Enzytec™ *Liquid* Citric acid

Art. No. E8230

Validation report

09/2022, page 1

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Enzytec™ *Liquid* Citric acid

Art. No. E8230

Contents

Enzytec™ *Liquid* Citric acid

Art. No. E8230

Abstract

Enzytec™ *Liquid* Citric acid is an enzymatic test kit for the determination of citric acid in food and other sample materials.

The kit contains all necessary reagents in a ready-to-use format and consists of only two components (reagent 1 and 2). The handling is very easy and due to ready-to-use reagents highly suitable for automation. The enzymatic reaction requires three enzymes (citrate lyase, malate dehydrogenase and lactate dehydrogenase) and NADH. Citrate present in the sample solution is cleaved into oxaloacetate and acetate by citrate lyase. In the presence of L-malate dehydrogenase (L-MDH) and reduced nicotinamide adenine dinucleotide (NADH), oxaloacetate reacts to L-malate, whereby NADH is consumed. In case oxaloacetate de-carboxylates, the formed pyruvate will react with NADH to L-lactate by L-lactate dehydrogenase (L-LDH). Consumed NADH is equivalent to the converted amount of citric acid and is measured at a wavelength of 340 nm within 20 minutes. The result is expressed as g/L of citric acid.

The test is specific to citric acid and shows no side activities or important interferences to different relevant acids. SO_2 and meso-tartaric acid do not interfere at or below 3.13 g/L. The LoD/LoQ is determined according to DIN 32645 (comparable to DIN ISO 11843-2) using aqueous citric acid control solutions. For a sample volume of 100 µL, LoD and LoQ were 15 mg/L and 40 mg/L citric acid, respectively. For a sample volume of 1000 µL LoD and LoQ will lower to 1.61 mg/L and 2.66 mg/L, respectively. The linear measurement range is from 40 mg/L to 1000 mg/L citric acid (100 µL sample volume). Samples with higher contents can be diluted with dist. water within the measurement range before measurement.

Trueness was checked by using materials from FAPAS (soft drink), NIST (cranberry juice), LGC (juice organic acids), and two control wines from the “Deutsche Weinanalytiker”. The recoveries ranged from 98 % to 105.9 %. Spiking of tomato ketchup, tomato paste, and orange juice resulted in blank corrected recoveries between 96.7 % and 98.1 %.

A special experiment was performed to calculate the contribution of each type of variation on intermediate precision (analyst, day, extraction and cuvette). For most of the samples the highest contribution to intermediate precision is the repeated measurement of each extract in a cuvette. Therefore, pipetting skills of one analyst will mainly drive the variation of results. Intermediate precision is between 6.2 % and 8.5 % for samples with extraction/centrifugation/dilution and below 4 % for samples that have to be diluted only.

After a storage of two weeks at 37 °C the stressed reagents were able to produce results between 97 % and 105 % recovery. Neither short freezing-thawing cycles, nor a harsh transport simulation affected the performance of the test system.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

1 Scope of method

1.1 Target analytes

The Enzytec™ *Liquid* Citric acid kit specifically targets citric acid.

1.2 Matrices

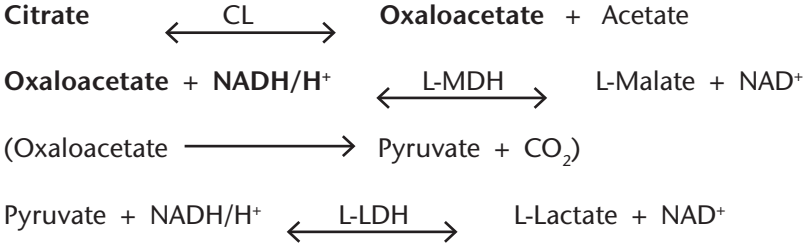
In course of this in-house validation, E8230 Enzytec™ *Liquid* Citric acid was tested with the following matrices:

- fruit juice
- soft drinks
- wine
- tomato ketchup
- tomato concentrate (paste)

2 Introduction

2.1 Principle

The enzymatic reaction requires three enzymes (citrate lyase, malate and lactate dehydrogenase) and NADH. Citrate present in the sample solution is cleaved into oxaloacetate and acetate by citrate lyase. In the presence of L-malate dehydrogenase (L-MDH) and reduced nicotinamide adenine dinucleotide (NADH), oxaloacetate reacts to L-malate, whereby NADH is consumed. In case oxaloacetate de-carboxylates spontaneously, the formed pyruvate will react with NADH to L-lactate by L-lactate dehydrogenase (L-LDH).



The amount of NADH consumed is equivalent to the converted amount of citrate and is measured at a wavelength of 340 nm due to its specific absorption. The result is expressed as g/L of citric acid.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

2.2 General information

Due to its excellent acidifier and preservative properties, citric acid (citrate; E330 - E333) is found in a broad range of foods and beverages, such as fruit juice and other soft drinks, beer, bread, candies, and dairy and meat products. Furthermore, it is used in the wine industry, with an allowable upper limit of just 1 g/L in the EU. The quantification of citric acid is also important in clinical chemistry for measurement in urine or seminal plasma.

3 Materials and methods

3.1 Test kit information

3.1.1 Kit name: Enyztec™ *Liquid* Citric acid

3.1.2 Article number: E8230

3.1.3 Reagents:
The test kit consists of 2 components; (see 3.1.4 - 3.1.5); all reagents are stable as indicated on the label at 2 - 8 °C (36 - 46 °F).

3.1.4 2 x 50 mL reagent 1 (L-MDH + L-LDH and NADH in buffer, pH 9.5)

3.1.5 2 x 12.5 mL reagent 2 (CL in buffer, pH 6.2)

3.2 Additional supplies, reagents, and apparatus

3.2.1 Variable micropipettes (10 - 100 µL and 500 - 5000 µL)

3.2.2 Multistepper pipette and tips for 100 µL, 500 µL, 2000 µL

3.2.3 Distilled water

3.2.4 5 M potassium hydroxide (KOH)

3.2.5 1 M potassium hydroxide (KOH)

