# Determination of xylanase activity in feeds by viscosimetry

## 1. Document history

Not relevant

## 2. Warnings

Refer to each product safety data sheet.
Wear suitable safety glasses and gloves.
Work under fume cupboard.

## 3. Scope

This method is applicable to determine endo-1,4-β-xylanase activity in feeds by viscosimetry.

## 4. Normative references

Not applicable.

## 5. Definitions

The words “feed” and “feeds” can be replaced with “feedingstuffs” in this document

## 6. Principle and reactions

In this method are described the different operations to be performed to determine the endo-1,4-β-xylanase activity in feeds supplemented with enzymatic feed additives, containing a xylanase activity, such as ROVABIO®, using a viscosimetric method.

### 6.1. Principle

This method is specific to the determination of endo-1,4-β-xylanase in feeds. Endo-1,4-β-xylanase hydrolyses the xylosidic linkages of the xylan. The test is set upon the enzymatic hydrolysis of xylosidic linkages of a solution containing soluble wheat arabinoxylan, a β-1,4-xylan polysaccharide substituted with arabinose. The enzymatic activity is proportional

to the viscosity reduction of a wheat arabinoxylan solution in the presence of the enzyme to be determined.

### 6.2. Definition of the unit

Note: The analysis is carried out in two steps.
Step 1: Determination of the activity of the feed additive (the one incorporated in the feed),
Step 2: Determination of the recovery of the feed additive in the feed.

Item activity is measured at pH = 5.5 and 30°C and expressed in xylanase visco units (X visco U).

For the recoveries, the endogenous activity of the feed matrix has to be taken into consideration, if not recoveries can be overestimated.

Investigations were undertaken to find conditions of extraction of the feeds leading to a negligible, indeed no expression of the endogenous activity as the added ROVABIO® enzyme would continue to exhibit its activity.

Such conditions were found by substituting the pH 6.0 MES/SDS (lauryl sulfate) buffer solution (0.1M MES, pH 6.0; 1 % (w/v) SDS (lauryl sulfate)) by a pH 3.30 citrate buffer in presence of BSA, as the endo–1,4-β-xylanase activity of the enzyme feed additive that is incorporated to the feed is still determined at 30°C in 1M sodium acetate buffer solution, pH 5.5.

Thus, the definition of the activity remains unchanged: one X visco U of endo-1,4-β-xylanase activity is defined as the amount of enzyme that hydrolyzes the substrate, thus reducing the solution viscosity, in order to change the relative fluidity by one unit without dimension per minute, according to the following conditions: pH 5.5 (or as indicated) and 30 °C (or as indicated).

### 6.3. Methodology

Significant interferences can occur during the endo-1,4-β-xylanase activity determination in feeds, especially when the feeds are formulated from wheat. The interference level has, therefore, to be determined for each analysis. This interference determination is performed using a control sample (feed containing no enzyme), to which a known quantity of enzyme is being added.
Thus, the quantification is based on a spiking procedure and uses:

- the feed additive with known activity. The type of the feed additive (powder or liquid), their batch number and incorporation rate (L/t or g/t) in the feed have to be known.
  [If the batch number is not provided, a retention enzyme, specific to the laboratory is used].
- the control feed sample (without enzyme = blank).
- the formulated feed sample (with the enzyme).

The endo-1,4-β-xylanase recovery percentage determination is performed in 5 steps:

1- Endo-1,4-β-xylanase activity determination in the feed additive.
2- Endo-1,4-β-xylanase activity determination in the control sample (without enzyme).
3- Spiking of the control feed with the feed additive at the same concentration than in the treated feed then determination of the endo-1,4-b-xylanase activity.
4- Endo-1,4-β-xylanase activity determination in the treated feed.
5- Deduction of the recovery percentage of the additive in the feed.

***Note: If the control sample is not provided, refer to section 13.***

## 7. Reagents and materials

All reagents must be of analytical grade. Except when specifically indicated, the water used is chromatographic grade water. The water used in the water bath can be tap or distilled water. The reagents used for the xylanase activity determination using the viscosimetric method are presented in annex 1. In every cases, the identity as well as the purity of the reagents are more important than the supplier criteria.

