
**Infant formula and adult
nutritionals — Determination of
vitamin B₁₂ by reversed phase high
performance liquid chromatography
(RP-HPLC)**

*Formules infantiles et produits nutritionnels pour adultes —
Détermination de la teneur en vitamine B₁₂ par chromatographie
liquide haute performance en phase inverse (CLHP-PI)*

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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).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is 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 the International Standard is equivalent to the AOAC Official Method 2011.10: *Vitamin B₁₂ in infant and pediatric formulas and adult nutritional*.

Infant formula and adult nutritionals — Determination of vitamin B₁₂ by reversed phase high performance liquid chromatography (RP-HPLC)

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

1 Scope

This International Standard specifies a method for the quantitative determination of vitamin B₁₂ in infant and adult formula (powders, ready-to-feed liquids and liquid concentrates) by reversed phase high performance liquid chromatography.

2 Terms and definitions

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

2.1

vitamin B₁₂

cyanocobalamin and other cobalt-containing corrinoids with vitamin B₁₂ biological activity, such as aquocobalamin, hydroxycobalamin, methylcobalamin and adenosylcobalamin, converted to cyanocobalamin

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2.2

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

2.3

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]

3 Principle

Cyanocobalamin and other cobalt-containing corrinoids are extracted from the sample using sodium acetate buffer (pH = 4,5) and the latter converted to cyanocobalamin using potassium cyanide at 105 °C. Extracts are purified and concentrated with C8 or C18 solid-phase extraction (SPE) cartridges and analysed with size-exclusion and reversed-phase chromatography. Determination of vitamin B₁₂ is made by liquid chromatography with visible detection at 550 nm.

4 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.

- 4.1 **Glacial acetic acid.**
- 4.2 **Acetonitrile**, HPLC grade.
- 4.3 **Drierite**, desiccant, anhydrous calcium sulfate, 8 mesh.
- 4.4 **Ethanol**, denatured.
- 4.5 **Formic acid**, 88 %.
- 4.6 **Potassium cyanide**, 97 %.
- 4.7 **Riboflavin**, 98 % to 102 % purity.
- 4.8 **Sodium acetate anhydrous or sodium acetate trihydrate**, ACS.
- 4.9 **Taka-Diastase**, Accurate Chemical Co.¹⁾ or equivalent.
- 4.10 **Triethylamine**, HPLC grade.
- 4.11 **Vitamin B₁₂ (cyanocobalamin) primary reference standard**, e.g. USP Reference 1152009 (approximately 10 µg/mg), Official lot¹⁾. Store in a desiccator protected from white light.

4.12 **Preparation of solutions and standard solutions**

4.12.1 **General**

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All solutions may be scaled up or down for convenience provided good laboratory practices are observed. Solutions can be stored refrigerated or at ambient temperature in tight, inert containers unless otherwise specified.

4.12.2 **Preparation of solutions**

4.12.2.1 **HPLC mobile phase A.** Dilute 4,0 ml of triethylamine with 1 000 ml of water. Adjust the pH to 5 to 7 with approximately 1,25 ml concentrated formic acid (4.5). Expiration: 1 week.

4.12.2.2 **HPLC mobile phase B.** Mix 4,0 ml of triethylamine and 250 ml of acetonitrile with 750 ml of water. Adjust the pH to 5 to 7 with approximately 1,25 ml concentrated formic acid. Expiration: 1 week in tightly stoppered container.

4.12.2.3 **HPLC mobile phase C.** Mix 4,0 ml of triethylamine and 750 ml of acetonitrile with 250 ml of water. Adjust the pH to 5 to 7 with approximately 1,25 ml concentrated formic acid. Expiration: 1 week in tightly stoppered container.

4.12.2.4 **HPLC mobile phase D.** Dilute 50 ml of acetonitrile to 2 000 ml with water. Expiration: 1 week in tightly stoppered container.

