

# Megazyme

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## D-SORBITOL/XYLITOL

### ASSAY PROCEDURE

KSORB 02/04

(58 Determinations per Kit)



### INTRODUCTION:

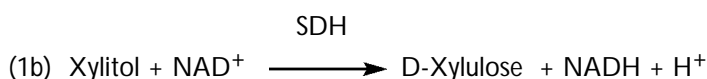
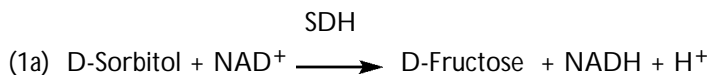
Sorbitol, a polyol (sugar alcohol), is a bulk sweetener found in numerous food products. Sorbitol is about 60% as sweet as sucrose with one-third the calories. It has a smooth mouthfeel with a sweet, cool and pleasant taste. Sorbitol is non-cariogenic and finds application in diatetic diets. It has been safely used in processed foods for almost half a century. It is also used in other products such as cosmetics and pharmaceuticals.

Sorbitol occurs naturally in a wide variety of fruits and berries. It is produced commercially by the hydrogenation of glucose. It has been affirmed GRAS (generally recognized as safe) by the U.S. Food and Drug Administration and has been approved for food use in Europe and many other countries around the world.

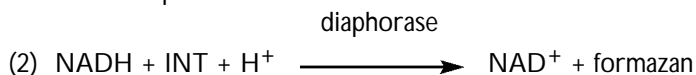
Sorbitol can cause gastrointestinal symptoms in adults when they consume approximately 10-50 g per day

### PRINCIPLE:

D-Sorbitol is oxidised by nicotinamide-adenine dinucleotide ( $\text{NAD}^+$ ) to D-fructose in the presence of sorbitol dehydrogenase (SDH) with the formation of reduced nicotinamide-adenine dinucleotide (NADH). In a parallel reaction, the enzyme also oxidises xylitol to D-xylulose.



However, since the equilibrium of the reaction lies in the favour of D-sorbitol or xylitol and  $\text{NAD}^+$ , a further reaction is required to utilise the NADH product. In this second reaction, in the presence of diaphorase, NADH reduces iodinitrotetrazolium chloride (INT) to a formazan compound.



The absorbance of the formazan is measured at 492 nm.

### KITS:

Kits suitable for performing 58 assays are available from Megazyme. The kits contain:

**Bottle 1:** Triethanolamine (TEA) buffer (40 mL, 0.2 M, pH 8.6), containing 50 mM potassium chloride, 2% Triton X-100 and 10% methanol. Store at 4°C.

**NOTE:** Methanol is a poisonous chemical, so the buffer should be handled with gloves. Methanol positively contributes to the assay by lowering the assay blank value ("creep" value). Buffer not containing methanol is available on request.

**Bottle 2:** (x2) NAD<sup>+</sup> (70 mg) + iodonitrotetrazolium chloride (INT) (3.5 mg); freeze dried powder. Stable for > 2 years at -20°C.

**Bottle 3:** Diaphorase (1.25 ml, 150 U/ml) in ammonium sulphate suspension. Stable for > 2 years 4°C.

**Bottle 4:** (x3) Sorbitol dehydrogenase lyophilizate (40 U/vial; SDH). Stable for > 2 years at -20°C.

**Bottle 5:** D-Sorbitol standard solution (5 ml, 0.15 mg/mL). Stable for > 2 years at 4°C. This solution is for assay control purposes only. Measurement of this solution is not necessary for calculating the results.

### 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 6.0 mL distilled water. Divide into appropriately sized aliquots and store in a dark container at -20°C between use and on ice during use. Stable for > 12 months at -20°C.
3. Use the contents of bottles 3 and 5 as supplied.
4. Dissolve the contents of one of bottle 4 in 1.0 ml of distilled water. Store at 4°C between use and on ice during use. On dissolution, this enzyme is stable for ~ 2 months at 4°C. **It is unstable at -20°C.**

#### **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 (10  $\mu$ l).
4. Positive displacement pipettor e.g. Eppendorf Multipette®  
- with 5.0 ml Combitip® (to dispense 0.6 ml aliquots of TEA buffer, 0.2 ml aliquots of NAD<sup>+</sup>/INT, 0.1-2.0 ml of sample solution and 0.1-2.0 ml of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 492 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.

