Megazyme

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L-ASCORBIC ACID
(L-ASCORBATE)

ASSAY PROCEDURE

K-ASCO 04/19

(*40 Manual Assays per Kit) or
(400 Auto-Analyser Assays per Kit)
(400 Microplate Assays per Kit) or

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

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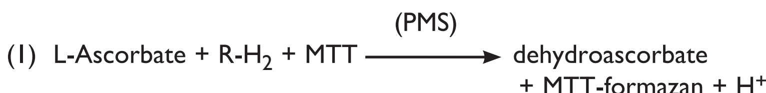
INTRODUCTION:

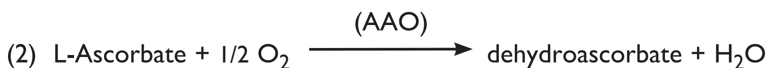
L-Ascorbic acid (Vitamin C), an anti-oxidant and free radical scavenger, is found ubiquitously in fruit and vegetables such as citrus fruits (oranges, lemons, limes, tangerines, etc.), melons, tomatoes, peppers, broccoli and green leafy vegetables such as spinach, potatoes and turnips. Its quantitative determination is especially important in the production of wine, beer, milk, soft drinks and fruit juices, where it can be a quality indicator. Given the essential role played in the human diet, L-ascorbic acid (E300) and salt derivatives (E301-303) are commonly used as food additives, with the additional advantage of their antioxidant and flavour enhancing properties. In the wine industry, L-ascorbic acid can be added to prevent oxidation of wine.

PRINCIPLE:

In the presence of the electron carrier PMS, 5-methylphenazinium methosulphate, and at pH 3.5, L-ascorbic acid (L-ascorbate), and some other reducing substances ($R\text{-}H_2$) present in the sample, reduce the tetrazolium salt MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] to a formazan compound. In assaying the sample, in the first reaction, both the ascorbic acid and the other reducing substances are measured (1).



Other reducing substances in the sample are then measured after selectively removing L-ascorbate using ascorbic acid oxidase (AAO) and atmospheric oxygen, giving a sample blank (2).



It is the MTT-formazan which is measured by the increase in absorbance at 578 nm. The difference in absorbance between the sample and sample blank is stoichiometric with the quantity of L-ascorbic acid in the sample.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

Under the conditions stated in this procedure, the assay is specific for L-ascorbic acid. However, iso-ascorbic acid (D-arabo-ascorbic acid), if present, will be determined simultaneously (but the rate of reaction is slower). In the measurement of iso-ascorbic acid, the incubation time with AAO needs to be increased to approx. 20 min.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.088 mg/L of sample solution at the maximum sample volume of 1.50 mL (or to 1.31 mg/L with a sample volume of 0.1 mL). The detection limit is 0.175 mg/L, which is derived from an absorbance difference of 0.010 and the maximum sample volume of 1.50 mL. The assay is linear over the range of 0.5 to 30 µg of L-ascorbic 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 1.50 mL, this corresponds to a L-ascorbic acid concentration of approx. 0.088 to 0.175 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 L-ascorbic acid has been completed within the time specified in the assay, it can be generally concluded that no interference has occurred. However, this can be further checked by adding L-ascorbic acid (approx. 15 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase 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. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding L-ascorbic acid to the sample in the initial extraction steps.

At concentrations up to 30 mg per assay, sugars that are found in foodstuffs do not interfere with the assay. D-Sorbitol does interfere, if present at levels above 20 mg/mL, as also do alcohols at concentrations of > 100 mg/mL (e.g. ethanol). These act by inhibiting the ascorbic acid oxidase but this can be resolved by increasing the incubation time with the enzyme. High levels of SO_2 (> 50 µg/assay) react with MTT and PMS which can lead to a creep reaction. This can be resolved by removal of the SO_2 (see example c).

