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Animal feeding stuffs — Determination of phytase activity

Aliments des animaux — Détermination de l'activité phytasique

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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Introduction

This International Standard has been developed to quantify phytase products in feed samples to enable the European Commission to control the phytase content of animal feed products. However, the method cannot be used to evaluate the *in vivo* efficacy of the phytase products.

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Animal feeding stuffs — Determination of phytase activity

1 Scope

This International Standard specifies the determination of phytase activity in feed samples.

The method does not distinguish between phytase added as a feed additive and endogenous phytase already present in the feed materials.

The method cannot be used to evaluate or compare the *in vivo* efficacy of the phytase product. It is not a predictive method of the *in vivo* efficacy of phytases present on the market as they can develop different *in vivo* efficacy per unit of activity.

The method is suitable and validated exclusively for the determination of phytase activity and exclusively in complete feeds.

NOTE The harmonized method was developed on the basis of the presently existing phytase products [E1600 (EC 3.1.3.8, 3-phytase), E1614 (EC 3.1.3.26, 4-phytase), and E1640 (EC 3.1.3.26, 4-phytase)]. Therefore, it might not necessarily be suitable as such for phytase products that are developed in the future. The harmonized method is thus a tool which is useful only to evaluate the total phytase activity in feed samples.

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2 Terms and definitions

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

2.1
phytase unit
U
amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under the reaction conditions specified in this International Standard

3 Principle

Phytase releases phosphate from the substrate *myo*-inositol hexakisphosphate (phytate). The released inorganic phosphate is determined by forming a yellow complex with an acidic molybdate/vanadate reagent. The optical density (OD) of the yellow complex is measured at a wavelength of 415 nm and the inorganic phosphate released is quantified from a phosphate standard calibration curve.

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4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

WARNING — This method requires the handling of hazardous substances. Observe local regulations for potentially hazardous chemicals to minimize risks to organizational, technical, and personal safety.

4.1 Ammonia solution, 25 % mass fraction; NH_3.

4.2 Ammonium heptamolybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$.

4.3 Ammonium monovanadate, NH_4VO_3.

4.4 Hydrochloric acid, 25 % mass fraction; HCl.

4.5 Nitric acid, 65 % mass fraction; HNO_3.

4.6 Potassium dihydrogenphosphate, KH_2PO_4.

4.7 Phytate, phytic acid, dodecasodium salt, $C_6H_6Na_{12}O_{24}P_6\cdot xH_2O$, from rice, Sigma® P0109[1].

4.8 Sodium acetate trihydrate, $CH_3COONa\cdot 3H_2O$.

4.9 Polysorbate 20[2].

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4.10 Dilute nitric acid. Dilute 1 volume nitric acid 65 % mass fraction (4.5) with 2 volumes water. Store at room temperature. The maximum storage time is indefinite.

4.11 Ammonium heptamolybdate reagent. Dissolve 100,0 g ammonium heptamolybdate tetrahydrate (4.2) in approximately 800 ml water. Add 10 ml 25 % mass fraction ammonia solution (4.1) and make up with water to 1 000 ml. Store at room temperature in the dark. The maximum storage time is 2 months.

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4.12 Ammonium vanadate reagent. Dissolve completely 2,35 g of ammonium monovanadate (4.3) in approximately 400 ml water (50 °C to 60 °C). Add 20 ml dilute nitric acid (4.10) and make up with water to 1 000 ml. Store at room temperature in the dark. The maximum storage time is 2 months.

4.13 Molybdate/vanadate STOP reagent. Mix 1 volume ammonium vanadate reagent (4.12) with 1 volume ammonium heptamolybdate reagent (4.11) and add 2 volumes dilute nitric acid (4.10). Mix and store at room temperature. The maximum storage time is 1 day.

4.14 Polysorbate 20, 10 % mass fraction. Dissolve 10,0 g of polysorbate 20 (4.9) with water and make up to 100 ml. Store at room temperature. The maximum storage time is 6 months.

