

# Megazyme

---

**D-MANNITOL  
L-ARABITOL**

**ASSAY PROCEDURE**

KMANOL 12/03

(60 Determinations per Kit)

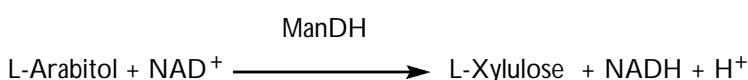
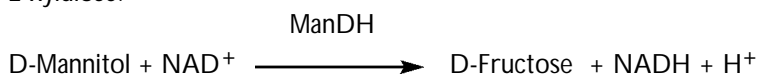


### INTRODUCTION:

D-Mannitol is an open-chain hexahydric polyol, produced by hydrogenation of fructose. It is used as a nutrient, dietary supplement and a texturizing agent. Due to its low hygroscopicity, 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<sup>+</sup>) to D-fructose in the presence of mannitol dehydrogenase (ManDH) with the formation of reduced nicotinamide-adenine dinucleotide (NADH). In a parallel reaction, the enzyme also oxidises L-arabitol to L-xylulose.



The absorbance increase is measured at 340 nm.

### KITS:

Kits suitable for performing 60 assays are available from Megazyme. The kits contain:

**Bottle 1:** Tris-HCl buffer, (50 ml, 100 mM, pH 9.0), containing 1.5 mg/ml BSA and 0.02% sodium azide. Store at 4°C. Stable for > 2 years at 4°C.

Caution ! Contains sodium azide.

**Bottle 2:** (x2) NAD<sup>+</sup> (150 mg); freeze dried powder. Stable for > 2 years at -20°C.

**Bottle 3:** Mannitol dehydrogenase (1.3 ml, 300 U/ml ManDH). Stable for > 2 years at 4°C.

**Bottle 4:** D-Mannitol standard solution (5 ml, 0.50 mg/ml). This solution is for assay control purposes only (see page 6). Measurement of this solution is not necessary for calculating the results. Stable for > 2 years at room temperature.

### PREPARATION OF REAGENTS:

1. Use the contents of bottle 1 as supplied.  
Stable for ~ 2 years at 4°C.
2. Dissolve contents of one of bottle 2 in 3.3 ml distilled water.  
Divide into appropriately sized aliquots and store at -20°C  
between use and on ice during use. Stable for 12 months at  
-20°C. Do not dissolve the contents of the second bottle  
until required.
3. Use the contents of bottle 3 as supplied. Store at 4°C  
between use and on ice during use.  
Stable for > 2 years at 4°C.
4. Use the contents of bottle 4 as supplied.

### EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Cuvettes. Disposable plastic cuvettes (1 cm light path, 3.0 ml).
3. Micro-pipettors, e.g. Gilson Pipetman (20 µl).
4. Positive displacement pipettor e.g. Eppendorf Multipette®  
- with 5.0 ml Combitip® (to dispense 0.8 ml aliquots of  
Tris-HCl buffer, 0.1 ml aliquots of NAD<sup>+</sup>, 0.1-2.0 ml of sample  
solution and 0.1-2.0 ml of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA YellowLab Test Tube Shaker TTS).
8. Thermostatted hot-block heater set at 25°C (optional).
9. Stop clock.
10. Whatman No.1 (9 cm) filter papers.

**PROCEDURE:**

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 2.92 ml

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

Sample solution: 2-75 µg D-mannitol per cuvette (in 0.10-2.0 ml sample volume)

Pipette into cuvettes	Blank	Sample
Solution 1 (Tris-HCl buffer + BSA):	0.80 ml	0.80 ml
Solution 2 (NAD <sup>+</sup> ):	0.10 ml	0.10 ml
Sample:	-	0.10 ml
Distilled water:	2.00 ml	1.90 ml
Mix*, after 2 min, read the absorbances of solutions (A <sub>1</sub> ). The reaction is then started by the addition of:		
Solution 3 (ManDH):	0.02 ml	0.02 ml
Mix*, wait until the reaction has stopped (approx. 3-4 min) and read absorbance (A <sub>2</sub> ).		

\* For example, with a plastic spatula or by gently inverting several times after sealing the cuvette with Parafilm<sup>R</sup>

### CALCULATIONS:

Determine the absorbance differences ( $A_2 - A_1$ ) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

The measured absorbance difference should, as a rule, be at least 0.10 absorbance units to achieve sufficiently precise results.

