Megazyme

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D-XYLOSE

INCLUDING

XYLAN & ARABINOXYLAN

ASSAY PROCEDURE

K-XYLOSE 04/18

(*100 Manual Assays per Kit) or
(1300 Auto-Analyser Assays per Kit) or
(1000 Microplate Assays per Kit)

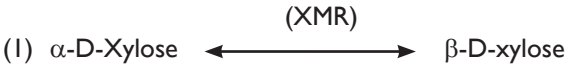
** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

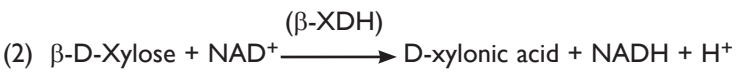
In nature, D-xylose occurs mainly in the polysaccharide form as xylan, arabinoxylan, glucuronoarabinoxylan, xyloglucan and xylogalacturonan. Mixed linkage D-xylans are also found in certain seaweed species and a similar polysaccharide is thought to make up the backbone of psyllium gum. Free D-xylose is found in guava, pears, blackberries, loganberries, raspberries, aloe vera gel, kelp, echinacea, boswellia, broccoli, spinach, eggplant, peas, green beans, okra, cabbage and corn. In humans, D-xylose is used in an absorption test to help diagnose problems that prevent the small intestine from absorbing nutrients, vitamins and minerals in food. D-Xylose is normally easily absorbed by the intestine. When problems with absorption occur, D-xylose is not absorbed and blood and urine levels are low. A D-xylose test can help to determine the cause of a child's failure to gain weight, especially when the child seems to be eating enough food. If, in a polysaccharide, the ratio of D-xylose to other sugars etc. is known, then the amount of the polysaccharide can be quantified from this knowledge plus the determined concentration of D-xylose in an acid hydrolysate. Xylans are a major portion of the polysaccharides that could potentially be hydrolysed to fermentable sugar for biofuel production.

PRINCIPLE:

Interconversion of the α- and β-anomeric forms of D-xylose is catalysed by xylose mutarotase (XMR) (1).



The β-D-xylose is oxidised by NAD^+ to D-xylonic acid in the presence of β-xylose dehydrogenase (β-XDH) at pH 7.5 (2).



The amount of NADH formed in this reaction is stoichiometric with the amount of D-xylose. It is the NADH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

All reported forms of β-xylose dehydrogenase also act on D-glucose. At concentrations of D-glucose similar to the levels of D-xylose measured in this assay, the current enzyme acts slowly. However, at high concentrations of D-glucose, the rate of reaction is very significant and thus problematic. In many instances, there will be a need to measure

D-xylose in the presence of high concentrations of D-glucose (e.g. in mixtures of sugars obtained on acid hydrolysis of wheat flour), thus it is essential to remove the D-glucose. In the current assay protocol, this is achieved by a short pre-incubation of the sample extract with hexokinase in the presence of an excess of ATP (as per the procedure on page 4).

β-Xylose dehydrogenase has no action on L-arabinose.

The smallest differentiating absorbance for the assay is 0.010 absorbance units, this corresponds to 0.350 mg/L of sample solution at the maximum sample volume of 2.00 mL (or to 7.01 mg/L with a sample volume of 0.1 mL). The detection limit is 0.701 which is derived from an absorbance difference of 0.020 and the maximum sample volume of 2.00 mL.

The assay is linear over the range of 2 to 100 µg of D-xylose per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to a D-xylose concentration of approx. 0.175 to 0.350 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-xylose has been completed within the time specified in the assay (approx. 6 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-xylose (approx. 25 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding D-xylose to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 100 assays in manual format (or 1300 assays in auto-analyser format or 1000 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (45 mL, pH 7.5) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+ plus ATP.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: Hexokinase suspension (2.2 mL).
Stable for > 2 years at 4°C.

Bottle 4: XDH/XMR solution (5.6 mL).
Stable for > 2 years below -10°C.

