UV assay for the determination of citric acid in foodstuffs and other sample materials
Test combination for 50 determinations

For *in vitro* use only
Store between 2 – 8 °C (36 – 46 °F)

This test was evaluated using selected samples of the following matrices: fruit juice, soft drinks, wine, tomato ketchup, tomato concentrate (paste).

Detailed results and information regarding associated validation data are found in the Validation Report.

This test has been approved as AOAC *Official Method of Analysis*℠ 2024.02 First Action. A publication is available in J. AOAC Int. 108(1), 29–46 (2025).

The test may be used with other foods or samples material, provided that these are subjected to individual validation by the user.

1. Test principle

Citric acid (citrate) is cleaved into oxaloacetate and acetate in the presence of the enzyme citrate lyase (CL):

Citric acid $\xrightarrow{CL}$ oxalacetate + acetate

The resulting oxaloacetate and its decarboxylation product pyruvate are reduced (in the presence of L-malate dehydrogenase (L-MDH) and L-lactate dehydrogenase (L-LDH) to L-malate and L-lactate respectively:

Oxalacetate + NADH + H^+ $\xrightarrow{L\text{-}MDH}$ L-malate + NAD^+

Pyruvate + NADH + H^+ $\xrightarrow{L\text{-}LDH}$ L-lactate + NAD^+

Reduced nicotinamide-adenine-dinucleotide (NADH) is oxidized to NAD. The amount of NADH consumed is equivalent to the amount of citric acid converted and is measured at a wavelength of 340 nm.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 2 x 50 mL with buffer, NADH, L-MDH, L-LDH
- Reagent 2: 2 x 12.5 mL with buffer, CL

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is the same.
- Bring samples to room temperature before measurement.
- Use liquid, clear and almost neutral sample solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data).
- Neutralize **strongly** acidic or alkaline samples by adding KOH/NaOH or HCl, respectively (pH approx. 6.5 – 7.5).
- For turbid test samples (e.g., juices): filter the test solution through a pleated paper or syringe filter. Alternatively, centrifuge the sample in a test tube at (3000 g for at least 5 minutes is recommended) until a clear filtrate or supernatant is obtained.
- Decolorize **strongly** colored samples that are measured undiluted (e.g., juices and wines) using polyvinylpolypyrrolidone (PVPP) if necessary. For example, add 0.1 g of PVPP to 10 mL of sample, stir for 1 minute, and then filter until a clear supernatant is obtained.
- Degas samples containing carbonic acid, e.g. by stirring in a beaker, filtration or centrifugation.
- Crush and homogenize solid and semi-solid samples and extract with water. Filter or centrifuge afterwards.
- Weigh samples with high fat content into a volumetric flask and extract with hot water; allow sample solution to cool down for fat separation (e.g. 15 minutes in an ice bath); fill volumetric flask up to the mark with water, filter aqueous solution before testing.
- Carrez clarification is **unsuitable**, as this absorbs citric acid!
- For clarification of protein-containing samples, preparation with perchloric acid or trichloroacetic acid is recommended.
- In case of higher sample volumes (up to 1000 µL), check the pH value of the test solution and neutralize in case of any doubt.

3.2. Representative Application Examples consistent with published AOAC methods and §64 52.01.01-5 LFGB

Note: The recommendations for sample preparation described below are in accordance with the published AOAC method and §64 Official Collection of Methods of Analysis, German Food and Feed Code (LFGB). Alternatives may be used if properly validated and verified by the user.

3.2.1. Tomato ketchup

- Accurately weigh approximately 1 g of test sample (± 1 mg) into a 100 mL beaker, add about 25 mL of distilled water and stir for about 10 minutes on a magnetic stirrer.
- Transfer the suspension quantitatively into a 50 mL volumetric flask and dilute to 50 mL with water.
- Mix the content and filter through a paper filter (discard the first 15 mL) or use a syringe filter; alternatively centrifuge the solution at 3000 g for at least 5 minutes in a reaction tube until a clear supernatant is obtained.

3.2.2. Tomato concentrate

- Accurately weigh approximately 1 g of test sample (± 1 mg) into a 100 mL beaker, add about 25 mL of distilled water and stir for about 10 minutes on a magnetic stirrer.
- Transfer the suspension quantitatively into a 100 mL volumetric flask and dilute to 100 mL with water.
- Mix the content and filter through a paper filter (discard the first 15 mL) or use a syringe filter; alternatively centrifuge the solution at 3000 g for at least 5 minutes in a reaction tube until a clear supernatant is obtained.

4. Manual test procedure

Wavelength: 340 nm
Temperature (measurement): 20 – 37 °C (68 – 99 °F)
Photometer alignment: against air (without cuvette)
Measuring range: 40 – 1000 mg/L (for 100 µL)

	Reagent blank	Samples / controls
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for **3 minutes at 20 – 37 °C (68 – 99 °F)**. **Read** absorbance **A_1**, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for **15 minutes at 20 – 37 °C (68 – 99 °F)** and **read** absorbance **A_2**.		