3.2.6 Centrifugal vials with a screw top

3.2.7 Graduated flasks (50 mL, 100 mL, 200 mL)

Enzytec™ *Liquid* Citric acid

Art. No. E8230

3.2.8 Beakers

3.2.9 Paper filter

3.2.10 Spectrophotometer for 4 mL cuvettes (set to 340 nm)

3.2.11 Single use acrylic cuvettes (4 mL)

3.2.12 Scale

3.2.13 pH-meter

3.2.14 Laboratory mincer/grinder, pestle and mortar, or Ultra-Turrax

3.2.15 Shaker (e.g. Roto Shaker Genie, Scientific Industries Inc.)

3.2.16 Centrifuge (e.g. Minifuge RF, Kendro, Hanau, Germany)

3.2.17 Refrigerator

3.3 Certified reference materials

1. LGC Dr. Ehrenstorfer Fruit Juice Organic Acid Mixture (CRM) (DRE-GS09000056WA, #2-H429382WA, exp. 31.08.2022 at 2 - 8 °C); 2023 mg/L citric acid (measurement uncertainty 18 mg/L)
2. NIST standard reference material 3282 Low Calorie Cranberry Juice Cocktail

   3221 mg/kg citric acid ± 0.053 mg/kg; k = 2
3. FAPAS Quality Control Material Soft Drink (T03167QC); assigned value 2870 mg/L; 150 mL
4. Standardwein der Deutschen Weinanalytiker (https://www.weinanalytiker.de/standard-testloesung/)
   i. Etikett “orange” lot 1081608: 1.005 +/- 0.0373 g/L; k = 1
   ii. Etikett “moosgrün“ lot 1071505: 0.457 +/- 0.0123 g/L; k = 1

Enzytec™ *Liquid* Citric acid

Art. No. E8230

3.4 Standard solution and spike solution

For the production of standard or spike solutions the following material was used:

- Citric acid monohydrate from Carl Roth, Art. No. 3958; ≥ 99.5 %, p.a., ACS, ISO
- Molecular weight (monohydrate): 210.14 g/mole
- Molecular weight (water-free): 192.13 g/mole
- Factor 1.0937

To obtain e.g. a solution with 5.000 g/L citric acid, 5.469 g of the monohydrate need to be weight and filled with water up to one L.

3.5 General preparation

3.5.1 This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed. No quality guarantee is accepted after expiry of the kit (see expiry label).

3.5.2 Store the kit at 2 - 8 °C (36 - 46 °F). Let all kit components come to room temperature 20 - 25 °C (68 - 77 °F) before use. Do not freeze any of the kit components.

3.5.3 Use separate tips for each sample extract (and control solutions) to avoid cross-contamination and pre-flush the tip before pipetting. Use a multistepper pipette for adding the reagent 1 and reagent 2 solution. Use a single tip for each of these components.

3.5.4 Components and procedures of the test kit have been standardized for use in this procedure. Do not interchange components between kits of different batches (lot numbers).

3.5.5 Store samples in a cold and dry room protected from light. Ensure that no cross-contamination takes place.

3.5.6 Keep in mind that solid samples can be inhomogeneous, therefore ground a representative part of the samples very well and homogenize before weighing.

3.6 Preparation of components

All reagents are ready to use.

3.7 Sample preparation

3.7.1 Use clear and colourless liquid samples directly, or after dilution to a citric acid concentration between 40 mg/L and 1000 mg/L.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

3.7.2 Filter or centrifuge turbid samples.

3.7.3 Degas samples containing carbon dioxide.

3.7.4 Tomato ketchup according to the German § 64 method 52.01.01-5:
Weight 1.000 g of sample (+/- 1 mg) in a 150 mL beaker, add about 50 mL of water and stir for about 10 min on a magnetic stirrer with a magnetic stir bar. Transfer this suspension quantitatively into a 100 mL volumetric flask and fill up to 100 mL with water. Mix the content of the flask and filter through a paper filter; discard the first 15 mL; alternatively: centrifuge. After a pre-test only small ΔOD values were observed. Therefore, 50 mL volumetric flasks were used and only 25 mL of water was used to suspend the sample.

3.7.5 Tomato concentrate according to the German § 64 method 26.11.03-5:
Weight 1.000 g of sample (+/- 1 mg) in a 100 mL beaker, add about 30 mL of water and stir for about 10 min on a magnetic stirrer with a magnetic stir bar. Transfer this suspension quantitatively into a 200 mL volumetric flask and fill up to 200 mL with water. Mix the content of the flask and filter through a paper filter; discard the first 15 mL; alternatively: centrifuge. After a pre-test only small ΔOD values were observed. Therefore, 100 mL volumetric flasks were used and only 25 mL of water was used to suspend the sample.

3.7.6 Use PVPP (Anafin Soft P; www.zefueg.de/Schoenung.html) in case of juices/wines with a strong dark color that are measured undiluted: add 0.1 g PVPP to 10 mL of juice or wine, stir for 1 min and filter.

3.8 Analysis

3.8.1 Bring all reagents to room temperature (20 - 25 °C/68 - 77 °F) before use.

3.8.2 It is recommended to use control samples like references or standard solutions.

3.8.3 Pipette the samples or control solution with a variable micropipette and the reagent 1 and 2 solution with a multistepper pipette to ensure good mixing.

3.8.4 Insert a sufficient number of cuvettes in a holder for all samples or control, for single determination. Record sample and control positions.

3.8.5 With each measurement, it is necessary to determine a reagent blank (RB) by using dist. water instead of sample or control solution.

3.8.6 Pipette 2 mL of reagent 1 (R1) in each cuvette.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

3.8.7 Add 100 µL of dist. water (blank), samples or control solutions; mix carefully using a plastic spatula.

3.8.8 Incubate for 5 min for temperatures between 20 °C and 37 °C.

3.8.9 Read and document absorbance A1 in a spectrophotometer set at 340 nm for each cuvette.

3.8.10 Add 500 µL of reagent 2 (R2) in each cuvette and mix well.

3.8.11 Incubate for 15 min for temperatures between 20 °C and 37 °C (64 - 77 °F).

3.8.12 Read and document absorbance A2 in a spectrophotometer set at 340 nm for each cuvette.

3.8.13 In case of higher sample volumes (up to 1000 µL), the volumes for R1 and R2 remain unchanged; remember changed calculation as described under 3.10.2; check pH value of the sample and neutralize in case of any doubt.

