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Animal feeding stuffs — Determination of phytase activity

Alimentation animale — Détermination de l'activité phytasique

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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.

Sample preparation is not part of the method specified in this document. A recommended sample procedure is given in ISO 6498 with the following clarifications:

— grinding of compound feed and feed raw materials < 1 mm if the $C_{V,r}$ is too high;

— grinding of mineral compound feed and premixture samples < 0,5 mm (see Reference [8]);

— coated feed additive products or products with a larger particle size can be pestled (see Reference [9]).

8 Sample extractions

8.1 For compound feeds (excluding mineral feeds)

Perform two weighings for each sample.

Weigh two portions of pellets or mash compound feed, of about 50 g each, into containers (500 ml conical flasks or 800 ml beakers or equivalent). Add 500 ml water and 0,5 ml of 10 % mass fraction polysorbate 20 (5.14) to the compound feed and mix vigorously for 45 min on a magnetic stirrer (6.3) with stirring bars (6.4).

Inhomogeneity, among other causes, in the sample can lead to high coefficients of variation of repeatability ($C_{V,r}$, see 11.3). For compound feed samples showing high $C_{V,r}$, such inhomogeneity can derive from inhomogeneous particle size distribution in products or inhomogeneous compound feed preparation. If compound feed samples show high $C_{V,r}$, grind the compound feed samples as described in ISO 6498 or by using an Ultra Centrifugal Mill[2)] with a sieve of nominal size of openings 1 mm. Grind 150 g of compound feed. Repeat the extraction with the ground samples as described in this clause.

8.2 For mineral feeds and premixtures

For phytase activities higher than 10 000 U/kg, in mineral feeds and premixtures, but lower than 2 000 000 U/kg:

— weigh 0,5 g ± 0,001 g of ground sample (which should be ground smaller than 0,5 mm) and 50 g ± 0,5 g of maize meal (5.22) in duplicate into containers (500 ml conical flasks or 800 ml beakers or equivalent);

— add 500 ml acetate buffer (5.16) in each container and mix vigorously for 1 h on a magnetic stirrer (6.3).

NOTE Ratio of mixture: In practice, mineral feed is added to complete feed at a rate of 1 % to 4 %, whereas premixtures are added at a rate of 0,2 % to 1 %. The sample mass of 0,5 g and 50 g maize correspond to a mixture rate of 1 %. This mixture rate is used as a convention for the comparability of the results of different laboratories, even if the declared mixture rate of mineral feed/premixture is lower or higher.

8.3 For feed additives

For phytase activities higher than 2 000 U/g (2 000 000 U/kg), in solid or liquid feed additives:

— for liquid feed additives samples:

  — weigh the samples (0,5 g or 1 g ± 0,001 g according to Table 6) in duplicate into a 100 ml volumetric flask;

  — add approximately 80 ml acetate buffer (5.16) into each flask and mix for 30 min on a magnetic stirrer (6.3);

  — remove the magnetic stirrer and fill up to 100,0 ml with acetate buffer (5.16);

— for solid feed additives samples:

  — weigh the samples (0,5 g or 1 g ± 0,001 g according to Table 6) in duplicate into a 250 ml beaker;

2) Ultra Centrifugal Mill 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.

— add accurately 100,0 ml acetate buffer (5.16) in each beaker and mix for 30 min on a magnetic stirrer (6.3).

NOTE If inhomogeneities or poor repeatabilities occur, an increase of sample mass and adequate adaptation of the extraction volume can be made.

9 Procedure

9.1 General

The volumes of reaction described in this clause can be multiplied if a photometer with a 1 cm cuvette or flow cell is used. For feeding stuff samples, a correspondingly larger volume of suspension (e.g. 10 ml) shall be centrifuged for 15 min at near 3 000*g* or 3 500*g* using a conventional laboratory centrifuge. Alternatively, larger sample volumes can also be filtered through folded filter. The first 5 ml should be then rejected. The supernatant, filtrate, phosphate standard solutions or phytase level control solution is then used with larger volumes of acetate buffer, phytate substrate solution and stop reagent.

For sample extracts and phytase level control, if the net measured absorption (absorption minus blank) is not within the calibration range (absorption/phosphate concentration) the determination shall be repeated with a suitable dilution.

Products containing phytase feed additives which were not included in the validation studies in the annexes shall be checked, especially on their linearity of response (release of phosphorus),

9.2 Blank solution

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.

