Animal feed additives – Determination of phytase activity – Colorimetric method

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Foreword

The method is very similar to the harmonised standard method IS0 30024:2009 [1] and follows the same principle i.e. the phytase releases phosphate from a phytate substrate and the released inorganic phosphate is determined by forming a yellow complex with an acidic molybdate-vanadate reagent. The optical density of the yellow complex is then measured at a wavelength of 415 nm and the inorganic phosphate released is quantified from a phosphate standard curve.

This method was developed according to the principles set out in ISO 9001 [2] and Good Laboratory Practice and has been written in accordance with the rules given in ISO 78-2:1999 [3].

Introduction

This in-house method of analysis has been developed to determine the activity of phytase in feed enzymes containing phytase derived from Schizosaccharomyces pombe or Trichoderma reesei. However, the method cannot be used to evaluate the in vivo efficacy of the phytase products.

Animal feed additives – Determination of phytase activity – Colorimetric method

1 Scope

This in-house Standard describes the determination of phytase activity in feed enzymes containing phytase derived from *Schizosaccharomyces pombe* or *Trichoderma reesei.*

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.

2 Terms and definitions

For the purposes of this document, the following definition applies.

2.1 Phytase unit (FTU)

One FTU is the amount of enzyme that releases 1µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37°C.

3 Principle

The phytase is incubated with sodium phytate, which results in the release of inorganic phosphate. The inorganic phosphate creates a yellow coloured complex when reacted with molybdate-vanadate reagent. The yellow colour of the complex is measured at a wavelength of 415 nm. The extent of colour formation can be directly related to the enzyme activity. Quantification of activity is made by an absolute method using a phosphate standard curve.

4 Reagents and materials

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralised 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. Refer to safety ***Annex 2 "(Safety - Danger classifications)"*** for further information and instructions.

Note: Collect and dispose of all solutions containing Vanadate as hazardous waste. Dispose of used gloves, paper tissues and plastic tubes in a plastic bag in the fume cupboard.

4.1 Tween 20 (Polysorbate 20), (Merck, Cat. No. 8.22184).

4.2 Acetic acid, 99.8%; CH_3COOH, (Sigma-Aldrich, Cat. No.33209).

4.3 Calcium chloride dihydrate, $CaCl_2$.2 H_20, (B&B, Cat. No.102382).

4.4 Phytate, phytic acid, dodecasodium salt, $C_6H_6Na_{12}O_{24}P_6.XH_20$, (from rice) (Sigma, Cat. No.: P0109).

4.5 Nitric acid, 65% mass fraction; HNO_3, (Merck, Cat. No.: 1.00456).

4.6 Ammonium heptamolybdate tetrahydrate, $(NH_4)_6MO_70_{24}$.4H2O, (Sigma, Cat. No. A7302).

4.7 Ammonia solution, 32% mass fraction; NH_3 , (Merck, Cat. No.1.05426).

4.8 Ammonium monovanadate, NH_4VO_3, (B&B, Cat. No.1012260250).

4.9 Sodium acetate trihydrate, $CH_3COONa.3H2O$, (B&B, Cat. No.106267).

4.10 Potassium dihydrogen phosphate, KH_2PO_4, (Allied Signal Riedel-de Haën, Cat. No. 30407).

4.11 Acetate buffer, pH 5.5; 0.25 M, 1000 ml.

Dissolve 30.02 g of sodium acetate trihydrate (4.9) and 0.147 g of Calcium chloride dihydrate (4.3) in approximately 900 ml water. Add 1.76 g of acetic acid (4.2) and then adjust the pH of the solution to 5.5 using 4 M acetic acid (4.19). Transfer the solution to a 1000 ml volumetric flask, add 1 m of 10% Tween 20 (4.21) and make up to 1000 ml with water. Store in a fridge at 2-5°C. The maximum storage time is 2 weeks.

4.12 Phytate substrate solution, 7.5mM.

Dissolve 2.05 g ±0.05 g of dodecasodium phytate (4.4) whose inorganic phosphorus content is ≤ 0.1 % mass fraction (see 8.3) in 200 ml acetate buffer (4.11). Adjust the pH of the solution to 5.5 using 4 M acetic acid (4.19) and make up to 250 ml with acetate buffer (4.11). Store in the dark and in a fridge at 2-5°C. The maximum storage time is 1 week.