3.9 Calculations

3.9.1 Calculate ΔA for every sample or control:

$$\Delta A = (df \times A1 - A2)_{sample\ or\ control} - (df \times A1 - A2)_{RB}$$

where df is a dilution factor calculated as follows:

df = (sample volume + R1)/(sample volume + R1 + R2) = 0.808 if 100 µL is used as sample volume

df = (sample volume + R1)/(sample volume + R1 + R2) = 0.857 if 1000 µL is used as sample volume

3.9.2 Calculate concentrations for every sample or control:

$$c = (V \times MW \times \Delta A)/(\varepsilon \times d \times v \times 1000)$$

where V = final volume; MW = molecular weight of citric acid; ε = absorption coefficient of NADH at 340 nm; d = light path within cuvette; v = sample volume

$$c\ [g/L\ citric\ acid] = (2.600\ mL \times 192.13\ g \times mole^{-1} \times \Delta A)/(6.3\ L \times mmole^{-1} \times cm^{-1} \times 1\ cm \times 0.1\ mL \times 1000)$$

$$c\ [g/L\ citric\ acid] = 0.7929 \times \Delta A$$

If a sample was diluted before measurement, this result has to be multiplied with the dilution factor.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

In case of a sample volume of 1000 µL, the calculation will change as follows:

c [g/L citric acid] = (3.500 mL x 192.13 g x $mole^{-1}$ x ΔA)/(6.3 L x $mmole^{-1}$ x cm^{-1} x 1 cm x 1.0 mL x 1000)

c [g/L citric acid] = 0.1067 x ΔA

3.9.3 Calculation in solid samples:

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

3.10 Criteria for acceptance

Recovery of aqueous standard solutions or reference solutions should be within 100 ± 5 %.

4 Summary of results and discussion

4.1 Selectivity: side activity

The following substances were tested for side reactivity (a positive reaction in the system in the absence of citric acid): ascorbic acid, D-tartaric acid, D-/L-malic acid, D-/L-isocitric acid, L-tartaric acid, L- and D-lactic acid, acetic acid, meso-tartaric acid, and oxalic acid. None of them showed side-activity. The substance were further tested at higher concentrations for characterisation of interference (see chapter 4.2).

4.2 Selectivity study: interference

Substances that have been tested for interference (influence on the recovery of citric acid) were: D-fructose, D-glucose, sucrose, lactose, sodium cyclamate, sucralose, xylitol, saccharin, acesulfam K, sorbitol, L-ascorbic acid, D-tartaric acid, L-tartaric acid, D-lactic acid, L-lactic acid, sorbic acid, acetic acid, meso-tartaric acid, D-/L-malic acid, SO_2, NaCl.

A concentration of e.g. 25 g/L of the interfering substance was mixed with 0.5 g/L citric acid solution and the recovery was then determined. In case of sulphur dioxide and meso-tartaric acid, there is no interference at or below 3.13 g/L. The sum of D- and L-malic acid does not interfere at or below 25 g/L. The other substances do not have an interfering effect in the determination of citric acid.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

4.3 Ruggedness study

These experiments were undertaken to show the influence of parameters on test kit results. These parameters are known to be subject of variation during use of the test kit. The parameters tested for their ruggedness were, incubation temperature (18 °C, 25 °C, 37 °C) and incubation times A1 (1 min, 3 min) and A2 (5 min, 10 min, 15 min, 20 min).

In order to achieve the required recoveries, the following incubation times are recommended:

A1 = 3 min + A2 = 15 min for incubation temperatures between 18 °C und 37 °C (64 - 99 °F). For practical reasons, the first incubation step can be decreased to 1 min when incubating at 37 °C (99 °F).

4.4 Stability studies

4.4.1 Accelerated stability study

Three independent lots of Enzytec™ *Liquid* Citric acid were tested with aqueous citric acid standard solutions and a wine sample to ensure consistent manufacturing between lots and to estimate the possible shelf life of the test kit by performing an accelerated stability study over 2 weeks at 37 °C (99 °F) storage temperature.

For all samples there was no trend towards lower or higher recoveries after 2 weeks at 37 °C. The mean recoveries from all samples tested under the specified condition is close to 100 %.

4.4.2 Stability study on transportation

To investigate the influence of harsh transport conditions, a simulated transport stability was performed. The conditions that were simulated included shaking and temperature changes. All components of one test kit lot were placed on a horizontal shaker at room temperature and agitated for 6 hours; 400 rpm were used at the beginning and changed later on to 150 rpms. Afterwards the components were refrigerated for 18 h at 2 - 8 °C (36 - 46 °F) followed by 7 h at room temperature on a horizontal shaker (150 rpm). Components were incubated at 37 °C (99 °F) for 18 h and after cooling down to room temperature measured, on the same day, with aqueous citric acid standards and a wine as usual.

4.4.3 Stability study on freezing

To simulate an unintended freezing of the test kit components, the whole test kit was frozen for 24 h at -20 °C (-4 °F). Afterwards the components were allowed to warm up to room temperature and were frozen again at -20 °C (-4 °F). After 24 h the components were thawed and after warm up to room temperature finally measured with aqueous citric acid standards and a wine sample against unstressed components. The repeated freezing overnight did not affect the functionality of the test system significantly.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

4.4.4 In-use stability study

To simulate the behavior when components are opened and used several times, bottles R1 and R2 of one test kit lot were opened and closed again after simulating a pipetting step. These test kits were given to the QC department for further regular check together with the regular stability test for un-opened test kits (real time stability). These experiments are running until April 2024. Please contact R-Biopharm AG if more information is required.

4.5 Matrix study

4.5.1 Estimation of Limit of Detection (LoD)

The Limit of Detection (LoD) was determined according to DIN 32645 (comparable to DIN ISO 11843-2). Each measurement was tested with 10 different aqueous citric acid solutions, with concentrations between 10 and 100 mg/L citric acid and a sample volume of 100 µL. This set of dilutions was tested two times independently with each of the three test kit lot. For a sample volume of 100 µL, the LoD is 15 mg/L citric acid.

Using the equation c (g/L) = ΔOD x 0.7929 and a minimum OD difference of 0.005 (see Roche IFU) the resulting LoD would be 4 mg/L. Using the same approach but increasing the sample volume to 1000 µL, the resulting LoD would be 0.53 mg/L.

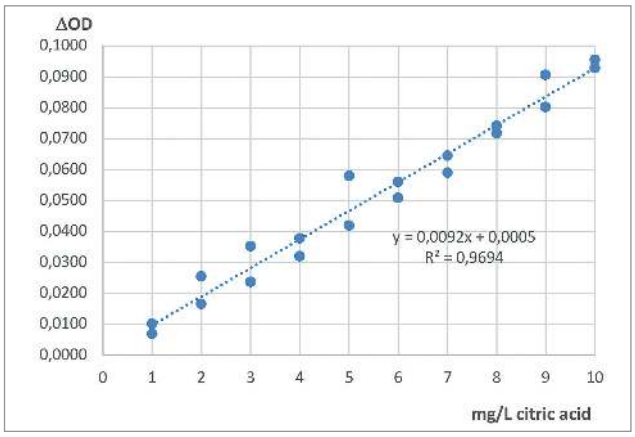


Figure 1: Measured OD values for aqueous solution with concentrations between 1 and 10 mg/L for two independent measurements for TC3 (blue dots); sample volume was 1000 µL.