Bottle 5: D-Xylose standard solution (5 mL, 0.25 mg/mL).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of one of bottle 2 in 21 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). Do not dissolve the contents of the second bottle until required.

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position.
Stable for > 2 years at 4°C.

4. Use the contents of bottle 4 as supplied. Before opening for the first time, shake the bottle to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position.
Stable for > 2 years below -10°C.

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years at 4°C.

NOTE: The D-xylose standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of D-xylose is determined directly from the extinction coefficient of NADH (page 5).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.4 mL aliquots of buffer (bottle 1) and NAD^+/ATP solution].
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.
10. Corning® Culture Tubes - screw cap, 16 x 125 mm [Fisher Scientific cat no. TKV-173-030B (tubes); TKV-178-020V (caps)]; Fisher Scientific, interact@fisher.co.uk.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.97 mL
Sample solution: 2-100 µg of D-xylose per cuvette (in 0.1-2.0 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.40 mL	0.40 mL
solution 2 (NAD^+/ATP)	0.40 mL	0.40 mL
suspension 3 (Hexokinase)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after at least 5 min and start the reaction by addition of:		
solution 4 (XDH/XMR)	0.05 mL	0.05 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (~ 6 min).		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-xylose}}$. The value of $\Delta A_{D\text{-xylose}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-xylose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D\text{-xylose}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-xylose [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-xylose:

$$c = \frac{2.97 \times 150.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-xylose}} \quad [g/L]$$

$$= 0.7076 \times \Delta A_{D\text{-xylose}} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of D-xylose

$$= \frac{c_{D\text{-xylose}}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

Content of Arabinoxylan

= Content of D-xylose (g/100 g)
x 100/(D-xylose content of polymer) [g/100 g]

e.g. for wheat flour arabinoxylan with a D-xylose content of 62%.

Arabinoxylan

= Content of D-xylose (g/100 g) x 100/62 [g/100 g]

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for D-xylose can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-xylose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water	35.8 mL
solution 1 (buffer)	8.4 mL
solution 2 (NAD^+/ATP)	8.4 mL (after adding 21 mL of H_2O to bottle 2)
suspension 3 (Hexokinase)	0.42 mL
Total volume	53.02 mL

Preparation of R2:

Component	Volume
distilled water	5.9 mL
solution 4 (XDH/XMR)	1.1 mL
Total volume	7.0 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 6 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 1 g/L of D-xylose using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-xylose can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-xylose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.297 mL
Linearity: 0.1-10 µg of D-xylose per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.040 mL	0.040 mL	0.040 mL
solution 2 (NAD^+/ATP)	0.040 mL	0.040 mL	0.040 mL
suspension 3 (Hexokinase)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:			
solution 4 (XDH/XMR)	0.005 mL	0.005 mL	0.005 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min).			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-xylose present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 2 and 100 µg. The sample solution must therefore be diluted sufficiently to yield a D-xylose concentration between 0.002 and 1.00 g/L.

Dilution Table

Estimated concentration of D-xylose (g/L)	Dilution with water	Dilution factor (F)
< 1.00	No dilution required	1
1.00-10.0	1 + 9	10
10.0-100	1 + 99	100
> 100	1 + 999	1000

If the value of $\Delta A_{D\text{-}xylose}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 7.5 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide should be degassed by increasing the pH to approx. 7.5 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no β-XDH, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-xylose in plant samples.

Mill plant materials to pass a 0.5 mm screen. Weigh out 1.0 g of sample and extract with 90 mL of water (heated to 80°C). Quantitatively transfer to a volumetric flask and dilute to the mark with distilled water. Mix, filter and use the appropriately diluted, clear solution for the assay.

(b) Determination of D-xylose in fermentation samples and cell culture medium.

Incubate an aliquot (approx. 10 mL) of the solution at approx. 90-95°C for 10 min to inactivate enzyme activity. Centrifuge or filter and use the supernatant or clear filtrate (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinisation can be performed with Carrez reagents. Homogenise gelatinous agar media with water and treat further as described above.