9.3 Standards

9.3.1 Phosphate standard solution

The phosphate stock standard solution (5.19) shall be diluted with 0,25 mol/l acetate buffer containing 0,01 % mass fraction polysorbate 20 (5.16) according to Table 1.

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

Standard solution	Volume of phosphate stock standard solution (5.19)	Volume of 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16)	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 (5.19).

9.3.2 Phytase level control

For each incubation of samples, a phytase level control is included. A phytase stock standard solution (5.21) 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 9.5.

9.4 Standard curve

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

The deviation between the maximum and minimum OD values observed for the three determinations of each phosphate dilution, expressed as maximum value minus minimum value divided by maximum value, does not exceed 15 %. If so, it is possible to discard one value out of the three. If the deviation between the maximum and the minimum on the two remaining OD values is still higher than 15 %, then proceed to three new determinations.

In order to keep phytase extracts in a linear range, the calibration curve should cover not more than 0,5 of OD.

For the phosphate standard solutions, pipette 360 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16) into a 2 ml tube (6.12). Add 40 µl phosphate standard solution (see Table 2).

For the phosphate standard blanks, pipette 400 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16) into a 2 ml tube (6.12).

In both cases, add 0,8 ml phytate substrate solution (5.18) and 0,8 ml STOP reagent (5.13). Mix the contents of the tubes and maintain them for 10 min at room temperature. Centrifuge (6.8) the tubes and their contents for 3 min at 11 000*g* to 20 000*g* 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 (5.16)	360 µl	400 µl
Phosphate standard solution (9.3.1)	40 µl	0 µl
Phytate substrate solution (5.18)	0,8 ml	0,8 ml
STOP reagent (5.13)	0,8 ml	0,8 ml
Mix	Yes	Yes
Time at room temperature	10 min	10 min
Centrifugation	3 min at 11 000*g* to 20 000*g*	3 min at 11 000*g* to 20 000*g*
Spectrophotometer (6.11)	415 nm (against water)	415 nm (against water)

9.5 Phytase level control

Perform three determinations 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 (5.16) into a 2 ml tube (6.12). Add 40 µl dilute phytase level control solution (see 9.3.2). Mix the sample. Pre-incubate the solutions for 5 min at 37 °C. Add 0,8 ml phytate substrate solution (5.18) preheated to 37 °C. Incubate for exactly 30 min at 37 °C. After 30 min, add 0,8 ml STOP reagent (5.13) and mix. Maintain the solutions for 10 min at room temperature and then centrifuge them for 3 min at 11 000*g* to 20 000*g*. Measure the *D*(415) of the clear supernatant.

For the phytase level control blank solutions, pipette 360 µl acetate buffer (5.15) into a 2 ml tube (6.12). Add 40 µl dilute phytase level control solution (see 9.3.2). 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 (5.13). As step 2, add phytate substrate solution (5.18) preheated to 37 °C. Then proceed with Table 3, column 3, row 9, onwards.

Table 3 — Procedure for level control

Assay steps	Level control samples	Blank
Acetate buffer 0,25 mol/l with 0,01 % mass fraction polysorbate 20 (5.16)	360 µl	360 µl
Dilute phytase level control solution (see 9.3.2)	40 µl	40 µl
Mix	Yes	Yes
Pre-incubation at 37 °C	5 min	5 min
Phytate substrate solution (5.18) at 37 °C	0,8 ml	0,8 ml: Step 2
Mix	No	No
Incubation at 37 °C	30 min	No
STOP reagent (5.13)	0,8 ml	0,8 ml: Step 1
Mix	Yes	Yes
Time at room temperature	10 min	10 min
Centrifugation	3 min at 11 000*g* to 20 000*g*	3 min at 11 000*g* to 20 000*g*
Spectrophotometer (6.11)	415 nm (against water)	415 nm (against water)

9.6 For compound feed (excluding mineral feeds)

Transfer 2 ml of the compound feed extract to a microcentrifuge tube (6.12) and centrifuge (6.8) for 3 min at 11 000*g* to 20 000*g*.

Perform three determinations for each extraction (see Clause 8) and two blanks, and average the results. The procedure is specified in Table 4.

