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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)

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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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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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).

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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 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].

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 µg/100 g to 1 500 µg/100 g for lycopene and from 1 µg/100 g to 2 250 µg/100 g for β-carotene. Based on the single-laboratory validation, the limit of detection (LOD) was 0,1 µg/100 g and the limit of quantification (LOQ) was 0,3 µg/100 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 Pyrogallic 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 pyrogallic acid in reagent alcohol.

Dissolve 6,3 g pyrogallic 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.

MTBE (5.1.3).

5.2 Standards

5.2.1 β-Carotene, USP (Rockville, MD) Part No. 1065480[1)] or equivalent.

5.2.2 Apocarotenal (β-Apo-8′-carotenal), USP Part No. 1040854[1)] or equivalent.

5.2.3 Lycopene, > 90 % by UV-Vis, Sigma-Aldrich (St. Louis, MO) Part No. L9879, Extrasynthese (Genay, France) Part No. 0305 S[1)], or equivalent.

5.2.4 β-Carotene system suitability reference standard, USP Part No. 1065491[1)].

1) 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 and endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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 (µg/100 ml)	400	200	1 200

5.3.2 Carotenoid stock solutions, ρ = 10 000 µg/100 ml to ρ = 20 000 µg/100 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 °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, ρ = 200 µg/100 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 µg/100 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 µl 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 µl 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, ρ = 200 µg/100 ml to 1 200 µg/100 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 (µg/100 ml)	96	96	96	96	96
β-carotene concentration (µg/100 ml)	240	128	80	32	4
Lycopene concentration (µg/100 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 µg/100 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 × 2,0 mm × 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 × 2,1 mm × 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.

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 this product. Equivalent products may be used if they can be shown to lead to the same results.

3) 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 this product. Equivalent products may be used if they can be shown to lead to the same results.

6.7 Analytical balance, capable of weighing to 0,01 mg.

6.8 Ultrasonic water bath, 40 kHz.

6.9 Reciprocating shaker, capable of 200 rpm.

6.10 Evaporator, with pure nitrogen supply.

6.11 Laboratory centrifuge, with adapters for 50 ml centrifuge tubes.

6.12 Centrifuge tubes, 50 ml, polypropylene.

6.13 Syringes, 1 ml, disposable.

6.14 Syringe filters, 0,2 µm, PTFE.

6.15 Class A volumetric flasks, various sizes, clear and amber.

6.16 Scintillation vials, 12 ml, amber.

6.17 HPLC vials, amber, with 300 µl inserts.

6.18 Class A volumetric pipets, various sizes.

7 Procedure

7.1 Sample preparation

7.1.1 General

While this method can quantify carotenoids in the range of 1 µg/100 g to 1 300 µg/100 g, it is recommended to only quantify a 100-fold difference with a single preparation. For example, the range of 1 µg/100 g to 100 µg/100 g works well for infant formula, but the range of 15 µg/100 g to 1 500 µg/100 g would work best for samples with the highest carotenoid concentrations.

This method is not applicable to materials that contain measurable levels of β-apo-8′-carotenal (apocarotenal). Because apocarotenal is used as an internal standard, its presence in the test material would inflate the amount of internal standard measured in the samples, leading to artificially low results for the analytes. Unknown samples should be prepared as blanks, using 5 ml pyrogallol solution in place of ISTD solution in 7.1.6, to demonstrate that they do not contain apocarotenal.

Prepare up to 12 samples at a time. Weigh all samples (powders and liquids) to 0,1 mg. At several points in the sample preparation, sample masses and dilutions may vary according to the concentration of an individual carotenoid in the product. If carotenoids are present in different ranges in the same sample, e.g. lycopene at ≤ 50 µg/100 g and β-carotene at 250 µg/100 g, then the sample should be prepared once for each concentration.

7.1.2 Powders

Record masses of both powder sample and water to four significant figures. Reconstitute 25 g powder sample with 200 ml 35 °C water in a reagent bottle and shake well. Mix on a spin plate for 1 min to 5 min until completely dispersed and no solids are visible. To ensure homogeneity, shake again immediately before transferring approximately 5,25 g reconstituted sample into a 50 ml centrifuge tube.

7.1.3 Liquid ready-to-feed (RTF) with individual carotenoid concentrations $\rho \leq 200$ µg/100 g

Shake bottle or can on a reciprocating shaker for 10 min before opening. Transfer approximately 5,25 g sample into a 50 ml centrifuge tube.

7.1.4 RTF sample with individual carotenoid concentrations > 200 µg/100 g

Shake bottle or can on a reciprocating shaker for 10 min before opening. Transfer approximately 2 g sample into a 50 ml centrifuge tube. Add 3 ml water, cap and vortex for 10 s. Let sit up to 15 min at room temperature.

7.1.5 Infant formula concentrate

Shake bottle or can on a reciprocating shaker for 10 min before opening. Transfer approximately 2,5 g sample into a 50 ml centrifuge tube. Add 2,5 ml water, cap and vortex for 10 s. Let sit up to 15 min at room temperature.

7.1.6 Volumetrically pipet 5,0 ml of the appropriate ISTD solution from (5.3.8) to each tube.

7.1.7 Add 1,5 ml KOH solution (5.1.13) to each tube with a repeater pipet.

7.1.8 Shake on a reciprocating shaker for 5 min.

7.1.9 Add 8 ml extraction solution (5.1.17) to each tube with a repeater pipet.

7.1.10 Shake for 10 min.

7.1.11 With a repeater pipet or dispenser, add 10 ml water and 10 ml hexane to each tube.

7.1.12 Shake for 1 min.

7.1.13 Centrifuge at 1 000 rpm (or equivalent to 200*g*) for 5 min.

7.1.14 Transfer a portion of the supernatant to a 12 ml scintillation vial. An adjustable pipette may be used.

For samples with individual carotenoid concentrations:

— ≤ 50 µg/100 g, use 10 ml supernatant;

— > 50 µg/100 g, use 3 ml supernatant.

