*Spectrophotometric method for assay of endo-1,4-beta-xylanase in the additive*

**EQUIPMENT:**

- Electronic balance
- Laboratory pH meter
- Magnetic stirrer
- Magnetic plate
- Laboratory centrifuge
- Water bath (thermostatic)
- Vibrating shaker
- UV/VIS Spectrophotometer
- Chronometer
- Automatic pipettes
- "Mohr" pipettes – 1; 2; 5; 10; 25 ml
- Volumetric flasks – 100; 500; 1000 ml.
- Glass test tubes – 18/180 mm
- Beakers – 300 ml
- Glass (plastic) funnels
- Filter paper

**REAGENTS:**

- Acetic acid (100%) – analytical grade
- Sodium hydroxide – analytical grade

- TWIN 80 (polysorbate) – analytical grade
- Tris (hydroximethyl)-aminomethane – analytical grade
- Distilled water
- Xylazyme – tablets (Megazyme)

**SOLUTIONS:**

**Acetate buffer with pH 4.7**

- Dissolve 1.4 mL glacial acetic acid in 900 ml distilled water in a Beher beaker of 1000mL.
- Adjust the pH at 4.7 with a 5 mol/l solution of sodium hydroxide
- Perform the adjustment using pH meter at constant stirring with magnetic stirrer
- Transfer the solution in a volumetric flask of 1000 mL and bring to volume with distilled water.
- The solution is prepared daily.

**Sodium hydroxide solution – 5mol/l**

- Dissolve 20g ± 0.01g sodium hydroxide with distilled water in a volumetric flask of 100 mL.
- After cooling down, bring to volume with distilled water.
- The solution is stable for 3 months at ambient temperature.

**TWIN 80 – 3%**

- Dissolve 6.0±0.1g TWIN 80 in 200 mL distilled water and stir for 10 minutes with magnetic stirrer.
- The solution is stable for 6 months at ambient temperature.

**Tris buffer – 3%**

- Dissolve 30±0.1g Tris with distilled water in a volumetric flask of 1000 mL.
- The solution is stable for 6 months at ambient temperature.

**Reference solution - Endo 1,4-β-xylanase derived from *Trichoderma* sp. with activity 1 250 000 EPU/g**

- Weigh 50±0.0001mg from the enzyme standard and dissolve in 50-60 ml acetate buffer with pH 4.7.
- After dissolution, bring the volume to 100 mL with the same buffer (*initial solution 1*).
- Dilute 1.6ml of *initial solution 1* with acetate buffer 4.7 in a volumetric flask of 100 mL. Bring to volume with the same buffer. The obtained solution is with xylanase activity of 10 000 mEPU/ml (*initial solution 2*)

Prepare series of dilutions with acetate buffer pH 4.7 in volumetric flasks of 100 mL in compliance with the following table:

| No of the solution | Xylanase content, mEPU/ml | Volume, ml Initial solution 2 | Final volume, ml |
|---|---|---|---|
| 1. | 200 | 2 | 100 |
| 2. | 400 | 4 | 100 |
| 3. | 600 | 6 | 100 |
| 4. | 800 | 8 | 100 |
| 5. | 1 000 | 10 | 100 |

All solutions are prepared daily.

**Stock test solution:**

- Weigh 1.0 g of the tested product with an accuracy of 0.0001g and transfer in a beaker
- Add 100 ml acetate buffer and stir for 15 min with magnetic stirrer (extraction of the enzyme).
- Leave the extract for settling down for about 10 minutes.
- Centrifuge 6 ml of the clear layer for 15 min at 4000 rpm in plastic tubes.
- Transfer the obtained supernatant in a clear test tube and use for measurement
- Dilute the sample being examined with acetate buffer until the enzyme content is within 400-800 mEPU/ml

**Placebo solution of wheat meal:**

- Weigh 1.0g of wheat meal with an accuracy of 0.0001g in a beaker
- Add 100 ml acetate buffer and stir for 15 min with magnetic stirrer (extraction of the enzyme
- Centrifuge 6 ml of the clear layer for 15 min at 4000 rpm in plastic tubes.
- Transfer the obtained supernatant in a clear test tube and use for measurement

