
**Infant formula and adult
nutritionals — Determination of
vitamin C by (ultra) high performance
liquid chromatography with
ultraviolet detection ((U)HPLC-UV)**

*Formules infantiles et produits nutritionnels pour adultes —
Détermination de la teneur en vitamine C par chromatographie
liquide à (ultra) haute performance avec détection dans l'ultraviolet
((CL(U)HP-UV)*



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Published in Switzerland

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this document is equivalent to the AOAC Official Method 2012.22: *Vitamin C in Infant Formula and Adult/Pediatric Nutritional Formula, Ultra-Performance Liquid Chromatography (UPLC) with Ultraviolet Detection*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Infant formula and adult nutritionals — Determination of vitamin C by (ultra) high performance liquid chromatography with ultraviolet detection ((U)HPLC-UV)

1 Scope

This document specifies a method for the determination of vitamin C (L-ascorbic acid) present in all forms of infant and adult formulas (powders, ready-to-feed liquids and liquid concentrates), using (ultra) high performance liquid chromatography with ultraviolet detection (U)HPLC-UV. The application range runs from 2,5 mg/100 g (limit of quantification) to 50 mg/100 g expressed in the product as consumed. The method is able to distinguish between D-ascorbic acid (isoascorbic- or erythorbic acid) and L-ascorbic acid.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

adult nutritional

nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein

3.2

infant formula

breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: CODEX STAN 72-1981]

4 Principle

Ascorbic acid is extracted from the sample using trichloroacetic acid (TCA) in the presence of tris [2-carboxyethyl]phosphine (TCEP) as a reducing agent and to protect ascorbic acid from oxidation. Ascorbic acid is then determined by Ultra High Performance Liquid Chromatography (UHPLC) or High Performance Liquid Chromatography (HPLC) with UV detection at 265 nm. Separation takes place on a C₁₈ column using decylamine as ion-pairing agent in a sodium acetate buffer solution (pH = 5,4) containing TCEP.

5 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

SAFETY PRECAUTIONS — Refer to material safety data sheets prior to use of chemicals. Use appropriate personal protective equipment when performing testing.

5.1 Reagents and standards

5.1.1 Acetonitrile, HPLC grade.

5.1.2 Ascorbic acid, purity > 99 %.

5.1.3 Decylamine.

5.1.4 Phosphoric acid, 85 %.

5.1.5 Ultra pure water, resistivity > 18 M Ω /cm.

5.1.6 Sodium acetate trihydrate.

5.1.7 Trichloroacetic acid (TCA).

5.1.8 Tris [2-carboxyethyl]phosphine (TCEP).

5.1.9 Isoascorbic acid.

5.1.10 Orotic acid.

5.2 Preparation of solutions

IMPORTANT — Vitamin C is sensitive to light and oxygen. Conduct operations under subdued light, or use amber glassware. Keep all solutions away from direct light.

5.2.1 **Sodium acetate solution**, substance concentration $c = 0,500$ mol/l (pH = 5,4).

Into a 500 ml volumetric flask, weigh 34,0 g of sodium acetate trihydrate (5.1.6), add about 400 ml of water and dissolve well. Adjust pH to 5,4 with phosphoric acid 85 % (5.1.4) and make up to volume with water.

5.2.2 **TCA solution**, 15 %.

Into a 500 ml volumetric flask, weigh 75,0 g of TCA (5.1.7), dissolve and make up to volume with water.

5.2.3 **TCEP solution**, mass concentration $\rho = 250$ μ g/ml.

Into a 500 ml volumetric flask, weigh 125 mg of TCEP (5.1.8), dissolve and make up to volume with water.

5.2.4 Mobile phase for UHPLC.

Into a 250 ml flask, mix 0,4 g of decylamine (5.1.3), 2,5 ml of acetonitrile (5.1.1), 25 ml of sodium acetate solution (5.2.1) and 205 ml of water. Do not make up to volume. Adjust pH to pH = 5,4 with phosphoric acid 85 % (5.1.4). Add 10 mg of TCEP (5.1.8).