7.1.15 Dry under nitrogen at ≤ 40 °C.

7.1.16 Reconstitute dried extract in sample solution (5.1.18) and vortex to mix, shaking if necessary to include any residue on the sides of the vial. An adjustable pipette may be used for the sample solution. Prepared sample extracts are stable for 24 h at room temperature.

7.1.17 For samples with individual carotenoid concentrations:

— ≤ 100 µg/100 g, add 0,5 ml;

— 100 µg/100 g to 500 µg/100 g, add 1 ml;

— 500 µg/100 g to 1 000 µg/100 g, add 2 ml;

— 1 000 µg/100 g to 1 500 µg/100 g, add 3 ml.

7.1.18 Filter through a 0,2 µm PTFE syringe filter prior to injection.

7.2 Chromatography

7.2.1 Chromatographic conditions

Set up the UHPLC system according to the specifications below and given in Table 4. Follow the manufacturer's instructions for column installation, cleaning and storage. 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 i.d. column. To minimize system dwell volume and extra-column volume, 0,12 mm i.d. connecting tubing and a low volume flow cell designed for UHPLC systems are recommended. On some LC systems, it is helpful to convert the pump to low delay volume mode.

Analytical column:	YMC C30 3 µm, 250 mm × 2,0 mm
Guard column:	YMC C30 3 µm, 10 mm × 2,0 mm
Column temperature:	30 °C
Mobile phases:	A: 20 mmol/l ammonium acetate in MeOH-Water (98+2) B: MTBE
Flow rate:	0,25 ml/min
Backpressure:	approximately 18 500 kPa (185 bar)
Injection volume:	5 µl
UV-Visible detection:	450 nm, ref = off

Table 4 — Gradient programme

Time (min)	**Mobile phase B** %
0	3
1	8
8	15
25	100
25,5	3
32	3

7.2.2 System suitability checks

7.2.2.1 Resolution between β-carotene *cis* and *trans* isomers and α-carotene

Inject the β-carotene system suitability working solution (5.3.7), and determine the resolution between the two major *cis* isomers of β-carotene, all-*trans*-β-carotene and α-carotene. Resolution should be ≥ 1,4 between 13-*cis*-β-carotene and *cis*/*trans* α-carotene, and ≥ 2,6 between all-*trans*- β-carotene and 9-*cis*-β-carotene. See Figure A.1.

7.2.2.2 Inject the calibration solutions before and after each set of sample injections (up to 12 samples in each set). Calculate the slope relative to the internal standard as shown in 8.3. The coefficient of determination (R^2) for each curve should be > 0,995. The slopes from the two curves should not differ

by more than 2 % for β-carotene and not by more than 10 % for lycopene. Use the average of the points from the two curves bracketing the samples for calculations.

Representative sample chromatograms are shown in Figures A.2 to A.5.

8 Calculations

8.1 Determination of purity

8.1.1 General

Determine the purity of β-carotene and lycopene standards by first determining the spectrophotometric purity and then the chromatographic purity of each. The overall purity is calculated as the product of the two measured purities.

8.1.2 Spectrophotometric purity (P_S)

Measure each standard measuring solution (5.3.3) against the appropriate solvent blank at its absorbance maximum (456 nm for β-carotene in cyclohexane and 476 for lycopene in cyclohexane). Calculate the spectrophotometric purity of each reference standard as the observed absorbance over the expected absorbance using Formula (1):

$$P_S = (A_{ms} \times V_{total} \times 1\,000)/(E \times V_{stock} \times \rho_m) \quad (1)$$

where

A_{ms} is the absorbance of the standard measuring solution;

V_{total} is the total volume of standard measuring solution made;

1 000 is the factor for g to mg;

E is the extinction coefficient, $E_{1\ \%,1cm}$ (β-carotene in cyclohexane: 2 505 at 456 nm, see Reference [11], lycopene in cyclohexane: 3 310at 476 nm, see Reference [12]);

V_{stock} is the volume of standard stock solution used, in ml;

ρ_m is the stock concentration by mass, in mg/100 ml.

Spectrophotometric purity is typically greater than 0,90 (i.e. 90 %).

8.1.3 Chromatographic purity (P_C)

Inject standard working solutions (5.3.4) at least three times. Calculate the chromatographic purity using Formula (2):

$$P_C = S_a/S_s \quad (2)$$

where

S_a is the area of the all-*trans*-carotenoid peak;

S_s is the sum of areas of all relevant peaks.

Relevant peaks include all peaks in the HPLC chromatogram with the exception of solvent peaks. Chromatographic purity is typically greater than 0,95 (i.e. 95 %).

8.1.4 Reference standard purity (P)

Calculate the purity of each reference standard using Formula (3):

$$P = P_S \times P_C \times 100 \quad (3)$$

where 100 is the factor for converting decimal to per cent.

8.2 Concentration of each carotenoid in standard solutions

8.2.1 Stock solution concentrations

Calculate the mass concentration of each carotenoid (e.g. β-carotene, ρ_{bcs}) in the all-*trans* form, in µg/100 ml, in each standard stock solution (5.3.2) using Formula (4):

$$\rho_{bcs} = (m_{bc}/V_{bc}) \times (P_{bc}/100) \times 1\,000 \times 100 \quad (4)$$

where

m_{bc} is the mass of β-carotene used to make the stock solution, in mg;

P_{bc} is the reference standard purity of all-*trans*-β-carotene calculated in (8.1.4);

100 is the conversion from per cent to decimal;

1 000 is the conversion of mg to µg;

100 is the conversion from µg/ml to µg/100 ml;

V_{bc} is the dilution volume of the stock solution.

8.2.2 Apocarotenal working solution concentration

Calculate the mass concentration of apocarotenal, ρ_{aws}, in the all-*trans* form, in µg/100 ml, in the apocarotenal working solution (5.3.4.4) using Formula (5):

$$\rho_{aws} = \rho_{as} \times (1/100) \quad (5)$$

where

ρ_{as} is the concentration of apocarotenal stock solution calculated in 8.2.1;

1/100 is the dilution of stock solution to working solution.