4.15 Acetate buffer, pH 5,5; 0,25 mol/l. Dissolve 34,0 g of sodium acetate trihydrate (4.8) in approximately 900 ml water. Adjust the pH with 25 % mass fraction hydrochloric acid (4.4) to 5,50 ± 0,02 and make up to 1 000 ml with water. Store at room temperature. The maximum storage time is 2 weeks.

4.16 Acetate buffer with 0,01 % mass fraction polysorbate 20, pH 5,5; 0,25 mol/l. Dissolve 34,0 g of sodium acetate trihydrate (4.8) in approximately 900 ml water. Adjust the pH with 25 % mass fraction hydrochloric acid (4.4) to 5,50 ± 0,02. Add 1 ml 10 % mass fraction polysorbate 20 (4.14) and make up to 1 000 ml with water. Store at room temperature. The maximum storage time is 2 weeks.

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1) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Tween 20 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

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4.17 Acetate buffer with 0,01 % mass fraction polysorbate 20, pH 5,5; 0,50 mol/l. Dissolve 68,0 g of sodium acetate trihydrate (4.8) in approximately 900 ml water. Adjust the pH with 25 % mass fraction hydrochloric acid (4.4) to 5,50 ± 0,02. Add 1 ml 10 % mass fraction polysorbate 20 (4.14) and make up to 1 000 ml with water. Store at room temperature. The maximum storage time is 2 weeks.

4.18 Phytate substrate solution, 7,5 mmol/l (5 mmol/l end-concentration in the reaction). Dissolve 2,00 g of dodecasodium phytate (4.7) whose inorganic phosphorus content is ⩽ 0,1 % mass fraction (see 9.3) in approximately 200 ml acetate buffer (4.15). Adjust the pH with 25 % mass fraction hydrochloric acid (4.4) to 5,50 ± 0,02 and make up with acetate buffer (4.15) to 250 ml. The maximum storage time is 2 weeks at 4 °C.

4.19 Phosphate stock standard solution, 50 mmol/l. Dry approximately 10 g of potassium dihydrogenphosphate (4.6) at 105 °C for 2 h and store it in a dessicator. Weigh approximately 682 mg of dried potassium dihydrogenphosphate, transfer it quantitatively to a 100 ml volumetric flask and make up to 100 ml with 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (4.16). Calculate the exact concentration of the phosphate stock standard solution. Store at room temperature. The maximum storage time is 2 weeks.

4.20 Phytase stock standard solution. Weigh 100,0 mg to 300,0 mg of a certified phytase standard, transfer it quantitatively to a 100 ml volumetric flask and dissolve it in 100 ml 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (4.16). Stir it for 15 min to 45 min. Store at room temperature. The maximum storage time is 1 day.

5 Apparatus

Usual laboratory apparatus, in particular, the following.

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

5.2 pH-meter, capable of being read to at least two places of decimals.

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5.3 Magnetic stirrers (⩾ 20 W power).

5.4 Egg-shaped stirring bars (40 mm × 20 mm).

5.5 Analytical balance, capable of being read to at least 0,1 mg.

5.6 Balance, capable of being read to at least 0,01 g.

5.7 Vortex mixer.

5.8 Centrifuge for microcentrifuge tubes (5.12), capable of 11 000g to 20 000g.

5.9 Electronic dispenser.

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

5.11 Spectrophotometer double beam or microplate reader.

5.12 Microcentrifuge tubes, capacity 2 ml.

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6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling procedure is given in ISO 6497 [1].

7 Sample preparation

Perform two weighings for each sample.

Weigh two portions of pellets or mash, of about 50 g each, into 500 ml conical flasks. Add 500 ml water and 0,5 ml of 10 % mass fraction polysorbate 20 (4.14) to the feed and mix vigorously for 45 min on a magnetic stirrer (5.3) with egg-shaped stirring bars (5.4). Transfer 2 ml of the feed extract to a microcentrifuge tube (5.12) and centrifuge (5.8) for 3 min at 11 000g to 20 000g.