4.1. Important notes for assay procedure

- The reagent blank value (water sample) must be determined **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated at 25 °C (77 °F). The test may generally perform within a range between **20 – 37 °C (68 – 99 °F)**.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- A multistepper pipette is recommended for adding reagents. Use a separate tip for each component.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance
- Always wait for the reaction to end or for the absorbance to stabilize (at least during the first test runs or validation). If the absorbance has not stopped after the recommended incubation time, continue measuring at 5-minute intervals, for example, until a constant absorbance value is reached.
- If a creep reaction occurs, the reaction will not have finished after stated incubation times and will typically show a constant increase of ΔA. Calculate the analyte-specific ΔA value by plotting the absorbance values against time and performing a linear regression to determine the rate of increase in ΔA per minute related to the creep reaction. Then, extrapolate the absorbance to the time at which reagent 2 is added.
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Concentration of citric acid

The extinction difference ΔA must be calculated for each sample:

$$\Delta A = (A_1 \times df - A_2)_{\text{sample or control}} - (A_1 \times df - A_2)_{RB}$$

df: Dilution factor
RB: Reagent blank

$$df = \frac{\text{sample volume + R1}}{\text{test volume}} = 0.808$$

The specified df value of **0.808 applies to a base application of 100 µL**. An increase in sample volume is possible (max. 1000 µL; refer to validation report). **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df)** accordingly.
Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of citric acid is calculated using Lambert-Beer's law:

$$\mathbf{C}_{\text{citric acid}}\ \mathbf{[g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.7929 \times \Delta A}$$

If the sample solution was diluted before measurement, this result has to be multiplied with the **sample pre-dilution factor F**.

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight citric acid [g/mol]	= 192.13
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation of solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

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

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. Therefore, we recommend Enzytec™ *Liquid* Multi-Acid Standard *low* (Art. No. E8460; with 0.250 g/L citric acid).

The recovery of this multi-standard *low* and other *aqueous* control solutions should be 100 ± 5 %.

As a certified (standard) reference material, we recommend:

- NIST Standard Reference Material 3282, *Low Calorie Cranberry Juice Cocktail*
- FAPAS Quality Control Material, *Soft Drink* (T03167QC)
- *Standardwein der Deutschen Weinanalytiker* (Standard wine of the German Wine Analysts); e.g. label “orange”; https://www.weinanalytiker.de/standard-testloesung/

6. Performance data

6.1. Specificity & side activities

The test is specific for citric acid. Several organic acids were tested for a potential side-activity at a concentration of 5.2 mmole/L (which is equivalent to 1 g/L of citric acid) in absence of citric acid (sample volume 100 µL): L-ascorbic acid, D- and L-tartaric acid, D-/L-malic acid, D/L-isocitric acid, L- and D-lactic acid, acetic acid, meso-tartaric acid, and oxalic acid. None of these substances showed side-activities at these concentrations.

6.2. Interferences

An interference study was conducted in the presence of 0.5 g/L citric acid. The tested substances included: D-glucose, D-fructose, sucrose and lactose, cyclamate, sucralose, xylitol, saccharin, acesulfame K, D-sorbitol, L-ascorbic acid, D- and L-tartaric acid, D- and L-lactic acid, sorbic acid, acetic acid, and NaCl (each 25 g/L). These substances do not have an interfering effect in the determination of citric acid.

D-/L-Malic acid (sum of both), meso-Tartaric acid and SO_2 (as Na_2SO_3) were tested in several concentration ranging from 1.56 to 25 g/L. In the case of SO_2 and meso-tartaric acid, no interference was observed at or below 3.13 g/L. The sum of D- and L-malic acid does not interfere at or below 25 g/L.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 1400 mg/L citric acid (sample volume of 100 µL) with a recommended measuring range of 40 – 1000 mg/L.

The limit of detection (LoD) was determined according to the DIN 32645:2008-11 method in a buffered aqueous solution. For a sample volume of 100 µL, the calculated LoD is 15 mg/L.

The limit of quantification (LoQ) was determined by precision profile. The calculated LoQ is 40 mg/L for a sample volume of 100 µL.

The smallest absorbance difference that the method can distinguish is ΔA = 0.005. For a sample volume of v = 1000 µL, this results in an LoD of 0.53 mg/L. Based on ΔA = 0.010, an LoQ of 1.06 mg/L was calculated.

6.4. Automation with Pictus 500

6.4.1. Limit of quantification (LoQ)

P500 application	LoQ
High Range	0.5 g/L
Basic Range	0.04 g/L
Sensitive Range	8 mg/L

6.4.2. Measuring ranges

P500 application	Measuring range
High Range	to 5 g/L
Basic Range	to 1 g/L
Sensitive Range	to 100 mg/L

6.4.3. Precision and accuracy

Data from the measurement of an aqueous solution are shown here.

High Range

Target concentration, g/L	1.0	0.25
Mean value, g/L	0.989	0.248
SD g/L	0.0104	0.0048
RSD, %	1.05	1.93
Recovery, %	98.9	99.1

Basic Range

Target concentration, g/L	1	0.5	0.25
Mean value, g/L	1.012	0.494	0.252
SD g/L	0.0061	0.0027	0.0028
RSD, %	0.60	0.54	1.13
Recovery, %	101.2	98.8	100.6

Sensitive Range

Target concentration, g/L	0.1	0.05	0.025
Mean value, g/L	0.100	0.048	0.024
SD g/L	0.0009	0.0007	0.0012
RSD, %	0.94	1.54	5.17
Recovery, %	100.0	95.8	95.2

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* Citric acid Validation reports
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Citric acid Excel template for results
- Enzytec™ Liquid Citric acid Technical Information
- Enzytec™ Liquid Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

On request, we offer the following services:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars
- Automation: application support & technical service

10. Disclaimer

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