8.2.3 Apocarotenal intermediate solution concentration

Calculate the mass concentration of apocarotenal, ρ_{ai}, in the all-*trans* form, in µg/100 ml, in the apocarotenal intermediate solution (5.3.5.1) using Formula (6):

$$\rho_{ai} = \rho_{as} \times (3/50) \quad (6)$$

where

ρ_{as} is the concentration of apocarotenal stock solution calculated in 8.2.1;

3/50 is the dilution of stock solution to intermediate solution.

8.2.4 Carotenoid concentrations in mixed carotenoid intermediate solution

Calculate the mass concentration of each carotenoid analyte (e.g. β-carotene, ρ_{bci}) in the all-*trans* form, in µg/100 ml, in the mixed carotenoid intermediate solution (5.3.5.2) using Formula (7):

$$\rho_{bci} = \rho_{bcs} \times (V_{bcs}/V_{mc}) \quad (7)$$

where

ρ_{bcs} is the concentration of β-carotene stock solution calculated in 8.2.1;

V_{bcs} is the volume of β-carotene stock solution used;

V_{mc} is the total volume of mixed carotenoid intermediate solution.

8.2.5 Concentrations of carotenoid analytes in calibrations solutions

Calculate the mass concentration of each carotenoid analyte (e.g. β-carotene, ρ_{bc}) in the all-*trans* form, in µg/100 ml, in each calibration solution (5.3.6) using Formula (8):

$$\rho_{bc} = \rho_{bci} \times (V_{mci}/V_t) \quad (8)$$

where

ρ_{bci} is the concentration of β-carotene in the mixed carotenoid solution calculated in 8.2.4;

V_{mci} is the volume of mixed carotenoid intermediate solution used;

V_t is the total volume of the calibration solution.

8.2.6 Concentration of apocarotenal internal standard in calibrations solutions

Calculate the mass concentration of apocarotenal, ρ_a, in µg/100 ml, in each calibration solution (5.3.6) using Formula (9):

$$\rho_a = \rho_{ai} \times (V_{ai} / V_t) \quad (9)$$

where

ρ_{ai} is the concentration of the apocarotenal intermediate solution calculated in 8.2.3;

V_{ai} is the volume of apocarotenal intermediate solution used;

V_t is the total volume of the calibration solution.

8.3 Calculate calibration curve

For each calibration solution calculate:

a) the peak area ratio for each analyte (peak area [all-*trans* β-carotene or lycopene])/(peak area [internal standard]);

b) the concentration ratio (concentration [all-*trans* β-carotene or lycopene])/(concentration [internal standard]).

Build a 5-point calibration curve with internal standard by plotting peak area ratios against concentration ratios, with relative concentration on the x-axis.

The accuracy on calibration points should be 100 % ± 10 % for calibration solutions C1 to C4, and the coefficient of determination (R^2) should be greater than 0,995.

The calibration and calculation may be achieved through data processing within the instrument software or off-line.

8.4 Mass of apocarotenal

Calculate the mass of apocarotenal, m_a, in µg, added to the test samples using Formula (10):

$$m_a = (\rho_a/100) \times V_a \times (4/50) \quad (10)$$

where

ρ_a is the mass concentration of apocarotenal in the intermediate (8.2.3) or working solution (8.2.2) used in the ISTD solution, in µg/100 ml;

100 is the conversion from µg/100 ml to µg/ml

V_a the volume of ISTD solution added to each sample, in ml;

4 the volume of apocarotenal intermediate or WS used in the ISTD solution, in ml;

50 is the volume of ISTD solution made, in ml.

8.5 Contents of all-*trans*-β-carotene, *cis* isomers of β-carotene and total β-carotene

Test sample concentrations calculated in this clause are expressed per 100 g on an as is basis for all samples, allowing each laboratory to apply appropriate reconstitution factors.

Calculate the contents of all-*trans*-β-carotene, *cis* isomers of β-carotene and total β-carotene in the test samples. For peak identification, refer to relative retention times of peaks in Figures A.1 to A.4.

Calculate the mass fraction of all-*trans*-β-carotene w_{atbc}, in µg/100 g, using Formula (11):

$$w_{atbc} = \frac{m_a}{m_s} \times \left(\frac{A_{atbc}}{A_a} - I_{atbc} \right) \times \frac{100}{S_{atbc}} \quad (11)$$

where

m_a is the mass of apocarotenal added to the test sample, in µg;

m_s is the mass of the test sample, in g;

A_{atbc} is the peak area of all-*trans*-β-carotene in the sample chromatogram, in arbitrary units;

A_a is the peak area of apocarotenal in the sample chromatogram, in arbitrary units;

I_{atbc} is the y-intercept of the calibration curve for all-*trans*-β-carotene;

S_{atbc} is the slope of the calibration curve for all-*trans*-β-carotene.

Calculate the mass fraction of *cis* isomers of β-carotene, w_{cisbc}, in µg/100 g, using Formula (12):

$$w_{cisbc} = \frac{m_a}{m_s} \times \left(\frac{((A_{15cisbc} \times 1,4) + (A_{13cisbc} \times 1,2) + A_{9cisbc})}{A_a} - I_{atbc} \right) \times \frac{100}{S_{atbc}} \quad (12)$$

where

m_a is the mass of apocarotenal added to the test sample, in µg;

m_s is the mass of the test sample, in g;

$A_{15cisbc}$ is the peak area of 15-*cis*-β-carotene in the sample chromatogram, in arbitrary units;

1,4 is the response factor for 15-*cis*-β-carotene relative to all-*trans*-β-carotene;

$A_{13cisbc}$ is the peak area of 13-*cis*-β-carotene in the sample chromatogram, in arbitrary units;

1,2 is the response factor for 13-*cis*-β-carotene relative to all-*trans*-β-carotene;

A_{9cisbc} is the peak area of 9-*cis*-β-carotene in the sample chromatogram, in arbitrary units;

A_a is the peak area of apocarotenal in the sample chromatogram, in arbitrary units;

I_{atbc} is the y-intercept of the calibration curve for all-*trans*-β-carotene;

S_{atbc} is the slope of the calibration curve for all-*trans*-β-carotene.