To confirm the theoretical 1000 µL-approach, the LoD experiment was repeated in one test kit lot with an increased sample volume of 1000 µL and a concentration range between 1 mg/L and 10 mg/L (see figure 1). The LoD calculated according to DIN 32645 using this data set is 1.61 mg/L. It was necessary to buffer the citric acid solutions in the same buffer as reagent 1. Therefore, increasing the sample volume up to 1000 µL will demand pH neutralization to prevent interference for samples matrices with a low pH value.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

4.5.2 Estimation of Limit of Quantification (LoQ)

The Limit of Quantification (LoQ) is also determined according to DIN 32645 (comparable to DIN ISO 11843-2). Each measurement was tested with 10 different aqueous citric acid solutions with concentrations between 10 and 100 mg/L citric acid. This set of dilutions was tested two times independently with each of the three test kit lots.

Using this approach, the LoQ is at 20 to 25 mg/L but data in fig. 2 clearly shows that at a citric acid level of 20 mg/L the RSD is still at 15 % and that the citric acid concentration with a RSD at 10 % is 40 mg/L. For a sample volume of 100 µL, an LoQ of 40 mg/L citric acid is reasonable.

Taken the data from the LoD experiment with a sample volume of 1000 µL the calculated LoQ is 2.66 mg/L. It should be noted that a using the 1000 µL approach will result in higher standard deviations.

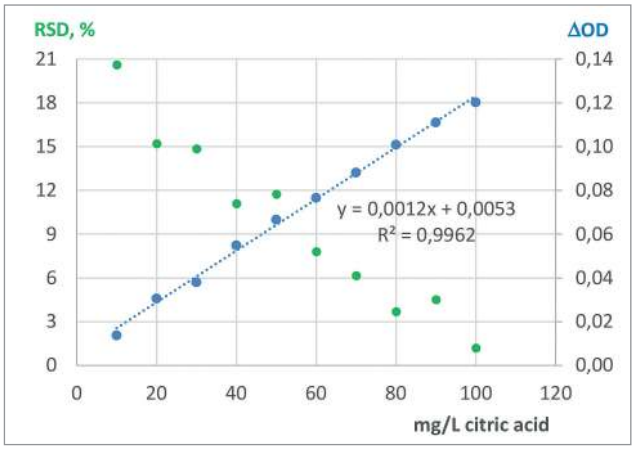


Figure 2: Measured OD values for aqueous solution with concentrations between 10 and 100 mg/L with standard deviation for two independent measurements for each lot (blue dots) and a sample volume of 100 µL; the relative standard deviation in % is also given (green dots).

4.5.3 Linearity/measurement range

Figure 3 shows the characterization of linearity using mean data from three lots with two independent runs each for concentration from 20 mg/L up to 1600 mg/L. For calculation of the linear regression only data for concentrations between 20 mg/L and 1400 mg/L were used. The resulting formula is ΔOD = 0.0012 x mg/L citric acid + 0.0013 with an R2 of 0.9999.

From the plot shown in fig. 3 it is clear that the upper limit of linearity is 1400 mg/L for new test kit lots whereas the lower limit of linearity is the LoQ (40 mg/L; see 4.5.2) by definition. It should be noted that stored test kit lots may not reach 1400 mg/L after a long time of storage and therefore a practical upper range of linearity of 1000 mg/L is stated to cover the whole shelf life.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

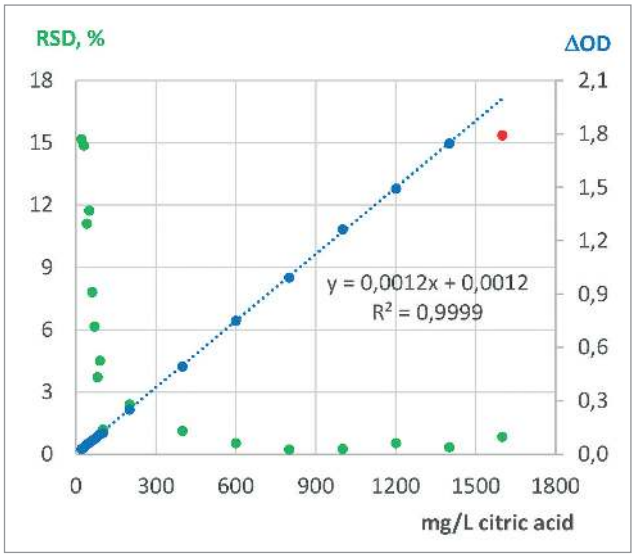


Figure 3: Linearity of the system between 20 mg/L and 1600 mg/L out of data derived from three test kit lots with n=2 replicates and a sample volume of 100 µL; the red dot was not included in linear regression.

4.5.4 Dilutability

Dilutability is characterized to check whether a high concentrated sample can be measured correctly when diluted within the measurement range. For the determination, one sample per matrix was used either directly (orange juice) or after spiking of the (extracted) matrix. Dilution was done with water to result in concentrations within or outside the measurement range. Each diluted extract was analyzed with two technical replicates per run. As can be seen in figures 4, 5, 6, and 7, samples diluted to a measured value of 1.2 g/L are already in the linear range of the system which perfectly match the characterization of linearity in chapter 4.5.3.