Nitrite ions lead to spontaneous decomposition of L-ascorbic acid, and metal ions above 100 µg/assay may inhibit the ascorbic acid oxidase. If oxalic acid is present at levels above 30 µg/assay, it should be removed from the sample with a slight excess of Ca^{2+}-ions in a weakly acid solution (pH 5-6).

If the sample to be analysed for L-ascorbic acid contains heavy metals (e.g. copper or iron), it is essential to prepare the sample solution immediately before pipetting into the cuvettes as L-ascorbic acid is unstable in solutions containing metal ions.

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 40 assays in manual format (or 400 assays in auto-analyser format or 400 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer (44 mL, pH 5.6). Stable for > 2 years at 4°C.
Bottle 2:	MTT (18 mL, pH 3.5). Stable for > 2 years in the dark at room temperature.
Bottle 3: (x2)	PMS. Stable for > 2 years in the dark at 4°C.
Bottle 4:	Ascorbic acid oxidase suspension (0.85 mL). Stable for > 2 years at 4°C.
Bottle 5:	L-Ascorbic acid (vitamin C) (~ 2 g). Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/ SUSPENSIONS:

1. Use the contents of bottle 1 as supplied. Warm to ~ 37°C before use. Stable for > 2 years at 4°C.
2. Use the contents of bottle 2 as supplied. Store in the dark at room temperature between use. Stable for 2 years in the dark at room temperature.
3. Dissolve the contents of one of bottle 3 in 8.8 mL of distilled water. **Do not** dissolve the contents of the second bottle until required. Store in a dark container (as supplied) at 4°C between use. Stable for > 6 months at 4°C.
4. Use the contents of bottle 4 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. **Swirl the bottle**

to mix contents before use. Subsequently, store the bottle in an upright position. Stable for > 2 years at 4°C.

5. Accurately weigh approx. 150 mg of L-ascorbic acid to the nearest 0.1 mg into a 100 mL volumetric flask. Fill to the mark with 3% (w/v) metaphosphoric acid plus 10 mM EDTA. Dilute an aliquot 1:10 (1 + 9) with 3% (w/v) metaphosphoric acid plus 10 mM EDTA buffer and mix thoroughly. This solution is stable for > 2 weeks at 4°C.

NOTE: The L-ascorbic 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 L-ascorbic acid is determined directly from the extinction coefficient of MTT-formazan (page 6).

METAPHOSPHORIC ACID BUFFER:

To prepare 3% (w/v) metaphosphoric acid plus 10 mM EDTA dissolve 3.0 g of metaphosphoric acid in 80 mL of distilled water and add 10 mL of 100 mM EDTA solution. Make to 100 mL with distilled water and store at 4°C between use.

To prepare 100 mM EDTA, weigh 2.92 g of EDTA and add to approx. 80 mL of distilled water, adjust pH to approx. 7 to dissolve the EDTA using 4 M NaOH and make to 100 mL.

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 and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of PMS solution, 0.2 mL of MTT Buffer solution and 0.5 mL of Buffer 1).
   - with 25 mL Combitip® (to dispense 1.5 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 578 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: 578 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 37°C
Final volume: 2.52 mL
Sample solution: 0.5-30 µg of L-ascorbic acid per cuvette (in 0.1-1.5 mL sample volume)

Read against air (without a cuvette in the light path) or against water
For each sample, a sample blank must be performed.

Pipette into cuvettes	Blank	Sample
distilled water (warmed to ~ 37°C) sample* solution 1 (buffer) suspension 4 (Ascorbic acid oxidase)	1.50 mL 0.10 mL 0.50 mL 0.02 mL	1.52 mL 0.10 mL 0.50 mL -
Mix** and incubate for 3 min at 37°C. While incubating, mix the contents of the blank and sample assays every 1 min for about 5 sec, then add:		
solution 2 (MTT buffer)***	0.20 mL	0.20 mL
Mix** and incubate for 2-3 min at 37°C. While incubating, mix the contents of the blank and sample assays every 1 min for about 5 sec, then read the absorbances of the sample blank and sample (A_1). Start the reactions by addition of:		
solution 3 (PMS)	0.20 mL	0.20 mL
Mix** and read the absorbances of the solutions (A_2) immediately one after the other at the end of the reaction (approx. 8 min at 37°C).		

