Megazyme

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D-SORBITOL/XYLITOL

ASSAY PROCEDURE

K-SORB 08/18

(*58 Manual Assays per Kit) or
(700 Auto-Analyser Assays per Kit) or
(580 Microplate Assays per Kit)

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

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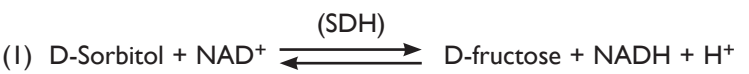
INTRODUCTION:

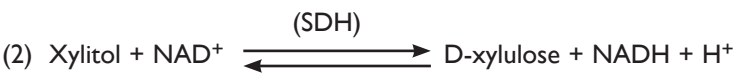
Sorbitol, a polyol (sugar alcohol), is a bulk sweetener found in numerous food products. Sorbitol is approximately 60% as sweet as sucrose with one-third of the calories. It has a smooth mouth feel with a sweet, cool and pleasant taste. Sorbitol is non-cariogenic and finds many dietetic applications, but can cause gastrointestinal problems in adults if large quantities are consumed (10-50 g per day). It has been safely used in processed foods for almost half a century and finds applications in the cosmetics and pharmaceuticals industries.

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 recognised 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.

PRINCIPLE:

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





However, since the equilibrium of reactions (1) and (2) lie in favour of D-sorbitol and xylitol, respectively, and NAD^+, a further reaction is required to utilise the NADH product. In this third reaction, in the presence of diaphorase, NADH reduces iodonitrotetrazolium chloride (INT) to an INT-formazan compound (3).

The amount of INT-formazan formed in this reaction is stoichiometric with the amount of D-sorbitol or xylitol. It is the INT-formazan which is measured by the increase in absorbance at 492 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

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 D-mannitol, L-arabitol and dulcitol do not react.

Under the assay conditions described on page 5, glycerol is oxidised very slowly (approx. 0.4% conversion with 100 µg of glycerol/assay), as is galactitol (approx. 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 "creep rate" of the reaction.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.066/0.055 mg of D-sorbitol or xylitol/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.197/0.165 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 1.0 to 20 µg of D-sorbitol (or xylitol) 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-sorbitol/xylitol concentration of approx. 0.066/0.055 to 0.13/0.11 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.

Coefficients of variation values between 1.0-3.3% (w/v or w/w) appear in the literature for measurement of D-sorbitol in solution or in baked goods.[1,2]

INTERFERENCE:

High concentrations of reducing substances, such as L-ascorbic acid in fruit juices, or SO_2 in jam, interfere with the assay as they react 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_2O_2/alkali and catalase [see Sample Preparation Example (a)].

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

If the conversion of D-sorbitol or xylitol has been completed within the time specified in the assay, it can be generally concluded that no interference has occurred. However, this can be further checked by

adding D-sorbitol or xylitol (approx. 10 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-sorbitol or xylitol 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 58 assays in manual format (or 700 assays in auto-analyser format or 580 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (40 mL, pH 8.6).
Stable for > 2 years at 4°C.

CAUTION: 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^+ plus INT.
Stable for > 2 years below -10°C.

Bottle 3: Diaphorase suspension (1.25 mL).
Stable for > 2 years at 4°C.

Bottle 4: (x3) Sorbitol dehydrogenase lyophilisate.
Stable for > 2 years below -10°C.

Bottle 5: D-Sorbitol standard solution (5 mL, 0.10 mg/mL) in 0.02% (w/v) sodium azide.
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 contents of one of bottle 2 in 6.0 mL of distilled water. Divide into appropriately sized aliquots and store in a dark container below -10°C between use and keep cool during use if possible. Stable for > 12 months below -10°C.

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

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 unstable to freezing but is stable for approx. 2 months at 4°C.**

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

NOTE: The D-sorbitol 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-sorbitol/xylitol is determined directly from the extinction coefficient of INT-formazan (page 6).