For the determinations, pipette 300 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16) into a 2 ml tube (6.12). Add 100 µl feed extract (see 8.1) as the test portion. Mix the contents of the tube. Pre-incubate for 5 min at 37 °C. Add 0,8 ml phytate substrate solution (5.18) preheated to 37 °C. Incubate for exactly 30 min at 37 °C. After 30 min, add 0,8 ml STOP reagent (5.13) and mix. Maintain the tube and its contents for 10 min at room temperature and then centrifuge for 3 min at 11 000*g* to 20 000*g*. Measure the *D*(415) of the clear supernatant.

For the blanks, pipette 300 µl 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16) into a 2 ml tube (6.12). Add 100 µl feed extract (see 8.1). The order of addition of solutions differs from that used for the test portion. Pre-incubate blanks for 5 min at 37 °C. Then, as step 1, add STOP reagent (5.13). As step 2, add phytate substrate solution (5.18) preheated to 37 °C. Then proceed with Table 4, column 3, row 9, onwards.

Table 4 — Procedure for compound feed (excluding mineral feeds)

Assay steps	Feed samples	Blank
Acetate buffer 0,25 mol/l with 0,01 % mass fraction polysorbate 20 (5.16)	300 µl	300 µl
Test portion	100 µl	100 µl
Mix	Yes	Yes
Pre-incubation at 37 °C	5 min	5 min
Phytate substrate solution (5.18) at 37 °C	0,8 ml	0,8 ml: Step 2
Mix	No	No
Incubation at 37 °C	30 min	No
STOP reagent (5.13)	0,8 ml	0,8 ml: Step 1
Mix	Yes	Yes

Table 4 *(continued)*

Assay steps	Feed samples	Blank
Time at room temperature	10 min	10 min
Centrifugation	3 min at 11 000*g* to 20 000*g*	3 min at 11 000*g* to 20 000*g*
Spectrophotometer (6.11)	415 nm (against water)	415 nm (against water)

For test portions with ≤ 200 phytase U/kg feed, 200 µl sample extract and 200 µl 0,50 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.17) are taken for the assay (1:2 dilution).

Samples with > 2 000 phytase U/kg feed shall be appropriately diluted with 0,25 mol/l acetate buffer with 0,01 % mass fraction polysorbate 20 (5.16).

9.7 For mineral feeds and premixtures

For phytase activities higher than 10 000 U/kg, in mineral feeds and premixtures, but lower than 2 000 000 U/kg:

— centrifuge (6.8) 2 ml of the extract coming from 8.2 for 3 min at 11 000*g* to 20 000*g*.

— the supernatant is used following Table 5.

Table 5 — Volumes for mineral feeds and premixtures

Expected activity of the sample U/kg	Volume of extract for the reaction µl	Volume of buffer in the reaction µl	Activity in the reaction U	Dilution factor *D* for calculation
10 000 to 25 000	300[a]	100	0,003 to 0,007 5	1,33
25 000 to 50 000	200	200	0,005 to 0,01	2
50 000 to 200 000	100	300	0,005 to 0,02	4
> 200 000	100	300	Suitable dilution	

[a] To increase the difference of absorption, due to high phosphate concentrations in the sample, in the range of 10 000 U/kg to 25 000 U/kg, the volume of extract can be increased to 400 µl (without an addition of buffer).

EXAMPLE Suitable dilution: samples with an activity of 400 000 U/kg are diluted 1:1 with extraction buffer (= two-fold predilution) before 100 µl feed extract is mixed with 300 µl buffer for the reaction (= four-fold dilution). Hence, the total dilution factor is 8.

Continue the procedure according to 9.6 with the selected volumes as given in Table 5.

9.8 For feed additives

For phytase activities higher than 2 000 U/g (2 000 000 U/kg), in solid or liquid feed additives:

— the clear extraction solution is diluted with acetate buffer (5.16) to a final activity of between 0,06 U/ml to 0,16 U/ml;

— 100 µl final solution is used in the same way as compound feed samples/extracts (see 9.6);

— continue the procedure according to 9.6 with the selected volumes as given in Table 6.

Table 6 — Volumes for feed additives

Expected activity of the sample U/g	Sample mass g	Dilution 1	Dilution 2	Final activity[a] in the solution U/ml	Dilution factor D[b]
approximately 2 000 to 2 500	1	1:25	1:10	0,1	100 000
5 000	1	1:25	1:20	0,1	200 000
10 000	1	1:50	1:20	0,1	400 000
> 10 000	0,5	Adequate dilution		0,1	

[a] The final activity refers to the solution used for incubation (further dilution effects during the incubation step are taken into account by the dilution factor).