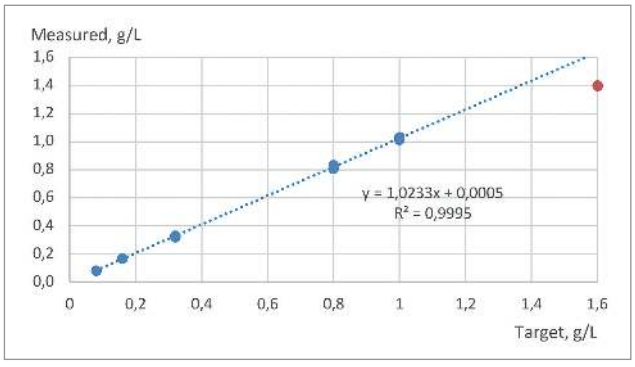


Figure 4: Results for dilutability of an orange juice (citric acid concentration was around 8 g/L before sample preparation); equation for regression was calculated without the data marked with red dots; n=2 per dilution were analyzed in two independent runs.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

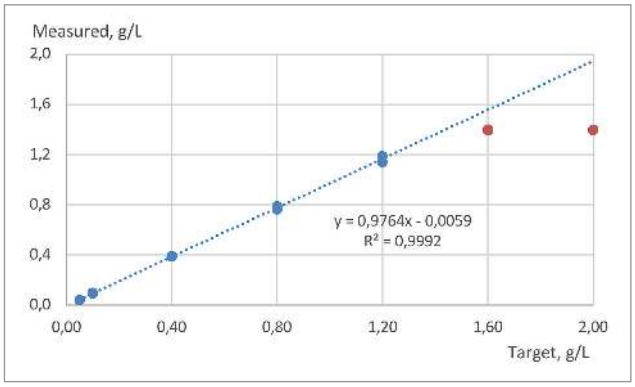


Figure 5: Results for dilutability of an extracted tomato ketchup (citric acid concentration was around 6.5 g/L before extraction); equation for regression was calculated without the data marked with red dots; n=2 per dilution were analyzed in two independent runs.

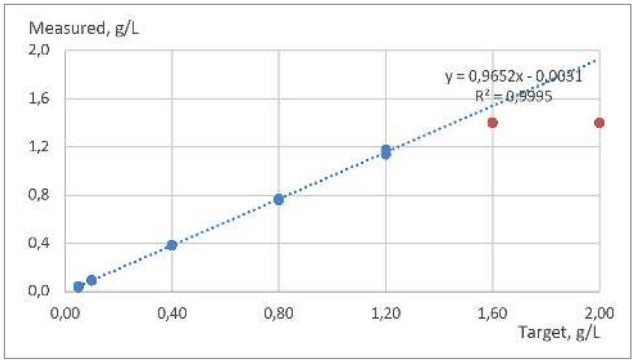


Figure 6: Results for dilutability of an extracted tomato paste (citric acid concentration was around 20 g/L before extraction); equation for regression was calculated without the data marked with red dots; n=2 per dilution were analyzed in two independent runs.

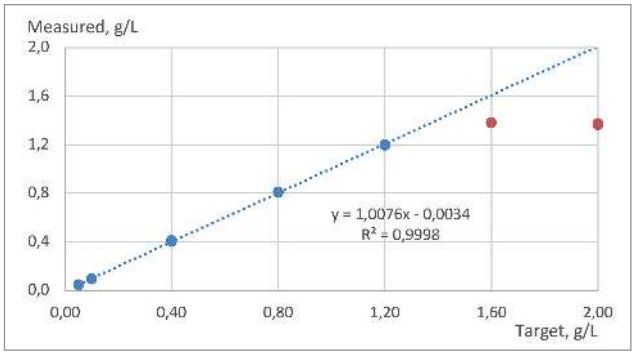


Figure 7: Results for dilutability of a spiked wine sample (citric acid concentration was around 0.3 g/L before spiking to 2.0 g/L); equation for regression was calculated without the data marked with red dots; n=2 per dilution were analyzed in two independent runs.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

4.5.5 Trueness

The trueness of the test system was checked during the characterization for laboratory-internal reproducibility (see chapter 4.5.10). Furthermore, the system was checked with two certified reference material (LGC Dr. Ehrenstorfer Fruit Juice Organic Acid Mixture (CRM); DRE-GS09000056WA; 2023 mg/L citric acid with a measurement uncertainty of 18 mg/L and NIST standard reference material 3282 Low Calorie Cranberry Juice Cocktail; 3221 mg/kg citric acid ± 0.053 mg/kg, k = 2; see chapter 3.3).

Table 1: Repeated measurement (n=12) of two certified reference materials for characterization of trueness during prototype verification.

	LGC Juice Organic Acid Target, 2.032 g/L		NIST Cranberry Juice Target, 3.221 g/L	
Replicate	g/L	rec. (%)	g/L	rec. (%)
1	2.047	100.7	3.45*	-
2	2.017	99.3	3.338	103.6
3	2.042	100.5	3.393	105.3
4	2.063	101.5	3.378	104.9
5	2.037	100.3	3.384	105.1
6	2.044	100.6	3.334	103.5
7	2.109	103.8	3.381	105.0
8	2.042	100.5	3.380	105.0
9	2.044	100.6	3.397	105.5
10	2.032	100.0	3.340	103.7
11	2.061	101.4	3.384	105.1
12	2.052	101.0	3.390	105.2
Mean, g/L	2.049	**100.8**	3.373	**104.7**
SD, g/L	0.0226		0.0235	
RSD (%)	1.10		0.70	

As can be seen in table 1, beside RSD value around 1 %, the mean recovery is at 100 % or close to 105 %. Both materials had to be dilute before measurement. Using the procedure described in the ERM application note no. 1 (available at https://crm.jrc.ec.europa.eu/graphics/cms_docs/erm1_english.pdf) both data sets were checked if there were significant differences to the certified value – this was not the case.

4.5.6 Recovery using spiked matrix samples

This performance characteristic was performed during validation for tomato ketchup, orange juice, and tomato paste because no (certified) reference samples were available for these matrices. All matrices showed quite high endogenous citric acid contents as can be seen in the upper part of table 2, 3, and 4. Each matrix was extracted/diluted as described in chapter 3.7 with six biological replicates each. Each of the existing extracts was measured twice so that in total 12 results were obtained.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

These extracts were also used for spiking purposes and measured. For spiking, 90 % volume of the extract was mixed with 10 % volume of the spiking solution which explains the "90 % value" in tables 2 to 4 to allow calculation of recovery. It was decided to spike levels that were at or the half of the original contents.

With the exception of a few results, recoveries were between 90 % and 110 % for tomato ketchup (table 2), orange juice (table 3), and tomato paste (table 4).

Table 2: Results of naturally incurred and spiked matrix samples; naturally incurred tomato ketchup sample extracts were spiked to result in a sample concentration of about 12 g/kg.

