Colorimetric method for approx. 24 assays

For *in vitro* use only
Store between +2 and +8°C

The method is recommended e. g. by MEBAK (Central European Commission for Brewing Technology).

Principle

L-Ascorbate + (x-H_2) + MTT ——— PMS ⟶ dehydro-ascorbate + X + MTT-formazan$^-$ + H^+

L-Ascorbate + ½ O_2 ——— AAO ⟶ dehydro-ascorbate + H_2O

Ref.: Beutler, H.-O. & Beinstingl, G. (1980) Bestimmung von L-Ascorbinsäure in Lebensmitteln, Deutsche Lebensmittel-Rundschau 76, 69-75

Assay performance

Wavelength:	578 nm (MTT-formazan) ε = 16.9 l x $mmol^{-1}$ x cm^{-1}
Light path:	1.00 cm (glass; plastic)
Temperature:	+37°C
Assay volume:	2.700 ml
Measurement:	against air, against water, or against sample blank
Sample solution:	0.5 to 20 µg L-ascorbic acid in 0.100 to 1.600 ml sample solution.

Reagents

1: 2 Bottles, each containing approx. 26 ml sodium phosphate / citrate buffer, pH approx. 3.5, approx. 80 mg 3-(4,5-dimethylthiazolyl-2) - 2,5-diphenyltetrazolium bromide, MTT (for stability see pack label). *The solution is ready for use*. Bring solution # 1 to +37°C before use. In order to guarantee the stability of solution # 1 (MTT buffer), take only the amount immediately needed from bottle # 1 and warm up to +37°C.

2: Lyophilizate with approx. 600 U ascorbate oxidase, AAO (for stability see pack label). *Dissolve contents of bottle # 2 with 0.5 ml redist. water*. The solution is stable for 6 weeks at +2 to +8 °C.

3: Bottle with approx. 5.5 ml PMS solution, approx. 25.3 mg 5-methylphenazinium methylsulfate (for stability see pack label). *The solution is ready for use.*

In addition (not contained in the kit):

Standard solution L-ascorbic acid, ultrapure, 0.2 g/l for test control only.

The reagents for the determination of L-ascorbic acid are not hazardous. The general safety rules for the work in chemical laboratories should be applied. After use the reagents can be disposed of with the laboratory waste. Packaging materials may be recycled.

Procedure

Pipette into cuvettes:	**Standard sample blank[1]**	**Standard assay[1]**	**Sample blank[2,3,4]**	**Sample assay[2,3,4]**	**Internal standard blank[5]**	**Standard and sample assay[5]**
Na-phosphate, citrate, MTT solution # 1	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml
Redist. Water	1.480 ml	1.500 ml	1.480 ml	1.500 ml	1.480 ml	1.500 ml
Sample solution[6] (e.g. 0.02 to 0.2 g L-ascorbic acid/l)	**-**	**-**	**0.100 ml**	**0.100 ml**	**0.050 ml**	**0.050 ml**
Standard solution[6] (e.g. 0.2 g L-ascorbic acid/l)	0.100 ml	0.100 ml	-	-	0.050 ml	0.050 ml
AAO solution # 2	0.020 ml	-	0.020 ml	-	0.020 ml	-
Mix[7] heavily (air to be mixed in), incubate at +37°C for 6 min. Read the absorbances (A_1). Add:						
PMS solution # 3	0.100 ml	0.100 ml	0.100 ml	0.100 ml	0.100 ml	0.100 ml
Mix[7], incubate in the dark (because the system is sensitive to light!) at +37°C for 15 min. Read the absorbances (A_2).						

Notes

1 Run a "standard" to see "accidents" in analysis. The measurement of the standard is not necessary for calculating results.
2 This assay together with the blank is a single determination.
3 In the case of a double determination, run two assays with different sample volumes. The absorbance differences measured have to be proportional to the sample volumes (calculate with resp. volume v).
4 Assay recommended in the case of trace level compound analysis, with sample volume increased up to 1.600 ml (0.0003 to 0.01 g L-ascorbic acid /l).
5 Recovery = [(2x$\Delta A_{sample+standard}$ - ΔA_{sample}) / $\Delta A_{standard}$] x 100 [%].
6 Before dispensing, rinse the enzyme pipette, resp. the tip of the piston pipette with sample resp. with standard solution
7 e.g. with a plastic spatula, or after closing the cuvette with Parafilm (trademark of American Can Co., Greenwich Ct., USA)

Calculation

$\Delta A = (A_2 - A_1)_{sample,\ resp.standard} - (A_2 - A_1)_{sample\ blank}$

The absorbance difference should be, as a rule, at least 0.100 ($\Delta A > 0.100$).