NOTE Mobile phases are hazy during preparation, but they become clear after pH adjustment.

5.2.5 Mobile phase for HPLC.

Into a 1 000 ml flask, mix 1,6 g of decylamine (5.1.3), 80 ml of acetonitrile (5.1.1), 100 ml of sodium acetate solution (5.2.1) and 820 ml of water. Do not make up to volume. Adjust pH to pH = 5,4 with phosphoric acid 85 % (5.1.4). Add 50 mg of TCEP (5.1.8).

5.2.6 Ascorbic acid stock solution, $\rho = 500 \mu\text{g/ml}$.

Into a 25 ml amber glass volumetric flask (6.2), weigh 12,5 mg of ascorbic acid (5.1.2). Dissolve and make up to volume with TCEP solution (5.2.3). This solution can be kept for 3 months if stored at 4 °C away from light.

5.2.7 Ascorbic acid intermediate standard solution, $\rho = 50 \mu\text{g/ml}$.

Into a 10 ml amber glass volumetric flask (6.2), pipet 1 ml of stock solution (5.2.6). Make up to volume with TCEP solution (5.2.3). This solution can be used for 1 month if stored at 4 °C away from light.

5.2.8 Ascorbic acid calibration standard solutions, $\rho = 0,5 \mu\text{g/ml}$, $\rho = 1,0 \mu\text{g/ml}$, $\rho = 2,0 \mu\text{g/ml}$, $\rho = 3,0 \mu\text{g/ml}$, $\rho = 5,0 \mu\text{g/ml}$, $\rho = 7,5 \mu\text{g/ml}$ and $\rho = 10 \mu\text{g/ml}$.

Into a 10 ml amber glass volumetric flask (6.2), pipet 0,1 ml, 0,2 ml, 0,4 ml, 0,6 ml, 1,0 ml, 1,5 ml and 2,0 ml of intermediate standard solution (5.2.7). Make up to volume with mobile phase to prepare the respective concentrations given above.

6 Apparatus

Usual laboratory glassware and equipment, and, in particular, the following.

6.1 Balances, with readability of 0,1 mg and 0,01 g.

6.2 Volumetric flasks, amber glass, 10 ml, 25 ml class A in accordance with ISO 1042.

6.3 Beaker, amber glass, 250 ml.

6.4 pH meter.

6.5 Paper filter¹⁾.

6.6 Membrane filters, syringe filters, 0,22 μm or 0,45 μm pore size.

1) Folded grade 5971/2 or Schleicher & Schuell are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

6.7 UHPLC column, Waters Acquity UPLC® ethylene bridged hybrid (BEH) C₁₈ column²⁾ length of 100 mm, internal diameter of 2,1 mm and particle size of 1,75 µm or equivalent.

6.8 HPLC column, Licrospher® RP-18 column²⁾, length of 250 mm, internal diameter of 4,6 mm and particle size of 5 µm or equivalent.

6.9 LC system, HPLC or UHPLC system equipped with a quaternary or binary pump, a sample injector, a UV-VIS detector (or optionally a diode array detector), a degassing system and data software.

7 Procedure

7.1 Sample preparation

7.1.1 General

If the product contains starch, add 50 mg α-amylase to the suspensions (7.1.2) and incubate for 15 min at 40 °C to decrease viscosity and facilitate handling. Mix liquid samples well to ensure homogeneity and continue directly to extraction.

7.1.2 Powder samples

Weigh 25,0 g (m_1) of powder into a brown glass 250 ml beaker (6.3), add 10 mg TCEP (5.1.8). Add 200,0 g (m_2) warm water (40 °C). Mix well until complete dissolution. Accurately weigh 2,0 g (m_3) of homogenized sample suspension into a 10 ml amber-glass volumetric flask (6.2). Calculate the sample mass (m_s) using Formula (1):

$$m_s = m_1 \times m_3 / (m_1 + m_2) \quad (1)$$

Proceed to the extraction (7.2) as soon as possible as vitamin C can degrade rapidly. Do not let reconstituted samples stand for longer than 30 min.

