International Standard

ISO 30024

Animal feeding stuffs — Determination of phytase activity

Alimentation animale — Détermination de l'activité phytasique

Second edition
2024-04

Reference number
ISO 30024:2024(en)

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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 327, *Animal feeding stuffs - Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 30024:2009), which has been technically revised.

The main changes are as follows:

— the scope has been extended to include complementary compound feeds, mineral feeds, premixtures and feed additives;

— phytic acid (phytate substrate) specifications have been added.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document quantifies phytase products in feeding stuff samples 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.

Animal feeding stuffs — Determination of phytase activity

1 Scope

This document specifies the determination of phytase activity in feeding stuff samples, including feed raw materials from plant origin, compound feeds (complete, complementary, mineral feeds), premixtures and feed additives.

The method is applicable to, and is collaboratively validated for, the determination of phytase activity in complete feed, complementary feed including mineral feed, premixtures and feed additives.

The method does not distinguish between phytase added as a feed additive and endogenous phytase already present in the feed materials. Therefore, the method is also applicable for feed materials from plant origin.

The method does not apply to evaluating or comparing 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.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
phytase unit
U
amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute in acetate buffer at pH 5,5 and 37 °C

Note 1 to entry: This is determined by the reaction conditions specified in this document.

3.2
premixture
premix
uniform mixture of one or more micro-ingredients/*feed additives* (3.4) with a diluent and/or carrier, and that is not intended for direct feeding to animals

Note 1 to entry: Premixtures are used to facilitate the uniform dispersion of the micro-ingredients/additives in a larger mix.

[SOURCE: ISO 20588:2019, 3.2.39]

3.3
mineral feed
mineral mix
mineral supplement
feed that mainly consists of mineral elements, which is as an entire mix free-flowing

Note 1 to entry: In European legislation, a mineral feed contains at least 40 % of crude ash.

Note 2 to entry: to entry : Mineral feed is a form of *compound feed* (3.5) and of complementary feed (see Note 1 to entry of 3.5).

[SOURCE: ISO 20588:2019, 3.2.37, modified — "mineral feed" replaced "mineral mix" as the preferred term. Notes to entry added.]

3.4
feed additive
substance intentionally added to feed and/or water, not consumed as feed by itself, whether or not it has a nutritional value, that affects the characteristics of feed including organoleptic properties, animal products, animal production or performance or welfare, or the environment

Note 1 to entry: Microorganisms, enzymes, acidity regulators, trace elements, vitamins and other products fall within the scope of this definition, depending on the purpose of use and the method of administration.

Note 2 to entry: Coccidiostats and histomonostats are a category of feed additives.

Note 3 to entry: Feed additive does not include *feed materials* (3.6) and *premixtures* (3.2).

[SOURCE: ISO 20588:2019, 3.2.18]

3.5
compound feed
formula feed
feed mixture
mixture of at least two *feed materials* (3.6), whether or not containing *feed additives* (3.4), for oral animal feeding in the form of a complementary feed or a complete feed

Note 1 to entry: Complementary feed is a form of compound feed as defined in ISO 20588:2019, 3.2.9.

Note 2 to entry: Complete feed is a form of compound feed as defined in ISO 20588:2019, 3.2.10.

[SOURCE: ISO 20588:2019, 3.2.11]

3.6
feed materials
products of vegetable or animal origin, whether or not containing *feed additives* (3.4), that are intended for use in oral animal feeding to meet animals' nutritional needs

Note 1 to entry: Feed materials can be in their natural state, fresh or preserved, or products derived from industrial processing, either organic or inorganic substances.

Note 2 to entry: Feed materials may be fed to animals either directly as such, or after processing, or in the preparation of *compound feed* (3.5), or as carrier of *premixtures* (3.2).

[SOURCE: ISO 20588:2019, 3.2.23, modified — "and products derived from industrial processing, either organic or inorganic substances" moved from the definition to Note 1 to entry.]

4 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.

5 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. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

5.1 Ammonia solution, 25 % mass fraction; NH_3.

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

5.3 Ammonium monovanadate, NH_4VO_3.

5.4 Hydrochloric acid, 25 % mass fraction; HCl.

5.5 Nitric acid, 65 % mass fraction; HNO_3.

5.6 Potassium dihydrogenphosphate, KH_2PO_4.

5.7 Phytate (anionic form of phytic acid), **phytic acid**:

— all forms of phytate or phytic acid are may be used, e.g. phytic acid (PA), phytic acid dodecasodium salt (Na-PA), phytic acid dodecapotassium salt (K-PA), phytic acid hexamagnesium salt n-hydrate (Mg-PA);

— ≤ 0,1 % mass fraction of inorganic phosphorus;

— assay ≥ 90 % phosphorus (P) basis (dry basis);

— the substrate should have a percentage of IP6 (= hexaphosphate inositol containing six phosphate groups) of more than 95.

Information about IP6 ratio or percentage should be available from the supplier upon request.

As phytic acid salt hydrates are supplied with different contents of crystallization water, ensure that the crystallization water is in the stoichiometric range of 10 mol to 13 mol. In cases of deviation, see 10.3.

For control of the phytate, the blank OD from the standard curve (see 9.4) shall be lower than 0,2. A higher OD value indicates phosphate or phytase contamination of used reagents.

5.8 Sodium acetate trihydrate, $CH_3COONa{\cdot}3H_2O$.

5.9 Polysorbate 20[1].

5.10 Diluted nitric acid.

Dilute one volume of nitric acid 65 % mass fraction (5.5) with two volumes water. Store at room temperature. The maximum storage time is two years.