**PROCEDURE:**

Process the supernatants according to the following scheme:

| **Solutions** | **Enzyme reference substance** | **Test solution** | **Control sample** | **Placebo Wheat meal** |
|---|---|---|---|---|
| Buffer, pH 4.7 | 4.5ml | 4.5ml | 5.0 ml - | 4.5ml |
| Enzyme reference solution | 0.5 ml | - | - | - |
| Test solution | - | 0.5 ml | - | 0.5 ml |
| Conditioning for 5 min at 50$^0$C | | | | |
| Substrate Xylazyme tablets | 1 tablet | 1 tablet | 1 tablet | 1 tablet |
| Tris solution 3% | 2ml | 2ml | 2ml | 2ml |
| Mixing on vibrating shaker | | | | |
| Cooling down, stirring | | | | |
| Filtration | | | | |
| Measuring the absorption at 590nm wavelength | | | | |

In order to assure accuracy of the measurement, prepare 4 parallel test tubes for each sample. Prior to measurement the test tubes are combined two by two.

**CALCULATIONS:**

The xylanase content in the additive expressed as EPU/g is calculated in the following way:

Determination of the absorption for 1 EPU/g by the means of linear regression: use the data from the measured absorption of the serial dilutions of the enzyme reference solutions prepared as given above.

Calculate the activity by the formula:

$$\text{Activity EPU/g} = \frac{E_{sample}}{E_{1EPU} \times m}$$

| | |
|---|---|
| $E_{sample}$ | Absorption of the sample being examined, corrected against the absorption of the control sample. |
| $E_{1\ EPU}$ | Absorption for 1 EPU, calculated as an average value form the absorptions of the different enzyme reference solution concentrations. |
| **m** | The weight of sample being examined, g/ml. |

*Spectrophotometric method for assay of endo-1,4-beta-xylanase in vitamin-mineral premixtures*

**EQUIPMENT:**

- Electronic balance
- Laboratory pH meter
- Magnetic stirrer
- Magnetic plate
- Laboratory centrifuge
- Water bath (thermostatic)
- Vibrating shaker
- UV/VIS Spectrophotometer
- Chronometer
- Automatic pipettes
- "Mohr" pipettes – 1; 2; 5; 10; 25 ml
- Volumetric flasks – 100; 500; 1000 ml.
- Glass test tubes – 18/180 mm
- Beakers – 300 ml
- Glass (plastic) funnels
- Filter paper

**REAGENTS:**

- Acetic acid (100%) – analytical grade
- Sodium hydroxide – analytical grade
- TWIN 80 (polysorbate) – analytical grade
- Tris (hydroximethyl)-aminomethane – analytical grade
- Distilled water

- Xylazyme – tablets (Megazyme)

**SOLUTIONS:**

**Acetate buffer with pH 4.7**

- Dissolve 1.4 mL glacial acetic acid in 900 ml distilled water in a Beher beaker of 1000mL.
- Adjust the pH at 4.7 with a 5 mol/l solution of sodium hydroxide
- Perform the adjustment using pH meter at constant stirring with magnetic stirrer
- Transfer the solution in a volumetric flask of 1000 mL and bring to volume with distilled water.
- The solution is prepared daily.

**Sodium hydroxide solution – 5mol/l**

- Dissolve 20g ± 0.01g sodium hydroxide with distilled water in a volumetric flask of 100 mL.
- After cooling down, bring to volume with distilled water.
- The solution is stable for 3 months at ambient temperature.

**TWIN 80 – 3%**

- Dissolve 6.0±0.1g TWIN 80 in 200 mL distilled water and stir for 10 minutes with magnetic stirrer.
- The solution is stable for 6 months at ambient temperature.

**Tris buffer – 3%**

- Dissolve 30±0.1g Tris with distilled water in a volumetric flask of 1000 mL.
- The solution is stable for 6 months at ambient temperature.

**Reference solution - Endo 1,4-β-xylanase derived from *Trichoderma* sp. with activity 1 250 000 EPU/g**

- Weigh 50±0.0001mg from the enzyme standard and dissolve in 50-60 ml acetate buffer with pH 4.7.
- After dissolution, bring the volume to 100 mL with the same buffer (*initial solution 1*).
- Dilute 1.6ml of *initial solution 1* with acetate buffer 4.7 in a volumetric flask of 100 mL. Bring to volume with the same buffer. The obtained solution is with xylanase activity of 10 000 mEPU/ml (*initial solution 2*)

Prepare series of dilutions with acetate buffer pH 4.7 in volumetric flasks of 100 mL in compliance with the following table:

| No of the solution | Xylanase content, mEPU/ml | Volume, ml Initial solution 2 | Final volume, ml |
|---|---|---|---|
| 1. | 200 | 2 | 100 |
| 2. | 400 | 4 | 100 |
| 3. | 600 | 6 | 100 |
| 4. | 800 | 8 | 100 |
| 5. | 1 000 | 10 | 100 |

All solutions are prepared daily.