7.1.3 Liquid samples

Weigh 2,0 g (m_s) of liquid sample into a 10 ml amber-glass volumetric flask (6.2).

Proceed to the extraction (7.2) as soon as possible as vitamin C can degrade rapidly. Do not let stand for longer than 15 min.

7.2 Extraction

To the prepared sample (7.1), add 4 ml of TCEP solution (5.1.8) and 2 ml of TCA solution (5.2.2). Bring up to volume with water. Filter the solution through a filter paper (6.5). Transfer 1 ml of filtrate into a 10 ml amber glass volumetric flask (6.2) containing 1 ml of acetate solution (5.2.1) and bring up to volume with mobile phase (5.2.4) or (5.2.5). Filter approximately 2 ml through a membrane filter (6.6) into a HPLC vial.

2) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

7.3 Analysis

7.3.1 Chromatographic conditions for UHPLC

Injection volume:	5 µl.
Auto sampler temperature:	10 °C.
Column temperature:	25 °C.
Flow rate:	0,35 ml/min.
Run time:	4,0 min.
Mobile phase:	Sodium acetate (50 mmol/l, pH = 5,4), decylamine (1,6 g/l), acetonitrile (1 %), TCEP (40 mg/l).
Detection:	UV at 265 nm.

At the end of each analytical series, rinse the column with a mixture of one part per volume of acetonitrile and one part per volume of water for 10 min at a flow rate of 0,4 ml/min.

7.3.2 Chromatographic conditions for HPLC

Injection volume:	25 µl.
Auto sampler temperature:	10 °C.
Column temperature:	25 °C.
Flow rate:	1,0 ml/min.
Run time:	20 min.
Mobile phase:	Sodium acetate (50 mmol/l, pH = 5,4), decylamine (1,6 g/l), acetonitrile (10 %), TCEP (40 mg/l).
Detection:	UV at 265 nm.

At the end of each analytical series, rinse the column with a mixture of one part per volume of acetonitrile and one part per volume of water for 60 min at a flow rate of 1,0 ml/min.

7.3.3 System suitability test

Equilibrate the chromatographic system for $\geq 0,5$ h. Inject any one of the standard solutions of L-ascorbic acid (5.2.8) at least six times and check peak retention times and response (peak height or area). Calculate the average response (peak height or area) and standard deviation. The coefficient of variation should not be higher than 2 %. Inject standard solutions (5.2.8) on a regular basis within a series of analyses. Ensure that orotic acid and isoascorbic acid are fully resolved from L-ascorbic acid by injecting separated standard solutions of both compounds (prepared as stated for L-ascorbic acid). If not resolved, the pH of the mobile phase may be decreased to pH = 5,0, or the amount of acetonitrile may be increased.

7.3.4 Calibration

Make single injections of all standard solutions (5.2.8) at least at the beginning and the end of each analytical series. Establish the calibration curve (seven points) by plotting peak response (height or area) versus ascorbic acid concentration of all the standard injections in the series. Perform linear regression. Calculate the slope (S) and intercept (I). Ensure the difference between slope and intercept

calculated at the beginning and the end of the series is not higher than 5 %. See [Clause 8](#) for the calibration curve.

7.3.5 Analysis

Make single injections of sample solutions.

7.3.6 Identification

Identify the L-ascorbic acid peak in the chromatograms of the sample solutions by comparison with the retention time of the corresponding peak in the standard solution. Identity can be confirmed by UV spectrum (200 nm to 400 nm) if a photo diode array (PDA) detector is used.

Example chromatograms are shown in [Annex A](#).

