Megazyme

www.megazyme.com

CITRIC ACID (CITRATE)

(EXTENDED STABILITY)

ASSAY PROCEDURE

K-CITR 04/20

(*72 Manual Assays per Kit) or
(840 Auto-Analyser Assays per Kit) or
(720 Microplate Assays per Kit)

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

INTRODUCTION:

Due to its excellent acidulant, flavourant and preservative properties, citric acid (citrate) is found in a large number of natural and processed foods and beverages, such as fruit juice and other soft drinks, beer, milk, bread, candies, and dairy and meat products. This acid also finds many other applications, such as in paper manufacture or in the wine industry, where the presence of significant quantities indicates the use of citric acid as an acidulant, a practice with an allowable upper limit of just 1 g/L (final concentration) in the EU. The quantification of citric acid is also important in clinical chemistry. Polyvinylpyrollidone (PVP) has been incorporated into the Megazyme assay format to prevent inhibition caused by tannins found in grape juice, fermenting must and wine. In addition to a > 2 years shelf life and competitive price, manual (see page 5 "A"), auto-analyser (see page 6 "B") and microplate (see page 8 "C") assay procedures are described, making this product ideal for citric acid determination applications in laboratories of any size.

PRINCIPLE:

Oxaloacetate and acetate are produced from citric acid (citrate) by the enzyme citrate lyase (1).

$$\text{(1)}\quad \text{Citrate} \xrightarrow{\text{(CL)}} \text{oxaloacetate} + \text{acetate}$$

The oxaloacetate product is converted to L-malate and NAD^+, in the presence of NADH and the enzyme L-malate dehydrogenase (L-MDH) (2).

$$\text{(2)}\quad \text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{(L-MDH)}} \text{L-malate} + NAD^+$$

However, if the enzyme oxaloacetate decarboxylase is present in the sample, some of the oxaloacetate product is converted to pyruvate. Thus, to ensure citric acid is measured quantitatively, D-lactate dehydrogenase (D-LDH) is employed to efficiently convert any pyruvate produced into D-lactate and NAD^+ (3).

$$\text{(3)}\quad \text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{(D-LDH)}} \text{D-lactate} + NAD^+$$

The amount of NAD^+ formed in the above reaction pathway is stoichiometric with the amount of citric acid. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for citric acid. In the analysis of commercial citric acid monohydrate, results of > 100% can be expected, due to partial loss of the water of crystallisation.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.246 mg/L of sample solution at the maximum sample volume of 1.7 mL. The detection limit is 0.491 mg/L, which is derived from an absorbance difference of 0.010 with a sample volume of 1.7 mL.

The assay is linear over the range of 1.0 to 100 µg of citric 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 1.7 mL, this corresponds to a citric acid concentration of approx. 0.246 to 0.491 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 citric acid has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding citric acid (approx. 40 µg in 0.2 mL) to the cuvette on completion of the reaction. A significant decrease 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. This is especially important when analysing samples containing free pyruvate, such as dark beer. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding citric 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 72 assays in manual format (or 840 assays in auto-analyser format or 720 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (40 mL, pH 7.5) plus sodium azide (0.02%) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: NADH plus PVP.
Stable for > 5 years below -10°C.

Bottle 3: L-Malate dehydrogenase plus D-lactate dehydrogenase, 1.5 mL.
Stable for > 2 years at 4°C.

Bottle 4: (x 3) Citrate lyase lyophilisate.
Stable for > 2 years below -10°C.

Bottle 5: Citric acid standard solution (5 mL, 0.20 mg/mL) in 0.02% (w/v) sodium azide.
Stable for > 2 years; store sealed 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 the contents of bottle 2 in 16 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. 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. Carefully dissolve the contents of one of bottle 4 in 0.55 mL of distilled water.
   Stable for 4 weeks at 4°C or > 6 months below -10°C.

NOTE: To ensure recovery of sufficient volume, do not invert bottle 4 during dissolution, and always store in an upright position.

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

NOTE: The citric 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 citric acid is determined directly from the extinction coefficient of NADH (page 5).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 500 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL and 25 mL Combitips® (to dispense aliquots of distilled water and buffer/NADH/PVP mixture).
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: 2.74 mL
Sample solution: 1.0-100 µg of citric acid per cuvette (in 0.20-1.0 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)	2.00 mL	1.80 mL
sample	-	0.20 mL
solution 1 (buffer)	0.50 mL	0.50 mL
solution 2 (NADH/PVP)	0.20 mL	0.20 mL
suspension 3 (L-MDH/D-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:		
solution 4 (CL)	0.02 mL	0.02 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (~ 5 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_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{citric\ acid}$.