* rinse the pipette tip with sample solution before dispensing the sample solution.

** for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

*** the reaction mixture is sensitive to light after addition of solution 2 (MTT buffer). Avoid leaving these cuvettes standing in the light.

NOTE: MTT and the reaction system containing MTT are sensitive to light. Consequently, reactions must be performed in the dark (e.g. in the spectrophotometer cuvette compartment with the photometer lid closed).

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both sample and sample blank. Subtract the absorbance difference of the sample blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{L\text{-ascorbic acid}}$. The value of $\Delta A_{L\text{-ascorbic acid}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of L-ascorbic acid can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of L-ascorbic acid [g/mol]
ε = extinction coefficient of MTT-formazan at 578 nm
= 16900 [$l \times mol^{-1} \times cm^{-1}$]
d = light path [cm]
v = sample volume [mL]

It follows for L-ascorbic acid:

$$c = \frac{2.52 \times 176.13}{16900 \times 1.0 \times 0.1} \times \Delta A_{L\text{-ascorbic acid}} \quad [g/L]$$

$$= 0.2626 \times \Delta A_{L\text{-ascorbic acid}} \quad [g/L]$$

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 L-ascorbic acid

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

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

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. All samples and standards must be tested with R1-Blank (with AAO) and R1-Sample (without AAO).
2. For each batch of samples that is applied to the determination of L-ascorbic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume	
	R1-Blank	R1-Sample
distilled water solution 1 (buffer) solution 2 (MTT buffer) suspension 4 (AAO)	28.5 mL 10.0 mL 4.0 mL 0.4 mL	28.9 mL 10.0 mL 4.0 mL -
Total volume	42.9 mL	42.9 mL

Preparation of R2:

Component	Volume
distilled water solution 3 (PMS)	2.8 mL 8.0 mL (after adding 8.8 mL of H_2O to bottle 3)
Total volume	10.8 mL

EXAMPLE METHOD:

R1: 0.200 mL
Sample: ~ 0.01 mL
R2: 0.025 mL

Reaction time: ~ 8 min at 37°C
Wavelength: 578 nm
Prepared reagent stability: > 2 days when refrigerated
Calculation: endpoint
Reaction direction: increase
Linearity: up to 279 mg/L of L-ascorbic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. All samples and standards must be tested with AAO (Blank) and without AAO (Sample).
2. For each batch of samples that is applied to the determination of L-ascorbic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 578 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 37°C
Final volume: 0.252 mL
Linearity: 0.1-3 µg of L-ascorbic acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample
distilled water sample/standard solution* solution 1 (buffer) suspension 4 (AAO)	0.150 mL 0.010 mL 0.050 mL 0.002 mL	0.152 mL 0.010 mL 0.050 mL -
Mix** intermittently for approx. 3 min then add:		
solution 2 (MTT buffer)***	0.020 mL	0.020 mL
Mix** intermittently for approx. 3 min and then read the absorbances of the solutions (A_1). Start the reactions by addition of:		
solution 3 (PMS buffer)	0.020 mL	0.020 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 8 min).		

* rinse the pipette/syringe with sample/standard solution before dispensing.
** 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).
*** the reaction mixture is sensitive to light after additon of MTT.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of L-ascorbic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.5 and 30 µg. The sample solution must therefore be diluted sufficiently to yield an L-ascorbic acid concentration between 0.005 and 0.30 g/L.

Dilution Table

Estimated concentration of L-ascorbic acid (g/L)	Dilution with phosphate buffer	Dilution factor (F)
< 0.3	No dilution required	1
0.3-3.0	1 + 9	10
3.0-30	1 + 99	100
> 30	1 + 999	1000

If the value of $\Delta A_{\text{L-ascorbic acid}}$ 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 1.50 mL, making sure that the sum of the sample and distilled water components in the reaction is 1.60 mL and using the new sample volume in the equation.

