

# Megazyme

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## **D-ISOCITRIC ACID (D-ISOCITRATE)**

### ASSAY PROCEDURE

K-ISOC 08/04

(70 Assays per Kit)

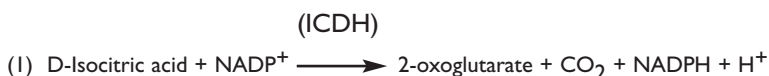


## INTRODUCTION:

D-Isocitric acid is a minor 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.

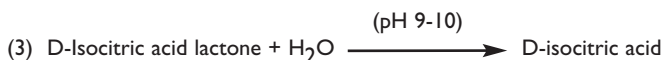
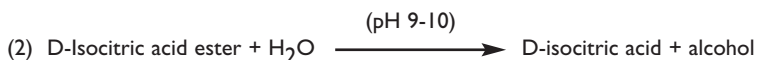
## PRINCIPLE:

D-Isocitric acid is oxidised by nicotinamide-adenine dinucleotide phosphate ( $\text{NADP}^+$ ) to 2-oxoglutarate and  $\text{CO}_2$  in the presence of isocitrate dehydrogenase (ICDH), with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (1).



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).



## 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 40  $\mu\text{g}$  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. 20 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 reagents used in the determination of D-isocitric acid are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

### KITS:

Kits suitable for performing 70 determinations are available from Megazyme. The kits contain the full assay method plus:

**Bottle 1:** Tris/HCl buffer (10 mL, 2 M, pH 7.6) containing magnesium chloride (100 mM) and sodium azide (0.02 % w/v) as a preservative.  
Stable for > 2 years at 4°C.

**Bottle 2:** NADP<sup>+</sup> (115 mg).  
Stable for > 5 years at -20°C.

**Bottle 3:** Isocitrate dehydrogenase suspension (1.5 mL, 18 U/mL). Stable for > 2 years at 4°C.

**Bottle 4:** D-Isocitric acid standard solution (5 mL, 0.3 mg/mL).  
Stable for > 2 years 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 7.2 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Once dissolved, the reagent is stable for > 2 years at -20°C.
3. Use the contents of bottle 3 as supplied.  
Stable for > 2 years at 4°C.
4. Use the contents of bottle 4 as supplied.  
Stable for > 2 years at room temperature.

**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, 100 µL and 200 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipipette®
  - 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 Tris/HCl buffer and NADP<sup>+</sup> 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) and GF/A glass fibre filter papers.

## PROCEDURE:

<b>Wavelength:</b>	340 nm
<b>Cuvette:</b>	1 cm light path (glass or plastic)
<b>Temperature:</b>	~ 25°C
<b>Final volume:</b>	2.32 mL
<b>Sample solution:</b>	1.0-40 µg of D-isocitric acid per cuvette (in 0.10-2.0 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 (Tris/HCl buffer)	0.10 mL	0.10 mL
solution 2 (NADP <sup>+</sup> )	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A <sub>1</sub> ) after approx. 3 min and start the reactions by addition of:		
suspension 3 (ICDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A <sub>2</sub> ) 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<sub>2</sub>-A<sub>1</sub>) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining  $\Delta A_{\text{D-isocitric acid}}$ . The value of  $\Delta A_{\text{D-isocitric acid}}$  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:

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

### where:

V	= final volume [mL]
MW	= molecular weight of D-isocitric acid [g/mol]
ε	= extinction coefficient of NADPH at 340 nm
	= 6300 [l × mol <sup>-1</sup> × cm <sup>-1</sup> ]

d = light path [cm]  
 v = sample volume [mL]

**It follows for D-isocitric acid:**

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

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

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**

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

**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 40 µg. The sample solution must therefore be diluted sufficiently to yield a D-isocitric acid concentration between 0.01 and 0.40 g/L.

**Dilution Table**

Estimated concentration of D-isocitric acid (g/L)	Dilution with water	Dilution factor (F)
< 0.4	No dilution required	1
0.4-4.0	1 + 9	10
> 4.0	1 + 99	100

If the value of  $\Delta A_{D\text{-isocitric acid}}$  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)  $\{K_4[Fe(CN)_6] \cdot 3H_2O\}$  (Sigma cat. no. P-9387) in 100 mL of distilled water. Store at room temperature.

**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. 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 an acidic sample is to be used undiluted (such as coloured 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 carbon dioxide 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, should be performed in the case of coloured samples.

**(e) Strongly coloured samples:** if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of activated carbon. Stir for 2 min and then filter.

**(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 20°C 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 1 g of activated charcoal, stir for 2 min and filter through Whatman GF/A glass fibre 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 20-25°C, adjust the pH to approx. 7.6 with 1 M HCl and the volume to 100 mL with distilled water. Add 1 g activated charcoal, stir for 2 min and filter through Whatman GF/A glass fibre 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. In *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 (A.I.J.N.)









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