Megazyme

CITRIC ACID (CITRATE)

(EXTENDED STABILITY)

ASSAY PROCEDURE

K-CITR 06/07

(72 Assays per Kit)

This Data Booklet is available at
www.megazyme.com in the following languages
French-German-Italian-Spanish-Portuguese

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INTRODUCTION:

Due to its excellent acidulant, flavorant 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, both manual (see page 5 "A") and auto-analyser (see page 6 "B") assay formats 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 0.460 mg/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 0.921 mg/L, which is derived from an absorbance difference of 0.010 with a sample volume of 1.00 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 1.00 mL, this corresponds to a citric acid concentration of approx. 0.460 to 0.921 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 reagents used in the determination of citric acid are not hazardous materials in the sense of the Hazardous Substances Regulations. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 72 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Tablets (18) containing glycylglycine buffer plus NADH, L-MDH, D-LDH and PVP.
Stable for > 2 years at -20°C.

NOTE: Maximum long-term stability of the tablet components is obtained by storage of the tablet bottle in a sealed container in the presence of a drying agent such as silica gel at -20°C.

Bottle 2: (x 3) Citrate lyase lyophilisate (27 U/vial).
Stable for > 2 years at -20°C.

Bottle 3: Citric acid standard solution (5 mL, 0.20 mg/mL) in 0.02 % (w/v) sodium azide.
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dissolve 1 tablet from bottle 1 in 8.0 mL of distilled water for every 4 assays planned. **This should be performed by crushing the tablet(s) with a small metal spatula and gently swirling (when fully crushed).** Once dissolved, the NADH absorbance will drop slightly over time, but the reagent is suitable for use for approx. 1 week when stored at 4°C or for approx. 4 weeks when stored at -20°C.

Warm the tablet bottle to room temperature (in a dessicator if possible) before removing the tablet(s) (to prevent condensation of moisture on the tablet container). Opening the tablet bottle while it is still cold will lead to absorption of moisture by the tablets which in turn will reduce the stability of the tablet components.

2. Carefully dissolve the contents of one of bottle 2 in 0.55 mL of distilled water. Stable for 4 weeks at 4°C or **> 6 months at -20°C.**

NOTE: To ensure recovery of sufficient volume, do not invert bottle 2 during dissolution, and always store in an upright position.

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years 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® (200 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.8 mL aliquots of distilled water].
   - with 25 mL Combitip® [to dispense 2.0 mL aliquots of NADH/L-MDH/D-LDH/PVP/glycyglycine buffer 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 FORMAT:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 3.02 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)	1.00 mL	0.80 mL
sample solution	-	0.20 mL
solution 1 (NADH/L-MDH/D-LDH/PVP/glygly buffer)	2.00 mL	2.00 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:		
solution 2 (CL)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 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:

$$c = \frac{3.02 \times 192.1}{6300 \times 1 \times 0.20} \times \Delta A_{\text{citric acid}} \quad [g/L]$$

$$= 0.4604 \times \Delta A_{\text{citric acid}} \quad [g/L]$$

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 FORMAT:

This kit is suitable for the preparation of 220.5 mL of reagent (equivalent to 1050 reactions of 0.212 mL). Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
Bottle 1 (NADH/L-MDH/D-LDH/PVP/glygly tablets)	3 tablets
H_2O	35.0 mL
Total volume	35.0 mL

Preparation of R2:

Component	Volume
Bottle 2 (CL)	add 1.8 mL of H_2O
Total volume	1.8 mL

EXAMPLE METHODS:

R1:	0.20 mL
Sample:	~ 0.002 mL
R2:	0.010 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

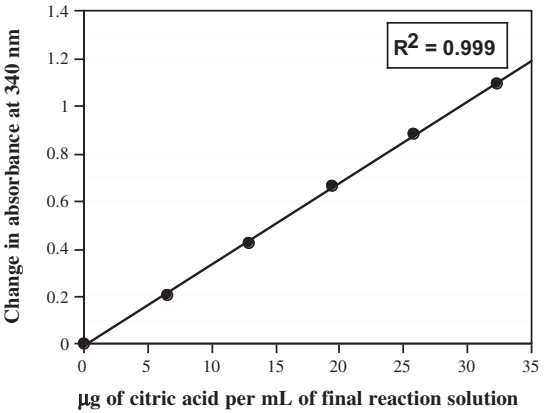

Figure 1. Calibration curve showing the linearity of reagent prepared from K-CITR. The reactions used to generate this calibration curve were performed at 25°C for 5 min, using a 10 mm path-length cuvette.

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 1.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 1.00 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. R-NADH2, 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 decolorising 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 Ultraturrax® 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 biological samples.

If necessary, heat biological samples at approx. 80°C for 20 min to

denature any enzymes present that may interfere with the assay (alternatively, deproteinise with perchloric acid as described on page 8). After centrifugation use the clear supernatant, with dilution if necessary, for the assay. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory.*

REFERENCE:

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

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