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

The concentration of citric acid can be calculated as follows:

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

where:

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

It follows for citric acid:

$$c = \frac{2.74 \times 192.1}{6300 \times 1.0 \times 0.20} \times \Delta A_{\text{citric acid}} \quad [g/L]$$

$$= 0.4177 \times \Delta A_{\text{citric acid}} \quad [g/L]$$

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 citric acid

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

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water solution 1 (buffer) bottle 2 (NADH/PVP) suspension 3 (L-MDH/D-LDH)	51.5 mL 13 mL 5 mL (after adding 16 mL of H_2O to bottle 2) 0.5 mL
Total volume	70 mL

Preparation of R2:

Component	Volume
bottle 4 (CL)	Add 7 mL of water to bottle 4
Total volume	7 mL

EXAMPLE METHODS:

R1:	0.25 mL
Sample:	~ 0.003 mL
R2:	0.025 mL

Reaction time:	5 min at either 25°C or 37°C
Wavelength:	340 nm
Prepared reagent stability:	5 days when refrigerated
Calculation:	endpoint
Reaction direction:	decrease
Linearity:	up to 33 µg/mL of citric acid in final reaction mixture

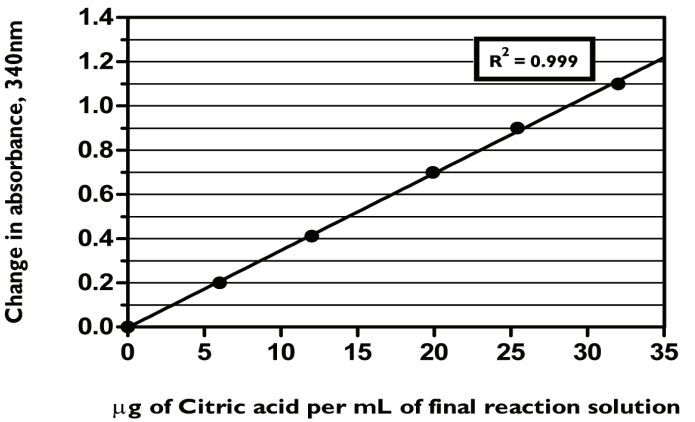

Figure 1. Calibration curve showing the linearity of reagent prepared from **K-CITR**. The reactions used to generate this calibration curve were performed at 25°C for 5 min, using a 10 mm path-length cuvette.

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for citric 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 citric 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: 0.274 mL
Linearity: 0.1-10 µg of citric acid per well (in 0.02-0.10 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.200 mL	0.180 mL	0.180 mL
sample solution	-	0.020 mL	-
standard solution	-	-	0.020 mL
solution 1 (buffer)	0.050 mL	0.050 mL	0.050 mL
solution 2 (NADH/PVP)	0.020 mL	0.020 mL	0.020 mL
suspension 3 (L-MDH/D-LDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:			
solution 4 (CL)	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 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 (for "manual format").

The amount of citric acid present in the cuvette (i.e. in the 0.20 mL of sample being analysed) should range between 1.0 and 100 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.005 and 0.50 g/L.

Dilution Table

Estimated concentration of citric acid (g/L)	Dilution with water	Dilution factor (F)
< 0.50	No dilution required	1
0.50-5.0	1 + 9	10
5.0-50	1 + 99	100
> 50	1 + 999	1000

If the value of $\Delta A_{citric\ acid}$ 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 1.70 mL, making sure that the sum of the sample and distilled water components in the reaction is 1.70 mL and using the new sample volume in the equation.

2. Sample clarification:

Carrez reagents cannot be used for deproteinisation as their use results in significantly reduced recoveries. Perchloric or trichloroacetic acid are used as alternatives (see specific examples).

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 > 0.2 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. 7.4 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing significant quantities of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 7.4 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 CL, 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 at 60°C. 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.

(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 trichloroacetic acid.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of free citric acid in wine.

In general, the concentration of free [F] citric acid in white and red wine can be determined without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:4 and sample volume of 0.2 mL are satisfactory.*

(b) Determination of citric acid and its esterified derivatives in wine.

The concentration of both free [F] and esterified [E] citric acid [F + E] in white and red wine can be determined as follows: add 6 mL of 2 M NaOH to 20 mL of wine and heat under reflux for 30 min with stirring. After cooling, carefully adjust the pH of the solution to 7.4 with 1 M H_2SO_4 and adjust the volume to 50 mL with distilled water. Then analyse the sample according to the general procedure. The concentration obtained is the sum of the free and esterified citric acid [F + E], and thus the esterified citric acid concentration alone [E] can be calculated as follows:

$$[E] = [F + E] - [F] \qquad [g/L]$$

(c) Determination of citric acid in beer.

After removal of carbon dioxide by stirring with a glass rod, dilute the sample according to the dilution table and analyse. **Note:** Dark beers are likely to contain free pyruvate, and thus additional NADH may be required (e.g. 0.2 mL of 2 mg/mL, using Megazyme cat. no. **C-NADH**, per assay). *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory.*

(d) Determination of citric acid in fruit juices, soft drinks, tea and other beverages.