### 7.1. Wheat arabinoxylan at 0.3% (w/V)

To prepare 100 mL of substrate, weigh exactly about 0.300 g to the nearest 0.001 g of wheat arabinoxylan. Place 70 mL of water (7.7.) in a beaker, add a magnetic stirring bar and place on a heating magnetic stirrer. Stir vigorously and start heating. When the water is hot (but not boiling), add the arabinoxylan in avoiding the formation of lumps. When the liquid starts to boil, reduce the heating until the simmering point, cover up the beaker with a glass beaker cover and continue the heating for 10 more minutes in order to completely dissolve the substrate. Stop the heating and continue the stirring until the solution reaches room temperature (at this step, the beaker can be placed onto a cold magnetic stirrer). Transfer the solution in a 100 mL volumetric flask. Rinse the beaker with water (7.7.) and transfer into the volumetric flask. Fill up the flask to volume using water (7.7.).
A fresh substrate solution has to be prepared each day of analysis.
Keep the substrate at room temperature.
At the end of the day, discard the excess substrate.

### 7.2. Citric acid monohydrated

### 7.3. Citrate buffer, pH3.30

In a 5 L beaker, weigh 446.5 g ± 0.1 g of citric acid (7.2.), then add water (7.7) to ¾ of the volume of the beaker. Stir until complete dissolution, then add 65.0 g ± 0.1 g of sodium hydroxide (7.4.). Stir until complete dissolution.
In a 100 mL beaker, weigh 2.500 g ± 0.005 g of BSA (7.6.), then add 40 mL of water (7.7.) and stir until dissolution. Transfer the BSA solution in the 5 L beaker, rinse the 100 mL beaker then make up to about 4.5 L volume with water (7.7.). Adjust to pH 3.30 with 5M sodium hydroxide solution (7.5.) using a calibrated pH-meter.
Transfer into a 5 L volumetric flask, rinse the 5 L beaker then fill up the flask to mark with water (7.7.).

Keep at room temperature for a maximum of 1 week.

### 7.4. Sodium hydroxide (pellets)

### 7.5. 5M sodium hydroxide solution

Weigh exactly about 200.0 g ± 0.1 g of sodium hydroxide (7.4) in a 1 L beaker, add a magnetic stirring bar and place onto a magnetic stirrer. Under continuous stirring, gently add 750 mL of water (7.7.). Transfer to a 1 L volumetric flask and fill up to volume using water (7.7.).
Keep at room temperature for a maximum of 2 months.

### 7.6. BSA: Bovine Serum Albumin

### 7.7. Water for chromatography, conductivity <0.1 S/cm (eg MilliQ water)

## 8. Apparatus

- Usual glassware and laboratory equipment.
- Grinder equipped with a 0.5 mm mesh opening grid.
- Disposable glass and plastic tubes with racks.
- P5000 and P1000 GILSON pipettes or equivalent.
- Stopwatch.
- Laboratory centrifuge (7000 rpm).
- Heating magnetic stirrer + magnetic stirring bars.
- Calibrated pH - meter and analytical balances, readabilities ± 0.3 mg for a capacity of 410 g and ± 0.1 g for a capacity of 4100 g.
- Cold water bath (cold pack).
- Water bath set at 30 °C (± 0.1 °C)
- Microviscosimeter (e.g. ANTON PAAR AMVn or LOVIS 2000M) + associated software.
- Ultrasonic bath.
- Multi-position magnetic stirrer.
- Filters and syringes.

References of the equipment used to date are listed in annex 2 for example.

The whole of the equipment has to be used in a clean environment in order to avoid any eventual contamination problems. The environment should be isolated from any concentrated enzyme sample and should be cleaned regularly. It is good practice to use pipettes specifically dedicated to this kind of analysis.

## 9. Sampling

Not applicable.

## 10. Procedure

### 10.1. Sample preparation

Feeds are ground with an ultra-centrifuge grinder equipped with a 0.5 mm mesh opening grid.