4.12.2.5 **Mixture of acetonitrile and water**, volume fraction 10 %. Dilute 150 ml of acetonitrile to 1 500 ml with water. Expiration: 1 month in tightly stoppered container.

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4.12.2.6 Mixture of acetonitrile and water, SPE elution solvent, volume fraction 30 %. Dilute 30 ml of acetonitrile to 100 ml with water. Expiration: 1 month in tightly stoppered container.

4.12.2.7 Mixture of acetonitrile and water, column cleaning and storage solution, volume fraction 50 %. Dilute 500 ml of acetonitrile to 1 000 ml with water in a volumetric flask. Expiration: 6 months.

4.12.2.8 Mixture of ethanol and water, volume fraction 25 %. Dilute 50 ml of ethanol to 200 ml with water. Expiration: 1 year in tightly stoppered container.

4.12.2.9 Potassium cyanide solution, mass concentration $\rho = 4$ g/l. Dissolve 0,02 g of potassium cyanide in and dilute to 5 ml with sodium acetate buffer (4.12.2.11) substance concentration $c = 0,25$ mol/l). Prepare fresh immediately before use.

4.12.2.10 Potassium cyanide solution, $\rho = 10$ g/l. Dissolve 0,25 g of potassium cyanide in water and dilute to 25 ml. Prepare fresh immediately before use.

4.12.2.11 Sodium acetate buffer, $c = 0,25$ mol/l. Dissolve 41,0 g of sodium acetate anhydrous or 68,0 g of sodium acetate trihydrate in approximately 1 800 ml of water. Adjust the pH to 4,5 with concentrated acetic acid (approximately 40 ml). Dilute to 2 000 ml with water. Expiration: 3 months.

4.12.2.12 Resolution test solution. Weigh approximately 0,005 g of riboflavin onto a weigh paper. Transfer to a 100 ml volumetric flask and bring to volume with 10 % acetonitrile solution. Stir to dissolve. Mix equal amounts of solution with the highest concentration of vitamin B₁₂ working standard solution. Expiration: 1 week.

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4.12.2.13 Taka-Diastase solution, $\rho = 60$ g/l. Dissolve 0,6 g of Taka-Diastase in 10 ml of water. Prepare fresh daily before use.

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4.12.3 Preparation of standard solutions

4.12.3.1 General. Prepare all standard solutions under UV shielded fluorescent lights and store at 2 °C to 8 °C in tightly stoppered volumetric flasks.

4.12.3.2 Vitamin B₁₂ stock standard solution, $\rho = 10\ 000$ µg/l. Accurately weigh the appropriate amount of vitamin B₁₂ standard (4.11) to give a stock standard concentration of 10 000 µg/l. Dissolve in and dilute to 100 ml with 25 % ethanol (4.12.2.8). Expiration: 6 months.

Calculate the amount of vitamin B₁₂ standard to be weighed, m_w , in milligrams using Formula (1):

$$m_w = 10\ 000 \times 0,1 \times \frac{1}{P} \quad (1)$$

where

10 000 is the desired stock standard solution concentration, in µg/l;

0,1 is the dilution volume, in l;

P is the purity of the vitamin B₁₂ standard (4.11) in µg of cyanocobalamin per mg of standard.

4.12.3.3 Vitamin B₁₂ intermediate standard solution, $\rho = 1\ 000$ µg/l. Dilute 10 ml of stock standard solution (4.12.3.2) to 100 ml with water. Expiration: 1 week.

4.12.3.4 Vitamin B₁₂ calibration standard solutions, $\rho = 2,5 \mu\text{g/l}$ to $25 \mu\text{g/l}$. Into separate volumetric flasks, dilute 0,5 ml, 1 ml, 2 ml, 3 ml, 4 ml and 5 ml of intermediate standard solution ([4.12.3.3](#)) to 200 ml with 10 % acetonitrile ([4.12.2.5](#)). Expiration: 1 month.