Note: Before using in assays, preheat to 37°C (minimum 5 minutes in water bath).

4.13 Phosphate stock standard solution, 18 mM, 1000 ml.

Dry approximately 5-10 g of potassium dihydrogenphosphate (4.10) at 60°C overnight in a heating cupboard (see annex 1: Procedure for Drying). Weigh precisely (4 digits) 2.45 g of dried potassium dihydrogen phosphate and dissolve in acetate buffer (4.11). Adjust the volume to 1000 ml with acetate buffer (4.11). Calculate the exact concentration of the phosphate stock standard solution. Store the solution in a fridge at 2-5°C. The maximum storage time is 2 weeks.

4.14 Dilute nitric acid, 24.5%, 200ml.

Dilute 75 ml nitric acid (65%) (4.5) with 100 ml water with continuous stirring. Transfer the solution to a 200 ml volumetric flask and adjust the volume to 200ml with water. Store at room temperature. The maximum storage time is indefinite.

Note: Nitric acid 24,5% is marked "Danger": Work in a fume cupboard. Wear gloves and glasses. Keep in a sealed bottle and store in a dry and well ventilated place.

4.15 Ammonia solution, 25%, 50ml.

Adjust the volume of 40 ml of 32% ammonia solution (4.7) to 50 ml with deionised water. Store at room temperature.

Ammonia solution, 25% is marked "Danger": Work in a fume cupboard. Wear gloves and glasses. Keep in a sealed bottle and store in a dry and well ventilated place.

4.16 Ammonium heptamolybdate reagent, 10%, 250ml.

Dissolve 25 g of ammonium heptamolybdate tetrahydrate (4.6) in 225 ml water. Heat the solution to dissolve the ammonium Heptamolybdate. Add 2.5 ml ammonia solution (25%) (4.15), turn off the heat and leave on the heater until totally dissolved. When cooled, transfer the solution to a 250 ml volumetric flask and adjust the volume to 250 ml with water. Mix well. Store at room temperature in the dark. The maximum storage time is one month.

Note: Ammonium Heptamolybdate reagent 10% is marked "Warning". Work in a fume cupboard. Wear gloves and glasses. Keep in a sealed bottle and store in a dry and well ventilated place.

4.17 Ammonium vanadate reagent, 0.24%, 250 ml.

Dissolve 0.5875 g of ammonium vanadate (4.8) in 100 ml water preheated to 60°C. Slowly add 5 ml of dilute nitric acid (4.14) with continuous stirring. When cooled, transfer the solution to a 250 ml volumetric flask and adjust the volume to 250 ml with water. Mix well. Store at room temperature in the dark. The maximum storage time is one month.

Pure ammonium Vanadate is extremely poisonous and experimentally mutagenic.

Note. Ammonium Vanadate 0.24% solution is marked "Warning". Avoid during pregnancy. Work in a fume cupboard. Wear gloves and glasses. Keep in a sealed bottle and store in a dry and well ventilated place.

4.18 Molybdate /vanadate COLOUR/STOP reagent, 200 ml.

Prepare just before use. Dispense 50 ml ammonium heptamolybdate reagent (4.16) in to a 200 ml volumetric flask followed by 50 ml ammonium vanadate reagent (4.17). Add 33 ml nitric acid (4.5) whilst swirling. Adjust the volume to 200 ml with water and mix. Store at room temperature in the dark. The maximum storage time is one day.

This amount will be enough for 100 tubes. If less colour/stop reagent is needed, the below table states the amount of reagents to be mixed:

Table 1: Colour/stop reagent preparation

Colour/Stop reagent ml	Ammonium Heptamolybdate (4.16) ml	Ammonium Vanadate (4.17) ml	Nitric Acid (4.5) ml
50 (25 tubes)	12.5	12.5	8.25
100 (50 tubes)	25	25	16.5
150	37.5	37.5	24.75
200	50	50	33
250	62.5	62.5	41.25
300	75	75	49.5

Note: Colour/Stop reagent is marked "Danger". Avoid during pregnancy. Work in a fume cupboard or in a well ventilated place. Wear gloves and glasses. Keep in a dry and well ventilated place.

4.19 Dilute acetic acid, 4M.

Add 22.9 ml acetic acid (4.2) into a 100 ml volumetric flask and fill to volume with water.