#### 10.1.1. Feed control sample preparation

1- Grind, whenever possible, a minimum of 100 g of control feed.
2- Weigh out 10.0 g to the nearest 0.5 g of ground feed and introduce them in a 250 mL conical flask. Add 100 mL of pH 3.30 citrate buffer (7.3), then a magnetic stirring bar, then place onto a multi-position magnetic stirrer. Stir for 30 minutes.
3- Transfer about 5 mL of this solution in a disposable plastic tube then centrifuge for 10 minutes at 7000 rpm at 15°C.
4- Filter then introduce 1 mL of filtrate in a hemolysis tube. Add 3 mL of pH 3.30 citrate buffer (7.3), mix using a vortex mixer, then perform the endo-1,4-β-xylanase activity determination as indicated in section 10.2.2 hereafter. The result will be called *control*.

Note: In the particular case of feeds with ROVABIO® AP T. Flex, weigh, whenever possible, 50 g to the nearest 1 g of control feed then add 500 mL of pH 3.30 citrate buffer (7.3.) then stir for 30 minutes before to proceed to steps 3 and 4 described above.

Remark: The supernatant and the dilution of the supernatant can be stored in the cold (15 °C maximum) before analysis.

#### 10.1.2. Spiked controlled feed sample preparation

1- Grind, whenever possible, a minimum of 100 g of control feed.
2- Weigh out 10.0 g to the nearest 0.5 g of ground feed and introduce it into a 250 mL conical flask.
3- Spikes preparation:
The spikes to be performed are dependent upon the type of the feed additive used.

| Spike with ROVABIO EXCEL LC (incorporation rate in the feed = 0.2 L/t). | Spike with ROVABIO EXCEL AP (incorporation rate in the feed = 50 g/t). |
|---|---|
| Introduce 0.5 mL of the additive in a 250 mL flask. | Introduce 0.25 g ± 10 mg of the additive in a 500 mL flask. |
| Fill up using pH 3.30 citrate buffer (7.3) then homogenize. Take 1 mL and introduce it in the flask containing the 10 g of control feed. | |

4- Add 100 mL of pH 3.30 citrate buffer solution (7.3.). Introduce a magnetic stirring bar and place onto a multi-position magnetic stirrer. Stir for 30 minutes.

5- Transfer about 5 mL of solution in a disposable plastic tube then centrifuge for 10 minutes at 7000 rpm at 15°C.
6- Filter then introduce 1 mL of filtrate into a hemolysis tube. Add 3 mL of pH 3.30 buffer citrate (7.3), mix with a vortex mixer then perform the endo-1,4-β-xylanase activity determination as indicated in section 10.2.2.. The result will be called spike.

Note: For the particular case of feeds with ROVABIO® AP T. Flex, weigh, whenever possible, 50 g to the nearest 1 g of control feed, then perform a 5 mL spike. Add 500 mL of pH 3.30 citrate buffer solution (7.3.) then stir for 30 minutes at room temperature (Steps 5 and 6 described before remain unchanged).

Remark: The supernatant and the dilution of the supernatant can be stored in cool conditions (15 °C maximum) before analysis.

### 10.1.3. Enzymed feed sample preparation

1- Grind, whenever possible, a minimum of 100 g of treated feed (with enzyme).
2- Weigh out 10.0 g to the nearest 0.5 g of ground feed and introduce them into a 250 mL conical flask. Add 100 mL of pH 3.30 citrate buffer (7.6.), then a magnetic stirring bar, then place onto a multi-position magnetic stirrer. Stir for 30 minutes.
3- Transfer about 5 mL of solution in a disposable plastic tube then centrifuge for 10 minutes at 7000 rpm at 15°C.
4- Filter then introduce 1 mL of filtrate into a hemolysis tube. Add 3 mL of pH 3.30 citrate buffer solution then perform the endo-1,4-β-xylanase activity determination as indicated in section 10.2.2.. The result will be called treated.

Note: For the particular case of feed with ROVABIO® AP T. Flex, weigh, whenever possible, 50 g to the nearest 1 g of treated feed. Add 500 mL of pH 3.30 citrate buffer (7.3) then stir for 30 minutes at room temperature (Steps 3 and 4 described above remain unchanged).

Remark: The supernatant and the dilution of the supernatant can be stored in cool conditions (15 °C maximum) before analysis.