5 Apparatus

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

5.1 HPLC system, consisting of a gradient pump, column switching valve and isocratic pump, UV-VIS detector equipped with a tungsten lamp capable of monitoring at 550 nm and autosampler capable of injecting 900 μl to 2 000 μl of sample.

5.2 HPLC column, analytical size exclusion column:

- 4 μm particle size, 250 mm \times 9,4 mm (e.g. Zorbax GF-250²⁾ P/N 884973-901);
- 5 μm , 300 mm \times 8 mm (Shodex Protein® KW-802.5²⁾, P/N F6989000), or equivalent.

5.3 HPLC column, analytical C18 column:

- 3 μm particle size, 100 mm \times 4,6 mm (e.g. Thermo Scientific Aquasil™²⁾ P/N 77503-104630) with C18 drop-in guard cartridges 3 μm , 10 mm \times 4,6 mm (e.g. Thermo Scientific Aquasil™²⁾ P/N 77503-014001);
- Epic Phenyl Hexyl, 3 μm , 120 Å, 100 mm \times 4,6 mm, (ES Industries 125191-EPHX)²⁾ with appropriate guard cartridge, or equivalent reversed-phase column compatible with 100 % aqueous mobile phase).

5.4 Oven, capable of maintaining temperatures of $95 \text{ }^\circ\text{C} \pm 5 \text{ }^\circ\text{C}$ and $105 \text{ }^\circ\text{C} \pm 5 \text{ }^\circ\text{C}$.

5.5 pH-meter, with calibration buffer. <https://standards.iteh.ai/catalog/standards/sist/b761c0c4-854f-424b-9d3f-aa088aeaf181/iso-20634-2015>

5.6 Analytical balance, accuracy to the nearest 0,000 01 g.

5.7 Beakers, glass, assorted sizes.

5.8 Bottle top dispenser, capable of dispensing 30 ml or equivalent.

5.9 Cylinders, graduated, glass, assorted sizes.

5.10 Desiccator.

5.11 Conical flasks, 125 ml capacity or equivalent glassware.

5.12 Filter paper, Whatman 2V²⁾ or equivalent.

5.13 Funnels, plastic, suitable to use with filter paper.

5.14 Gloves, disposable.

5.15 Pipettor, variable volume, 100 μl to 1 000 μl .

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.

5.16 Laboratory light shields, yellow or clear shields with a cut off of at least 385 nm.

5.17 Solid phase extraction (SPE) cartridges, C8, 900 mg (e.g. Alltech/Grace Davidson P/N 20966²), C18, 900 mg (e.g. Alltech/Grace Davidson P/N 20942²), or equivalent. An example of a SPE cartridge qualification procedure is given in [Annex C](#).

5.18 Syringes, disposable, assorted sizes.

5.19 Syringe filters, 0,45 µm nylon.

5.20 Vacuum manifold, 24 ports with stopcocks or equivalent.

5.21 Volumetric pipets, assorted sizes.

5.22 Volumetric flasks, assorted sizes.

6 Procedure

6.1 General

Prepare all samples under UV shielded fluorescent lights. Prepared product samples can be analysed for up to 14 days after preparation if stored at 2 °C to 8 °C in tightly stoppered volumetric flasks. All product samples should be as uniform and representative as possible. This should be accomplished by thoroughly mixing or stirring products before sampling.

6.2 Sample preparation

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6.2.1 General

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For products containing starch, add 1 ml of Taka-Diastase solution ([4.12.2.13](#)). Allow Taka-Diastase to react with the samples for at least 30 min before continuing with the extraction.

6.2.1.1 Liquid samples

For ready-to-feed liquids, mix samples well to ensure homogeneity and accurately weigh approximately 20,0 g of adult nutritionals or 25,0 g of infant formula into a 100 ml volumetric flask. Continue with extraction.

6.2.1.2 Powder samples

If the powder sample homogeneity is unknown, assume that it is non-homogenous and proceed as for dry blended/non-homogenous powder samples ([6.2.1.3](#)).

