΔOD 510 ($OD\ 510_{standard}$ – $OD\ 510_{blank}$) obtained with the Ronozyme® RumiStar standards (8.1 and 8.2) on the y-axis and the calculated alpha-amylase activity on the x-axis. The best fitting line is calculated by linear regression, y = *m*x, (set intercept at 0) as shown in Figure 1.

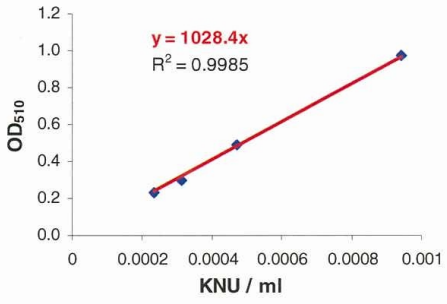

Figure 1 — Ronozyme® RumiStar standard (Lot 97-12102)

9.2 Calculation alpha-amylase activity

The alpha-amylase activity ($a_{\alpha\text{-amylase}}$) is calculated as follows:

$$a_{\alpha\text{-amylase}} = \frac{\Delta OD \times D}{m \times W}$$

where

ΔOD is OD 510_{sample} – OD 510_{blank}

m is the slope of the standard curve, in KNU^{-1} ml OD 510

D is the dilution factor (extraction volume x dilution of the extract), in ml

W is the weight of the sample, in g resp. kg

9.3 Examples

9.3.1 Example 1 – Concentrated feed

ΔOD = 0.549 OD_{510}

m = 1028.4 KNU^{-1} ml OD_{510}

D = 40 000 ml (500 ml extraction volume x 80 [1:80 dilution of extract])

W = 0.050 kg

$$a = \frac{0.549 \times 40\,000}{1028.4 \times 0.05} = 427\ KNU kg^{-1}$$

9.3.2 Example 2 –Solid Ronozyme® RumiStar samples

ΔOD = 0.573 OD_{510}

m = 1028.4 KNU^{-1} ml OD_{510}

D = 600 000 ml (100 ml extraction volume x 6000 [1:6000 dilution of extract])

W = 0.987 g

$$a = \frac{0.449 \times 600000}{1028.4 \times 0.987} = 265\ KNU g^{-1}$$

9.4 Remarks

- It is recommended to use nitrile gloves and plastic spoons when handling with imidazole hydrochloride.
- Components in feed extracts may alter the Ronozyme® RumiStar alpha-amylase activity. Therefore, **all concentrated feed extracts have to be at least 1:10 diluted with phosphate buffer (4.13)**. Blank concentrated feed extracts have to be 1:10 diluted.

10. Precision

10.1 Limit of Detection and Limit of Quantification

The determination of the Detection Limit ($L_D = 3\sigma$) and Quantification Limit ($L_Q = 10\sigma$) are according to the IUPAC nomenclature [2] and are given as ΔOD_{510}.

Detection Limit	L_D = 0,018 = 2 KNU / kg feed
Quantification Limit	L_Q = 0,061 = 6 KNU / kg feed

10.2 Intralaboratory test

The values derived from this intralaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.3 Repeatability

The relative standard deviation of repeatability (RSD_r) is the average coefficient of variation from two independent results obtained from the same sample at the same day, from the same technician, with the same equipment and method.

The relative standard deviation of repeatability (RSD_r) is estimated to 4% for feed mash and feed pellets and 2% for per se CT and liquid samples.

10.4 Reproducibility

The relative standard deviation of reproducibility (RSD_R) is the average coefficient of variation from results obtained with the same sample using the same method but measured at different days.

The relative standard deviation of reproducibility (RSD_R) is estimated to 6% for feed mash and feed pellets and 4% for per se CT and liquid samples.

Bibliography

[1] ISO 6497, Animal feeding stuffs — Sampling

[2] Nomenclature in Evaluation of Analytical Methods Including Detection and Quantification Capabilities. Pure & Appl. Chem., Vol. 67, No. 10, pp. 1699-1723 (1995)