## 10.2. Sample assay

### 10.2.1. Xylanase activity determination in the feed additives as preparations

A determination of endo-1,4-β-xylanase activity is carried out on the enzyme sample that was added to the feedingstuff using method T004. To do this, either:

- The batch number was provided by the customer, then the retention sample stored in the laboratory is used to perform the spike. In this case, the enzymatic activity determination of the additive provided by the customer is not performed and the laboratory value is used instead.
- The batch number xas indicated by the customer, then a retention sample specifically selected by the laboratory for this purpose is used for the spike.

### 10.2.2. Xylanase activity determination in the samples

**Example for AMVn or LOVIS 2000M type ANTON PAAR viscosimeter:**

First, the micro viscosimeter has to be set and an appropriate capillary and ball have to be chosen. Data are available in annex 3.

Before to start an enzymatic analysis, the capillary and the ball have to be calibrated against water (**water blank**) and against the substrate (**substrate blank**). These calibrations against water and against the substrate are performed every day an analysis is performed.

Between each analysis, rinse with the solution to be analyzed.

All the cleaning has to be performed using water.

At the end of the day, clean the capillary and the ball using water, ethanol, then acetone then dry with a flux of nitrogen.

**10.2.2.1.** Calibration against water - Water blank determination

**Example for AMVn type ANTON PAAR viscosimeter:**

Fill up the capillary (containing a calibrated ball) with water (7.7.). Make sure that no air bubble is present in the capillary, then place it into the microviscosimeter.
The microviscosimeter will perform 60 measurements. The average drop time for the ***40 last measurements*** for water is labelled Tw (value equal to about 12 s).

**Example for LOVIS 2000M type ANTON PAAR viscosimeter:**

Fill up the capillary (containing a calibrated ball) with water (7.7.). Make sure that no air bubble is present in the capillary, then place it into the microviscosimeter.
The microviscosimeter will perform 21 measurements cycle (one cycle corresponding to ways 1 and 2). The average drop time for the ***16 last measurements of way 2*** for water is labelled Tw (value equal to about 9 s).

**10.2.2.2.** Calibration against the substrate - Substrate blank determination

**Example for AMVn type ANTON PAAR viscosimeter:**

Place 3.2 mL of 0.3% wheat arabinoxylan (7.1.) in a disposable tube. Sonicate for 5 seconds then place in a thermostated water bath at 30 °C and leave to equilibrate for (at least) 5 minutes.
*Note: At the beginning of the day, it is possible to prepare several tubes containing some substrate solution (3.2 mL) and to leave them to incubate in the 30 °C water bath.*

Add 0.8 mL of pH 3.30 buffer citrate (7.3) into the substrate incubated at 30°C (final concentration = 0.24% w/V). Mix using a vortex then fill up the capillary containing a calibrated ball with the mixture (make sure that no air bubble is present in the capillary). Insert the capillary into the viscosimeter.
The viscosimeter will perform 30 measurements cycle. The average drop time for the ***20 last measurements*** for the substrate is labelled Ts (value equal to about 26 s).

If the obtained value is too low or if the drop time decrease is lower than -10 msec/min, discard the substrate and prepare a new substrate solution.

**Example for LOVIS 2000M type ANTON PAAR viscosimeter:**

Place 3.2 mL of 0.3% wheat arabinoxylan (7.1.) in a disposable tube. Sonicate for 5 seconds then place in a thermostated water bath at 30 °C and leave to equilibrate for (at least) 5 minutes.
*Note: At the beginning of the day, it is possible to prepare several tubes containing some substrate solution (3.2 mL) and to leave them to incubate in the 30 °C water bath.*

Add 0.8 mL of pH 3.30 buffer citrate (7.3) into the substrate incubated at 30°C (final concentration = 0.24% w/V). Mix using a vortex then fill up the capillary containing a calibrated ball with the mixture (make sure that no air bubble is present in the capillary). Insert the capillary into the viscosimeter.
The viscosimeter will perform 21 measurements cycle (one cycle corresponding to ways 1 and 2). The average drop time for the ***16 last measurements*** **of way 2** for the substrate is labelled Ts (value equal to about 20 s).

If the obtained value is too low or if the drop time decrease is lower than -10 msec/min, discard the substrate and prepare a new substrate solution.