Inhomogeneity in the sample can lead to high coefficients of variation (CVs). For feed samples showing CVs > 15 %, such inhomogeneity can derive from inhomogeneous particle size distribution in products or inhomogeneous feed preparation. If feed samples show high CVs, grind the feed samples using an Ultra centrifugal mill[3] with a sieve of nominal size of openings 1 mm. Grind 150 g feed and extract as described in this clause.

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8 Procedure

8.1 Blank solutions

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Inorganic phosphate in the sample contributes to colour formation. Therefore, blanks are included for each sample. For calculation of phytase activity, subtract blank values from the test values.

8.2 Standards

8.2.1 Phosphate standard solutions

The phosphate stock standard solution (4.19) is diluted with 0,25 mol/l acetate buffer containing 0,01 % mass fraction polysorbate 20 (4.16) according to Table 1.

Table 1 — Dilution steps to obtain standard colorimetric solutions for the phosphate curve

Standard solution	Volumes phosphate stock standard solution (4.19)	Volumes 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (4.16)	Dilution factor	Concentration µmol/ml [a]
A	1	1	2	25
B	1	3	4	12,5
C	1	7	8	6,25
D	1	15	16	3,125

[a] Calculate the exact concentrations (4.19).

3) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

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8.2.2 Phytase level control

For each incubation of samples, a phytase level control is included. A phytase stock standard solution (4.20) with known activity is diluted to a final activity of 0,15 U/ml to 0,25 U/ml and the exact activity is determined as specified in 8.4.

8.3 Standard curve

Perform three determinations for each phosphate dilution and two blanks, and average the results. The procedure is specified in Table 2.

For the phosphate standard solutions, pipette 360 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (4.16) into a 2 ml tube (5.12). Add 40 µl phosphate standard solution (Table 1).

For the phosphate standard blanks, pipette 400 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (4.16) into a 2 ml tube (5.12).

In both cases, add 0,8 ml phytate substrate solution (4.18) and 0,8 ml STOP reagent (4.13). Mix the contents of the tubes and maintain them for 10 min at room temperature. Centrifuge (5.8) the tubes and their contents for 3 min at 11 000g to 20 000g and measure the OD of the clear supernatant at 415 nm, D(415).

Table 2 — Procedure for standard curve

Assay steps	Standard colorimetric solutions	Blank
Acetate buffer 0,25 mol/l with 0,01 % mass fraction polysorbate 20 (4.16)	360 µl	400 µl
Phosphate standard solution (8.2.1)	40 µl	0 µl
Phytate substrate solution (4.18)	0,8 ml	0,8 ml
STOP reagent (4.13)	0,8 ml	0,8 ml
Mix	Yes	Yes
Room temperature	10 min	10 min
Centrifugation	3 min at 11 000g to 20 000g	3 min at 11 000g to 20 000g
Spectrophotometer (5.11)	415 nm (against water)	415 nm (against water)

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8.4 Phytase level control

Perform three determinations for each dilution and two blanks, and average the results. The procedure is specified in Table 3.

For the phytase level control determination solutions, pipette 360 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (4.16) into a 2 ml tube (5.12). Add 40 µl dilute phytase level control solution (8.2.2). Mix the sample. Pre-incubate the solutions for 5 min at 37 °C. Add 0,8 ml phytate substrate solution (4.18) preheated to 37 °C. Incubate for exactly 30 min at 37 °C. After 30 min, add 0,8 ml STOP reagent (4.13) and mix. Maintain the solutions for 10 min at room temperature and then centrifuge them for 3 min at 11 000g to 20 000g. Measure the D(415) of the clear supernatant.

For the phytase level control blank solutions, pipette 360 µl acetate buffer (4.15) into a 2 ml tube (5.12). Add 40 µl dilute phytase level control solution (8.2.2). The order of addition of solutions differs from that used for the determinations. Pre-incubate blanks for 5 min at 37 °C. Then, as step 1, add STOP reagent (4.13); as step 2, add phytate substrate solution (4.18) preheated to 37 °C. Then proceed with Table 3, column 3, row 9, onwards.

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