
**Infant formula and adult
nutritionals — Determination of
 β -carotene, lycopene and lutein
by reversed-phase ultra-high
performance liquid chromatography
(RP-UHPLC)**

*Formules infantiles et produits nutritionnels pour adultes —
Détermination du bêta-carotène, du lycopène et de la lutéine par
chromatographie liquide ultra haute performance à phase inversée*

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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).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 2016.13: *Determination of Lutein, β -Carotene, and Lycopene in Infant Formula and Adult Nutritionals by Ultra-High-Performance Liquid Chromatography: Final Action (β -Carotene and Lycopene Only)*.

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.

Introduction

Lutein, β -carotene and lycopene are among the carotenoids present in human milk and are added to infant formula and adult nutritionals^{[1][2][3]}. Lutein may play a role in vision and cognitive function, and β -carotene has provitamin A activity^{[4][5]}. Accurate and precise measurements of these added ingredients are important for ensuring their presence in the allowable ranges.

This analytical method was originally presented to the Stakeholder Panel on Infant Formula and Adult Nutritionals through AOAC International, and a single-laboratory validation was previously published^[6]. It was recommended as an AOAC Final Action method for β -carotene and lycopene after the collaborative study data was reviewed by the same panel^[7].

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Infant formula and adult nutritionals — Determination of β -carotene, lycopene and lutein by reversed-phase ultra-high performance liquid chromatography (RP-UHPLC)

WARNING — The use of this method can involve hazardous materials, operations and equipment. This method does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this method to establish appropriate safety and health practices.

1 Scope

This document specifies a method for the quantitative determination of β -carotene and lycopene in infant formula and adult nutritionals in solid (i.e. powders) or liquid (i.e. ready-to-feed liquids and liquid concentrates) forms using reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) and UV-visible detection. The application range runs from 1 $\mu\text{g}/100\text{ g}$ to 1 500 $\mu\text{g}/100\text{ g}$ for lycopene and from 1 $\mu\text{g}/100\text{ g}$ to 2 250 $\mu\text{g}/100\text{ g}$ for β -carotene. Based on the single-laboratory validation, the limit of detection (LOD) was 0,1 $\mu\text{g}/100\text{ g}$ and the limit of quantification (LOQ) was 0,3 $\mu\text{g}/100\text{ g}$ for each carotenoid.

The method does not apply to materials that contain measurable levels of β -apo-8'-carotenal. The reproducibility data meets the requirements given in References [8] and [10].

[Annex C](#) specifies the determination of lutein. The reproducibility data does not meet the requirements given in Reference [9].

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 Standard 72-1981]

4 Principle

Test samples (reconstituted powders, liquid ready-to-feed and liquid concentrates) are spiked with an internal standard and treated with potassium hydroxide. Samples are then extracted with methyl tert-butyl ether (MTBE) and tetrahydrofuran (THF), followed by hexane. The supernatants from the liquid-liquid extraction are dried under nitrogen and reconstituted in 2-propanol. Separation is performed by reversed-phase chromatography on a C30 column. All-*trans* β -carotene and lycopene are separated from their major *cis* isomers, as well as from lutein, zeaxanthin and α -carotene. Although this method does not involve the high system backpressure normally associated with UHPLC, the low system volume is recommended for resolution with a 2,0 mm internal diameter (i.d.) column.

Throughout this method, estimated sample concentrations for standard and sample preparations are stated per 100 g on a reconstituted basis (as is for ready-to-feed liquids, 25 g sample + 200 g water for powder samples, or diluted 1:1 by weight for liquid concentrates) in accordance with References [8], [9] and [10].

5 Reagents and materials

During the analysis, unless otherwise stated, only use reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity. Reagent volumes may be scaled up or down provided good laboratory practices are followed.