[b] The dilution factor *D* also includes the factor of the reaction (100 µl sample + 300 µl buffer; corresponds to a 1:4 dilution) and 100 ml extraction volume, e.g. sample with 5 000 U/g → D = 100 × 25 × 20 × 4 = 200 000.

If high absorption values occur, the linear liberation of phosphate shall be proven. For this purpose, the sample solution is diluted several times in order to meet absorption differences in the range of the calibration curve. Calculated results should fulfil the criteria of repeatability.

EXAMPLE Calculation of dilution on the basis of the expected activity:

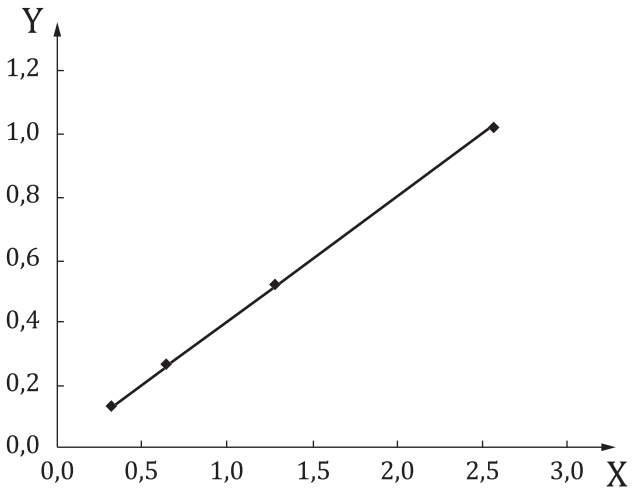
Expected activity: 7 500 U/g
Activity of the final solution: 0,1 U/ml
Sample mass: 1 g
Volume of extraction: 100 ml
Dilution = (1,0 g × 7 500 U/g)/(100 ml × 0,1 U/ml) = (1,0 g × 7 500 U × ml)/(g × 100 ml × 0,1 U) = 750
The sample should be diluted 1:750 in order to reach the final activity.
Dilution 1: 1:50 (e.g. 100 µl + 4 900 µl)
Dilution 2: 1:15 (e.g. 100 µl + 1 400 µl)

10 Calculations

10.1 Formulation of standard curve

Plot a standard curve with $\Delta D(415)$ $[D(415)_s - D(415)_b]$, where $D(415)_s$ and $D(415)_b$ are the mean standard and blank ODs, respectively, obtained with the phosphate standards (see 9.3.1 and 9.4), on the ordinate and the calculated phosphate concentration on the abscissa. The 1→10 dilution in the microcentrifuge tube [40 µl diluted standard (see Table 1) plus 360 µl buffer] for reaction should take the phosphate concentration of the standard into account for the calculation. The best fitting line is calculated by linear regression, $\Delta D(415) = kc(PO_4^{3-})$, (set intercept at 0). An example is shown in Figure 1.

For that linear regression, the coefficient of determination (r^2) should be at least 0,998.



Key

X	$c(PO_4^{3-})$/(µmol/ml)	
Y	$D(415)$	
with		
$D(415)$	OD at 415 nm	$D(415) = 0{,}400\ 8\ c(PO_4^{3-})$
$c(PO_4^{3-})$	concentration of phosphate	$r^2_{c(PO_4^{3-})\,D(415)} = 0{,}999\ 6$

Figure 1 — Example of plot of OD versus concentration for phosphate standard colorimetric solutions buffered with 0,25 mol/l acetate containing 0,01 % mass fraction polysorbate 20

10.2 Calculation of phytase activity

The phytase activity, a_p, in U/g or U/kg, is calculated as shown in Formula (1):

$$a_p = \frac{\Delta D(415) V_d}{k\, m\, t} \tag{1}$$

where

$\Delta D(415)$ is the net OD at 415 nm, calculated from:

$$D(415)_t - D(415)_b$$

in which $D(415)_t$ and $D(415)_b$ are the mean test portion and blank ODs (see 9.6, 9.7 or 9.8), respectively;

k is the slope of the standard curve, in reciprocal micromole millilitres, at $D(415)$ with four digits;

V_d is the volume, in millilitres, corrected for dilution (extraction volume times dilution of the compound feed extract or D factor for premixtures, mineral feeds and feed additives);

m is the mass, in grams or kilograms, of the sample;

t is the incubation time, in min.

