Megazyme

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TREHALOSE

ASSAY PROCEDURE

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K-TREH 01/20

(*100 Manual Assays per Kit) or
(1100 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*

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INTRODUCTION:

Trehalose is a naturally occurring disaccharide containing two glucose molecules bound in an α,α-1,1 linkage. This structure results in a chemically stable, non-reducing sugar with many important functional characteristics. Trehalose is commonly found in nature, provides a source of energy, and has been shown to be a primary factor in stabilising organisms during times of freezing, drying and heating. Trehalose is consumed as part of a normal diet in mushrooms, honey, lobster, shrimp and foods produced using baker's and brewer's yeast.

Trehalose has been determined to be generally recognised as safe (GRAS) as a multipurpose ingredient for all uses in food, including as a sweetener, stabiliser, thickener and flavour enhancer, in general accordance with current good manufacturing practices. It has a uniquely high glass transition temperature for a disaccharide, is non-browning, and will not hydrolyse at low pH or high temperatures. This results in more stable colours and flavours in foods to which it is added. Trehalose is 45% as sweet as sucrose when compared in a 10% (w/v) solution and is stable at up to 94% relative humidity. The low hygroscopic nature of trehalose dihydrate results in a free-flowing, stable and dry product. As a non-reducing sugar, it does not react with amino acids or proteins to initiate Maillard browning.

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PRINCIPLE:

Trehalose is hydrolysed to D-glucose by trehalase (1), and the D-glucose released is phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (2).

(1) Trehalose + H_2O $\xrightarrow{\text{(trehalase)}}$ 2 x D-glucose

(2) D-Glucose + ATP $\xrightarrow{\text{(HK)}}$ G-6-P + ADP

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

(2) G-6-P + $NADP^+$ $\xrightarrow{\text{(G6P-DH)}}$ gluconate-6-phosphate + NADPH + H^+

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose and thus with twice the amount of

trehalose. It is the NADPH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for trehalose.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. With a sample volume of 0.20 mL, this corresponds to 3.45 mg/L (non-reduced assay procedure) or 18.98 mg/L (reduced assay procedure) of trehalose in the sample solution. The detection limit is 6.9 mg/L (non-reduced assay procedure) or 37.52 mg/L (reduced assay procedure) of trehalose in the sample solution, which is derived from an absorbance difference of 0.020 with a sample volume of 0.2 mL.

The assay is linear over the range of 4 to 80 µg of trehalose 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 0.20 mL, this corresponds to 1.73 to 3.45 mg/L (non-reduced assay procedure) or 9.49 to 18.98 mg/L (reduced assay procedure) of trehalose in the sample solution. If the sample is diluted during sample preparation (as well as that which occurs in the borohydride reduction step), 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.

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INTERFERENCE:

If the hydrolysis of trehalose and the conversion of D-glucose has been completed within the time specified in the assay (approx. 5 min for each incubation), it can be generally concluded that no interference has occurred. However, this can be further checked by adding trehalose or D-glucose (approx. 40 µg of either in 0.2 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 trehalose 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 1100 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 (25 mL, pH 7.6) plus magnesium chloride and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$ plus ATP.
Stable for > 5 years below -10°C.

Bottle 3: Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 mL.
Stable for > 2 years at 4°C.

Bottle 4: Trehalase suspension (2.25 mL).
Stable for > 2 years at 4°C.

Bottle 5: D-Glucose standard solution (5 mL, 0.4 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 4 years; store sealed at room temperature.

Bottle 6: Trehalose dihydrate control (~ 2 g).
Stable for > 4 years; store sealed at room temperature.

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 bottle 2 in 12 mL of distilled water. **Stable for ~ 4 weeks 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 & 4. Use the contents of bottles 3 and 4 as supplied. Before opening for the first time, shake the bottles to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position.
Stable for > 2 years at 4°C.

5. Use the contents of bottle 5 as supplied.
Stable for > 4 years; store at room temperature.

6. Dissolve 200 mg of trehalose dihydrate (which corresponds to approx. 180.9 mg of trehalose) in 1 L of distilled water. Add 0.2 mL of this solution to assays to check the activity of trehalase enzyme. Stable for > 4 years below -10°C.

NOTE: The D-glucose standard solution (solution 5) and the trehalose preparation (solution 6) are only assayed when 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 trehalose is determined directly from the extinction coefficient of NADPH (page 6).

REQUIRED REAGENTS (not enclosed):

1. **Reagent 1. Sodium hydroxide (50 mM)**
   Dissolve 2.0 g of sodium hydroxide in 900 mL of distilled water. Adjust the volume to 1 L. Store at room temperature.