5.1 Reagents

5.1.1 Laboratory water, 18 megaohm-cm.

5.1.2 Methanol (MeOH), HPLC grade.

5.1.3 Methyl tert-butyl ether (MTBE), HPLC grade.

5.1.4 *n*-Hexane, HPLC grade.

5.1.5 Cyclohexane, HPLC grade.

5.1.6 Potassium hydroxide (KOH), pellets, ACS grade.

5.1.7 Reagent alcohol (ROH), denatured, 90 % ethanol, HPLC grade.

5.1.8 α -Tocopherol (Vitamin E), synthetic, 95 %.

5.1.9 Pyrogalllic acid (Pyrogallol), ACS grade.

5.1.10 2-Propanol (IPA), HPLC grade.

5.1.11 Tetrahydrofuran (THF), 99,9 %, stabilized with butylated hydroxytoluene (BHT).

CAUTION — THF can form peroxides and only THF stabilized with BHT should be used. Refer to safety data sheets when using any reagent. Use appropriate personal protective equipment when performing analyses.

5.1.12 Ammonium acetate, HPLC grade, 98 %.

5.1.13 Potassium hydroxide solution, a mass fraction of 50 %.

Add 50 ml water to a 250 ml beaker. Weigh 50 g KOH and slowly transfer to the beaker under constant stirring. When dissolved and cooled, transfer to a media bottle and store at room temperature for up to six months.

5.1.14 Vitamin E solution in MTBE, substance concentration $c = 10$ mmol/l.

Dissolve 1,1 g α -tocopherol in 250 ml MTBE. Store in a refrigerator for up to one month.

5.1.15 Vitamin E solution in THF, $c = 10$ mmol/l.

Dissolve 1,1 g α -tocopherol in 250 ml THF. Store in a refrigerator for up to one month.

5.1.16 Pyrogallol solution, $c = 0,2$ mol/l pyrogallol acid in reagent alcohol.

Dissolve 6,3 g pyrogallol acid in 250 ml ROH. Store in a refrigerator for up to one month. Solution should be clear at room temperature; discard if coloured.

5.1.17 Extraction solution, $c = 1$ mmol/l vitamin E in MTBE-THF (1 + 1).

Dissolve 0,22 g α -tocopherol in 250 ml MTBE and 250 ml THF. Store in a refrigerator for up to one month.

5.1.18 Sample solution, $c = 10$ mmol/l vitamin E in IPA.

Dissolve 4,4 g α -tocopherol in 1 000 ml IPA. Store in a refrigerator for up to one month.

5.1.19 Mobile phase A for LC system, $c = 20$ mmol/l ammonium acetate in methanol–water (98 + 2).

Combine 980 ml MeOH, 20,0 ml water and 1,54 g ammonium acetate, and mix to dissolve.

5.1.20 Mobile phase B for LC system. [ISO 23443:2020](https://standards.iteh.ai/catalog/standards/iso/3fd8fb2b-7987-492a-988a-4af25dfd86b6/iso-23443-2020)

MTBE ([5.1.3](#)).

5.2 Standards

5.2.1 β -Carotene, USP (Rockville, MD) Part No. 1065480¹⁾ or equivalent.

5.2.2 Apocarotenal (β -Apo-8'-carotenal), USP Part No. 1040854¹⁾ or equivalent.

5.2.3 Lycopene, > 90 % by UV-Vis, Sigma-Aldrich (St. Louis, MO) Part No. L9879, Extrasynthese (Genay, France) Part No. 0305 S¹⁾, or equivalent.

5.2.4 β -Carotene system suitability reference standard, USP Part No. 1065491¹⁾.

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5.3 Standards preparation

5.3.1 Standard solution preparation is summarized in [Table 1](#) and detailed below. Use glass volumetric pipettes and flasks for preparation of all standard solutions unless otherwise noted.