#### **REFERENCES:**

1. Bergmeyer, H.U., (1988) in Methods of Enzymatic Analysis (Bergmeyer, H.U. ed) 3rd ed., Vol.VI, pp. 356-362, VCH Publishers (UK) Ltd., Cambridge, U.K.
2. Beutler, H.-O. (1988) in Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.) 3rd ed., Vol.VI, pp. 484-490, VCH Publishers (UK) Ltd., Cambridge, U.K.
3. International Federation of Fruit Juice Producers (IFU, Methods of Analysis, no. 62-1995); contained in "Code of Practice for Evaluation of Fruit and Vegetable Juices". (1996) edited by Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community (A.I.J.N.).

**PROCEDURE:**

Wavelength: 492 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 2.87 mL

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

Sample solution: 1.0-20 µg D-sorbitol or xylitol per cuvette (in 0.10-2.0 ml sample volume)

Pipette into cuvettes	Blank	Sample
Solution 1 (TEA buffer mix):	0.60 ml	0.60 ml
Distilled water:	2.00 ml	1.90 ml
Sample:	-	0.10 ml
Solution 2 (NAD <sup>+</sup> /INT):	0.20 ml	0.20 ml
Solution 3 (diaphorase):	0.02 ml	0.02 ml
Mix* and after 2 min read the absorbances of solutions (A <sub>1</sub> ). Repeat the measurement after 2 min. If a change in absorbance greater than 0.010 is observed, the sample must be treated to remove reducing substances (see below). The reaction must be started immediately by the addition of:		
Solution 4 (SDH):	0.05 ml	0.05 ml
Mix*, wait until the reaction has stopped (approx. 20 min) and read absorbances of the solutions (A <sub>2</sub> ). If the reaction has not stopped after 20 min, continue to read the absorbances at 5 min intervals until the absorbance increases constantly for 5 min (see Figure 2).		

\* For example, with a plastic spatula or by gentle inversion after sealing the cuvette with Parafilm<sup>R</sup>

If the absorbance (A<sub>2</sub>) increases constantly, extrapolate the absorbance to the time of the addition of solution 4 (SDH).

**NOTE:**

1. INT and the reaction system containing INT is sensitive to light. Consequently, reactions must be performed in dark.
2. Cap the cuvettes and store in a dark cupboard or in the spectrophotometer cuvette compartment with the photometer lid closed.

### 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 differences 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:

$$\begin{aligned} V &= \text{final volume [ml]} \\ v &= \text{sample volume [ml]} \\ MW &= \text{molecular weight of the substance being assayed [g/mol]} \\ d &= \text{light path [cm]} \\ &= \text{extinction coefficient of INT-formazan at 492 nm} \\ &= 19.9 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]} \end{aligned}$$

It follows for D-sorbitol:

$$\begin{aligned} c &= \frac{2.87 \times 182.17}{19.9 \times 1.0 \times 0.10 \times 1000} \times A_{\text{D-sorbitol}} \quad [\text{g/l}] \\ &= 0.2627 \times A_{\text{D-sorbitol}} \quad [\text{g D-sorbitol/l sample solution}] \end{aligned}$$

for xylitol:

$$\begin{aligned} c &= \frac{2.87 \times 152.15}{19.9 \times 1.0 \times 0.10 \times 1000} \times A_{\text{xylitol}} \quad [\text{g/l}] \\ &= 0.2194 \times A_{\text{xylitol}} \quad [\text{g xylitol/l sample solution}] \end{aligned}$$

This calculation is correct only if just D-sorbitol or xylitol 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-sorbitol

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

Content of xylitol

$$= \frac{C_{\text{xylitol}} [\text{g/L sample solution}]}{\text{weight}_{\text{sample}} [\text{g/L sample solution}]} \times 100 [\text{g/100g}]$$

### **SPECIFICITY:**

As well as D-sorbitol and xylitol, sorbitol dehydrogenase also oxidises other polyols such as tibitol, iditol and allitol, but with a lower rate. Polyalcohols such as mannitol, arabitol and dulcitol do not react.

Under the assay conditions described on page 5, glycerol is oxidised very slowly (approximately 0.4% conversion with 100 µg of glycerol/assay), as is galactitol (approximately 3% conversion with 10 µg of galactitol/assay). However, the contribution of these substances, if present in the sample, can be taken into account by extrapolation back to the time of addition of the sorbitol dehydrogenase, using the linear "end-rate" of reaction.