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, 100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.5 mL aliquots of buffer 1 and 0.2 mL aliquots of NAD^+/INT solution).
   - with 25.0 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 492 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: 492 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.87 mL
Sample solution: 1.0-20 µg of D-sorbitol plus xylitol 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)	0.50 mL	0.50 mL
solution 2 (NAD^+/INT)	0.20 mL	0.20 mL
suspension 3 (Diaphorase)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 2 min. Repeat the measurement after another 2 min. If a change in absorbance greater than 0.010 is observed, the sample must be treated to remove reducing substances (see "interference" section on page 2). Start the reactions immediately by addition of:		
solution 4 (SDH)	0.05 mL	0.05 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 15 min). If the reaction has not stopped after 20 min, continue to read the absorbances at 5 min intervals until the absorbances increase constantly over 5 min** (see Figures 1 and 2, page 13).		

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

** if this "creep" rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of the addition of solution 4 (SDH).

NOTE: INT and the reaction system containing INT are sensitive to light. Consequently, reactions must be performed in the dark (e.g. in the spectrophotometer cuvette compartment with the photometer lid closed).

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 $\Delta A_{D\text{-sorbitol/xylitol}}$.

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

The concentration of D-sorbitol/xylitol 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 being assayed [g/mol]
ε = extinction coefficient of INT-formazan at 492 nm
= 19900 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-sorbitol:

$$c = \frac{2.87 \times 182.17}{19900 \times 1.0 \times 0.1} \times \Delta A_{D\text{-sorbitol}} \quad [g/L]$$

$$= 0.2627 \times \Delta A_{D\text{-sorbitol}} \quad [g/L]$$

for xylitol:

$$c = \frac{2.87 \times 152.15}{19900 \times 1.0 \times 0.1} \times \Delta A_{xylitol} \quad [g/L]$$

$$= 0.2194 \times \Delta A_{xylitol} \quad [g/L]$$

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 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

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

Content of xylitol

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

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-sorbitol/xylitol 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-sorbitol/xylitol **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^+/INT) suspension 3 (Diaphorase)	34.5 mL 10.0 mL 4.0 mL (after adding 6 mL of H_2O to bottle 2) 0.4 mL
Total volume	48.9 mL

Preparation of R2:

Component	Volume
solution 4 (SDH)	6.1 mL (after adding 6.1 mL of H_2O to bottle 4)
Total volume	6.1 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 15 min at 37°C
Wavelength:	492 nm
Prepared reagent stability:	> 7 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 163 mg/L of D-sorbitol/ xylitol using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-sorbitol/xylitol 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-sorbitol/xylitol **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 492 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.287 mL
Linearity: 0.1-2 µg of D-sorbitol/xylitol 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)	0.050 mL	0.050 mL	0.050 mL
solution 2 (NAD^+/INT)	0.020 mL	0.020 mL	0.020 mL
suspension 3 (Diaphorase)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
solution 4 (SDH)	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. 15 min). If the reaction has not stopped after 15 min, continue to read the absorbances at 5 min intervals until the absorbances increase constantly over 5 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 solution 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:

1. Sample dilution.

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. The sample solution must therefore be diluted sufficiently to yield a D-sorbitol or xylitol concentration between 0.010 and 0.20 g/L.

Dilution Table

Estimated concentration of D-sorbitol or xylitol (g/L)	Dilution with water	Dilution factor (F)
< 0.20	No dilution required	1
0.2-2.0	1 + 9	10
2.0-20	1 + 99	100
> 20	1 + 999	1000

If the value of $\Delta A_{D\text{-sorbitol/xylitol}}$ 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. 8.6 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, such as beer, should be degassed by increasing the pH to approx. 8.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: a sample blank, i.e. sample with no SDH, 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 polyvinylpolypyrrolidone (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 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 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.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-sorbitol/xylitol in fruit juice.

Pipette 5.0 mL of blackcurrant juice or 1.0 mL of apple juice (filtered if necessary) into a 50 mL volumetric flask. Add successively 30 mL of distilled water, 1 mL KOH (2 M) and 0.05 mL of H_2O_2 solution (30% v/v), mix and incubate for 15 min at 25°C. Adjust the pH to approx. 8.0 by the addition of H_2SO_4 (1 M). Add 0.01 mL of catalase, mix and incubate for 20 min at approx. 25°C, 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 blackcurrant juice preparation there is an immediate linear increase in absorbance, but this is not due to D-sorbitol. When D-sorbitol was added at 1-10 µg/assay, the absorbance increased rapidly and then continued increasing at the same rate as the background reaction (blackcurrant juice + no added D-sorbitol). These results indicate that this particular blackcurrant juice contains little, if any, D-sorbitol.