Note: 4 M acetic acid reagent is marked "Danger". Work in a fume cupboard. Wear gloves and glasses. Keep in a sealed bottle and store in a dry and well ventilated place.

4.20 Phytase stock control solution (activity approx. 0.03 FTU/ml).

Use as appropriate, a well characterised phytase product derived from either *Schizosaccharomyces pombe* (e.g. lot 160728, activity 5980 FTU/g) or from *Trichoderma reesei* (e.g. lot 3017116, activity 42788 FTU/g) as the control sample.

Weigh 1g of phytase control into a 100ml volumetric flask and fill with acetate buffer (4.11) to a final volume of 100ml. Record the exact weight and use in calculation (8.2). Take a sub sample and further dilute with acetate buffer (4.11) to give a final activity of about 0.03 FTU/ml. If stored, store at 2-5°C for no longer than 1 hour.

4.21 Tween 20, 10% mass fraction.

Dissolve 10,0 g of tween 20 (4.1) with water and make up to 100 ml. Store at room temperature. The maximum storage time is 2 weeks.

5 Apparatus

Usual laboratory apparatus, in particular, the following.

5.1 Heating cupboard, 60°C.

5.2 Water bath, thermostatically controlled set at 37.0°C ±0.1°C.

5.3 Spectrophotometer, 415 nm.

5.4 Centrifuge for 15 ml glass test tubes, max G: 3645, max rpm 4500.

5.5 Vortex mixer.

5.6 Alarm clock.

5.7 Electronic and manual pipettes in the range 1ml to 10ml.

5.8 Magnetic stirrer.

5.9 pH-meters, with two decimal digital readout.

5.10 Magnetic bars.

5.11 Analytical balance, sensitivity 0.03 mg.

5.12 Technical balance, sensitivity 0.3 mg.

5.13 Dispensette Brand pipettes, 2ml, 10ml, 25 ml.

5.14 Electronic pipettes in 1ml to 10ml range.

5.15 Glass fiber filter circles, GA-55 (110mm).

5.16 Hamilton diluter, Microlab 500 series (optional).

5.17 Disposal plastic test tubes, 15.5 x 100 mm (15 ml).

5.18 Volumetric flasks, 10, 200, 250 and 1000ml.

5.19 Measuring glass, 100, 200, 250, 1000ml.

6 Sample preparation

Mix dry enzyme products manually with a spoon and turn liquid enzyme product samples upside down 3 times before sampling.

6.1 Dry enzyme samples

Perform two weighing for each sample.

Weigh two portions of dry enzyme, of about 1 g each, into 100 ml volumetric flasks and fill to the mark with acetate buffer (4.11). Record the weights and use them in final calculations (8.2). Stir the mixtures on a magnetic stirrer for 20 minutes and then filter it through a glass filter. Dilute the filtrates with acetate buffer (4.11) to an expected activity of about 0.03 FTU/ml.

NOTE: If samples are coated with a thermo protection technology (TPT) coating, DO NOT FILTER. Leave to precipitate for 5 mins before use.

6.2 Liquid enzyme samples

Perform two weighing for each sample.

Weigh two portions of liquid feed enzyme, of about 1 g, into 10 ml volumetric flasks and fill to the mark with acetate buffer (4.11). Record the weights and use them in final calculations (8.2). Mix the solutions. Dilute with acetate buffer (4.11) to an expected activity of about 0.03 FTU/ml.

Note: Use a Hamilton Diluter to make all standard, control and sample dilutions.

7 Procedure

Analyze all samples (standards (7.1), control (7.2), enzyme product samples (7.3) and blank (7.4)) at the same time. The assay run is performed as a continuous flow.

7.1 Phosphate Standard solutions

Using 50 ml volumetric flasks, dilute the phosphate stock standard solution (4.13) with acetate buffer (4.11) to give the following final phosphate concentrations.

$C_{dilution}$ =
0.0 mM
0.5 mM
1.0 mM
1.5 mM
2.0 mM
2.5 mM
3.0 mM
4.0 mM

Use the following equation to determine the volume of stock solution required:

$$C_{stock\ solution} * V_{stock\ solution} = C_{dilution} * V_{dilution}$$

$$V_{stock\ solution} = (C_{dilution} * V_{dilution})/C_{stock\ solution}$$

Example for 0.5 mM: $V_{stock\ solution} = (0.5\ mM * 50\ ml)/18\ mM$

$V_{stock\ solution}$ = 1.389 ml stock solution

Table 2 shows the dilutions steps needed to obtain the final phosphate concentrations of 0.0 to 4.0mM using 18mM phosphate stock standard solution (4.13).