**10.2.2.3.** Enzymatic activity determination for the sample

**Example for AMVn type ANTON PAAR viscosimeter:**

Place 3.2 mL of 0.3% wheat arabinoxylan solution (7.1.) in a disposable sterile tube. Sonicate for 5 seconds then place the tube in a thermostated water bath at 30 °C and leave to equilibrate for (at least) 5 minutes.
Add 0.8 mL of the enzymatic preparation to the 30 °C incubated substrate. Start the stopwatch.
Mix using a vortex then fill up the capillary containing a calibrated ball with the mixture (make sure that no air bubble is present in the capillary).
Insert the capillary into the microviscosimeter.
Start the measurement after exactly 1 minute.

The viscosimeter will perform 30 measurements cycle. The average drop time for the 20 last measurements for the sample is labelled Tt.

At the end of the analysis, remove the capillary and rinse with the solution to be analyzed.

**Example for LOVIS 2000M type ANTON PAAR viscosimeter:**

Place 3.2 mL of 0.3% wheat arabinoxylan solution (7.1.) in a disposable sterile tube.
Sonicate for 5 seconds then place the tube in a thermostated water bath at 30 °C and leave to equilibrate for (at least) 5 minutes.
Add 0.8 mL of the enzymatic preparation to the 30 °C incubated substrate. Start the stopwatch.
Mix using a vortex then fill up the capillary containing a calibrated ball with the mixture (make sure that no air bubble is present in the capillary).
Insert the capillary into the microviscosimeter.
Start the measurement after exactly 1 minute.

The viscosimeter will perform 21 measurements cycle (one cycle corresponding to ways 1 and 2). The average drop time for the ***16 last measurements*** **of way 2** for the sample is labelled Tt.

At the end of the analysis, remove the capillary and rinse with the solution to be analyzed.

# 11. Calculation

## 11.1. Determination of the enzymatic activity

| | | |
|---|---|---|
| Fr | = | relative fluidity |
| Tw | = | average drop time for the water (msec) |
| Ts | = | average drop time for the substrate (msec) |
| Tt | = | drop time during the assay at a given t time (msec) |
| t | = | assay time (minutes) |
| $T_{1/2}$ | = | assay time (t) plus half (½) of Tt (minutes) |
| DF | = | dilution factor |
| $V_T$ | = | total test volume (4 mL) |
| $V_S$ | = | enzyme solution volume in the sample (0.8 mL) |
| FaS | = | substrate factor (specific to each substrate batch) |

① For the selected value (20 or 16 last measurements), calculate Fr for each Tt.

$$Fr = \frac{Ts - Tw}{Tt - Tw}$$

② Plot Fr = f ($T_{1/2}$).
③ Determine the linear regression slope.

④ From it, find out the activity: the obtained slope is proportional to the enzymatic activity present in the sample.

$$\text{Activity (U/g)} = F_r \, \text{Slope} \times DF \times \frac{V_T}{V_S} \times FaS$$

### 11.2. Recovery percentage calculation

The enzymatic activity recovery percentage of the sample is deduced by applying the following calculation formula for different activities:

$$\%\text{Recovery} = \frac{\text{Treated} - \text{Control}}{\text{Spike} - \text{Control}} \times 100$$

### 11.3. Normalised activity calculation

Activity is corrected due to interferences:

$$\text{Normalised Activity (U/kg)} = \%\text{Recovery} \times \text{Theoretical activity (U/kg)}$$

where

$$\text{Theoretical Activity (U/kg)} = \text{Incorporation rate in Feed} \times \text{Item Activity}$$

### 11.4. Results validation

Usually, samples are analyzed in duplicate.
The difference between the two results for the treated and the spike feeds is calculated as follows:

$$\text{Difference} = \frac{\text{Value}_{max.} - \text{Value}_{min.}}{\text{Value}_{min.}} \times 100$$

The difference has to be lower than 30%. If not, perform a new analysis.

### 11.5. Non compliant results

**For example for AMVn or LOVIS 2000M type ANTON PAAR viscosimeter:**
**Substrate blank**: The viscosity reduction maximum (dropping time) that should be obtained during the calibration is - 10 msec / min. Lower values could indicate a contamination of the substrate or of the capillary with an enzyme. Clean thoroughly the capillary and repeat the calibration. If the problem remains, prepare a new substrate solution.