2. Sample clarification.

The Carrez-clarification protocol cannot be used in sample preparation for L-ascorbic acid determination due to the resultant low recovery of the analyte.

In place of Carrez-reagents, samples are clarified and protein is precipitated and removed by a combination of treatment with 3% (w/v) metaphosphoric acid plus 10 mM EDTA buffer and filtration. For the preparation of 3% (w/v) metaphosphoric acid plus 10 mM EDTA buffer, see page 4.

3. General considerations.

(a) Liquid samples: clear, slightly coloured liquid samples can be used directly in the assay.

(b) Alkaline samples: if an alkaline sample is to be used undiluted, the pH of the solution should be adjusted to approx. 3.5 using 2 M HCl.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by filtration or by stirring with a glass rod.

(d) Coloured samples: lightly coloured samples can be analysed directly.

(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 3% (w/v) metaphosphoric acid plus 10 mM EDTA and filter if necessary.

(g) Samples containing fat: extract such samples with 3% (w/v) metaphosphoric acid plus 10 mM EDTA at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask at 60°C. Cool to allow the fat to separate and make up to the mark with 3% (w/v) metaphosphoric acid plus 10 mM EDTA. Filter the solution, discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for the assay.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume 1 M potassium phosphate buffer (pH 3.5) with mixing. Centrifuge at 1,500 *g* for 10 min and dilute with 3% (w/v) metaphosphoric acid plus 10 mM EDTA.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of L-ascorbic acid in wine.

Adjust the pH of the wine to 3.5-4.0 with 1 M HCl and dilute with water or 3% (w/v) metaphosphoric acid plus 10 mM EDTA to a suitable L-ascorbic acid concentration (see dilution table). Decolourise red wine with PVPP as follows: add 0.2 g of PVPP to 10 mL of wine and mix for 5 min. Filter. Wine containing sulphur dioxide (SO_2) is treated with formaldehyde as follows: add one drop of formaldehyde solution (approx. 5% v/v) to 10 mL of wine, mix and incubate for 5 min at 20-25°C. Adjust the pH to 3.5-4.0, dilute if necessary with 3% (w/v) metaphosphoric acid plus 10 mM EDTA (according to the dilution table) and use this solution for the assay. If a sample volume of 0.2-0.5 mL is required, increase the incubation time with the ascorbic acid oxidase to 5 min. *Typically, no dilution is required and a sample volume of 0.1-0.5 mL is satisfactory.*

(b) Determination of L-ascorbic acid in beer.

After removal of carbon dioxide by stirring approx. 10 mL of beer with a glass rod or by filtration, adjust the pH to 3.5-4.0 with 1 M HCl (if necessary). *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(c) Determination of L-ascorbic acid in fruit and vegetable juices and beverages.

The L-ascorbic acid concentration of clear solutions can generally be determined without any sample treatment except adjustment of pH to 3.5-4.0 (if necessary) and dilution according to the dilution table.

Perform dilutions with 3% (w/v) metaphosphoric acid plus 10 mM EDTA (see dilution table). However, if coloured solutions require analysis undiluted, they may need decolourising as follows: stir 10 mL of liquid sample for 5 min with 0.2 g of PVPP and then filter. Use the clear/slightly coloured filtrate directly in the assay. Filter turbid juices. *Typically, for orange juice, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(d) Determination of L-ascorbic acid in milk.

Adjust the pH of 100 mL of milk to 3.5-4.0 by addition of approx. 1 g of citric acid (monohydrate). Filter the solution and discard the first 5 mL of filtrate. Use the slightly opalescent solution directly in the assay. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(e) Determination of L-ascorbic acid in meat products.

