BS ISO 23443:2020

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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)

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# National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/34, Food Technical Committee Chairmen.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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Published by BSI Standards Limited 2020

ISBN 978 0 539 05212 1

ICS 67.050

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 July 2020.

**Amendments/corrigenda issued since publication**

| Date | Text affected |
|---|---|

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INTERNATIONAL STANDARD

# ISO 23443

First edition
2020-07

# 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*

Reference number
ISO 23443:2020(E)

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Published in Switzerland



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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.


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# 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 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]

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## 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 %.


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**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.

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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 (µg/100 ml) | 400 | 200 | 1 200 |

**5.3.2 Carotenoid stock solutions,** $\rho$ = 10 000 µg/100 ml to $\rho$ = 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,** $\rho$ = 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.


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#### 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, $\rho$ = 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.

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