## 12. Precision

Not applicable

## 13. Special cases

For a set to be complete, it must contain:

- a control sample
- the treated feed
- the enzyme (batch number) that was used during the feed production

However, the set may not contain any control feed. Two cases are then possible:

- The feed is essentially composed of wheat or corn. Then use a reference wheat or maize as control.
- The composition is unknown. In this situation, spike the treated with the same concentration as the theoretical concentration. Use an arbitrary value comprised between 0 and 400 for the control.

When the control is absent, the provided values are only indicative.

## 14. Annexes

Annex 1: Equipment list.
Annex 2: AMVn type microviscosimeter settings
Annex 3: LOVIS 2000M type microviscosimeter settings

## 15. Bibliography

N/A

# Annex 1

## Equipment used and associated references for example

### Consumables

| | | |
|---|---|---|
| P1000 cones | GILSON | Ref.: D1000ST |
| 13 mL tubes | SARSTEDT | Ref:: 55-518 PP |
| Hemolysis tubes | VWR | Ref.: 0512102 |
| 2.5 mL combitips | VWR | Ref.: 613-3521 |
| Glassfiber Multigrade syringe filters (Ø 0.45µm) | Millipore | Ref.: SLHVM25NK |
| Stickers | DIPÔLE | Ref.: ETQ5020TCGW |
| Resin film | DIPÔLE | Ref.: FR65AXR7 |

### Equipment

Stopwatch
METTLER AE200 analytical balance, 0.1 mg precision
Mettler Toledo MP230 pH meter, 0.1 pH unit precision

| | | | |
|---|---|---|---|
| GILSON pipettes | P1000 | VWR | Ref: 01-285-077 |
| | P5000 | VWR | Ref: 01-285-102 |
| 4780 Eppendorf multipette | | VWR | Ref: 01-302-011 |

Water bath, 0.1 °C precision
ANTON PAAR AMVn or LOVIS 2000M microviscosimeter + associated software
Ultra-centrifuge grinder
Centrifuge
Ultrasonic bath
Multi-position stirrer

# Annex 2

**Measurement equipment used with AMVn microviscosimeter settings**

| | | |
|---|---|---|
| Apparatus type | AMVn type Anton Paar microviscosimeter | Supplier: ANTON PAAR |
| Capillary | 1.6 mm internal diameter capillary | Supplier: ANTON PAAR |
| Ball | 1.5 mm diameter steel ball | Supplier: ANTON PAAR |
| Measurement angle | 75° | |
| Measurement temperature | 30 °C | |
| Number of measurements for the water blank | 60 | The 20 first values are not taken into account in the calculations |
| Expected value for the water blank | About 12 seconds | |
| Number of measurements for the substrate blank | 30 | The 10 first values are not taken into account in the calculations |
| Expected value for the substrate blank | About 26 seconds | |
| Number of measurements for the sample assay | 30 | The 10 first values are not taken into account in the calculations |

# Annex 3

**Measurement equipment used with LOVIS 2000M microviscosimeter settings**

| | | |
|---|---|---|
| Apparatus type | LOVIS 2000M type Anton Paar microviscosimeter | Supplier: ANTON PAAR |
| Capillary | 1.59 mm internal diameter capillary | Supplier: ANTON PAAR |
| Ball | 1.5 mm diameter steel ball | Supplier: ANTON PAAR |
| Measurement angle | 75° | |
| Measurement temperature | 30 °C | |
| Number of measurements for the water blank | 21 cycles – cycle was composed to way 1 and way 2 | Only the 16 last values in way 2 are taken into account in the calculations |
| Expected value for the water blank | About 9 seconds | |
| Number of measurements for the substrate blank | 21 cycles – cycle was composed to way 1 and way 2 | Only the 16 last values in way 2 are taken into account in the calculations |
| Expected value for the substrate blank | About 20 seconds | |
| Number of measurements for the sample assay | 21 cycles – cycle was composed to way 1 and way 2 | Only the 16 last values in way 2 are taken into account in the calculations |