Pass meat samples through a meat mincer three times with a 1 min stand between passes. Homogenise approx. 4 g of sample in 10 mL of 1 M potassium phosphate buffer (pH 3.5) with an Ultra-turrax® or Polytron® homogeniser (or equivalent) for 20 sec to effect complete disintegration of meat particles. Adjust the pH to 3.5-4.0, if necessary, with 2 M KOH and quantitatively transfer the slurry to a 100 mL volumetric flask. Rinse the homogeniser shaft with water and add these washings to the flask. Fill to the mark with 100 mM potassium phosphate buffer (pH 3.5), mix, place in a refrigerator (or ice-water) for approx. 20 min to allow separation of the fat and then filter. *Typically, no dilution is required and a sample volume of 1.0 mL is satisfactory.*

(f) Determination of L-ascorbic acid in potatoes.

Mince approx. 50 g of potato with 50 mL of 1 M potassium phosphate buffer (pH 3.5) and 0.1 mL of *n*-octanol (to minimise foaming) with a household mixer for approx. 1 min. Adjust the pH to 3.5-4.0 with 2 M KOH. Quantitatively transfer the mixture to a 500 mL volumetric flask with distilled water, fill up to mark with distilled water, mix and filter. *Typically, no dilution is required and a sample volume of 1.0 mL is satisfactory.*

(g) Determination of L-ascorbic acid in flour.

Accurately weigh approx. 20 g of flour into a 100 mL Erlenmeyer flask. Add 100 mL of 3% (w/v) metaphosphoric acid plus 10 mM EDTA. Shake the suspension until it is homogeneous. Filter an aliquot of the

solution through Whatman No. 1 filter paper and use the clear filtrate directly for the assay. Assays should be performed as soon as possible after filtration to avoid precipitation of starch in the assay system. *Typically, no dilution is required and a sample volume of 1.0 mL is satisfactory.*

(h) Determination of L-ascorbic acid in vitamin tablets.
Dissolve 1 g of the L-ascorbic acid containing tablets in 100 mL of 3% (w/v) metaphosphoric acid plus 10 mM EDTA. Dilute according to the dilution table. *Typically, a sample volume of 0.1 mL is satisfactory.*

(i) Determination of iso-ascorbic acid in meat products.
Since ascorbic acid oxidase reacts less rapidly with iso-ascorbic acid under the assay conditions described here (see Specificity, Sensitivity, Linearity and Precision, page 1), the incubation time with this enzyme must be increased to 20 min. Meat products are prepared and extracted as described for L-ascorbic acid. *Typically, no dilution is required and a sample volume of 0.5-1.0 mL is satisfactory.*

REFERENCE:

Beutler, H. O. (1988). L-Ascorbate and L-Dehydroascorbate. "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 376-385, VCH Publishers (UK) Ltd., Cambridge, UK.

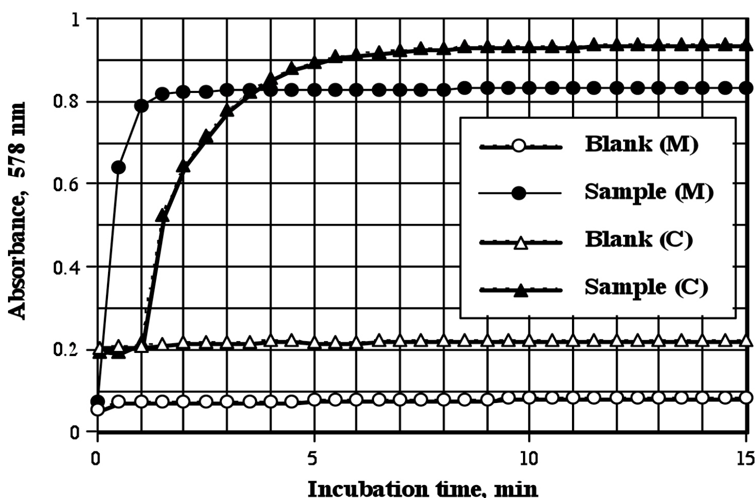


Figure 1. Increase in absorbance at 578 nm on incubation of 20 µg of ascorbic acid with ascorbic acid oxidase in the presence of PMS. (M) Megazyme kit; (C) competitor kit.

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