8 Calculations

Calculate the vitamin C mass fraction, w , in mg ascorbic acid/100 g, using [Formula \(2\)](#):

$$w = \frac{(A - I) \times V_1 \times V_3 \times 100}{S \times m_s \times V_2 \times 1\,000} \quad (2)$$

where

- A is the response (height or area) of the ascorbic acid peak obtained for the sample solution;
- I is the intercept of the calibration curve, see [7.3.4](#);
- V_1 is the volume of the test solution (volume used to dissolve the test portion), in ml ($V_1 = 10$ ml);
- V_3 is the volume of the final sample dilution, in ml ($V_3 = 10$ ml);
- 100 is the conversion to 100 g basis;
- S is the slope of the calibration curve, see [7.3.4](#);
- m_s is the mass of the test portion, in g ($m_s = 2,0$ g of liquid or as calculated in [Formula \(1\)](#) for powder samples);
- V_2 is the volume used in the sample dilution, in ml ($V_2 = 1,0$ ml);
- 1 000 is the conversion from μg to mg.

9 Precision

9.1 General

Details of the interlaboratory test of the precision of the method are summarized in [Annex B](#). The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than those given in [Annex B](#).

9.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases.

The values for adult nutritional powder milk based are:

$$\bar{x} = 6,33 \text{ mg/100 g} \quad r = 0,25 \text{ mg/100 g}$$

The values for infant formula powder partially hydrolysed soy based are:

$$\bar{x} = 19,5 \text{ mg/100 g} \quad r = 1,30 \text{ mg/100 g}$$

The values for SRM 1849a are:

$$\bar{x} = 8,12 \text{ mg/100 g} \quad r = 0,80 \text{ mg/100 g}$$

The values for adult nutritional powder low fat are:

$$\bar{x} = 17,6 \text{ mg/100 g} \quad r = 0,75 \text{ mg/100 g}$$

The values for infant formula powder soy based are:

$$\bar{x} = 10,3 \text{ mg/100 g} \quad r = 0,48 \text{ mg/100 g}$$

The values for adult nutritional RTF high fat are:

$$\bar{x} = 17,6 \text{ mg/100 g} \quad r = 2,06 \text{ mg/100 g}$$

The values for infant formula RTF milk based are:

$$\bar{x} = 12,0 \text{ mg/100 g} \quad r = 3,58 \text{ mg/100 g}$$

The values for infant elemental powder are:

$$\bar{x} = 34,0 \text{ mg/100 g} \quad r = 6,96 \text{ mg/100 g}$$

The values for child formula powder are:

$$\bar{x} = 4,92 \text{ mg/100 g} \quad r = 0,36 \text{ mg/100 g}$$

The values for adult nutritional RTF high protein are:

$$\bar{x} = 19,7 \text{ mg/100 g} \quad r = 0,93 \text{ mg/100 g}$$

9.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases.

The values for adult nutritional powder milk based are:

$$\bar{x} = 6,33 \text{ mg/100 g} \quad R = 0,57 \text{ mg/100 g}$$

The values for infant formula powder partially hydrolysed soy based are:

$$\bar{x} = 19,5 \text{ mg/100 g} \quad R = 4,38 \text{ mg/100 g}$$

The values for SRM 1849a are:

$$\bar{x} = 8,12 \text{ mg/100 g} \quad R = 0,84 \text{ mg/100 g}$$

The values for adult nutritional powder low fat are:

$$\bar{x} = 17,6 \text{ mg/100 g} \quad R = 3,19 \text{ mg/100 g}$$

The values for infant formula powder soy based are:

$$\bar{x} = 10,3 \text{ mg/100 g} \quad R = 1,73 \text{ mg/100 g}$$

The values for adult nutritional RTF high fat are:

$$\bar{x} = 17,6 \text{ mg/100 g} \quad R = 5,58 \text{ mg/100 g}$$

The values for infant formula RTF milk based are:

$$\bar{x} = 12,0 \text{ mg/100 g} \quad R = 4,42 \text{ mg/100 g}$$

The values for infant elemental powder are:

$$\bar{x} = 34,0 \text{ mg/100 g} \quad R = 10,88 \text{ mg/100 g}$$

The values for child formula powder are:

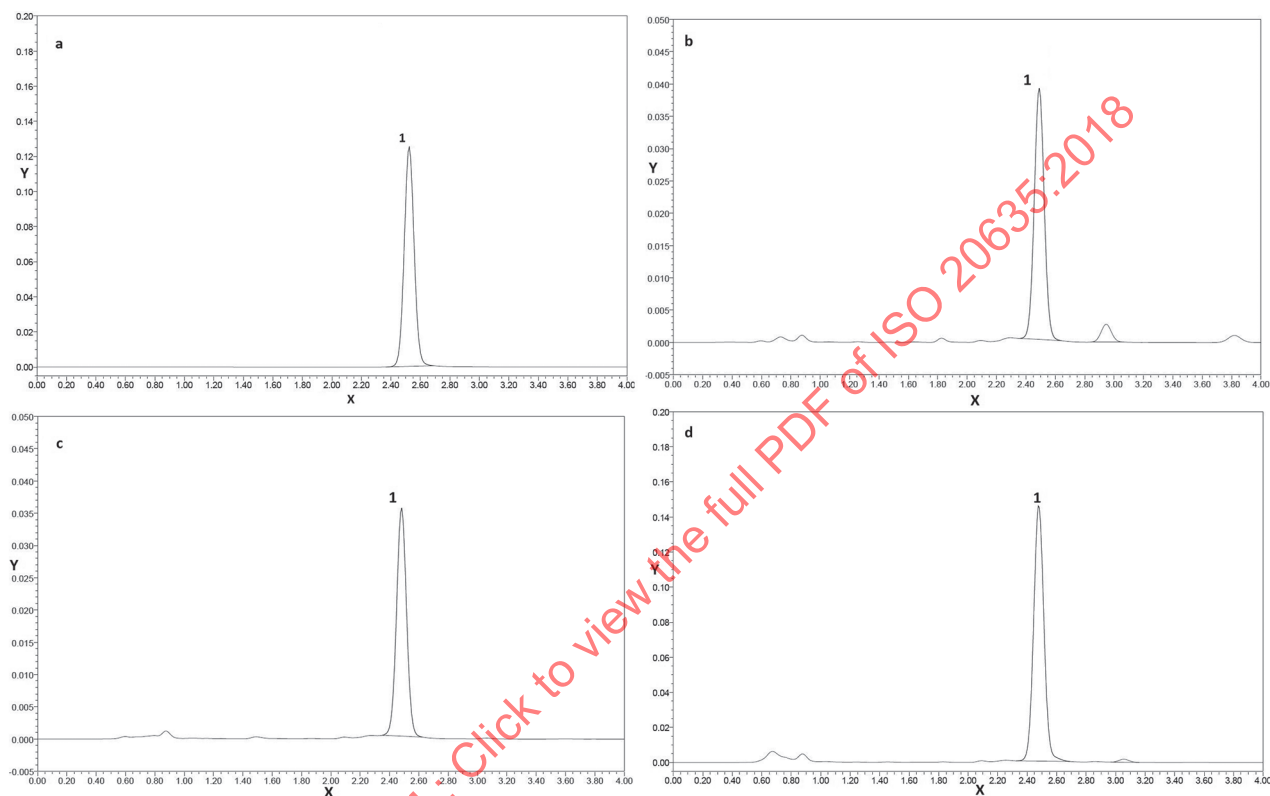
$$\bar{x} = 4,92 \text{ mg/100 g} \quad R = 0,62 \text{ mg/100 g}$$

The values for adult nutritional RTF high protein are:

$$\bar{x} = 19,7 \text{ mg/100 g} \quad R = 5,14 \text{ mg/100 g}$$

Annex A (informative)