2. **Reagent 2. Alkaline borohydride (10 mg/mL sodium borohydride in 50 mM sodium hydroxide)**
   Accurately weigh approx. 50 mg of sodium borohydride (Sigma cat. no. S9125) into polypropylene containers (10 mL volume with screw cap). Record the exact weight on the tubes, seal the tubes and store them in a desiccator with silica gel for future use. When weighing the borohydride, it is suggested that about 10 lots are prepared for convenience.

   Immediately before use, dissolve the sodium borohydride to a concentration of 10 mg/mL in 50 mM sodium hydroxide (Reagent 1). This solution is stable for 4-5 h at room temperature.

3. **Reagent 3. Acetic acid (200 mM)**
   Add 11.6 mL of glacial acetic acid to 600 mL of distilled water and adjust the volume to 1 L. Store at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Volumetric flasks (50 and 100 mL capacity).
4. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
5. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of buffer 1 and 0.1 mL aliquots of $NADP^+$/ATP solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
6. Analytical balance.
7. Spectrophotometer set at 340 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Whatman No. 1 (9 cm) filter paper.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.54 mL
Sample solution: 4-80 µg of trehalose or D-glucose per cuvette (in 0.20 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)	2.20 mL	2.00 mL
sample solution	-	0.20 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 ($NADP^+$/ATP)	0.10 mL	0.10 mL
suspension 3 (HK/G-6-PDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 5 min and start the reactions by addition of:		
suspension 4 (trehalase)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min**.		

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

** if the absorbance continues to increase, this may be due to effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

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

The concentration of trehalose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times \frac{1.1}{0.2} \times \Delta A_{trehalose} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of trehalose [g/mol]
ε = extinction coefficient of NADPH at 340 nm
= 6300 [$l \times mol^{-1} \times cm^{-1}$]
d = light path [cm]
v = sample volume [mL]
2 = 2 molecules of D-glucose released from each molecule of trehalose hydrolysed
1.1/0.2 = 0.2 mL of the sample extract is treated with borohydride and the final volume after treatment and neutralisation is 1.1 mL

[watermark]

It follows for trehalose:

$$c = \frac{2.54 \times 342.3}{6300 \times 1 \times 0.2 \times 2} \times \frac{1.1}{0.2} \times \Delta A_{trehalose} \quad [g/L]$$

$$= 1.8976 \times \Delta A_{trehalose} \quad [g/L]$$

If the sample has been diluted during preparation (other than that involved in the borohydride reduction step), 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 trehalose

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

When analysing pure trehalose or where the concentration of trehalose is significantly larger than that of free D-glucose, the borohydride reduction step can be omitted. In such cases, the concentration of trehalose can be calculated as follows:

$$c = \frac{2.54 \times 342.3}{6300 \times 1 \times 0.2 \times 2} \times \Delta A_{trehalose} \quad [g/L]$$

$$= 0.3450 \times \Delta A_{trehalose} \quad [g/L]$$

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 trehalose

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

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 trehalose 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 trehalose **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 ($NADP^+$/ATP) suspension 3 (HK/G-6-PDH)	40.60 mL 4.40 mL 2.20 mL (after adding 12 mL of H_2O to bottle 2) 0.44 mL
Total volume	47.64 mL

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Preparation of R2:

Component	Volume
distilled water suspension 4 (trehalase)	5.50 mL 0.44 mL
Total volume	5.94 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 5 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.74 g/L of trehalose using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for trehalose 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 trehalose **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:	0.254 mL
Linearity:	0.1-8.0 μg of trehalose or D-glucose per well (in 0.02 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.220 mL	0.200 mL	0.200 mL
sample solution	-	0.020 mL	-
standard solution	-	-	0.020 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 ($NADP^+$/ATP)	0.010 mL	0.010 mL	0.010 mL
suspension 3 (HK/G-6-PDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 5 min and start the reactions by addition of:			
suspension 4 (trehalase)	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. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 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 4.

CALCULATION (Microplate Assay Procedure):

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

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

SAMPLE PREPARATION:

Sample dilution.

The amount of trehalose present in the cuvette (i.e. in the 0.2 mL of sample being analysed) should range between 4 and 80 µg. Thus, the sample solution before the borohydride reduction step must be diluted sufficiently to yield a trehalose concentration between 0.12 and 2.4 g/L.

Dilution Table

Estimated concentration of trehalose (g/L)	Dilution with water	Dilution factor (F)
< 2.4	No dilution required	1
2.4-24	1 + 9	10
24-240	1 + 99	100

If the value of $\Delta A_{trehalose}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly.

1. PREPARATION OF SAMPLE EXTRACTS and REMOVAL OF FREE D-GLUCOSE

A. Trehalose Extraction:

Mill dry samples to pass a 0.5 mm screen; cut solid fatty samples (e.g. chocolate) into fine shavings with a sharp knife; analyse soft food products (e.g. spreads) without further preparation. All samples should be at room temperature before they are weighed.

Samples containing 0-12% trehalose

1. Accurately weigh approx. 1.0 g of the sample into a dry pyrex beaker (100 mL capacity) and add 40 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).