	Tomato ketchup			
Dilution	Spike	Measured	90 % value	
	g/kg	g/kg	g/kg	
50	0	6.38	5.74	
50	0	6.75	6.07	
50	0	6.75	6.07	
50	0	6.58	5.92	
50	0	6.88	6.19	
50	0	6.92	6.23	
50	0	6.53	5.87	
50	0	6.23	5.61	
50	0	6.54	5.88	
50	0	6.39	5.75	
50	0	6.51	5.86	
50	0	6.63	5.97	
Mean		**6.59**		
SD		0.21	spike*	
RSD (%)		**3.14**	g/kg	rec. (%)
50	6.48	12.11	6.37	98.3
50	6.48	12.09	6.02	92.9
50	6.48	12.27	6.20	95.7
50	6.48	11.67	5.75	88.8
50	6.48	12.32	6.13	94.6
50	6.48	12.15	5.92	91.4
50	6.48	12.60	6.73	103.8
50	6.48	12.51	6.90	106.5
50	6.48	12.37	6.48	100.1
50	6.48	12.33	6.57	101.4
50	6.48	12.44	6.59	101.7
50	6.48	12.73	6.77	104.4
Mean			**6.37**	**98.3**
SD			0.36	5.63
RSD (%)			**5.73**	

*Measured (spiked) minus 90 % value

Enzytec™ *Liquid* Citric acid

Art. No. E8230

Dilution	Orange juice			
	Spike g/kg	Measured g/kg	90 % value g/kg	
25	0	7.87	7.08	
25	0	8.02	7.22	
25	0	8.08	7.27	
25	0	7.92	7.13	
25	0	8.61	7.75	
25	0	7.85	7.07	
25	0	8.30	7.47	
25	0	8.41	7.57	
25	0	8.37	7.53	
25	0	8.09	7.28	
25	0	9.02	8.12	
25	0	8.39	7.55	
Mean		**8.25**		
SD		0.34	spike*	
RSD (%)		**4.18**	g/kg	rec. (%)
25	4	10.87	3.79	94.6
25	4	11.09	3.87	96.8
25	4	10.95	3.67	91.9
25	4	11.13	4.01	100.1
25	4	11.37	3.63	90.6
25	4	10.98	3.91	97.8
25	4	11.41	3.94	98.5
25	4	11.37	3.80	95.0
25	4	11.49	3.95	98.8
25	4	11.30	4.02	100.4
25	4	11.99	3.87	96.7
25	4	11.50	3.95	98.8
Mean			**3.87**	**96.7**
SD			0.12	3.10
RSD (%)			**3.21**	
25	8	14.58	7.49	93.7
25	8	14.78	7.56	94.4
25	8	15.18	7.91	98.9
25	8	14.74	7.61	95.1
25	8	16.31	8.56	107.0
25	8	14.86	7.79	97.4
25	8	14.93	7.46	93.2
25	8	15.21	7.64	95.5
25	8	15.28	7.74	96.8
25	8	15.22	7.94	99.3
25	8	16.17	8.05	100.6
25	8	15.16	7.61	95.1
Mean			**7.78**	**97.2**
SD			0.31	3.84
RSD (%)			**3.95**	

*Measured (spiked) minus 90 % value

Table 3: Results of naturally incurred and spiked matrix samples; naturally incurred orange juice sample extracts were spiked to result in a sample concentration of about 11 g/L and 15 g/L.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

Dilution	Tomato paste			
	Spike g/kg	Measured g/kg	90 % value g/kg	
100	0	20.70	18.63	
100	0	22.55	20.30	
100	0	21.29	19.16	
100	0	21.17	19.05	
100	0	21.73	19.56	
100	0	21.91	19.72	
100	0	21.18	19.06	
100	0	21.00	18.90	
100	0	19.96	17.96	
100	0	21.14	19.02	
100	0	21.64	19.47	
100	0	21.39	19.25	
Mean		**21.30**		
SD		0.64	spike*	
RSD (%)		**3.02**	g/kg	rec. (%)
100	21	39.66	21.03	100.1
100	21	38.30	18.00	85.7
100	21	38.95	19.79	94.2
100	21	39.36	20.30	96.7
100	21	39.95	20.39	97.1
100	21	39.05	19.33	92.1
100	21	40.22	21.15	100.7
100	21	40.89	21.99	104.7
100	21	40.42	22.46	106.9
100	21	40.43	21.40	101.9
100	21	39.37	19.89	94.7
100	21	40.63	21.37	101.8
Mean			**20.59**	**98.1**
SD			1.23	5.88
RSD (%)			**6.00**	
100	42	59.71	41.08	97.8
100	42	59.69	39.39	93.8
100	42	58.34	39.18	93.3
100	42	62.47	43.42	103.4
100	42	59.31	39.75	94.7
100	42	59.25	39.53	94.1
100	42	60.55	41.49	98.8
100	42	60.89	41.99	100.0
100	42	60.89	42.93	102.2
100	42	60.58	41.56	99.0
100	42	60.89	41.42	98.6
100	42	61.19	41.94	99.8
Mean			**41.14**	**98.0**
SD			1.40	3.33
RSD (%)			**3.40**	

*Measured (spiked) minus 90 % value

Table 4: Results of naturally incurred and spiked matrix samples; naturally incurred tomato paste sample extracts were spiked to result in a sample concentration of about 40 g/kg and 60 g/kg.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

4.5.7 Precision of extraction

To characterize the influence of extraction (and the following pipetting into cuvettes) refer to tables 2, 3, and 4 for the naturally incurred matrices tomato ketchup, orange juice and tomato paste. RSDs are in all cases at or below 4 %. If a liquid sample need to be diluted only, RSD values around 1 % can be obtained (table 1).

4.5.8 Precision of repeatability

As seen for other enzymatic assays, pipetting of one extract into different cuvettes seems to be the main contributor to imprecision. To characterize the citric acid assay, sample extracts from tomato ketchup and paste were pipetted in 6 technical replicates into cuvettes and measured. The experiment was repeated on two more days. As can be seen in table 5, RSD values are quite comparable to the one showed in table 2, 3, and 4. For more data on repeatability see "4.5.10 Laboratory-internal reproducibility".

Table 5: Results of naturally incurred matrix samples measured with n=6 technical replicates by one person in one test kit lot on three different days; an asterisk marks an outlying value according to single Grubbs.

	Day 1		Day 2		Day 3	
	Paste	Ketchup	Paste	Ketchup	Paste	Ketchup
Replicate	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
1	21.13	6.11	22.04	6.76	15.67*	6.24
2	21.93	6.75	21.71	6.56	21.93	6.18
3	21.27	6.46	21.65	6.83	22.30	6.84
4	21.59	6.35	21.49	6.13	20.33	6.59
5	21.60	6.34	21.69	6.94	20.78	6.13
6	21.00	6.38	21.27	6.70	18.58	6.44
Mean	21.42	6.40	21.64	6.66	20.78	6.40
SD	0.35	0.21	0.25	0.29	1.47	0.27
RSD (%)	**1.62**	**3.26**	**1.18**	**4.31**	**7.08**	**4.27**

*A1 quite high; outlier (Grubbs)

4.5.9 Interlot precision

To characterize for differences between test kit lots, an interlot precision experiment was set up by analyzing two different aqueous solution and a wine with n=6 biological replicates on one day by one person in all three kit lots.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

Table 6: Interlot precision tested with two different aqueous solution and a wine with n=6 biological replicates on one day by one person in three different lots.