Calculate the mass fraction of total β-carotene w_{tbc}, in µg/100 g, using Formula (13):

$$w_{tbc} = w_{atbc} + w_{cisbc} \tag{13}$$

8.6 Contents of total lycopene

Test sample concentrations calculated in this clause are expressed per 100 g on an as is basis for all samples, allowing each laboratory to apply appropriate reconstitution factors.

Calculate the content of total lycopene in the test samples. For peak identification, refer to relative retention times of peaks in Figure A.5. The first *cis* lycopene peak has a relative retention time of 0,87 compared to all-*trans*-lycopene and any peaks between the first *cis* peak and all-*trans*-lycopene are also included as *cis* lycopene.

Calculate the mass fraction of total *trans* and *cis* isomers of lycopene, w_{tlyc}, in µg/100 g, using Formula (14):

$$w_{tlyc} = \frac{m_a}{m_s} \times \left(\frac{\left(A_{atlyc} + A_{cislyc}\right)}{A_a} - I_{atlyc} \right) \times \frac{100}{S_{atlyc}} \tag{14}$$

where

m_a is the mass of apocarotenal added to the test sample, in µg;

m_s is the mass of the test sample, in g;

A_{atlyc} is the peak area of all-*trans*-lycopene in the sample chromatogram, in arbitrary units;

A_{cislyc} is the peak area of *cis* isomers of lycopene in the sample chromatogram, in arbitrary units;

A_a is the peak area of apocarotenal in the sample chromatogram, in arbitrary units;

I_{atlyc} is the y-intercept of the calibration curve for all-*trans*-lycopene;

S_{atlyc} is the slope of the calibration curve for all-*trans*-lycopene.

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 difference between the results of duplicate portions of the same sample tested at the same day/batch should not exceed 8 % of the mean result as measured by the coefficient of variation. The values of *r* are given in Table 5.

9.3 Reproducibility

The difference between the results of duplicate determinations tested on different batches/days should not exceed 15 % of the mean result as measured by the coefficient of variation. The values of *R* are given in Table 5.

Table 5 — Precision data

Sample	$\bar{x}$ µg/100 g	*r* µg/100 g	*R* µg/100 g
Precision data for all-*trans*- β-carotene			
NIST SRM 1869	6,9	0,6	1,4
Toddler formula powder milk-based	7,7	0,8	2,5
Infant formula powder milk-based	10,3	0,6	4,5
Infant formula powder soy-based	11,7	1,4	3,9
Infant formula powder milk-based	14,1	0,8	5,0
Infant formula ready-to-feed	15,9	0,8	4,2
Precision data for total β-carotene			
NIST SRM 1869	10,9	0,8	3,4
Toddler formula powder milk-based	13,5	2,2	3,1
Infant formula powder milk-based	15,3	2,0	5,9
Infant formula powder soy-based	18,6	2,2	5,9
Infant formula powder milk-based	20,3	1,1	7,0
Infant formula ready-to-feed	25,1	0,8	6,2
Precision data for total lycopene			
NIST SRM 1869	30,1	1,4	4,5

10 Test report

The test report shall contain the following data:

a) all information necessary for the identification of the sample (type of sample, origin and designation of the sample);

b) a reference to this document, i.e. ISO 23443;

c) the date and type of sampling procedure (if known);

d) the date of receipt;

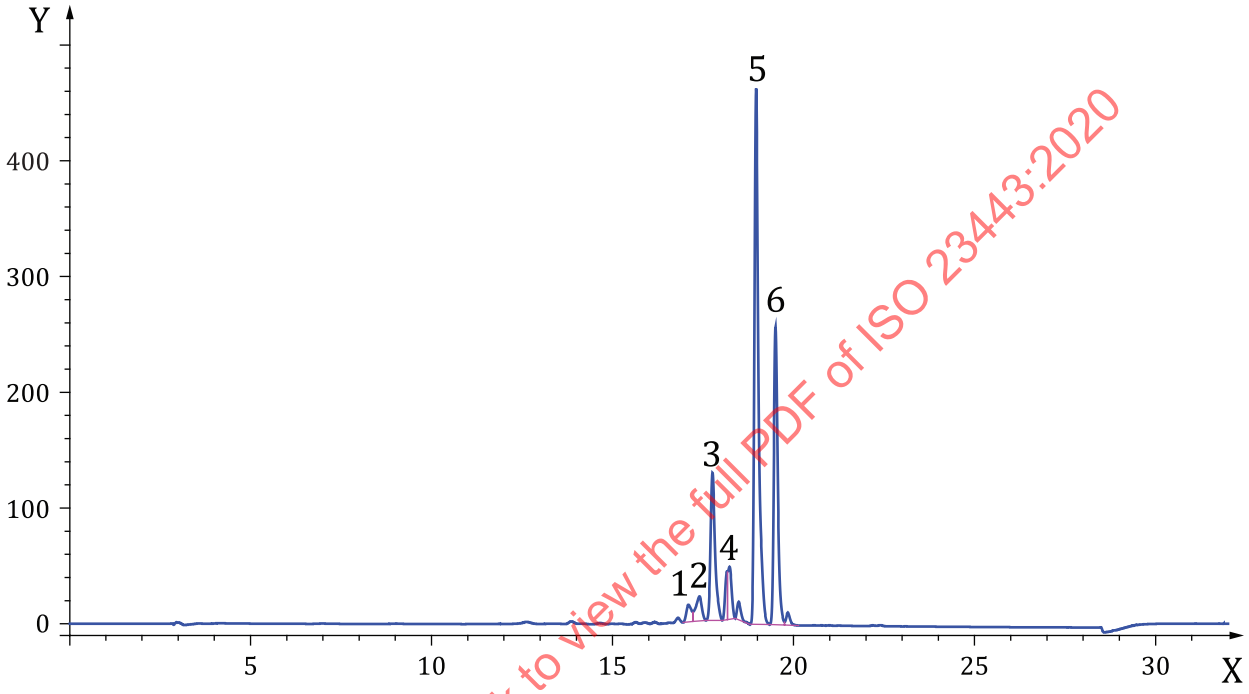
e) the date of the test;

