Megazyme

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D-MANNITOL/ L-ARABITOL

ASSAY PROCEDURE

K-MANOL 04/18

(*60 Manual Assays per Kit) or
(580 Auto-Analyser Assays per Kit) or
(600 Microplate Assays per Kit)

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

INTRODUCTION:

D-Mannitol is an open-chain hexahydric polyol, produced by hydrogenation of fructose. It is used as a nutrient, a dietary supplement and a texturising agent. Due to its low hydroscopicity, D-mannitol is often used as a dusting powder to protect products against moisture pick-up. A major use of D-mannitol is in chewing gum. D-Mannitol is also widely used in intestinal permeability tests.

PRINCIPLE:

D-Mannitol is oxidised by nicotinamide-adenine dinucleotide (NAD^+) to D-fructose in the presence of mannitol dehydrogenase (ManDH) with the formation of reduced nicotinamide-adenine dinucleotide (NADH) (1). In a parallel reaction, the enzyme also oxidises L-arabitol to L-xylulose (2).

$$\text{(1)}\quad \text{D-Mannitol} + NAD^+ \xrightarrow{\text{(ManDH)}} \text{D-fructose} + NADH + H^+$$

$$\text{(2)}\quad \text{L-Arabitol} + NAD^+ \xrightarrow{\text{(ManDH)}} \text{L-xylulose} + NADH + H^+$$

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

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

As well as D-mannitol and L-arabitol, ManDH also slowly oxidises sorbitol; at 50 µg per assay, it is not detected in the standard determination. However at much higher concentrations (e.g. 250 µg per assay), it does contribute to the final absorbance value and must be allowed for (see discussion under "Interference" and refer to Figure 1, on page 12). No activity could be detected on xylitol or glycerol at 3,000 µg in the assay mixture.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.17/0.14 mg of D-mannitol/L-arabitol sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.50/0.42 mg/L, which is derived from an absorbance difference of 0.015 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 5 to 75 µg of D-mannitol/L-arabitol 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-mannitol concentration of approx. 0.17 to 0.34 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:

As well as D-mannitol and L-arabitol, ManDH also slowly oxidises sorbitol (see Figure 1, page 12).

If the conversion of D-mannitol or L-arabitol has been completed within the time specified in the assay (approx. 4 min), it can be generally concluded that no interference has occurred. If the sample contains high levels of D-sorbitol, after the rapid oxidation of the D-mannitol or L-arabitol is complete (3-4 min), there is a second, less rapid and linear increase in absorbance at 340 nm. This is readily observed from a kinetic curve (see Figure 1, page 12), but can also be observed and accounted for without the need for a recording spectrophotometer. In the latter case, simply take an absorbance reading at 4 min, and then at 2 min intervals over the next 8 min. Plot the absorbance values and extrapolate back to the time of addition of ManDH. From this absorbance value, calculate the D-mannitol/L-arabitol content. Lack of interference in the assay can be further checked by adding D-mannitol or L-arabitol (approx. 30 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-mannitol or L-arabitol 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 60 assays in manual format (or 580 assays in auto-analyser format or 600 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (10 mL, pH 9.0) plus sodium azide (0.02% w/v) as preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+.
Stable for > 2 years below -10°C.

Bottle 3: Mannitol dehydrogenase suspension (1.3 mL).
Stable for > 2 years at 4°C.

Bottle 4: D-Mannitol standard solution (5 mL, 0.30 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 3.3 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).

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.
Stable for > 2 years at 4°C.

NOTE: The D-mannitol 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-mannitol is determined directly from the extinction coefficient of NADH (see 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 0.1 mL aliquots of buffer 1 and of NAD^+ solution).
   - with 25.0 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.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.32 mL
Sample solution: 2-75 µg of D-mannitol per cuvette (in 0.10-2.00 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)	0.10 mL	0.10 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:		
suspension 3 (ManDH)	0.02 mL	0.02 mL
Mix* and read absorbances of the solutions (A_2) at the end of the reaction (approx. 4 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 $\Delta A_{D\text{-mannitol}}$.

The value of $\Delta A_{D\text{-mannitol}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-mannitol and/or L-arabitol can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of the substance to be assayed [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-mannitol:

$$c = \frac{2.32 \times 182.17}{6300 \times 1.0 \times 0.10} \times \Delta A_{D\text{-mannitol}} \quad [g/L]$$

$$= 0.6708 \times \Delta A_{D\text{-mannitol}} \quad [g/L]$$

for L-arabitol:

$$c = \frac{2.32 \times 152.15}{6300 \times 1.0 \times 0.10} \times \Delta A_{L\text{-arabitol}} \quad [g/L]$$

$$= 0.5603 \times \Delta A_{L\text{-arabitol}} \quad [g/L]$$

This calculation is correct only if just D-mannitol or L-arabitol is present in the sample solution.