6.2.1.3 Dry blended powder samples

For dry blended/non-homogenous powder samples, accurately weigh approximately 25,0 g. Add 200,0 g water at 40 °C before mixing until a homogeneous suspension is obtained. A homogenizer can be used when necessary. Accurately weigh approximately 20,0 g of adult nutritionals or 25,0 g of infant formula into a 100 ml volumetric flask.

6.2.1.4 Wet blended powder samples

For wet blended homogenous powder samples, accurately weigh approximately 3,0 g of powder into a 100 ml volumetric flask. Add 25 ml of water and mix until all of the powder dissolves.

6.2.1.5 Elemental formulas

For products containing free amino acids and no intact protein, add 0,5 g of calcium caseinate or non-fat dry milk to 3,0 g of a wet blended homogeneous powder diluted with 25 ml water or 20 g to 25 g of a liquid or reconstituted dry blended nonhomogeneous powder. Mix well to dissolve the protein. Immediately add 30 ml of sodium acetate buffer (4.12.2.11) and 1 ml of 1 % potassium cyanide. Dilute to volume with water, filter, and clean up and concentrate up to 60 ml of filtrate on a preconditioned 900 mg C8 or C18 cartridge. Do not heat the sample in the 105 °C oven.

6.2.2 Extraction

Add 30 ml of sodium acetate buffer (4.12.2.11) to each sample extract and swirl to mix. In a fume hood, add 1 ml of freshly prepared 1 % potassium cyanide (4.12.2.10) to each sample and swirl to mix. Heat samples in a 105 °C oven for at least 60 min, but for no more than 120 min. The oven temperature will drop when the door is opened. Start timing when the oven temperature returns to 105 °C.

Remove samples from the oven and immediately cool in an ice bath. Dilute samples to volume with water. Mix well. Filter samples through a filter paper (5.12) into 125 ml conical flasks or equivalent glassware. If necessary, filter papers can be changed if they become clogged. If prepared samples are opaque and contain very small insoluble particles, centrifuge samples and transfer the liquid layer to funnels lined with filter paper. Do not heat any samples to which calcium caseinate has been added, see 6.2.1.5.

6.2.3 Sample concentration

To clean up and concentrate the sample, insert a 900 mg SPE cartridge (5.17) onto the stopcock of the vacuum manifold and attach a 30 ml disposable syringe barrel to the top of each cartridge. Condition each cartridge with at least 20 ml of acetonitrile (elute by gravity) and rinse each cartridge with at least 10 ml of water. Using volumetric pipets, transfer sample filtrates to cartridges using the guidelines in Table 1. If the vitamin B₁₂ concentration is unknown, use guidelines for RTF products containing 1 µg/l to 10 µg/l. If necessary, apply enough vacuum so that the samples drip steadily through the cartridges. Sample filtrates should pass through the cartridges at a rate of no more than 120 drops/min. Discard eluent.

Table 1 — Guidelines for loading sample filtrates onto SPE cartridges

Vitamin B ₁₂ in RTF products µg/l	Volume of filtrate loaded onto SPE cartridge ml	Final dilution volume ml
< 1	80	5
1 – 10	70 – 80	10
11 – 20	50 – 60	10
21 – 50	20 – 40	10

NOTE Do not load more than 60 ml of adult nutritionals onto an Alltech C8 or C18 cartridge.

After all of the sample filtrate has passed through the cartridge, rinse each cartridge with 5 ml water and discard eluent. Air dry each cartridge by pulling a vacuum until no more eluent is observed. Close each stopcock.

Using the guidelines in Table 1, place a 5 ml or 10 ml volumetric flask under each cartridge. Add 4,4 ml of the 30 % mixture of acetonitrile and water (4.12.2.6) to all 900 mg SPE cartridges. Open each stopcock and elute vitamin B₁₂ into the volumetric flasks with vacuum. Eluent should pass through the cartridges at a rate of no more than 120 drops/min.