f) the test results and the units in which they have been expressed;

g) any operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

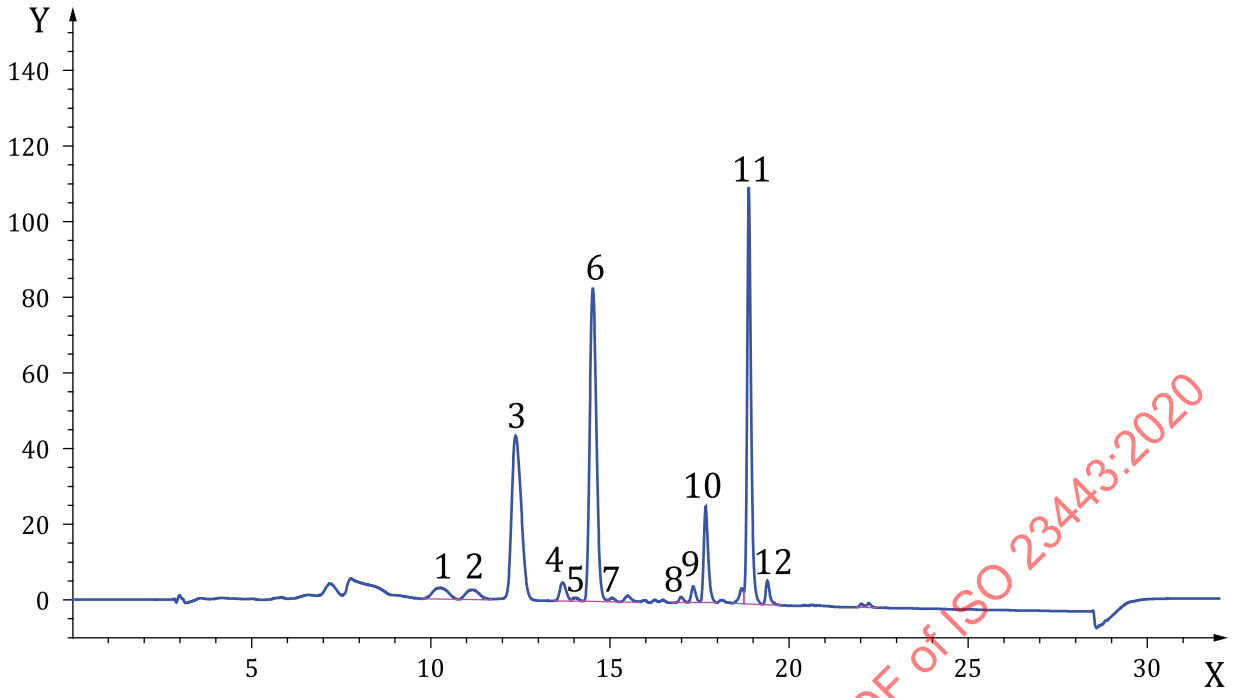
Example chromatograms



Key

X	time, in min	3	13-*cis*-β-carotene
Y	arbitrary units	4	*cis*-α-carotene
1	*cis*-α-carotene / *cis*-β-carotene	5	all-*trans*-β-carotene
2	15-*cis*-β-carotene	6	9-*cis*-β-carotene

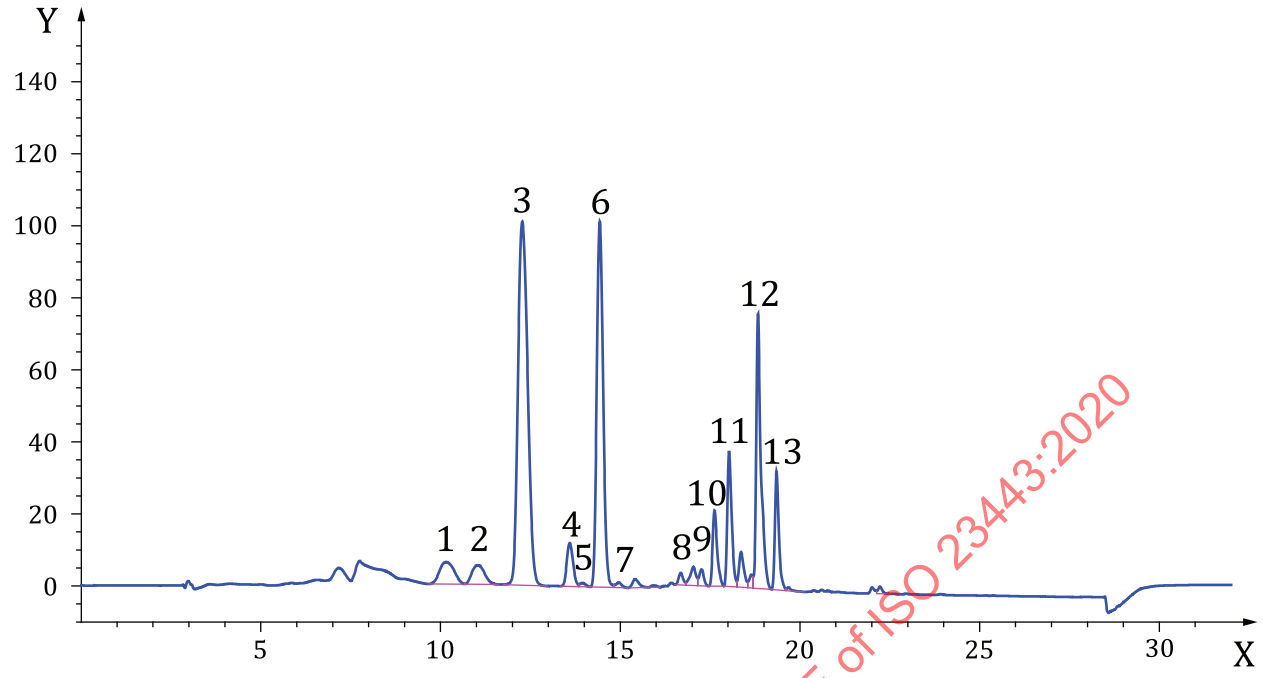
Figure A.1 — Chromatogram of β-carotene system suitability solution