Table 1 — Composition and nominal concentrations of carotenoid standard solutions

Standard solution	β -carotene	Lycopene	Apocarotenal
Stock solutions			
Standard (mg)	5,0	2,5	5,0
Total volume (ml)	25	25	25
Concentration (mg/100 ml)	20	10	20
UV-Visible solutions (200 μg/100 ml)			
Stock solution (ml)	1,0	2,0	—
Total volume (ml)	100	100	—
Working solutions (200 μg/100 ml) in sample solvent			
Stock solution (ml)	0,1	0,2	1,0
Total volume (ml)	10	10	100
Intermediate solutions in sample solvent			
Stock solution (ml)	2,0	2,0	3,0
Total volume (ml)	100	—	50
Concentration (μ g/100 ml)	400	200	1 200

5.3.2 Carotenoid stock solutions, $\rho = 10\,000\ \mu\text{g}/100\ \text{ml}$ to $\rho = 20\,000\ \mu\text{g}/100\ \text{ml}$.

Weigh (to 0,01 mg) approximately 5 mg each of β -carotene ([5.2.1](#)) and apocarotenal ([5.2.2](#)) reference standard into separate 25 ml volumetric flasks. Add approximately 20 ml vitamin E solution in MTBE ([5.1.14](#)) to each, sonicate for 2 min to 3 min, and dilute to volume with vitamin E solution in MTBE.

Weigh (to 0,01 mg) approximately 2,5 mg lycopene ([5.2.3](#)) reference standard into a 25 ml volumetric flask. Add approximately 20 ml vitamin E solution in THF ([5.1.15](#)), sonicate for 2 min to 3 min, and dilute to volume with vitamin E solution in THF.

Store stock solutions at $-20\ ^\circ\text{C}$ for up to six months and check their purity each time new standard solutions are made from them. When taken from the freezer, stock solutions should be sonicated for 2 min and vortexed to bring all carotenoids into solution.

5.3.3 UV-Visible solutions for spectroscopy potency check, $\rho = 200\ \mu\text{g}/100\ \text{ml}$.

Transfer 1,0 ml β -carotene standard stock solution ([5.3.2](#)) to a 100 ml volumetric flask and dilute to volume with cyclohexane.

Transfer 2,0 ml lycopene standard stock solution ([5.3.2](#)) to a 100 ml volumetric flask and dilute to volume with cyclohexane.

Immediately measure solutions by UV-visible spectroscopy and calculate purity according to [8.1.2](#).

5.3.4 Individual carotenoid working solutions for chromatographic purity check (200 $\mu\text{g}/100\ \text{ml}$).

5.3.4.1 Analyse working solutions by UHPLC on the same day they are prepared and calculate the chromatographic purity of each according to [8.1.3](#).

5.3.4.2 β -carotene working solution.

With an adjustable pipet, transfer 100 μ l of standard stock solution (5.3.2) to a 10 ml volumetric flask and dilute to volume with sample solution.

5.3.4.3 Lycopene working solution.

With an adjustable pipet, transfer 200 μ l standard stock solution (5.3.2) to a 10 ml volumetric flask and dilute to volume with sample solution.

5.3.4.4 Apocarotenal working solution.

Transfer 1,0 ml standard stock solution (5.3.2) to a 100 ml volumetric flask and dilute to volume with sample solution. Store at -20°C for up to one month and use for internal standard (5.3.8).

5.3.5 Intermediate solutions, $\rho = 200\ \mu\text{g}/100\ \text{ml}$ to $1\ 200\ \mu\text{g}/100\ \text{ml}$.

5.3.5.1 Apocarotenal intermediate solution.

Transfer 3,0 ml apocarotenal stock solution (5.3.2) to a 50 ml volumetric flask and dilute to volume with sample solution. Store at -20°C for up to one month.

5.3.5.2 Mixed carotenoid intermediate solution.

Combine 2,0 ml each of β -carotene and lycopene standard stock solutions (5.3.2) in a 100 ml volumetric flask and dilute to volume with sample solution. Store at -20°C for up to one month.