2. Quantitatively transfer the solution to a 50 mL volumetric flask. Adjust the volume to the mark with distilled water, mix the contents thoroughly and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

Samples containing 12-40% trehalose

1. Accurately weigh approx. 500 mg of the sample into a dry pyrex beaker (100 mL capacity) and add 80 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).

2. Quantitatively transfer the solution to a 100 mL volumetric flask. Adjust the volume to the mark with distilled water, mix the contents thoroughly and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

Further treatment of extracts

3. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle. *This solution may be slightly turbid, depending on the nature of the sample extracted.* Analyse this solution immediately or store at 4°C until analysed. If a turbidity forms in the solution, filter it again before analysis.

> **Note:** For samples containing 40-100% trehalose, adjust the volume to 250 mL.

B. Removal of Reducing Sugars:

1. Accurately dispense a 0.2 mL aliquot of the solution to be analysed (containing approx. 0.2 to 2.4 mg/mL of trehalose) into the bottom of a glass test-tube (16 x 100 mm).
2. Add 0.2 mL of **Reagent 2** (alkaline borohydride solution) to the tube, stir the mixture vigorously and store at 40°C for 30 min to effect complete reduction of reducing-sugars to sugar alcohols.
3. Add 0.5 mL of **Reagent 3** (200 mM acetic acid) to the tube with vigorous stirring on a vortex mixer. **A vigorous effervescence should be observed** (This treatment removes excess borohydride and adjusts the pH to approx. 4.5).
4. After 5 min, add 0.2 mL of solution 1 (2 M imidazole buffer, pH 7.0) to adjust the pH of the solution to approx. 7.
   This is the **sample solution** and should be analysed as described on page 5.

2. PREPARATION OF SAMPLE EXTRACTS NOT REQUIRING REMOVAL OF FREE D-GLUCOSE

Trehalose Extraction:

Mill dry samples to pass a 0.5 mm screen; cut solid fatty samples (e.g. chocolate) into fine shavings with a sharp knife; analyse soft food products (e.g. spreads) without further preparation. All samples should be at room temperature before they are weighed.

Samples containing 0-12% trehalose

1. Accurately weigh approx. 1.0 g of the sample into a dry pyrex beaker (300 mL capacity) and add 200 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).
2. Quantitatively transfer the solution to a 250 mL volumetric flask. Adjust the volume to the mark with distilled water and mix the contents thoroughly, and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

Samples containing 12-40% trehalose

1. Accurately weigh approx. 250 mg of the sample into a dry pyrex beaker (300 mL capacity) and add 200 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).
2. Quantitatively transfer the solution to a 250 mL volumetric flask. Adjust the volume to the mark with distilled water, mix the contents thoroughly and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

Note: For samples containing 40-100% trehalose, adjust the volume to 500 mL.

Further treatment of extracts

3. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle. *This solution may be slightly turbid, depending on the nature of the sample extracted.* This is the **sample solution** and should be immediately analysed as described on page 5. Analyse this solution immediately or store at 4°C until analysed. If a turbidity forms in the solution, filter it again before analysis.

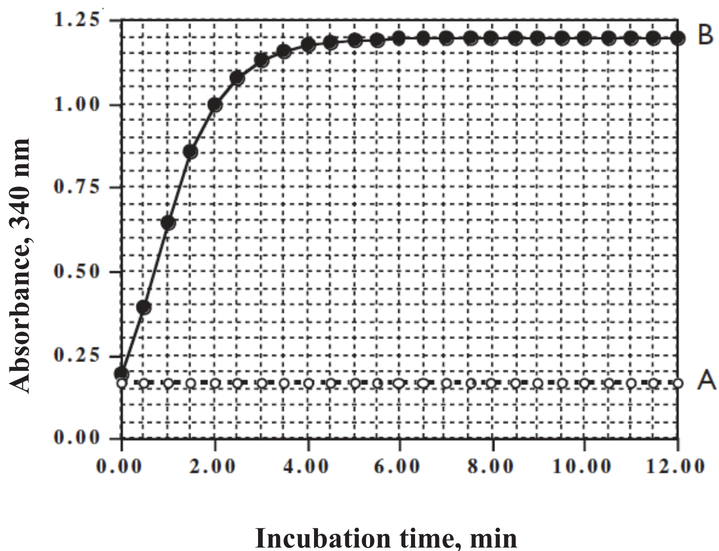

Figure 1. Measurement of trehalose using trehalase enzyme.
A. Incubation mixture with trehalose (100 µg) but no trehalase enzyme.
B. Incubation mixture containing trehalose (100 µg) and trehalase enzyme (9.8 U).

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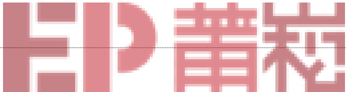

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