	TC 1		TC 2		TC 3		All TCs
Sample	Measured (mg/L)	rec. (%)	Measured (mg/L)	rec. (%)	Measured (mg/L)	rec. (%)	Measured (mg/L)
Aqueous solution 1000 mg/L	1026	102.6	984	98.4	976	97.6	
	1024	102.4	966	96.6	978	97.8	
	1020	102.0	990	99.0	981	98.1	
	1004	100.4	972	97.2	1023	102.3	
	1001	100.1	956	95.6	1002	100.2	
	1001	100.1	974	97.4	984	98.4	
Mean	1013	101.3	974	97.4	991	99.1	992
SD	11.9		12.1		18.5		21.4
RSD (%)	**1.18**		**1.24**		**1.87**		2.15
Multi-acid standard low 250 mg/L	253	101.1	240	96.0	245	97.9	
	243	97.1	252	100.9	245	97.9	
	241	96.4	258	103.3	243	97.4	
	248	99.2	249	99.5	246	98.3	
	240	95.9	249	99.5	249	99.5	
	242	96.9	258	103.1	241	96.3	
Mean	244	97.8	251	100.4	245	97.9	247
SD	5.0		6.8		2.62		5.7
RSD (%)	**2.03**		**2.73**		**1.07**		2.32
Wine (standard) #1081608 (orange) 1005 mg/L	942	93.8	958	95.3	948	94.3	
	968	96.3	986	98.1	988	98.3	
	959	95.4	949	94.4	964	95.9	
	964	95.9	986	98.1	984	97.9	
	978	97.3	969	96.4	956	95.2	
	981	97.6	983	97.8	972	96.7	
Mean	965	96.0	972	96.7	969	96.4	969
SD	14.0		15.9		15.6		14.5
RSD (%)	**1.45**		**1.64**		**1.61**		1.50

The results are shown in table 6 and prove that all three lots were comparable over the whole measurement range. All RSD values were at or below 2 % with one exception in one lot for one matrix.

4.5.10 Precision of laboratory-internal reproducibility (intermediate precision)

To get an idea about intermediate or laboratory-internal reproducibility, one test kit lot was tested on two different days using three different photometers by three persons (table 7). In contrast to former validation studies for enzymatic test kits, the use of three different test kit lots was omitted because it was shown several times before that the parameter 'lot' does not contribute very much to the overall variation of results. The measurement was made with two certified reference materials (FAPAS Soft Drink and NIST Cranberry Juice), two control wines (Deutsche Weinanalytiker; see 3.3), and two native samples from local retailers (tomato ketchup and orange juice). All samples were extracted by each analyst with n=3 on each of the two days and analyzed in two cuvettes per extract. Each analyst made the experiment on different days within a period of three weeks.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

Table 7: Characterization of laboratory-internal reproducibility (intermediate precision) by three analyst extracting or diluting the materials and analyzed them on two different days with three extracts per day and analyzing each extract in two cuvettes.

				FAPAS Soft drink	NIST Cranberry juice	Wine A	Wine B	Ketchup	Orange juice
				2.87 g/L	3.221 g/L	1.005 g/L	0.457 g/L	n.a.	n.a.
Analyst	Day	Extract	Cuvette	g/L	g/L	g/L	g/L	g/L	g/L
1	1	1	1	2.778	3.194	1.058	0.449	5.871	6.477
1	1	1	2	2.902	3.293	1.035	0.456	6.252	6.786
1	1	2	1	2.793	3.288	1.014	0.442	6.176	6.743
1	1	2	2	2.834	3.355	0.941	0.491	6.525	7.140
1	1	3	1	2.722	3.357	0.968	0.471	6.011	6.736
1	1	3	2	2.779	3.435	0.948	0.470	6.618	7.009
1	2	4	1	2.847	3.378	0.982	0.498	5.914	6.812
1	2	4	2	2.897	3.372	1.033	0.460	6.322	6.733
1	2	5	1	2.889	3.434	0.953	0.479	6.174	7.022
1	2	5	2	2.825	3.476	0.990	0.513	6.644	6.914
1	2	6	1	2.877	3.292	1.005	0.465	6.037	6.961
1	2	6	2	2.867	3.472	1.015	0.456	6.673	6.932
2	1	1	1	2.825	3.281	0.979	0.463	6.427	6.749
2	1	1	2	2.787	3.510	0.990	0.472	6.681	6.278
2	1	2	1	2.778	3.353	0.986	0.457	6.849	6.711
2	1	2	2	2.758	3.473	1.003	0.476	6.242	6.388
2	1	3	1	2.813	3.496	0.984	0.473	6.852	6.228
2	1	3	2	2.749	3.565	0.979	0.473	6.813	6.224
2	2	4	1	2.799	3.307	0.954	0.461	6.227	6.244
2	2	4	2	2.776	3.353	0.963	0.456	6.233	6.034
2	2	5	1	2.748	3.319	0.964	0.488	6.387	6.167
2	2	5	2	2.717	3.336	0.987	0.463	5.845	5.986
2	2	6	1	2.752	3.340	0.955	0.479	6.473	6.001
2	2	6	2	2.691	3.501	0.994	0.470	6.219	6.197
3	1	1	1	2.892	3.329	0.973	0.467	5.443	6.766
3	1	1	2	2.826	3.406	0.945	0.465	6.016	7.196
3	1	2	1	2.868	3.483	1.127	0.486	5.899	7.153
3	1	2	2	2.892	3.567	0.930	0.459	6.016	7.167
3	1	3	1	2.793	3.491	0.994	0.474	5.887	7.083
3	1	3	2	2.852	3.491	1.005	0.476	6.260	7.053
3	2	4	1	2.889	3.363	0.985	0.460	6.532	6.128
3	2	4	2	2.811	3.430	0.967	0.457	7.109	6.379
3	2	5	1	2.875	3.541	0.940	0.469	6.763	6.336
3	2	5	2	2.870	3.473	0.957	0.457	7.130	6.440
3	2	6	1	2.898	3.467	0.989	0.466	7.064	6.612
3	2	6	2	2.889	3.544	0.956	0.466	7.180	6.489
			Mean, g/L	2.821	3.410	0.985	0.469	6.382	6.619
			SD, g/L	0.060	0.094	0.038	0.014	0.420	0.376
			RSD, %	2.11	2.75	3.81	2.98	6.58	5.67