**Stock test solution:**

- Weigh 1.0 g of the tested product with an accuracy of 0.0001g and transfer in a beaker
- Add 100 ml acetate buffer and stir for 15 min with magnetic stirrer (extraction of the enzyme).
- Leave the extract for settling down for about 10 minutes.
- Centrifuge 6 ml of the clear layer for 15 min at 4000 rpm in plastic tubes.
- Transfer the obtained supernatant in a clear test tube and use for measurement
- Dilute the sample being examined with acetate buffer until the enzyme content is within 400-800 mEPU/ml

**Placebo solution of vitamin-mineral premixture:**

- Weigh 1.0g of vitamin-mineral premixture with an accuracy of 0.0001g in a beaker
- Add 100 ml acetate buffer and stir for 15 min with magnetic stirrer (extraction of the enzyme
- Centrifuge 6 ml of the clear layer for 15 min at 4000 rpm in plastic tubes.
- Transfer the obtained supernatant in a clear test tube and use for measurement

**PROCEDURE:**

Process the supernatants according to the following scheme:

| **Solutions** | **Enzyme reference substance** | **Test solution** | **Control sample** | **Placebo Vitamin-mineral premixture** |
|---|---|---|---|---|
| Buffer, pH 4.7 | 4.5ml | 4.5ml | 5.0 ml - | 4.5ml |
| Enzyme reference solution | 0.5 ml | - | - | - |
| Test solution | - | 0.5 ml | - | 0.5 ml |
| Conditioning for 5 min at $50^0$C | | | | |
| Substrate Xylazyme tablets | 1 tablet | 1 tablet | 1 tablet | 1 tablet |
| Tris solution 3% | 2ml | 2ml | 2ml | 2ml |
| Mixing on vibrating shaker | | | | |
| Cooling down, stirring | | | | |
| Filtration | | | | |
| Measuring the absorption at 590nm wavelength | | | | |

In order to assure accuracy of the measurement, prepare 4 parallel test tubes for each sample. Prior to measurement the test tubes are combined two by two.

**CALCULATIONS:**

The xylanase content in the vitamin-mineral premixture expressed as EPU/g is calculated in the following way:

Determination of the absorption for 1 EPU/g by the means of linear regression: use the data from the measured absorption of the serial dilutions of the enzyme reference solutions prepared as given above.

Calculate the activity by the formula:

$$\text{Activity EPU/g} = \frac{E_{sample}}{E_{1EPU} \times m}$$

| | |
|---|---|
| $E_{sample}$ | Absorption of the sample being examined, corrected against the absorption of the control sample. |
| $E_{1\ EPU}$ | Absorption for 1 EPU, calculated as an average value form the absorptions of the different enzyme reference solution concentrations. |
| **m** | The weight of sample being examined, g/ml. |

*Spectrophotometric method for assay of endo-1,4-beta-xylanase in feedingstuff*

**EQUIPMENT:**

- Electronic balance
- Laboratory pH meter
- Magnetic stirrer
- Magnetic plate
- Laboratory centrifuge
- Water bath (thermostatic)
- Vibrating shaker
- UV/VIS Spectrophotometer
- Chronometer
- Automatic pipettes
- "Mohr" pipettes – 1; 2; 5; 10; 25 ml
- Volumetric flasks – 100; 500; 1000 ml.
- Glass test tubes – 18/180 mm
- Beakers – 300 ml
- Glass (plastic) funnels
- Filter paper

**REAGENTS:**

- Acetic acid (100%) – analytical grade
- Sodium hydroxide – analytical grade
- Tris (hydroxymethyl)-amino methane - analytical grade
- TWIN 80
- Distilled water
- Xylazyme – tablets (Megazyme)

**SOLUTIONS:**

**Acetate buffer with pH 4.7(for extraction of the feeding stuffs):**

- Dissolve 1.4 mL glacial acetic acid in 900 ml distilled water in a Beher beaker of 1000mL.
- Adjust the pH at 4.7 with a 5 mol/l solution of sodium hydroxide
- Perform the adjustment using pH meter at constant stirring with magnetic stirrer
- Transfer the solution in a volumetric flask of 1000 mL and bring to volume with distilled water.
- The solution is prepared daily.