Key

X	time, in min	6	apocarotenal
Y	arbitrary units	7	9′-*cis*-lutein
1	13-*cis*-lutein	8	*cis*-β-carotene
2	13′-*cis*-lutein	9	15-*cis*-β-carotene
3	all-*trans*-lutein	10	13-*cis*-β-carotene
4	zeaxanthin	11	all-*trans*-β-carotene
5	9-*cis*-lutein	12	9-*cis*-β-carotene

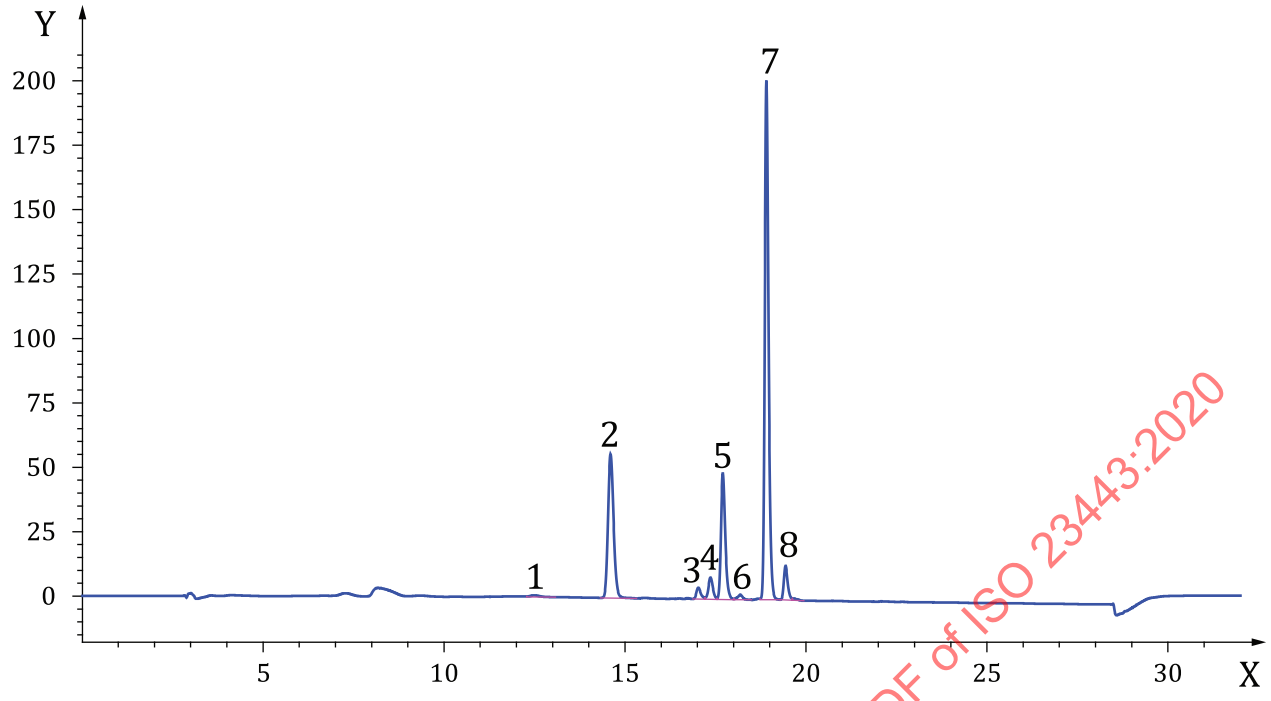
Figure A.2 — Chromatogram of a milk-based infant formula sample



Key

X	time, in min	7	9′-*cis*-lutein
Y	arbitrary units	8	*cis*-α-carotene / cis-β-carotene
1	13-*cis*-lutein	9	15-*cis*-β-carotene
2	13′-*cis*-lutein	10	13-*cis*-β-carotene
3	all-*trans*-lutein	11	*cis*/*trans*-α-carotene
4	zeaxanthin	12	all-*trans*-β-carotene
5	9-*cis*-lutein	13	9-*cis*-β-carotene
6	apocarotenal		

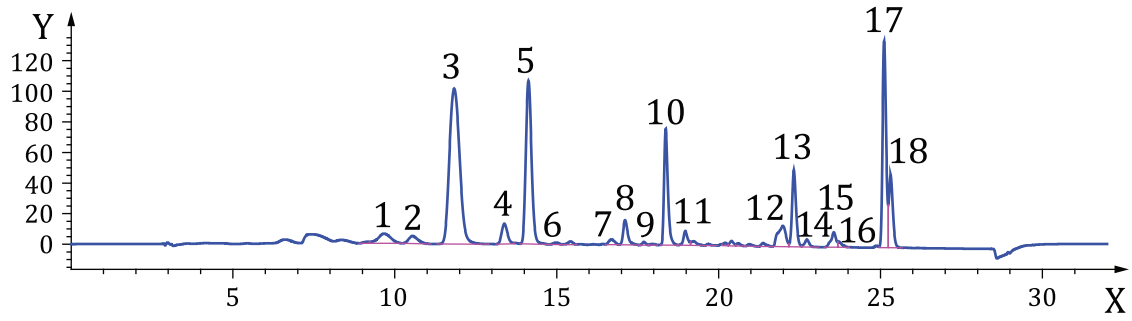
Figure A.3 — Chromatogram of a toddler formula sample