Enzytec™ *Liquid* Citric acid

Art. No. E8230

As can be seen in table 7, the analysis of all originally "clear" matrices (soft drink, cranberry juice, wine) resulted in an overall RSD of around 3 % with the exception of wine A with an RSD of 4 % which can be attributed to the use of the undiluted wine with a high concentration of citric acid. Tomato ketchup had to be extracted before measurement. This explains the quite high RSD of about 6.5 % because several handling steps had to be performed before measurement. Orange juice had to be centrifuged before measurement due to a high content of particulated pulp in the juice. For this matrix there was a clear difference for analyst 3 when comparing day 1 and day 2.

This experiment was especially designed together with an AOAC statistical expert (Paul Wehling, ChemStats Consulting, Minneapolis, MN, USA) to calculate repeatability, intermediate precision and the contribution of each type of precision (analyst, day, extraction, and cuvette) by a nested ANOVA design. Table 8 shows the results for repeatability s(r) and intermediate precision s(i) together with their relative measures given in percentage (RSD). Except for ketchup and orange juice, both RSD values are below 4 %. Since both performance characteristics are quite close together for each of these four matrices, it can be concluded that repeatability is the main driver of total precision (see also table 9 for more explanations). Ketchup showed higher values for both types of precision, which is mainly attributed to the fact that the extraction is much more complicated than that for orange juice which is centrifuged and diluted only. The other matrices had to be diluted only. The quite high value for intermediate precision in case of orange juice is mainly driven by analyst 3 and the differences between both days of measurement as already mentioned above (see table 7).

Table 8: Characterization of intermediate and repeatability precision from the nested analysis of variance.

Performance characteristic	FAPAS Soft drink	NIST Cranberry juice	Wine A	Wine B	Ketchup	Orange juice
	2.87 g/L	3.221 g/L	1.005 g/L	0.457 g/L	n.a.	n.a.
Mean, g/L	2.821	3.410	0.985	0.469	6.382	6.619
s(r), g/L	0.040	0.084	0.039	0.014	0.297	0.178
RSD(r), %	1.41	2.46	3.95	3.02	4.66	2.68
s(i), g/L	0.065	0.097	0.039	0.015	0.539	0.411
RSD(i), %	2.32	2.85	3.94	3.17	8.45	6.22

Table 9 shows that the highest contribution to total precision within a laboratory is depending on the sample type. For cranberry juice and wines it is obvious that the pipetting step into the cuvette has the highest contribution to the total precision. The FAPAS sample, which is also an easy matrix seems to be different form the other easy matrices. In this case, the intermediate precision (2.32 %) is already very good (see table 7 and 8) and therefore small differences due to the analyst may end up in a high percentage of contribution. Tomato ketchup and orange juice had to be extracted/centrifuged before measurement and since this step was done every day of measurement, it was expectable that the day has the highest contribution to total precision.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

As a take-home message it can be concluded that the pipetting skills of the analyst will mainly drive the variation of results in case of citric acid measurement.

Table 9: Characterization of contribution of each variance component (analyst, day, extract, and cuvette) to total precision within one lab.

Contributor to total precision	FAPAS Soft drink	NIST Cranberry juice	Wine A	Wine B	Ketchup	Orange juice
	2.87 g/L %	3.221 g/L %	1.005 g/L %	0.457 g/L %	n.a. %	n.a. %
Analyst	45.9	15.7	0.0	0.0	0.0	29.2
Day	17.0	10.0	0.0	9.7	69.6	52.2
Extract	2.9	20.6	0.0	0.0	0.0	1.2
Residual (cuvette)	34.2	53.7	100.0	90.3	30.4	17.4
RSD(i), %	2.32	2.85	3.94	3.17	8.45	6.22

4.6 Method comparison

Soft drinks, juices, re-constituted juices, and wines were chosen to characterize the new method in comparison to the Roche test kit and an important competitor test kit. As can be seen in table 10 it is quite clear that all three methods are able to measure citric acid in soft drinks, juices, and white wine. When spiking red wine samples with 0.3 g/L citric acid (table 11), the recoveries are good.

Enzytec™ *Liquid* Citric acid

Art. No. E8230

Table 10: Method comparison using soft drinks, juices, re-constituted juices, and wines analyzed as received with two technical replicates; the new Enzytec™ *Liquid* Citric acid was compared to the Roche Kit (10 139 076 035) and a competitor citric acid kit.

			Enzytec™ *Liquid*	Competitor	Roche
Category	**Sample**	**Dilution**	Mean, g/L	Mean, g/L	Mean, g/L
Soft drinks	Orange lemonade	5	1.64	1.62	1.66
	Isotonic lemonade	20	4.77	4.87	4.88
	Isotonic lemonade	20	4.77	4.50	4.94
	Ice tea peach	10	2.61	2.52	2.53
	Energy drink	20	8.22	8.11	8.17
Juices	Energy drink	3	1.36	1.33	1.33
	Energy drink	10	3.01	2.94	3.01
	Energy drink	20	7.53	7.58	7.57
	Energy drink	10	3.25	3.18	3.23
	Energy drink	10	3.57	3.40	3.59
	Energy drink	10	3.57	3.28	3.59
	Energy drink	25	9.56	9.49	9.53
Concentrates (diluted to juices)	Energy drink	25	9.06	8.80	9.05
	Energy drink	100	35.97	35.09	35.96
Wines	Energy drink	1	0.17	0.14	0.15
	Energy drink	1	0.28	0.25	0.26
	Energy drink	1	0.30	0.05	0.27
	Energy drink	1	0.09	0.01	0.07

Table 11: Results for spiking of two different red wine samples with 0.3 g/L; a further dilution of 1+1 was also analyzed using all three test kits.

	Spike	Dilution	Enzytec™ *Liquid*	Competitor	Roche
Cabernet Syrah	+ 0.3 g/L	-	100 %	87 %	100 %
Cabernet Syrah	+ 0.3 g/L	1+1	104 %	102 %	105 %
Cabernet Merlot	+ 0.3 g/L	-	100 %	85 %	99 %
Cabernet Merlot	+ 0.3 g/L	1+1	100 %	112 %	104 %