(c) Determination of D-xylose in polysaccharides and fibrous plant material.

Mill plant material or polysaccharide to pass a 0.5 mm screen using a Retsch centrifugal mill, or similar. Accurately weigh approx. 100 mg of material into a Corning® screw-cap culture tube (16 x 125 mm). Add 5 mL of 1.3 M HCl to each tube and cap the tubes. Incubate the tubes at 100°C for 1 h. Stir the tubes intermittently during the incubation. Cool the tubes to room temperature, carefully loosen the caps and add 5 mL of 1.3 M NaOH. Quantitatively transfer the contents of the tube to a 100 mL volumetric flask using distilled water and adjust the volume to 100 mL with distilled water. Mix thoroughly by inversion and filter an aliquot of the solution through Whatman No. 1 filter paper or centrifuge at 1,500 *g* for 10 min. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

(d) Determination of D-xylose in whole blood samples.

a. Solutions:

Concentrated Carrez I solution. Dissolve 30 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P9387) in 200 mL of distilled water. Store at room temperature.

Concentrated Carrez II solution. Dissolve 60 g of zinc sulphate {$ZnSO_4.7H_2O$} (Sigma cat. no. Z4750) in 200 mL of distilled water. Store at room temperature.

b. Procedure:

Heat 1 mL of whole blood sample at approx. 80°C for 20 min in a microfuge tube then centrifuge at 13,000 x *g* for 10 min and recover the supernatant. Add 20 µL Carrez Reagent II and mix thoroughly, then add 20 µL Carrez Reagent I and mix thoroughly. Centrifuge the sample again at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The final volume of the clarified supernatant will be approximately one quarter of the starting volume of the original sample. Therefore, adjust the volume of the starting material as required to obtain sufficient volume of clarified sample for the test.

(e) Determination of D-xylose in biological tissue samples.

Accurately weigh approx. 5 g of representative biological tissue into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using an Ultra-turrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above"

the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat (if present). Centrifuge an appropriate volume of the sample at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the clear filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The amount of starting material and volumes used can be adjusted accordingly depending on the amount of analyte present in the sample.

(f) Determination of D-xylose in biological fluid samples (e.g. urine and serum).

For some biological fluid samples it may be sufficient to test them directly without any sample preparation other than appropriate dilution in distilled water. If this is not adequate then deproteinisation with either perchloric acid or trichloracetic acid may be required.

Deproteinise biological samples by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge an appropriate volume of the sample at 1,500 x *g* for 10 min and recover the supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay. Alternatively, use 50% (w/v) trichloroacetic acid instead of perchloric acid.

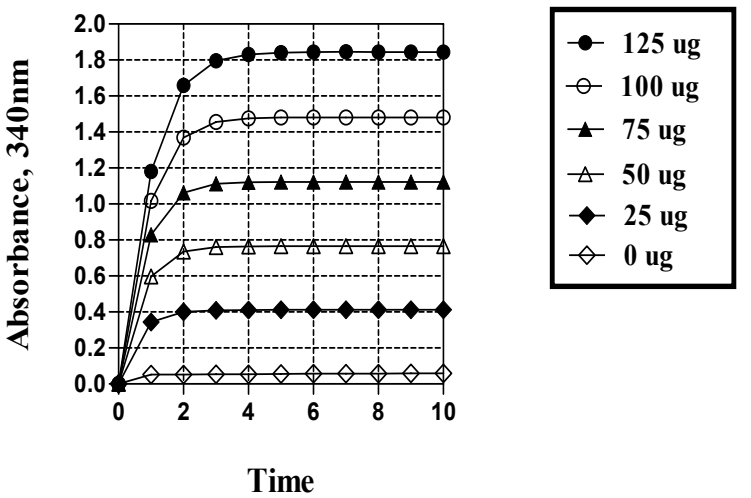


Figure 1. Increase in absorbance at 340 nm on incubation of 0-125 µg of D-xylose with β-xylose dehydrogenase plus xylose mutarotase.

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