Table 2 – Dilution steps to obtain standard colorimetric solutions for the phosphate curve

Final Phosphate concentration in mM	Volume of KH_2PO_4 (18 mM) into 50 ml volumetric flask	Volume of Acetate buffer
0	0.0 ml	50.0 ml
0.5	1.4 ml	48.6 ml
1.0	2.8 ml	47.2 ml
1.5	4.2 ml	45.8 ml
2.0	5.6 ml	44.4 ml
2.5	6.9 ml	43.1 ml
3.0	8.3 ml	41.7 ml
4.0	11.1 ml	38.9 ml

Description of assay (standards):

Perform two determinations for each phosphate dilution and average the results.

For each phosphate standard solution, pipette 1.00 ml into a plastic tube. Add 2.00 ml Colour/Stop reagent (4.18) followed by 2.00 ml phytate substrate solution (4.12) preheated to 37°C. Put on a lid and turn upside down five times. Incubate the samples at room temperature for 5-10 min. Centrifuge for 10 minutes at 3500 rpm. Measure the absorbance of the solution at 415nm. Adjust the spectrophotometer to zero with water.

7.2 Phytase control solution

Perform two determinations and average the results.

Description of assay (control):
Pipette 1.00 ml control sample (4.20) into a plastic tube. Pre-incubate the samples in the water bath at 37.0°C for 5 minutes. Using five second time intervals, add 2.00 ml of phytate substrate solution (4.12) preheated to 37°C. Incubate the tubes in the water bath at 37.0°C for exactly 60 minutes. Then add 2.00 ml Colour/Stop reagent (4.18), put on a lid and turn the sample upside down five times. Centrifuge for 10 minutes at 3500 rpm. Measure the absorbance of the solutions at 415nm. Adjust the spectrophotometer to zero with water.

Note: The assay procedure is the same as for the enzyme product samples (7.3).

7.3 Enzyme product samples

Description of assay:

Perform two determinations and average the results.

Pipette 1.00 ml diluted enzyme product sample (6.1 or 6.2 as appropriate) into a plastic tube. Pre-incubate the samples in the water bath at 37.0°C for 5 minutes. Using five second time intervals, add 2.00 ml phytate substrate solution (4.12) preheated to 37°C. Incubate the tubes in the water bath at 37.0°C for exactly 60 minutes. Then add 2.00 ml Colour/Stop reagent (4.18) to the samples in the same order as before with a time interval of exactly five seconds. Put on a lid and turn upside down five times. Centrifuge for 10 minutes at 3500 rpm. Measure the absorbance of the solution at 415nm. Adjust the spectrophotometer to zero with water.

Note: The assay procedure is the same as for the control (7.2).

7.4 Blank

Perform two determinations and average the results.

Pipette 1.00 ml acetate buffer(4.11) into a plastic tube. Pre-incubate the samples in the water bath at 37.0°C for 5 minutes. Using five second time intervals, add 2.00 ml of phytate substrate solution (4.12) preheated to 37°C. Incubate the tubes in the water bath at 37.0°C for exactly 60 minutes. Then add 2.00 ml Colour/Stop reagent (4.18), put on a lid and turn the sample upside down five times. Centrifuge for 10 minutes at 3500 rpm. Measure the absorbance of the solutions at 415nm. Adjust the spectrophotometer to zero with water.

Note: The assay procedure is the same as for the enzyme product samples (7.3).

8 Calculations

8.1 Standard curve

Plot a standard curve with the OD_{415} readings on the ordinate (y-axis) and the calculated phosphate concentrations on the abscissa (x-axis). The best fitting line is calculated by linear regression. An example is shown in Figure 1.