5.11 Ammonium heptamolybdate reagent.

Dissolve 100,0 g of ammonium heptamolybdate tetrahydrate (5.2) in approximately 800 ml water (hot water at 50 °C to 60 °C may be used to facilitate salt dissolution). Add 10 ml 25 % mass fraction ammonia solution

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

(5.1) and make up with water to 1 000 ml. Store at room temperature in the dark. The maximum storage time is two months.

5.12 Ammonium vanadate reagent.

Dissolve completely 2,35 g of ammonium monovanadate (5.3) in approximately 400 ml of water (hot water at 50 °C to 60 °C may be used to facilitate salt dissolution). Add 20 ml diluted nitric acid (5.10) and make up with water to 1 000 ml. Store at room temperature in the dark. The maximum storage time is two months.

5.13 Molybdate/vanadate STOP reagent.

Mix one volume of ammonium vanadate reagent (5.12) with one volume of ammonium heptamolybdate reagent (5.11) and add two volumes diluted nitric acid (5.10). Mix and store at room temperature. The maximum storage time is one day.

5.14 Polysorbate 20, 10 % mass fraction.

Dissolve 10,0 g of polysorbate 20 (5.9) with water and make up to 100 ml. Store at room temperature. The maximum storage time is six months.

5.15 Acetate buffer, pH 5,5; 0,25 mol/l.

Dissolve 34,0 g of sodium acetate trihydrate (5.8) in approximately 900 ml water. Adjust the pH with 25 % mass fraction hydrochloric acid (5.4) to 5,50 ± 0,02 and make up to 1 000 ml with water. Store at room temperature. The maximum storage time is two weeks.

5.16 Acetate buffer with 0,01 % mass fraction polysorbate 20, pH 5,5; 0,25 mol/l.

Dissolve 34,0 g of sodium acetate trihydrate (5.8) in approximately 900 ml water. Adjust the pH with 25 % mass fraction hydrochloric acid (5.4) to 5,50 ± 0,02. Add 1 ml 10 % mass fraction polysorbate 20 (5.14) and make up to 1 000 ml with water. Store at room temperature. The maximum storage time is two weeks.

5.17 Acetate buffer with 0,01 % mass fraction polysorbate 20, pH 5,5; 0,50 mol/l.

Dissolve 68,0 g of sodium acetate trihydrate (5.8) in approximately 900 ml water. Adjust the pH with 25 % mass fraction hydrochloric acid (5.4) to 5,50 ± 0,02. Add 1 ml 10 % mass fraction polysorbate 20 (5.14) and make up to 1 000 ml with water. Store at room temperature. The maximum storage time is two weeks.

5.18 Phytate substrate solution, 7,5 mmol/l (3 mmol/l end-concentration in the reaction).

Dissolve 2,00 g of phytate (5.7) in approximately 200 ml acetate buffer (5.15). Depending of the purity and/or the water content of phytic acid (see 10.3), if necessary, adjust slightly the 2,00 g mass. Adjust the pH to 5,50 ± 0,02, e.g. with 25 % mass fraction hydrochloric acid (5.4), and make up with acetate buffer (5.15) to 250 ml. The maximum storage time is two weeks at 4 °C.

5.19 Phosphate stock standard solution, 50 mmol/l.

Dry approximately 10 g of potassium dihydrogenphosphate (5.6) at 105 °C for 2 h and store it in a desiccator. 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 (5.16). Calculate the exact concentration of the phosphate stock standard solution. Store at 4 °C. The maximum storage time is two weeks.

5.20 Phytase standard, as a quality control sample, with phytase activity not less than 3 500 U/g.

5.21 Phytase stock standard solution.

Weigh 100,0 mg to 300,0 mg of a phytase standard (5.20), transfer it quantitatively to a 100 ml volumetric flask and dissolve it in approximately 80 ml 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16). Stir it for 15 min to 45 min. After removing the magnetic stirrer, fill up to the mark with 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16). The maximum storage time is one day at room temperature, or if aliquoted, the maximum storage time at –18 °C is six months.

5.22 Maize meal

Unprocessed maize grain/broken maize is ground smaller than 1 mm or 2 mm and used as a dilution matrix for the analysis of mineral feeds and premixtures (see 8.2). Conventional maize flour can also be used. The phytase activity in maize meal/flour is in itself negligible, but to exclude it completely and to prevent a significant influence by multiplying the dilutions in the result calculation, heating the meal overnight at 130 °C is recommended.

Equivalent matrix to maize, such as soy protein concentrate, without phytase activity, is possible but should be validated in-house.

6 Apparatus

Usual laboratory apparatus, in particular, the following shall be used.

6.1 Water bath, thermostatically controlled at 37 °C ± 0,2 °C (with inserts for 2 ml tubes).

6.2 pH-meter, capable of being read to at least two decimal places.

6.3 Magnetic stirrers (≥ 20 W power).

6.4 Egg-shaped stirring bars (40 mm × 20 mm) or **cylindrical stirring bars** (60 mm × 10 mm) or equivalent.

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

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

6.7 Vortex mixer.

6.8 Centrifuge, for microcentrifuge tubes (6.12), capable of 11 000*g* to 20 000*g*.

6.9 Electronic dispenser or **mechanical dispenser.**

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

6.11 Spectrophotometer, double beam or microplate reader.

6.12 Microcentrifuge tubes, capacity 2 ml.

7 Sampling and sample preparation

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 document. A recommended sampling procedure is given in ISO 6497.