Megazyme

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D-ISOCITRIC ACID (D-ISOCITRATE)

ASSAY PROCEDURE

K-ISOC 11/19

(*100 Manual Assays per Kit) or
(1000 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*

INTRODUCTION:

D-Isocitric acid is an organic acid found in most fruit juices. It is an important marker in multicomponent procedures for the evaluation of authenticity and quality of fruit products; high citric/isocitric acid ratios can be used as an indicator of citric acid addition in some juices.

PRINCIPLE:

D-Isocitric acid is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to 2-oxoglutarate and CO_2 in the presence of isocitrate dehydrogenase (ICDH), with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (1).

$$\text{(1)}\quad \text{D-Isocitric acid} + NADP^+ \xrightarrow{\text{(ICDH)}} \text{2-oxoglutarate} + CO_2 + NADPH + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-isocitric acid. It is the NADPH which is measured by the increase in absorbance at 340 nm.

Bound D-isocitric acid is released by alkaline hydrolysis (2), (3), and then measured using the same principle (1).

$$\text{(2)}\quad \text{D-Isocitric acid ester} + H_2O \xrightarrow{\text{(pH 9-10)}} \text{D-isocitric acid} + \text{alcohol}$$

$$\text{(3)}\quad \text{D-Isocitric acid lactone} + H_2O \xrightarrow{\text{(pH 9-10)}} \text{D-isocitric acid}$$

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for D-isocitric acid. D-malic acid, L-lactic acid, L-aspartic acid and fumaric acid do not react.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.177 mg/L of sample solution at the maximum sample volume of 2.00 mL (or to 3.54 mg/L with a sample volume of 0.1 mL). The detection limit is 0.354 mg/L, which is derived from an absorbance difference of 0.010 and the maximum sample volume of 2.00 mL.

The assay is linear over the range of 1 to 80 µg of D-isocitric acid 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-isocitric acid concentration of 0.177 to 0.354 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:

If the conversion of D-isocitric acid has been completed within the time specified in the assay (approx. 3 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-isocitric acid (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-isocitric acid 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 1000 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 (12 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2:	$NADP^+$. Stable for > 5 years below -10°C.
Bottle 3:	Isocitrate dehydrogenase suspension (2.1 mL). Stable for > 2 years at 4°C.
Bottle 4:	D-Isocitric acid standard solution (5 mL, 0.3 mg/mL in 0.02% sodium azide). Stable for > 2 years below -10°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 bottle 2 in 10.2 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 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 at 4°C.

4. Use the contents of bottle 4 as supplied.
   Stable for > 2 years below -10°C.

NOTE: The D-isocitric acid 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-isocitric acid is determined directly from the extinction coefficient of NADPH (see pages 4 and 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 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of Buffer 1 and $NADP^+$ solution).
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: 1.0-80 µg of D-isocitric acid 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 ($NADP^+$)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 3 (ICDH)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 3 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 $\Delta A_{D\text{-isocitric acid}}$. The value of $\Delta A_{D\text{-isocitric acid}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-isocitric acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D\text{-isocitric acid}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-isocitric acid [g/mol]
ε = extinction coefficient of NADPH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-isocitric acid:

$$c = \frac{2.32 \times 192.10}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{D-isocitric acid}} \quad [g/L]$$

$$= 0.7074 \times \Delta A_{\text{D-isocitric acid}} \quad [g/L]$$

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 D-isocitric acid

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

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-isocitric acid 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-isocitric acid **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
bottle 1 (buffer) bottle 2 ($NADP^+$) distilled water	2.2 mL 2.2 mL (after adding 10.2 mL of H_2O to bottle 2) 39 mL
Total volume	43.4 mL

Preparation of R2:

Component	Volume
bottle 3 (ICDH) distilled water	0.41 mL 4.7 mL
Total volume	5.11 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 3 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.8 g/L of D-isocitric acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-isocitric acid 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-isocitric acid **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: 0.1-8 µg of D-isocitric acid 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 ($NADP^+$)	0.010 mL	0.010 mL	0.010 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:			
suspension 3 (ICDH)	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. 3 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).

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-isocitric acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 1.0 and 80 µg. The sample solution must therefore be diluted sufficiently to yield a D-isocitric acid concentration between 0.01 and 0.80 g/L.

Dilution Table

Estimated concentration of D-isocitric acid (g/L)	Dilution with water	Dilution factor (F)
< 0.8	No dilution required	1
0.8-8.0	1 + 9	10
> 8.0	1 + 99	100

If the value of $\Delta A_{\text{D-isocitric acid}}$ 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. 7.6 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. 7.6 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 ICDH, 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 distilled 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-isocitric acid in fruit juices.

Adjust 25 mL of filtered sample to a pH of approx. 7.6 using 2 M NaOH. Quantitatively transfer the solution to a 50 mL volumetric flask and adjust to volume with distilled water. Add 0.5 g of PVPP, stir for 5 min and filter through Whatman No. 1 (9 cm) filter paper. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(b) Determination of D-isocitric acid and its derivatives (i.e. total D-isocitric acid).

Add 50 mL of filtered sample solution or juice to a 100 mL Erlenmeyer flask and adjust the pH of the solution to approx. 11.0 with 1 M NaOH (monitor with a pH meter). If reducing substances are present, add 0.01 mL of hydrogen peroxide solution (30% v/v). Incubate the solution for 20 min in a boiling water bath. Check the

pH with a pH test strip, and adjust with 1 M NaOH if necessary. Cool the solution to room temperature, adjust the pH to approx. 7.6 with 1 M HCl and the volume to 100 mL with distilled water. Add 0.5 g of PVPP, stir for 5 min and filter through Whatman No. 1 (9 cm) filter paper. Use the clear solution for the assay. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

REFERENCES:

1. Beutler, H. O. (1989). D-Isocitrate. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VII**, pp. 13-19, VCH Publishers (UK) Ltd., Cambridge, UK.

2. International Federation of Fruit Juice Producers (IFU, Methods of Analysis, no. 54-1984) (1984). Contained in *Code of Practice for Evaluation of Fruit and Vegetable Juices* (1996). Edited by Association of the Industry of Juices and Nectars from Fruit and Vegetables of the European Economic Community (AIJN).

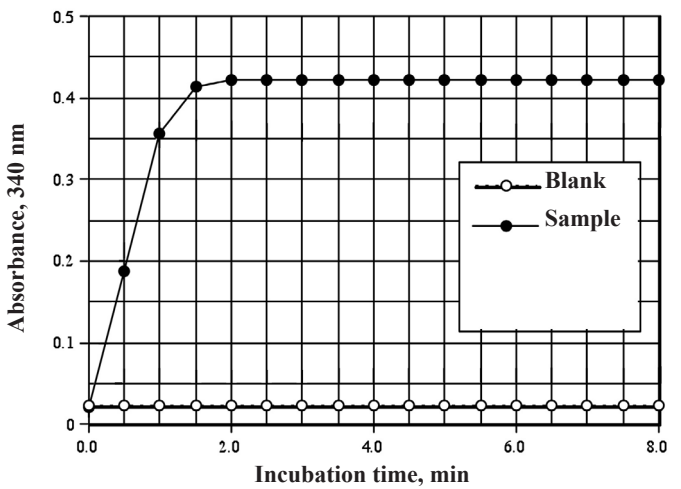

Figure 1. Increase in absorbance at 340 nm on incubation of 30 µg of isocitric acid with isocitrate dehydrogenase in the presence of $NADP^+$.

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