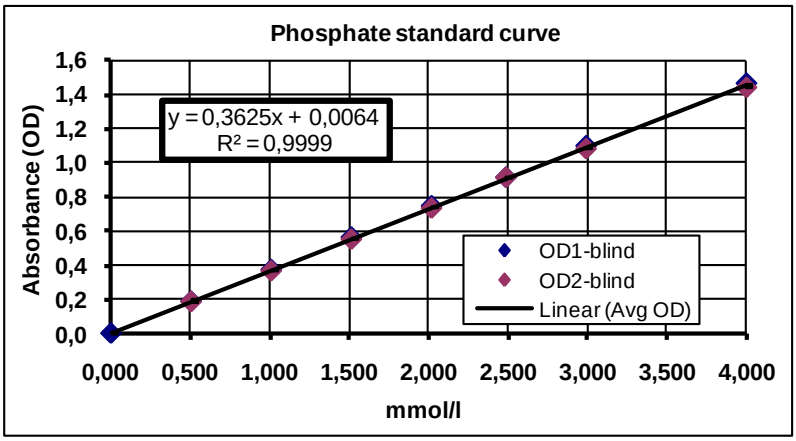

Figure 1 – Example of plot of optical density versus concentration of phosphate standard colorimetric solutions.

Notes:
a) OD of the blank needs to be ≤ 0.13 (0mM phosphate); if not, the results are rejected and the analysis is repeated;
b) the intercept (β) must not exceed $|0.02|$;
c) the correlation coefficient (R^2) should be ≥0.99;
d) the absorbance reading must be within the linear range (R^2 = 0.99) of the calibration curve and above the quantification limit. The OD range is set to be 0.01-2.0 which corresponds to up to 0-6 mM phosphate.

8.2 Calculation of phytase activity

The phytase activity is calculated as follows:

$$\text{Activity (FTU/g)} = \frac{(OD_{sample} - OD_{blank} - \beta) \times D}{Incubation time \times \alpha \times W_{sample}}$$

Where

OD_{sample} = mean absorbance of enzyme product (7.3) or absorbance of control sample (7.2), as appropriate;

OD_{blank} = mean absorbance of blank sample (7.4);

D = dilution factor for the sample (extraction volume x dilution of extract) in ml

Incubation time= 60 minutes;

W_{sample} = weight of sample or control in g

α = Slope of the standard curve;

β = Intercept of standard curve.

Example 1 Phytase control sample

ΔOD = OD_{sample} - OD_{blank} = 1.491 OD_{415}

α = 0.3625 OD_{415} ml / µmol

β = 0.0064 OD_{415}

D = 100000 ml (100 ml extraction volume x 1000 further dilution)

W_{sample} = 1.0101 g

$$\text{Activity (FTU/g)} = \frac{(1.491 - 0.0064) \times 100000}{60 \times 0.3625 \times 1.0101} = 6{,}770 \text{ µmol / (min*g)} = \underline{6{,}770 \text{ FTU/g}}$$

Example 2 Product enzyme sample

ΔOD = OD_{sample} - OD_{blank} = 1.739 OD_{415}

α = 0.3625 OD_{415} ml / µmol

β = 0.0064 OD_{415}

D = 100000 ml (100 ml extraction volume x 1000 further dilution)

W_{sample} = 1.0301 g

$$\text{Activity (FTU/g)} = \frac{(1.739 - 0.0064) \times 100000}{60 \times 0.3625 \times 1.0301} = 7{,}730 \text{ µmol / (min*g)} = \underline{7{,}730 \text{ FTU/g}}$$

8.3 Correction for phytic acid purity and water content

The purity and water content from phytic acid varies from batch to batch and has therefore to be included in the calculations for the substrate preparation.

Example Phytic acid dodecasodium salt (0.008 % mass fraction inorganic phosphorous e.g. P0109 from Sigma Lot 057K0049).

For a substrate concentration of 7.5 mM phytic acid, the correction is given by:

$$\text{Weight of phytic acid} = \frac{0.0075 mol \; x \; M_w}{V \; x \; P \; x \; (1 - C_w)} \rightarrow \frac{0.0075 mol \; x \; 923.8 g/mol}{1 l \; x \; 0.97 g/g \; x \; (1 - 0.126 g/g)} = 8.17 \text{ g/l}$$

Where
0.0075 mol/l is the substrate concentration (=7,5 mmol/l)
M_w is molar weight of phytic acid (in g/mol) = 923.8 g/mol
V is the final volume (in l)
P is purity of the phytic acid (in g/g) = 0.97 g/g
C_w is water content (in g/g) = 0.126/g

9 Precision

9.1 Limit of detection and limit of quantification

The determination of the detection limit (L_D = 3σ) and the quantification limit (L_Q = 10σ) are according to the IUPAC nomenclature [4] and are given by ΔOD_{415}.