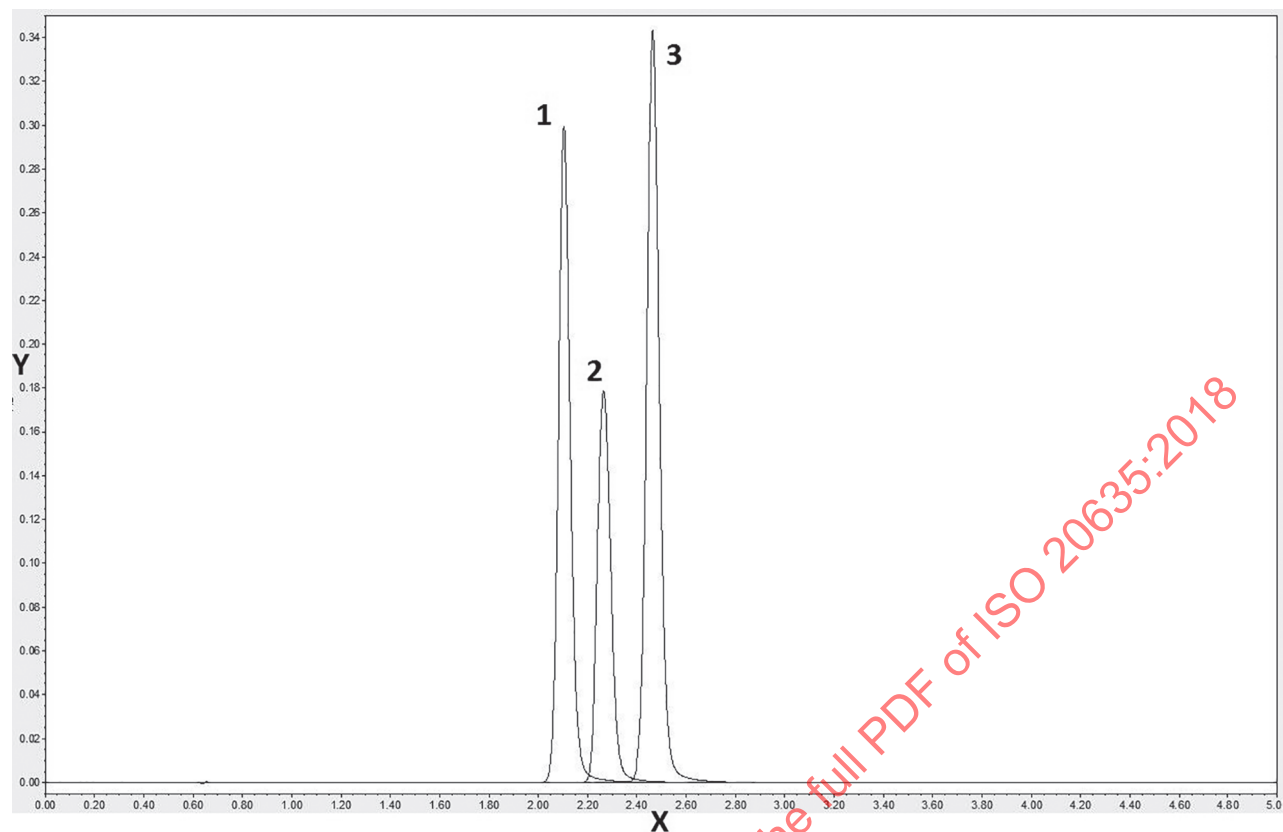
Typical chromatograms



Key

- | | | | |
|---|-----------------------------|---|-----------------|
| a | Standard solution. | 1 | ascorbic acid |
| b | Infant formula, milk based. | X | time, in min |
| c | Infant formula, soy based. | Y | arbitrary units |
| d | Adult nutritional. | | |

Figure A.1 — Example HPLC separation on a BEH, C₁₈, 1,7 μ m, 100 mm $\times$ 2,1 mm



Key

- | | | | |
|---|------------------|---|-----------------|
| 1 | ascorbic acid | X | time, in min |
| 2 | orotic acid | Y | arbitrary units |
| 3 | isoascorbic acid | | |

Figure A.2 — Example of separation of ascorbic acid, orotic acid and isoascorbic acid