EXAMPLE 1 Phytase level control:

— $\Delta D(415) = 0{,}216$

— k = 0,375 7 µmol^{-1} ml

— V_d = 30 000 ml (100 ml extraction volume × 30 [1→30 dilution of phytase stock standard solution (5.21)] × 10 [40 µl diluted phytase stock standard solution (5.21) + 360 µl buffer = 1→10])

— m = 0,107 4 g

— t = 30 min

$$a_p = \frac{0{,}216 \times 30\,000}{0{,}375\,7 \times 0{,}107\,4 \times 30} = 5\,353\ \mu\text{mol min}^{-1}\ \text{g}^{-1} = 5\,353\ \text{U g}^{-1}$$

EXAMPLE 2 Compound feed test portion:

— $\Delta D(415)$ = 0,183

— k = 0,375 7 µmol^{-1} ml

— V_d = 2 000 ml (500 ml extraction volume × 4 [100 µl extract + 300 µl buffer = 1→4])

— m = 0,050 kg

— t = 30 min

$$a_p = \frac{0{,}183 \times 2\,000}{0{,}375\,7 \times 0{,}050 \times 30} = 650\ \mu\text{mol min}^{-1}\ \text{kg}^{-1} = 650\ \text{U kg}^{-1}$$

10.3 Correction for phytic acid purity and water content

The purity and water content of phytic acid (5.7) varies from batch to batch and therefore shall be included in the calculation for the substrate preparation.

EXAMPLE 1 Phytic acid (0,008 % mass fraction inorganic phosphorus).

The correction is given by Formula (2):

$$C = \frac{C_{\text{theo}} \times M}{w_p \times \left(1 - w_{H_2O}\right)} \tag{2}$$

where

C is the mass concentration, in grams per litre, of phytate, after corrections, used to produce the 5.18 phytate substrate solution;

C_{theo} is the requested molar concentration, in mole per litre, of phytate in (5.18) at 7,5 mmol/l;

M is the molar mass, in grams per mole, of phytate (as salt forms);

w_p is the purity, as a mass fraction, of phytate;

w_{H_2O} is the water content, as a mass fraction, of phytate.

EXAMPLE 2 Using Formula (2):

— C_{theo} = 7,5 mmol/l;

— M = 923,8 g/mol;

— w_p = 97 % mass fraction;

— w_{H_2O} = 12,6 % mass fraction:

C = 0,007 5 × 923,8 / [0,97 × (1 – 0,126)] = 8,17 g/l

10.4 Interference with blank values

High levels of, for example, MCP [monocalciumphosphate, $Ca(H_2PO_4)_2$] in feeding stuffs samples can lead to high blank values [$D(415)_b$ > 1,6]. Some spectrophotometers are not necessarily linear in the high OD range (close to 2). Phytase values obtained from feeding stuffs samples with such high blank values shall be interpreted carefully since the high OD values can lead to an over- or underestimation of the phytase content. A 1→2 or 1→4 dilution of the feeding stuffs extract can help to reduce the OD value. However, $\Delta D(415)$ should be higher than $\Delta D(415)$ of the lowest calibration standard point.

11 Precision

11.1 Limit of detection and limit of quantification

There are two suitable ways to calculate the limit of detection (L_D) and the limit of quantification (L_Q), as follows:

— according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature (see Reference [5]) and blank measurements, the detection limit is defined as $L_D = 3s$, and the quantification limit as $L_Q = 10\sigma$, where s and σ are the estimated and absolute standard deviations, respectively;

— according to tools based on DIN standards (see Reference [6]), linear calibration curves and measurements.

In terms of $\Delta D(415)$, the detection limit is given by Formula (3):

$$L_D = 0{,}011\ \Delta D(415) \quad (3)$$

or 20 U/kg.

The quantification limit is given by Formula (4):

$$L_Q = 0{,}036\ \Delta D(415) \quad (4)$$

or 60 U/kg.

11.2 Interlaboratory tests

The values derived from the interlaboratory tests are not necessarily applicable to concentration ranges and matrices other than those given. See Annexes A, B and C.

The precision data given in 11.3 and 11.4 were calculated from the pooled results of the studies reported in References [7], [8], [9], [10] and [11].

11.3 Repeatability

The coefficient of variation of repeatability, $C_{V,r}$, is the coefficient of variation from independent results obtained from the same sample on the same day, from the same technician, with the same equipment and method.