5.3.6 Calibration solutions.

Transfer apocarotenal intermediate solution (5.3.5.1) and mixed carotenoid intermediate solution (5.3.5.2) to volumetric flasks according to Table 2 and dilute to volume with sample solution. Store at -20°C for up to one month. Solutions may be aliquoted to HPLC vials prior to storing in the freezer.

Table 2 — Composition and nominal concentrations of carotenoid calibration solutions

Calibration solution	C1	C2	C3	C4	C5
Apocarotenal intermediate (5.3.5.1) added (ml)	2,0	2,0	2,0	2,0	8,0
Mixed carotenoid intermediate (5.3.5.2) added (ml)	15,0	8,0	5,0	2,0	1,0
Total volume (ml)	25	25	25	25	100
Apocarotenal concentration ($\mu\text{g}/100\ \text{ml}$)	96	96	96	96	96
β -carotene concentration ($\mu\text{g}/100\ \text{ml}$)	240	128	80	32	4
Lycopene concentration ($\mu\text{g}/100\ \text{ml}$)	120	64	40	16	2

5.3.7 β -Carotene system suitability solutions.

Preparation of β -carotene system suitability solutions is summarized in Table 3 and detailed below. To make the stock solution, transfer approximately 20 mg β -carotene system suitability reference standard (5.2.4) to a 50 ml volumetric flask. Add 1 ml water and 4 ml THF and sonicate for 5 min. Dilute to volume with IPA and sonicate for 5 min. Cool to room temperature and filter the cloudy suspension through a 0,2 μm polytetrafluoroethylene (PTFE) syringe filter.

To make the working solution, dilute 5 ml of the filtered stock solution to 25 ml with IPA. Store in a refrigerator for up to three months or at -20°C for up to six months.

Table 3 — Composition of β -carotene system suitability solutions

Suitability solution	β -carotene
Stock solution composition	
Standard added (mg)	20
Total volume (ml)	50
Working solution composition	
Stock solution added (ml)	5
Total volume (ml)	25

5.3.8 Internal standard solution (ISTD).

5.3.8.1 Prepare immediately before use. The apocarotenal solutions used to make the ISTD should be made from the same apocarotenal stock solution (5.3.2) as that used to make the calibration solutions (5.3.6).

5.3.8.2 Infant formula and samples with low carotenoid concentrations (up to 100 μg of an individual carotenoid per 100 g).

Transfer 4,0 ml apocarotenal working solution (5.3.4.4) to a 50 ml volumetric flask and dilute to volume with pyrogallol solution (5.1.16). This is enough solution for nine samples.

5.3.8.3 Samples with individual carotenoid concentrations > 100 $\mu\text{g}/100\text{ g}$.

Transfer 4,0 ml apocarotenal intermediate solution (5.3.5.1) to a 50 ml volumetric flask and dilute to volume with pyrogallol solution.

6 Apparatus

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

6.1 UHPLC system, consisting of a binary or quaternary pump, autosampler, thermostatted column compartment, UV-Vis detector and data acquisition software. A high sensitivity flow cell (e.g. 60 mm) in the detector provides the best results, but a standard 10 mm flow cell may be used if system suitability criteria can be met.

6.2 Analytical column, C30 carotenoid column, 250 mm $\times$ 2,0 mm $\times$ 3 μm (Part No. CT99S03-2502WT; YMC, Kyoto, Japan)²⁾. Other columns may be used if the system suitability criteria (7.2.2) can be met.

6.3 Guard column, C30 guard column, 10 mm $\times$ 2,1 mm $\times$ 3 μm (Part No. CT99S03-01Q1GC; YMC)³⁾.

6.4 Guard cartridge holder, Part No. XPGCH-Q1 (YMC)³⁾.

6.5 Spectrophotometer, wavelength range of 200 nm to 700 nm, with 1 cm quartz cells.

6.6 Top loading balance, capable of weighing to 0,1 g.

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