(b) Determination of D-sorbitol/xylitol in diabetic honey.

Accurately weigh approx. 5 g of honey into a 100 mL volumetric flask, add approx. 70 mL of distilled water and incubate for 10 min at approx. 60°C. Allow to cool and fill to the mark with distilled water. Dilute the solution according to the dilution table and assay.

(c) Determination of D-sorbitol/xylitol in samples containing protein.

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.

(d) Determination of D-sorbitol 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 1,500 *g* in sealed polypropylene tubes, 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.

(e) Further applications.

For details of sampling, treatment and stability of other samples, such as pharmaceuticals, cosmetics, etc., refer to Bergmeyer (1988).[1]

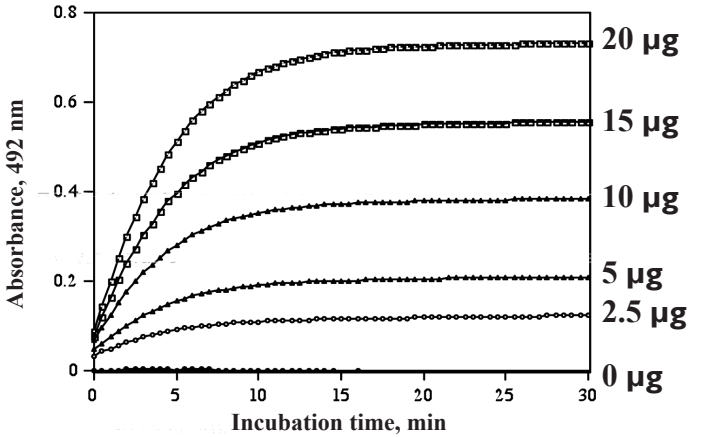


Figure 1. Increase in absorbance at 492 nm on incubation of D-sorbitol with SDH under standard assay conditions. Levels of added D-sorbitol were: 0, 2.5, 5, 10, 15 and 20 µg.

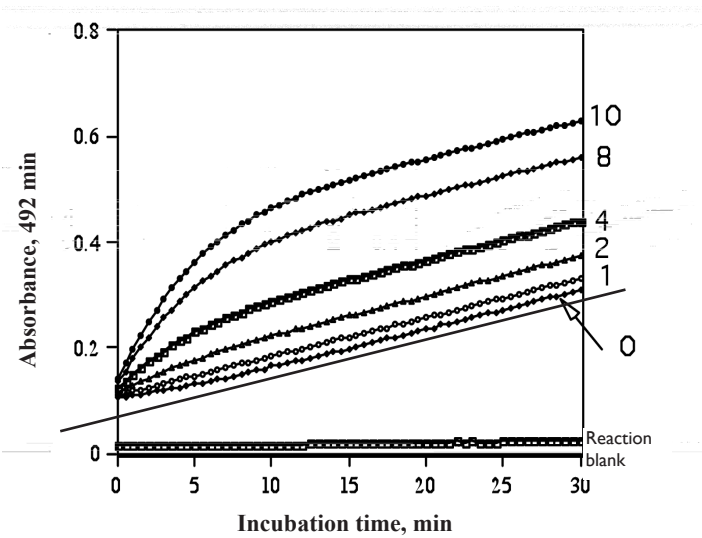


Figure 2. Increase in absorbance at 492 nm on incubation of blackcurrent preparation [see sample preparation example (a)] with and without added D-sorbitol with SDH. Levels of added D-sorbitol were: 0, 1, 2, 4, 8 and 10 µg.

NOTES:

REFERENCES:

1. Bergmeyer, H. U. (1988). D-Sorbitol. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U. ed.), 3rd ed., **Vol. VI**, pp. 356-362, VCH Publishers (UK) Ltd., Cambridge, UK.

2. Beutler, H. O. (1988). Xylitol. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U. ed.), 3rd ed., **Vol. VI**, pp. 484-490, VCH Publishers (UK) Ltd., Cambridge, UK.

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.).

Megazyme

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

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