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-mannitol

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

Content of L-arabitol

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

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-mannitol/L-arabitol 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-mannitol/L-arabitol **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 solution 1 (buffer) solution 2 (NAD^+)	58.6 mL 3.3 mL 3.3 mL (after adding 3.3 mL of H_2O to bottle 2)
Total volume	65.2 mL

Preparation of R2:

Component	Volume
distilled water suspension 3 (ManDH)	6.9 mL 0.6 mL
Total volume	7.5 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 4 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.75 g/L of D-mannitol/L-arabitol using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-mannitol/L-arabitol 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-mannitol/L-arabitol **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: 0.232 mL
Linearity: 0.1-7.5 µg of D-mannitol/L-arabitol 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)	0.010 mL	0.010 mL	0.010 mL
solution 2 (NAD^+)	0.010 mL	0.010 mL	0.010 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 3 (ManDH)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 4 min). If the reaction has not stopped after 4 min, continue to read the absorbances at 1 min intervals until the absorbances increase constantly over 1 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).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 3.

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-mannitol present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 5 and 75 µg. The sample solution must therefore be diluted to yield a D-mannitol concentration between 0.05 and 0.75 g/L.

Dilution Table

Estimated concentration of D-mannitol (g/L)	Dilution with water	Dilution factor (F)
< 0.75	No dilution required	1
0.75-7.5	1 + 9	10
> 7.5	1 + 99	100

If the value of $\Delta A_{D\text{-mannitol}}$ 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 an acidic sample is to be used undiluted (such as red wine or coloured fruit juice), the pH of the solution should be increased to approx. 9.0 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing carbon dioxide should be degassed by increasing the pH to approx. 9.0 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 ManDH, should be performed in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of polyvinylpolypyrrolidone (PVPP). Stir for 2 min and then filter.

(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 20°C and fill the volumetric flask to the mark with 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-mannitol in chewing gum.

Accurately weigh approx. 2 g of representative chewing gum sample into a 50 mL Duran® bottle. Add 10 mL of toluene and 20 mL of distilled water and stir the slurry on a magnetic stirrer for about 20 min (until the gum is fully dispersed). Centrifuge the suspension at approx. 1,500 *g* in sealed polypropylene tubes for 10 min, and carefully remove the upper phase (toluene) and discard with waste solvents. Transfer the lower phase (aqueous) to a 100 mL volumetric flask and adjust to volume. Use 0.1 mL for assay.

(b) Determination of D-mannitol in protein-containing samples.

Deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, use Carrez reagents.

(c) Determination of D-mannitol 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.

(d) Determination of D-mannitol 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.

(e) Determination of D-mannitol 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.

REFERENCE:

Beutler, H. O. (1988). Xylitol. "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U. ed.), 3rd ed., **Vol VI**, p. 487, VCH Publishers (UK) Ltd., Cambridge, UK.

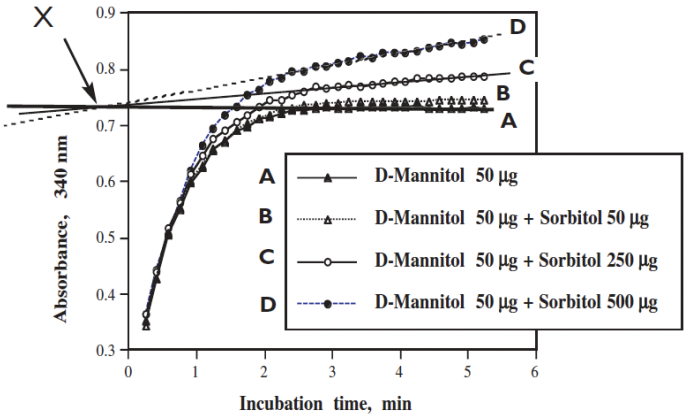

Figure 1. Increase in absorbance on oxidation of D-mannitol or D-mannitol/D-sorbitol mixtures by ManDH in the presence of NAD^+. **A.** D-mannitol (50 µg); **B.** D-mannitol (50 µg) + D-sorbitol (50 µg); **C.** D-mannitol (50 µg) + D-sorbitol (250 µg); or **D.** D-mannitol (50 µg) + D-sorbitol (500 µg). "**X**" marks the time of addition of ManDH.

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Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com