Key

X	time, in min	4	15-*cis*-β-carotene
Y	arbitrary units	5	13-*cis*-β-carotene
1	all-*trans*-lutein	6	*cis*/*trans*-α-carotene
2	apocarotenal	7	all-*trans*-β-carotene
3	*cis*-β-carotene	8	9-*cis*-β-carotene

Figure A.4 — Chromatogram of a ready-to-feed adult nutritional sample



Key

X	time, in min	9	*cis*/*trans*-α-carotene
Y	arbitrary units	10	all-*trans*-β-carotene
1	13-*cis*-lutein	11	9-*cis*-β-carotene
2	13′-*cis*-lutein	12	*cis*-lycopene
3	al-*trans*-lutein	13	*cis*-lycopene
4	zeaxanthin	14	*cis*-lycopene
5	apocarotenal	15	*cis*-lycopene
6	9′-*cis*-lutein	16	*cis*-lycopene
7	15-*cis*-β-carotene	17	all-*trans*-lycopene
8	13-*cis*-β-carotene	18	5-*cis*-lycopene

Figure A.5 — Chromatogram of NIST SRM 1869 sample

Annex B
(informative)

Precision data

The data given in Tables B.1 to B.5 were obtained in an interlaboratory study conducted in 2018[7] in accordance with ISO 5725-2[15] and the AOAC-IUPAC guidelines for collaborative study procedures[16]. The study was performed based on requirements outlined in References [8], [9], and [10].

Table B.1 — Precision data for all-*trans*-β-carotene in infant and toddler formula

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]
Year of interlaboratory test	2018	2018	2018	2018	2018	2018
Number of laboratories	10	10	10	10	10	10
Number of non-compliant laboratories	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	8	8	9	9	9	8
Number of outliers (laboratories)	2	2	1	1	1	2
Number of accepted results	8	8	9	9	9	8
Mean value, $\bar{x}$ (µg/100 g)	6,9	7,7	10,3	11,7	14,1	15,9
Repeatability standard deviation, s_r	0,2	0,3	0,2	0,5	0,3	0,3
Reproducibility standard deviation, s_R	0,5	0,9	1,6	1,4	1,8	1,5
Coefficient of variation of repeatability, $C_{V,r}$, %	2,9	4,2	2,0	4,1	2,2	2,0
Coefficient of variation of reproducibility, $C_{V,R}$, %	7,4	11,1	15,3	11,8	12,5	9,4
Repeatability limit, r [$r = 2{,}8 \times s_r$]	0,6	0,8	0,6	1,4	0,8	0,8
Reproducibility limit, R [$R = 2{,}8 \times s_R$]	1,4	2,5	4,5	3,9	5,0	4,2
HorRat value	0,31	0,47	0,68	0,53	0,58	0,45

[a] NIST SRM 1869; [b] Toddler formula powder milk-based; [c] Infant formula powder milk-based 1; [d] Infant formula powder soy-based; [e] Infant formula powder milk-based 2; [f] Infant formula ready-to-feed.

Table B.2 — Precision data for total β-carotene in infant and toddler formula

Samples	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]
Year of interlaboratory test	2018	2018	2018	2018	2018	2018
Number of laboratories	10	10	10	10	10	10
Number of non-compliant laboratories	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	9	8	10	9	9	8
Number of outliers (laboratories)	1	2	0	1	1	2
Number of accepted results	9	8	10	9	9	8
Mean value, $\bar{x}$ (µg/100 g)	10,9	13,5	15,3	18,6	20,3	25,1
Repeatability standard deviation, s_r	0,3	0,8	0,7	0,8	0,4	0,3
Reproducibility standard deviation, s_R	1,2	1,1	2,1	1,9	2,5	2,2
Coefficient of variation of repeatability, $C_{V,r}$, %	2,3	6,0	4,5	4,5	1,8	1,1
Coefficient of variation of reproducibility, $C_{V,R}$, %	11,3	8,5	13,7	10,4	12,2	8,8
Repeatability limit, r [$r = 2{,}8 \times s_r$]	0,8	2,2	2,0	2,2	1,1	0,8
Reproducibility limi,t R [$R = 2{,}8 \times s_R$]	3,4	3,1	5,9	5,9	7,0	6,2
HorRat value	0,51	0,39	0,65	0,50	0,60	0,45

[a] NIST SRM 1869; [b] Toddler formula powder milk-based; [c] Infant formula powder milk-based 1; [d] Infant formula powder soy-based; [e] Infant formula powder milk-based 2; [f] Infant formula ready-to-feed.

Table B.3 — Precision data for total lycopene in infant formula

Samples	1[a]
Year of interlaboratory test	2018
Number of laboratories	10
Number of non-compliant laboratories	0
Number of laboratories retained after eliminating outliers	8
Number of outliers (laboratories)	2
Number of accepted results	8
Mean value, $\bar{x}$ (µg/100 g)	30,1
Repeatability standard deviation, s_r	0,5
Reproducibility standard deviation, s_R	2,2
Coefficient of variation of repeatability, $C_{V,r}$, %	1,6
Coefficient of variation of reproducibility, $C_{V,R}$, %	7,4
Repeatability limit, r [$r = 2{,}8 \times s_r$]	1,4
Reproducibility limit, R [$R = 2{,}8 \times s_R$]	4,5
HorRat value	0,39

[a] NIST SRM 1869.