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{d \times v \times 1000} \times A \quad [\text{g/l}]$$

where:

- V = final volume [ml]
- v = sample volume [ml]
- MW = molecular weight of the substance being assayed [g/mol]
- d = light path [cm]
- = extinction coefficient of NADH at 340 nm
- = 6.3 [l x mmol<sup>-1</sup> x cm<sup>-1</sup>]

It follows for D-mannitol:

$$c = \frac{2.92 \times 182.17}{6.3 \times 1.0 \times 0.10 \times 1000} \times A_{\text{D-mannitol}} \quad [\text{g/l}]$$
$$= 0.844 \times A_{\text{D-mannitol}} \quad [\text{g D-mannitol/l sample solution}]$$

It follows for L-arabitol:

$$c = \frac{2.92 \times 152.15}{6.3 \times 1.0 \times 0.10 \times 1000} \times A_{\text{L-arabitol}} \quad [\text{g/l}]$$
$$= 0.705 \times A_{\text{L-arabitol}} \quad [\text{g L-arabitol/l sample solution}]$$

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 appropriate 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

$$= \frac{C_{\text{D-mannitol}} [\text{g/l sample solution}]}{\text{weight}_{\text{sample}} [\text{g/l sample solution}]} \times 100 \quad [\text{g/100g}]$$

Content of L-arabitol

$$= \frac{C_{\text{L-arabitol}} [\text{g/l sample solution}]}{\text{weight}_{\text{sample}} [\text{g/l sample solution}]} \times 100 \quad [\text{g/100g}]$$

#### **SPECIFICITY:**

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). No activity could be detected on xylitol or glycerol at a concentration of 3000 µg in the assay mixture.

#### **SENSITIVITY:**

The smallest measureable absorbance for the procedure is 0.005 absorbance units. With a sample volume of 2.00 ml this corresponds to a D-mannitol/L-arabitol concentration of 0.21/18 mg/litre of sample solution (with a sample volume of 0.1 ml, this corresponds to 4.2/3.5 mg/litre of sample solution). The detection limit is 0.59/49 mg/litre, and this is derived from an absorbance difference of 0.014 with maximum sample volume (2.00 ml).

#### **LINEARITY:**

Linearity occurs between 5.0 µg D-mannitol/L-arabitol per assay (2.5 mg D-mannitol/L-arabitol per l of sample solution; sample volume = 2.00 ml) to 75 µg D-mannitol/L-arabitol per assay (0.75 g D-mannitol/L-arabitol per l of sample solution; sample volume = 0.100 ml).

#### **ACCURACY:**

In duplicate determinations of a particular sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.10 ml, this corresponds to a D-mannitol concentration of 4.2-8.4 mg/l. If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor, F. If the sample is

weighed for sample preparation, e.g.using 1 g/100 ml, a difference of 0.015-0.03 g/100 g can be expected.

### RECOGNIZING INTERFERENCE:

a. If the conversion of D-mannitol or L-arabitol has been completed within the time stated in the assay procedure, it can be concluded that, in most cases, 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), 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 to the time of addition of the enzyme. From this absorbance value, calculate the D-mannitol/ L-arabitol content.

b. Possible interference by substances contained in the sample can be recognized by including an internal standard as a control: this is performed by running a determination with the sample, and a second determination in which a set volume of standard solution is added in addition e.g. 0.05 ml of D-mannitol standard solution (50 µg/0.1 ml). Alternatively, the D-mannitol standard solution can be added after the reaction has reached its end point (taking into consideration the new total volume of the reaction mixture, in the calculations).

The D-mannitol standard solution can be used as an internal standard in order to check the measurement for interfering substances as follows:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
Solution 1	0.80 ml	0.80 ml	0.80 ml	0.80 ml
Solution 2	0.10 ml	0.10 ml	0.10 ml	0.10 ml
Sample soln.	-	0.10 ml	-	0.05 ml
D-mannitol standard soln.	-	-	0.10 ml	0.05 ml
Distilled water	2.00 ml	1.90 ml	1.90 ml	1.90 ml

Mix, and read the absorbances of the solutions ( $A_1$ ) after approx. 2 min. Continue as per the described procedure (page 3). Follow the instructions given under "Procedure".

The recovery of the standard is calculated according to the following equation:

$$\text{Recovery} = \frac{[2 \times A_{(\text{sample} + \text{standard})}] - A_{\text{sample}}}{A_{\text{standard}}} \times 100 (\%)$$

### SAFETY:

In general the reagents used in the D-mannitol/L-arabitol determination procedure are not hazardous as defined by EC Regulation 67/548EEC and updates. However, a low concentration of sodium azide is added to the buffer to prevent microbial spoilage.

### SAMPLE PREPARATION:

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, and therefore the sample solution should be diluted, if necessary to a concentration of between 0.0025 and 0.75 g/l, as illustrated below.