Dilute the sample to yield a citric acid concentration of less than 0.50 g/L (see dilution table). Clear, neutral solutions can generally be determined without any sample treatment (except dilution). Turbid liquids generally only require filtering before the dilution step. Coloured solutions are usually suitable for analysis after dilution to an appropriate citric acid concentration. However, if coloured solutions require analysis undiluted, they may need decolourising as follows: adjust 25 mL of liquid sample to approx. pH 7.4 with 1 M NaOH and increase the volume to 50 mL with distilled water. Add 0.5 g of PVPP, stir for 5 min and filter through Whatman No. 1 filter paper. Use the clear, slightly coloured filtrate directly in the assay. *Typically, a further dilution of 1:20 and sample volume of 0.2 mL are satisfactory.*

(e) Determination of citric acid in cheese, meat, bread, vegetable and fruit products.

Accurately weigh approx. 5 g of representative material into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using an Ultra-turrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat (if present). Filter, discarding the first 3-5 mL, and use the clear filtrate for the assay. *Typically, no further dilution is required and a sample volume of 0.2 mL is satisfactory.*

(f) Determination of citric acid in edible oils, margarines and salves.

Accurately weigh approx. 5 g of representative material into a 200 mL glass beaker, add 60 mL of distilled water and stir vigorously on a hot-plate magnetic stirrer until boiling. Transfer the aqueous phase with a pipette into a 100 mL volumetric flask. Repeat the extraction with 30 mL of distilled water. Bring the temperature of

the volumetric flask to 20-25°C and fill to the mark with distilled water. Place the volumetric flask in an ice-bath or refrigerator for 15 min and filter an aliquot of the solution through Whatman No. 1 filter paper. *Typically, no further dilution is required and a sample volume of 0.2 mL is satisfactory.*

(g) Determination of citric acid in paper.

Accurately weigh approximately 2 g of paper into a 100 mL volumetric flask. After addition of approx. 60 mL of distilled water, stir the contents vigorously (magnetic stirrer) for approx. 1 h at room temperature. Remove the magnetic stirrer bar and fill up to the mark with distilled water. Mix and filter through Whatman No. 1 filter paper. Use the clear filtrate directly in the assay. *Typically, no further dilution is required and a sample volume of 0.2 mL is satisfactory.*

(h) Determination of citric acid in hard and soft candies.

Accurately weigh approx. 3 g of representative material into a 100 mL volumetric flask containing approx. 70 mL of distilled water and heat at 60°C with occasional shaking for 20 min, or until fully dispersed. After cooling to room temperature, fill up to the mark with distilled water, mix and filter through Whatman No. 1 filter paper. *Typically, no further dilution is required and a sample volume of 0.2 mL is satisfactory.*

(i) Determination of citric acid in whole blood samples.

a. Solutions:

Concentrated Carrez I solution. Dissolve 30 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P9387) in 200 mL of distilled water. Store at room temperature.

Concentrated Carrez II solution. Dissolve 60 g of zinc sulphate {$ZnSO_4.7H_2O$} (Sigma cat. no. Z4750) in 200 mL of distilled water. Store at room temperature.

b. Procedure:

Heat 1 mL of whole blood sample at approx. 80°C for 20 min in a microfuge tube then centrifuge at 13,000 x *g* for 10 min and recover the supernatant. Add 20 µL Carrez Reagent II and mix thoroughly, then add 20 µL Carrez Reagent I and mix thoroughly. Centrifuge the sample again at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The final volume of the clarified supernatant will be approximately one quarter of the starting volume of the original sample. Therefore adjust the volume of the starting material as required to obtain sufficient volume of clarified sample for the test.

(j) Determination of citric acid in biological tissue samples.

Accurately weigh approx. 5 g of representative biological tissue into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using a Ultra-turrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat (if present). Centrifuge an appropriate volume of the sample at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the clear filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The amount of starting material and volumes used can be adjusted accordingly depending on the amount of analyte present in the sample.

(k) Determination of citric acid in biological fluid samples (e.g. urine and serum).

For some biological fluid samples it may be sufficient to test them directly without any sample preparation other than appropriate dilution in distilled water. If this is not adequate then deproteinisation with either perchloric acid or trichloracetic acid may be required.

Deproteinise biological samples by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge an appropriate volume of the sample at 1,500 x *g* for 10 min and recover the supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay. Alternatively, use 50% (w/v) trichloroacetic acid instead of perchloric acid.

REFERENCE:

Mollering, H. (1989). Citrate. "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VII**, pp. 2-12, VCH Publishers (UK) Ltd., Cambridge, UK.

NOTES:

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