In preparing the final dilution, for samples collected in 10 ml volumetric flasks, dilute to volume with water.

For samples collected in 5 ml volumetric flasks, in a hood add 0,1 ml of freshly prepared 0,4 % potassium cyanide solution (4.12.2.9) to each volumetric flask. Place prepared samples in a 95 °C oven for at least

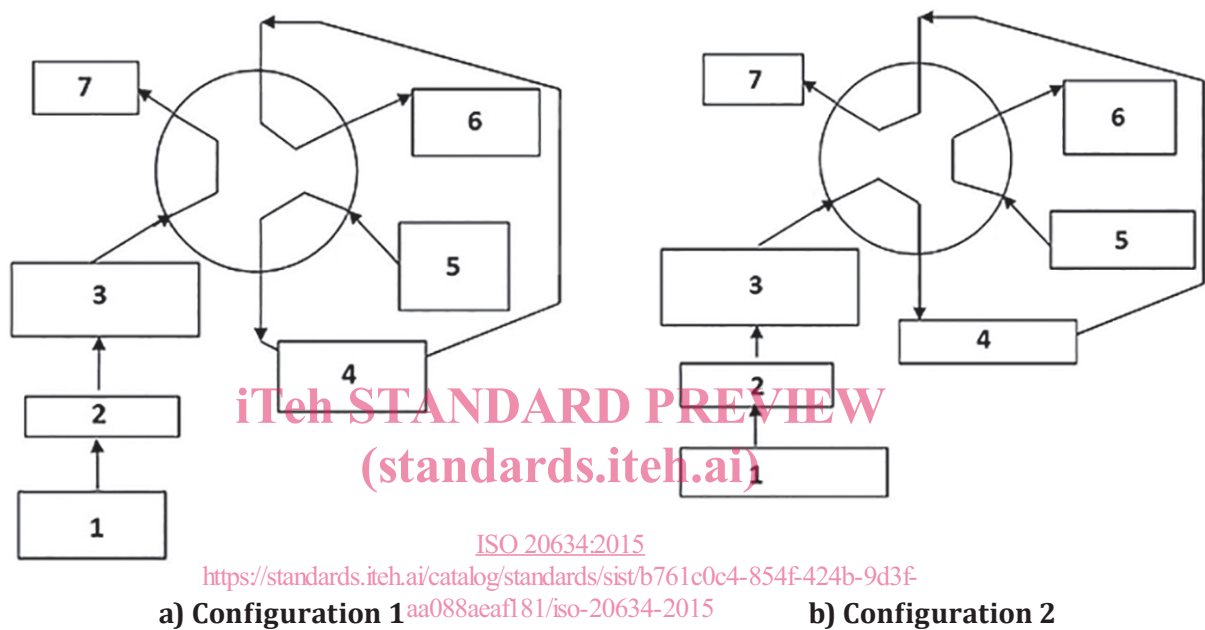
1,5 h, but for no more than 4 h. Remove samples from the oven and cool to room temperature. Dilute to volume with water.

Filter an aliquot of each standard solution and prepared sample solution through a syringe filter (5.19) into an autosampler vial.

6.3 HPLC analysis

6.3.1 System setup and configuration

The system setup and configuration are given in Figure 1.



Key

- | | |
|-------------------------|-------------------|
| 1 isocratic pump | 5 gradient pump |
| 2 autosampler | 6 UV/VIS detector |
| 3 size exclusion column | 7 waste |
| 4 C18 analytical column | |

Figure 1 — System setup and configuration of the HPLC system

6.3.2 Instrument operation conditions

- Run time: 30 min to 35 min
- Injection volume: 0,9 ml to 2,0 ml
- System configuration, see Table 2

Table 2 — System configuration

Time min	Valve configuration (see Figure 1)
0 - 10,5	1
10,5 - 14,5	2
14,5 - 30,0 to 35,0	1