In terms of OD_{415},

Detection limit L_D = 3 x 0.0139 ΔOD_{415} = 0.0417 which equates to 0.16 FTU/g.

Quantification limit L_Q = 10 x 0.0139 ΔOD_{415} = 0.139 which equates to 0.61 FTU/g.

10 Quality Assurance and control

For each test run, a double determination on the phytase control is performed as a quality control check. If the measured activity of the double determination has a coefficient variation (CV_r) above 10%, results are rejected and the analysis is repeated.

11 Test report

The test report shall specify:

a) information necessary for the complete identification of the sample
b) test method used
c) results of the test
d) the date of the test

Annex 1

Procedure for drying of KH_2PO_4

1. Adjust the heating cupboard to 60°C.
2. Weigh 5-10 g of KH_2PO_4 into a large beaker or a container with a large bottom (this is important, as the powder will dry only on the surface if using a beaker with a small bottom).
3. Let the KH_2PO_4 dry (unsealed) overnight!
4. Upon drying, place the beaker containing KH_2PO_4 in a desiccator (unsealed) for 15-30 minutes until cooled. Store in desiccator.

Annex 2

Safety (Danger classification)

- **Acetic acid (23%) 4M**

	**Danger** H226: Flammable liquid and vapour. H314: Causes severe skin burns and eye damage. P210: Keep away from heat/sparks/open flames/hot surfaces. — No smoking. P260: Do not breathe dust/fume/gas/mist/vapours/spray. P280: Wear protective gloves/protective clothing/eye protection/face protection. P304: IF INHALED: P340: Remove victim to fresh air and keep at rest in a position comfortable for breathing. P310: Immediately call a POISON CENTER or doctor/physician.

- **Ammonia solution (25%)**

	**Danger** H314: Causes severe skin burns and eye damage. P260: Do not breathe dust/fume/gas/mist/vapours/spray. P280: Wear protective gloves/protective clothing/eye protection/face protection. P273: Avoid release to the environment. P304: IF INHALED: P340: Remove victim to fresh air and keep at rest in a position comfortable for breathing. P310: Immediately call a POISON CENTER or doctor/physician.

- **Ammoniummolybdate10%:**

	**Warning** P280: Wear protective gloves/protective clothing/eye protection/face protection. P302: IF ON SKIN: P352: Wash with plenty of soap and water. P332:IF SKIN IRRITATION: P313 : Get medical advice/attention.

- **Ammoniumvanadat 0,24%**

	**Warning** H332: Harmful if inhaled. P261: Avoid breathing dust/fume/gas/mist/vapours/spray. P271: Use only outdoors or in a well-ventilated area. P304: IF INHALED: P340: Remove victim to fresh air and keep at rest in a position comfortable for breathing. P312: Call a POISON CENTER or doctor/physician if you feel unwell.

- **Stop solution**

	**Danger** H314: Causes severe skin burns and eye damage. P260: Do not breathe dust/fume/gas/mist/vapours/spray. P280: Wear protective gloves/protective clothing/eye protection/face protection. P304: IF INHALED: P340: Remove victim to fresh air and keep at rest in a position comfortable for breathing. P310: Immediately call a POISON CENTER or doctor/physician.

- **Nitric acid 24,5%**

	**Danger** H314: Causes severe skin burns and eye damage. P260: Do not breathe dust/fume/gas/mist/vapours/spray. P280: Wear protective gloves/protective clothing/eye protection/face protection. P304: IF INHALED: P340: Remove victim to fresh air and keep at rest in a position comfortable for breathing. P310: Immediately call a POISON CENTER or doctor/physician.

Bibliography

[1] ISO 30024: Animal feeding stuffs – Determination of phytase activity, 2009.

[2] ISO 9001: 2008 Quality Management systems.

[3] ISO 78-2: Chemistry – Layouts for standards – Part 2: Methods of chemical analysis, 1999.

[4] Nomenclature in Evaluation of Analytical methods including Detection and Quantification Capabilities. Pure & Applies Chem., Vol 67. No. 10, PP 1699-1723 (1995).