**Acetate buffer with pH 4.7(for dilution of the enzyme standard):**

- Dissolve 1.4 mL glacial acetic acid in 900 ml distilled water in a Beher beaker of 1000mL.
- Adjust the pH at 4.7 with a 5 mol/l solution of sodium hydroxide
- Perform the adjustment using pH meter at constant stirring with magnetic stirrer.
- Add 10 mL 3% solution of TWIN80
- Transfer the solution in a volumetric flask of 1000 mL and bring to volume with distilled water.
- The solution is prepared daily.

**Sodium hydroxide solution – 5 mol/L:**

- Dissolve 20g ± 0.01g sodium hydroxide with distilled water in a volumetric flask of 100 mL.
- After cooling down, bring to volume with distilled water.
- The solution is stable for 3 months at ambient temperature.

**TWIN 80 – 3%:**

- Dissolve 6.0±0.1g TWIN 80 in 200 mL distilled water and stir for 10 minutes with magnetic stirrer.
- The solution is stable for 6 months at ambient temperature.

**Tris buffer – 3%:**

- Dissolve 30±0.1g Tris with distilled water in a volumetric flask of 1000 mL.
- The solution is stable for 6 months at ambient temperature.

**Reference solution - Endo 1,4-β-xylanase derived from *Trichoderma* sp. with activity 1 250 000 EPU/g):**

- Weigh 50±0.0001mg from the enzyme standard and dissolve in 50-60 ml acetate buffer with pH 4.7 (*for dilution of the enzyme standard*).
- After dissolution, bring to volume to 100 mL with the same buffer (*initial solution 1*).
- Dilute 1.6ml of *initial solution 1* with acetate buffer 4.7 in a volumetric flask of 100 mL. Bring to volume with the same buffer. The obtained solution is with xylanase activity of 10 000 mEPU/ml.( *initial solution 2*)
- Prepare a series of solutions with different enzyme content in compliance with the following table:

| Volume of initial solution 2, ml | Final volume, ml | Xylanase content, mEPU/ml | Xylanase content, EPU/kg |
|---|---|---|---|
| 0 | 200 | 0 | 0 |
| 1 | 200 | 50 | 1000 |
| 2 | 200 | 100 | 2000 |
| 3 | 200 | 150 | 3000 |
| 4 | 200 | 200 | 4000 |

The solution is prepared daily.

**Extraction of feeding stuffs:**

- Grind and homogenize the feeding stuff.
- In 5 beakers weigh 10 g of the grinded and homogenized feeding stuff.
- Add the 200 mL portions of the 5 standard solutions (table 1) into the respective beakers, resulting in spiking levels of 0, 1000, 2000, 3000 and 4000 EPU/kg.
- Stir on a magnetic stirrer for 15 min.
- Let settle and centrifuge 6 mL of the extract for 15 min at 5000 rpm.
- Decant the supernatant and use for the assay described in item 6.

**PROCEDURE:**

Process the supernatants according to the following scheme:

| Solutions | Test solution | Control |
|---|---|---|
| Buffer 4.7 | 4.5ml | 5.0ml |
| Feeding stuff extract | 0.5ml | - |
| Conditioning for 5 min at $50^0$C | | |
| Substrate - Xylazyme-tabl | 1 tablet | 1 tablet |
| Incubation for 2.5 h (150 min.) at $50^0$C | | |
| TRIS 3% solution | 2.0ml | 2.0ml |
| Stirring on vibration shaker | | |
| Cooling down, stirring | | |
| Filtrations | | |
| Measure the absorbance at 590nm wavelength | | |

In order to assure better accuracy of the measurement, prepare 4 parallel test tubes for each sample. Prior to measurement the test tubes are combined two by two.

**CALCULATIONS:**

Calculate the average absorbance for each sample solution.

Compute the regression line (y=ax+b) with the spiking levels as "x" and the corrected sample absorbance as "y".

Calculate the value of "x" for y = 0. "x" is the calculated enzymatic activity in the feedingstuff.