C = (V x MW x ΔA) / (ε x d x v x 1000) [g L-ascorbic acid/l sample solution]

c = (2.700 x 176.13 x ΔA) / (16.9 x 1.00 x 0.100 x 1000) = **0.2814 x ΔA [g L-ascorbic acid/l sample solution]**

If the sample has been diluted during preparation, multiply the result with dilution factor F.

When analyzing samples which are weighed out for sample preparation, calculate the content from the amount weighed:

$$Content_{L\text{-ascorbic acid}} = \frac{C_{L\text{-ascorbic acid}}\ [g/l\ sample\ solution]}{weight_{sample}\ [\ in\ g/l\ sample\ solution]} \times 100\ [g/100\ g]$$

Sample preparation

If the sample has one of the characteristics below, which hamper the test, please follow the corresponding sample preparation procedure.

1. Dilute *clear, colorless and colored liquid samples* with meta-phosphoric acid, 1.5 % (w/v); pH 3.5 to get a sample solution with 0.02 to 0.2 g of L-ascorbic acid/l.
2. Filter or centrifuge *turbid solutions*, dilute (see pt. 1).
3. Degas *samples containing carbon dioxide*, e. g. by filtration, dilute (see pt. 1).
4. Treat "*strongly colored solutions*" used undiluted with PVPP, e. g. 1 g/100 ml, mix, incubate a few minutes, filter.
5. Crush (corn size < 0.3 mm) or homogenize *solid or semi-solid (pasty) samples*, extract with meta-phosphoric acid, 1.5 % (w/v), pH 3.5; or dissolve in meta-phosphoric acid, 1.5 % (w/v), pH 3.5; filter and dilute (see pt. 1) if necessary.
6. Extract *fat containing samples* with meta-phosphoric acid, 1.5 % (w/v), pH 3.5, at a temperature above the melting point of fat, e. g. in a 100 ml volumetric flask. Adjust to +20 °C, fill volumetric flask to the mark. Store in ice or in refrigerator for approx. 15 resp. 30 min, filter.
7. *Deproteinize samples* with meta-phosphoric acid, 15 % (w/v); adjust pH to 3.5 to 4.0 with KOH (2 M), dilute with redist. water to get a meta-phosphoric acid concentration of 1.5 % (w/v).

Note: The Carrez clarification cannot be used in sample preparation because of too low results (ascorbic acid is unstable under alkaline conditions).

Assay characteristics

1. *Specificity:* Specific for L-ascorbic acid. (Iso-ascorbic acid reacts with reduced speed: 20 min reaction time instead of 6 min for L-ascorbic acid). In the analysis of commercial L-ascorbic acid results of < 100 % have to be expected depending on the age of the material and the storage conditions (oxidation of L-ascorbic acid takes place).
2. *Sensitivity:* 0.1 mg/l ($\Delta A = 0.005$; v = 1.600 ml; V = 2.700 ml)
3. *Detection limit*: 0.3 mg/l ($\Delta A = 0.015$; v = 1.600 ml; V = 2.700 ml)
4. *Linearity*: 0.5 µg/assay (v = 1.600 ml; V = 2.700 ml)
   to 20 µg/assay (v = 0.100 ml; V = 2.700 ml)
5. *Precision:* ΔA = +/- 0.005 to 0.010 absorbance units
   CV = approx. 1 to 3 %.
6. *Interferences*: > 20 mg D-sorbitol/assay and > 100 mg ethanol/assay inhibit AAO. Amounts of > 50 µg sulfite/assay react with MTT/PMS by a creep reaction. Sulfite in wine should be removed by the addition of formaldehyde.
7. *Technical Information*:

   a) Systems with MTT and especially with PMS are sensitive to light.
   b) Mix heavily after the addition of AAO in order to replace the oxygen consumed by AAO by oxygen from the air.
   c) Use meta-phosphoric acid (e. g. from Merck, cat. N° 546), 1.5 % (w/v), pH adjusted to 3.5 up to 4.0 by the addition of KOH (10 M).