Table B.4 — Precision data for all-*trans*-lutein in infant formula and adult nutritionals

Samples	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]
Year of interlaboratory test	2018	2018	2018	2018	2018	2018
Number of laboratories	10	10	10	10	10	10
Number of non-compliant laboratories	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	10	10	9	10	9	9
Number of outliers (laboratories)	0	0	1	0	1	1
Number of accepted results	10	10	9	10	10	9
Mean value, $\bar{x}$ (µg/100 g)	24,6	25,0	72,0	9,7	4,7	123,1
Repeatability standard deviation, s_r	0,6	2,0	1,1	1,0	0,1	3,5
Reproducibility standard deviation, s_R	3,5	3,7	7,8	1,1	0,5	16,5
Coefficient of variation of repeatability, $C_{V,r}$, %	2,4	8,0	1,5	10,0	2,3	2,8
Coefficient of variation of reproducibility, $C_{V,R}$, %	14,0	14,8	10,9	10,9	10,5	13,4
Repeatability limit, r [$r = 2{,}8 \times s_r$]	1,7	5,6	3,1	2,8	0,3	9,8
Reproducibility limit, R [$R = 2{,}8 \times s_R$]	9,8	10,4	21,8	3,1	1,4	46,2
HorRat value	0,71	0,75	0,65	0,48	0,41	0,86

[a] NIST SRM 1869; [b] Toddler formula powder milk-based; [c] Infant formula powder milk-based 1; [d] Infant formula powder soy-based; [e] Infant formula powder milk-based 2; [f] Infant formula ready-to-feed.

Table B.5 — Precision data for total lutein in infant formula and adult nutritionals

Samples	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]
Year of interlaboratory test	2018	2018	2018	2018	2018	2018
Number of laboratories	10	10	10	10	10	10
Number of non-compliant laboratories	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	10	10	9	10	10	8
Number of outliers (laboratories)	0	0	1	0	0	2
Number of accepted results	10	10	9	10	10	8
Mean value, $\bar{x}$ (µg/100 g)	31,3	30,5	100,4	12,8	6,7	142,9
Repeatability standard deviation, s_r	0,9	2,0	2,2	1,5	0,7	3,6
Reproducibility standard deviation, s_R	5,2	5,3	11,5	2,2	1,3	17,6
Coefficient of variation of repeatability, $C_{V,r}$, %	3,0	6,6	2,2	12,0	9,8	2,5
Coefficient of variation of reproducibility, $C_{V,R}$, %	16,6	17,5	11,5	17,0	19,9	12,3
Repeatability limit, r [$r = 2{,}8 \times s_r$]	2,5	5,6	6,2	4,2	2,0	10,1
Reproducibility limit, R [$R = 2{,}8 \times s_R$]	14,6	14,8	32,2	6,2	3,6	49,3
HorRat value	0,87	0,91	0,72	0,78	0,83	0,81

[a] NIST SRM 1869; [b] Toddler formula powder milk-based; [c] Infant formula powder milk-based 1; [d] Infant formula powder soy-based; [e] Infant formula powder milk-based 2; [f] Infant formula ready-to-feed.

Annex C
(informative)

Determination of lutein

C.1 General

Because the reproducibility data for lutein does not meet the requirements given in Reference [9], this annex is for information only. This annex outlines the use of the method for the quantitative determination of lutein in infant formula and adult nutritionals in solid (i.e. powders) or liquid (i.e. ready-to-feed liquids and liquid concentrates) forms using RP-UHPLC and UV-visible detection. The application range runs from 1 µg/100 g to 2 250 µg/100 g for lutein. Based on the single-laboratory validation, the LOD was 0,1 µg/100 g and the LOQ was 0,3 µg/100 g. The method is not applicable to materials that contain measurable levels of β-apo-8′-carotenal.

C.2 Additional reagents and materials

C.2.1 Reagents and standards

C.2.1.1 Ethanol (EtOH), pure, HPLC grade.

C.2.1.2 Lutein, ChromaDex (Irvine, CA) Part No. ASB-00012453[4)] or equivalent.

C.2.1.3 Lutein containing approximately 10 % zeaxanthin, USP Part No. 1370804[4)].

C.2.2 Standards preparation

C.2.2.1 General

Standard solution preparation is summarized Table C.1. Use glass volumetric pipettes and flasks for the preparation of all standard solutions unless otherwise noted.

4) 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 this product. Equivalent products may be used if they can be shown to lead to the same results.

Table C.1 — Composition and nominal concentrations of carotenoid standard solutions

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

C.2.2.2 Lutein stock solution, ρ = 20 000 µg/100 ml

Weigh (to 0,01 mg) approximately 5 mg of lutein (C.2.1.2) reference standard into a 25 ml volumetric flask. Add approximately 20 ml vitamin E solution in MTBE (5.1.14), sonicate for 2 min to 3 min and dilute to volume with vitamin E solution in MTBE.

Store the stock solution at −20 °C for up to six months and check its purity each time new standard solutions are made from it. When taken from the freezer, the stock solution should be sonicated for 2 min and vortexed to bring all the lutein into the solution.

C.2.2.3 UV-Visible solutions for spectroscopy potency check, ρ = 200 µg/100 ml

Transfer 1,0 ml lutein standard stock solution (C.2.2.2) to a 100 ml volumetric flask and dilute to volume with pure ethanol.

Immediately measure solutions by UV-visible spectroscopy and calculate purity according to C.4.1.2.

C.2.2.4 Lutein working solution for chromatographic purity check, ρ = 200 µg/100 ml

C.2.2.4.1 General

Analyse working solutions by UHPLC on the same day they are prepared and calculate the chromatographic purity of each according to C.4.1.3.

C.2.2.4.2 Lutein working solution

With an adjustable pipet, transfer 100 µl of lutein stock solution (C.2.2.2) to a 10 ml volumetric flask and dilute to volume with sample solution.

C.2.2.5 Mixed carotenoid intermediate solution, ρ = 200 µg/100 ml to 1 200 µg/100 ml

Combine 2,0 ml each of lutein, β-carotene, and lycopene standard stock solutions (5.3.2, C.2.2.2), in a 100 ml volumetric flask and dilute to volume with sample solution. Store at −20 °C for up to one month.