Dilution Table

Estimated concentration of D-mannitol (g/l)	Dilution with water	Dilution factor (F)
< 0.05	-	1
0.05 – 0.5	1 + 9	10
0.5 – 5	1 + 99	100
> 5	1 + 999	1000

If the absorbance difference measured  $A (A_2 - A_1)$  for the sample is less than 0.10 absorbance units larger than that of the blank, the sample volume may be increased to 2.0 ml (making sure the sum of the sample and distilled water components in the reaction is 2.0 ml and using the new sample volume “v” in the equation). If  $A$  is still too low (e.g. less than 0.1), weigh out more sample, or dilute it less strongly.

**Liquid samples:** Clear/slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

**Acidic samples:** If an acidic sample is to be used undiluted (such as red wine or coloured fruit juice), increase the pH of the solution to approx. 9.0 using 2 M NaOH.

**Carbon dioxide:** Degass samples containing carbon dioxide either by stirring, e.g. with a glass rod for 5 min, or by filtration.

**Coloured samples:** Perform a sample blank, i.e. sample with no ManDH enzyme, in the case of coloured samples.

**Strongly coloured samples:** If used undiluted, decolorise strongly-coloured samples by adding 1 g activated charcoal per 100 ml of solution. Stir for 1 min and filter.

**Solid samples:** Homogenise or crush solid samples in distilled water and filter if necessary.

**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, 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.

**Samples containing protein:** Deproteinize samples containing protein with perchloric acid; alternatively, clarify with Carrez reagents.

**Carrez I solution.**- Dissolve 3.60 g of potassium hexacyanoferrate (II)  $\{K_4[Fe(CN)_6] \cdot 3H_2O\}$  (Sigma Cat. no. P-9387) in 100 ml of distilled water.

**Carrez II solution.**- Dissolve 7.20 g of zinc sulphate ( $ZnSO_4 \cdot 7H_2O$ ) (Sigma Cat. no. Z-4750) in 100 ml of distilled water.

**Sodium Hydroxide (100 mM).**- Dissolve 4 g of sodium hydroxide in 1 litre of distilled water.

#### EXAMPLES:

##### 1. Determination of D-mannitol in chewing gum.

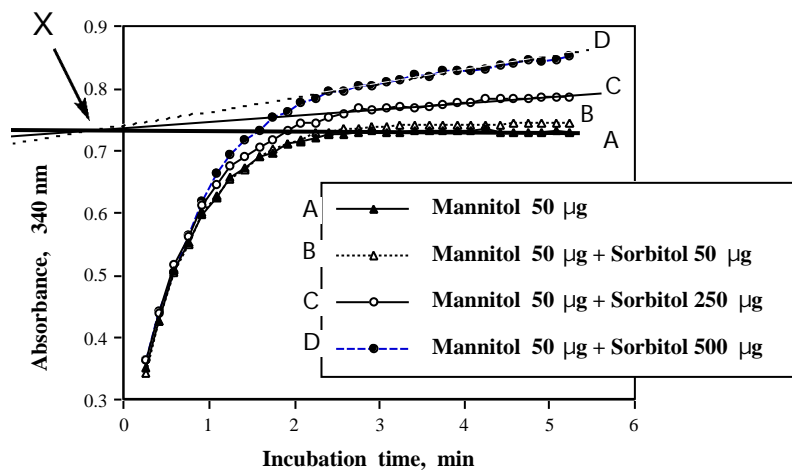
Accurately weigh approx. 2 g of representative chewing gum sample into a 50 ml Duran bottle. Add 10 ml of toluene and 20 ml of distilled water and stir the slurry on a magnetic stirrer for about 20 min (until the gum is dispersed). Centrifuge the suspension at 3,000 rpm (~1,500 g) in sealed polypropylene tubes, 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.

##### 2. Determination of D-mannitol in other food materials, beverages or confectionery.

Samples should be prepared in a manner similar to that employed for D-sorbitol, L-malic acid, D-malic acid or acetic acid, as described in the relevant Megazyme test booklets (see [www.megazyme.com](http://www.megazyme.com)).

#### REFERENCE:

Beutler, H.-O. (1988) in Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.), 3rd ed., Vol VI, pp. 487, VCH Publishers (UK) Ltd., Cambridge, U.K.



**Figure 1.** Increase in absorbance on oxidation of D-mannitol, or D-mannitol/D-sorbitol mixtures by ManDH in the presence of NAD<sup>+</sup>. **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.



Megazyme International Ireland Ltd.,  
 Bray Business Park, Bray,  
 Co. Wicklow,  
 IRELAND

Telephone: (353.1) 286 1220  
 Facsimile: (353.1) 286 1264  
 Internet: [www.megazyme.com](http://www.megazyme.com)  
 E-Mail: [info@megazyme.com](mailto:info@megazyme.com)

**WITHOUT GUARANTEE**

The information contained in this booklet is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.