### **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-sorbitol (or xylitol) concentration of ~ 0.06 mg/litre of sample solution (with a sample volume of 0.1 ml, this corresponds to ~ 1.2 mg/litre of sample solution). The detection limit is ~ 0.18 mg/litre, and this is derived from an absorbance difference of 0.015 with maximum sample volume (2.00 ml).

### **LINEARITY:**

Linearity occurs between 1.0 µg D-sorbitol (or xylitol)/assay (0.5 mg D-sorbitol (or xylitol) /l of sample solution; sample volume = 2.00 ml) to 20 µg D-sorbitol (or xylitol) /assay (0.2 g D-sorbitol (or xylitol)/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-sorbitol/xylitol concentration of ~ 1.2-2.4 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.

Coefficients of variation between 1.0% to 3.3% appear in literature for measurement of sorbitol in solution and for measurement in baked goods.<sup>1,2</sup>

#### **SAFETY:**

In general, the reagents used in the sorbitol/xylitol assay determination procedure are not hazardous as defined by EC Regulation 67/548EEC and updates. However, the optimal buffer mix contains 10% methanol, which is hazardous. Buffer free of methanol is available on request, but with this reaction blanks will be higher.

However, the general safety procedures that apply to the use of chemical substances should be followed.

#### **INTERFERENCE:**

High concentrations of reducing substances, such as L-ascorbic acid in fruit juices or SO<sub>2</sub> in jam interfere with the assay as they interact with INT causing a "creep" reaction. Concentrations of L-ascorbic acid higher than 5 µg/ml should be removed by treating the sample with H<sub>2</sub>O<sub>2</sub> and alkali.

#### **Perform this treatment as follows:**

Weigh or pipette sample, diluted if necessary, into a 50 ml volumetric flask. Add water to a volume of about 40 ml, plus 1 ml of KOH (2M) and 0.01 ml of H<sub>2</sub>O<sub>2</sub> (30% w/v). Incubate solution for 10 min at approx. 70°C. Cool to 20-25°C, adjust to pH 7-8 with sulphuric acid (1 M). Fill to the mark with water, mix well, filter and use the solution for the assay.

Reducing sugars (e.g. D-fructose) up to 1 mg/assay do not interfere with the assay (over a period of 60 min).

#### **RECOGNIZING INTERFERENCE:**

- a. If the conversion of D-sorbitol or xylitol has been completed within the time stated in the assay procedure, it can be concluded that, in most cases, no interference has occurred.
- b. Possible interference by substances contained in the sample can be recognized by including an internal standard as a control: this is performed by performing a determination with the sample, and a second determination in which a set volume of assay control solution is added e.g. 0.1 ml of D-sorbitol control solution (10 µg/0.1 ml).

The assay control solution can be used as an internal standard in order to check the measurement for interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
Solution 1	0.60 ml	0.60 ml	0.60 ml	0.60 ml
Solution 2	0.20 ml	0.20 ml	0.20 ml	0.20 ml
Solution 3	0.02 ml	0.02 ml	0.02 ml	0.02 ml
Sample solution	-	0.10 ml	-	0.05 ml
Assay control sln.	-	-	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<sub>1</sub>) after approx. 2 min. Continue as described in the procedure on page 5. Follow the instructions given under "Procedure".

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

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

### SAMPLE PREPARATION:

The amount of D-sorbitol or xylitol present in the cuvette (i.e. in the 0.1 ml of sample being analysed) should range between 1.0 and 20 µg, and therefore the sample solution should be diluted, if necessary to a concentration of between 0.010 and 0.10 g/l, as illustrated below.

Dilution Table

Estimated concentration of D-sorbitol or xylitol, (g/l)	Dilution with water	Dilution factor (F)
< 0.10	-	1
0.1 – 1.0	1 + 9	10
1.0 – 10	1 + 99	100
> 10	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. 8.0 using 2 M NaOH.

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

**Coloured samples:** Perform a sample blank, i.e. sample with no SDH enzyme, in the case of coloured samples (see procedure).