The average pondered maximum coefficient of variation of repeatability (coming from Annexes A, B and C, respectively, for compound feeds, mineral feeds and premixtures, and feed additives), $C_{V,r}$, is set at:

— 10 % for compound feeds (excluding mineral feeds);

— 8 % for mineral feeds and premixtures;

— 6 % for feed additives.

11.4 Reproducibility

The coefficient of variation of reproducibility, $C_{V,R}$, is the mean coefficient of variation from results obtained with the same sample using the same method, but received from different laboratories, measured on different days, with different equipment and technicians.

The average pondered maximum coefficient of variation of reproducibility (coming from Annexes A, B and C, respectively, for compound feeds, mineral feeds and premixtures, and feed additives), $C_{V,R}$, is set at:

— 13 % for compound feeds (excluding mineral feeds);

— 17 % for mineral feeds and premixtures;

— 16 % for feed additives.

12 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the test method used, with reference to this document, i.e. ISO 30024;

c) any operating details not specified in this document, or regarded as optional, together with details of any incidents which can have influenced the test result(s);

d) the test result obtained;

e) the date of the test.

Annex A
(informative)

Interlaboratory study results for compound feeds (excluding mineral feeds)

A.1 First interlaboratory test

An international collaborative test (see Reference [7]), conducted in 2005, involving fourteen laboratories in seven countries, was carried out on eight feed samples. The fourteen laboratories comprised six which are National Reference Laboratories (NRL) [Austria, Denmark, France, Germany (2), Hungary], one outside the European Union [i.e. the Food Inspection Agency (Ottawa, Canada)], two private laboratories in France and Switzerland, and five company laboratories. The samples were compound feeds composed of typical ingredients and using a realistic recipe enriched with the phytase enzyme from different sources and in different forms, i.e. the phytase was produced by different companies and added in either a solid or liquid form.

Eight compound feed samples (A to H) were analysed, which contained individually one of four different phytase products, either as liquid or as solid formulation. Each material was analysed in blind duplicates.

Precision data for the method from Reference [7] are given in Table A.1.

Table A.1 — Precision data of the method during the first/initial collaborative study

Variable	**500** U/kg	**750** U/kg	**1 000** U/ kg	**1 250** U/kg	**1 500** U/kg	**1 500** U/kg	**1 500** U/kg	**1 500** U/kg
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Mean, U/kg	519	726	772	1 219	1 498	1 621	1 364	1 199
No. of outliers	0	3	1	0	0	2	2	2
No. of laboratories after outlier elimination	14	11	13	14	14	12	12	12
Standard deviation of repeatability, s_r, U/kg	43	43	62	88	159	164	119	26
Coefficient of variation of repeatability, $C_{V,r}$, %	8,3	6,0	8,0	7,2	10,6	10,1	8,8	2,2
Standard deviation of intermediate precision, s_{ir}, U/kg	66	51	89	127	164	164	131	39
Coefficient of variation of intermediate precision, $C_{V,ir}$, %	12,7	7,0	11,5	10,4	11,0	10,1	9,6	3,3
Standard deviation of reproducibility, s_R, U/kg	78	59	115	155	182	164	153	65
Coefficient of variation of reproducibility, $C_{V,R}$, %	15,0	8,1	14,9	12,8	12,2	10,1	11,2	5,4

A.2 Second interlaboratory test

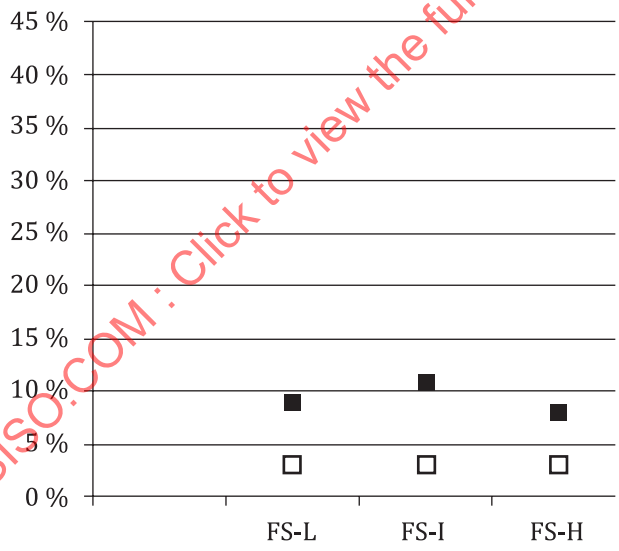
In 2014 to 2016, several NRL requested to the European Union Reference Laboratory for Feed Additives (EURL-FA) a study be conducted on the applicability of this document to feeding stuffs samples containing a specific 6-phytase produced by *Komagataella pastoris* (registered as 4a16 in the European Union Register of Feed Additives). A specific intercomparison study was performed in 2017 to 2018. That interlaboratory test, from EURL FA-NRL network and the holder of the feed additive authorization in Europe (with eleven volunteers from nine European countries, nine effective participants, eight laboratories reporting results for compound feeds and analyses in blind duplicate), indicated that the method given in this document is fit for purpose to also determine the phytase activity in three broiler complete compound feeds containing the

specific 4a16 phytase. Precision data from References [10] and [11], for three compound feeds, are given in Table A2.

Table A.2 — Results of phytase activity determination in three complete compound feeds, supplemented with 4a16 phytase feed additive

Variable	Broiler complete compound feed FS-L	Broiler complete compound feed FS-I	Broiler complete compound feed FS-H
Robust mean, U/kg	1 115	1 795	3 611
No. of outliers/rejected results	0	0	0
No. of laboratories after outlier elimination	8	8	8
Robust standard deviation of repeatability, s_r, U/kg	29	49	104
Coefficient of variation of repeatability, $C_{V,r}$, %	2,6	2,7	2,9
Robust standard deviation of reproducibility, s_R, U/kg	102	196	277
Coefficient of variation of reproducibility, $C_{V,R}$, %	9,1	11,0	7,7

For each of the three samples, robust coefficients of variation of repeatability and reproducibility, $C_{V,r}$ and $C_{V,R}$, respectively, are given in Figure A.1. For these complete compound feeds, containing the specific 4a16 phytase product, repeatability is constant with a $C_{V,r}$ around 3 %. Reproducibility is also quite homogenous, with a $C_{V,R}$, ranging from 7,7 % to 11, 0 %.



Key

■ robust reproducibility relative standard deviation

□ robust repeatability relative standard deviation

Ordinate = Robust repeatability (open squares) and reproducibility (black filled squares) relative standard deviation

Abscissa = reference sample name.

Figure A.1 — Plot of repeatability and reproducibility coefficients of variation versus sample identification during EURL FA-NRL interlaboratory

A.3 Third interlaboratory test on complementary compound feeds

The procedure for compound feeds (excluding mineral feeds), as described in 8.1 and 9.6, was investigated in interlaboratory study No. 443/M (2016) of VDLUFA Section VI. Ten laboratories participated, from three countries (Austria, Germany and Switzerland). Five complementary compound feeds, containing 4a1600 or 4a1640 phytase, were tested. The results were evaluated in accordance with the ISO 5725 series. Precision data are given in Table A.3.

Table A.3 — Results of phytase activity determination in five complementary compound feeds supplemented with 4a1600 or4a1640 phytase feed additive

Variable	Sample 1[a]	Sample 2[b]	Sample 3[c]	Sample 4[d]	Sample 5[e]
Mean, U/kg	3 799	2 874	8 103	3 378	7 699
No. of outliers/rejected laboratories	2	2	2	2	2
No. of laboratories after outlier elimination	10	10	10	10	10
No. of results without outliers	40	40	40	40	40
Standard deviation of repeatability, s_r, U/kg	262	246	354	187	185
Coefficient of variation of repeatability, $C_{V,r}$, %	6,89	8,56	4,37	5,53	2,41
Standard deviation of reproducibility, s_R, U/kg	715	265	798	335	500
Coefficient of variation of reproducibility, $C_{V,R}$, %	18,82	9,23	9,85	9,92	6,49

[a] Protein rich complementary feed for pigs for fattening with a 4a1640 phytase declared value of 4 000 U/kg.

[b] Complementary feed for breeding sows with a 4a1640 phytase declared value of 2 250 U/kg.

[c] Complementary feed for piglets with a 4a1600 phytase declared value of 8 667 U/kg.

[d] Protein rich complementary feed for pigs with a 4a1600 phytase declared value of 3 000 U/kg.

[e] Protein rich complementary feed for pigs with a 4a1600 phytase declared value of 6 850 U/kg.