**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 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.

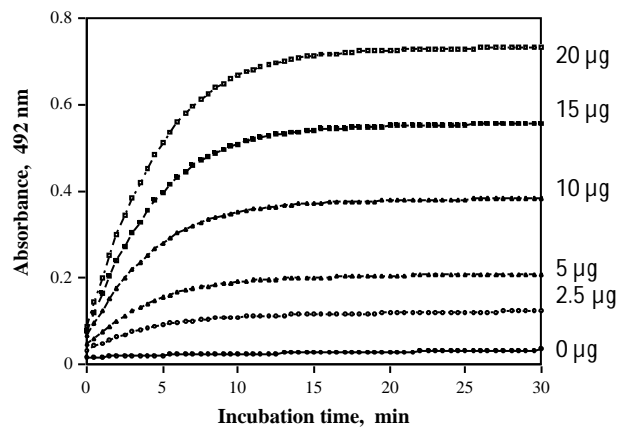
**Samples containing fat:** Extract such samples with hot water at a temperature above the melting point of the fat e.g. in a 100 ml volumetric flask. Adjust to 20°C and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min, filter. Discard the first few ml of filtrate, and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

## EXAMPLES:

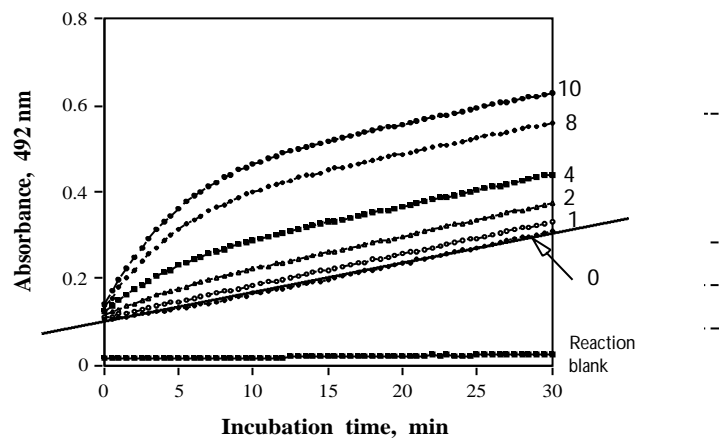
### 1. Determination of D-sorbitol/xylitol in fruit juice.

Pipette 5.0 ml of blackcurrent juice or 1.0 ml of apple juice (filtered if necessary) into a 50 ml volumetric flask. Add successively 30 ml of redistilled water, 1 ml KOH (2 M) and 0.05 ml of hydrogen peroxide solution (30% w/v), mix and incubate for 15 min at 25°C. Adjust the pH to approx 8 by the addition of sulphuric acid (1 M). Add 0.01 ml of catalase (Megazyme Cat. no. E-CATYL), mix and incubate for 20 min (10 min in the case of apple juice), fill the volumetric flask to the mark

with water and filter. Use the filtrate for the assay. As can be seen from Figure 2, with blackcurrent preparation there is a quite rapid increase in absorbance, but this is not due to D-sorbitol. When D-sorbitol was added at 1-10 mg/assay, the absorbance increased rapidly and then continued increasing at the same rate as the background reaction (blackcurrent juice + no added sorbitol). These results indicate that this particular blackcurrent juice contains little, if any, D-sorbitol.



**Figure 1.** Increase in absorbance at 492 nm on incubation of D-sorbitol with SDH under standard assay conditions. Levels of added D-sorbitol were: 0 µg, 2.5 µg, 5 µg; 10 µg; 15 µg; and 20 µg.



**Figure 2.** Increase in absorbance at 492 nm on incubation of blackcurrent preparation (see method) with and without added D-sorbitol with SDH. Levels of added D-sorbitol were: 0 µg; 1 µg; 2 µg; 4 µg, 8 µg, or 10 µg.

**2. Determination of D-sorbitol/xylitol in diabetic honey:**

Accurately weigh approx. 5 g of honey into a 100 ml volumetric flask and add approx. 70 ml water. Incubate for 10 min at approx 70°C. Allow to cool and fill to the mark with water. Dilute solution according to the dilution table and use it for the assay.

**3. Determination of D-sorbitol/xylitol in protein-containing samples:**

Deproteinize protein-containing sample solutions with ice-cold perchloric acid (1 M) in a ratio of 1:2 (1 + 1), centrifuge and neutralize the supernatant with KOH (1 M).

**4. 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.

**5. Further applications:**

For details of sampling, treatment and stability of other samples such as pharmaceuticals, cosmetics etc., refer to Bergmeyer (1988)<sup>1</sup>.



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