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Infant formula and adult nutritionals — Determination of *trans* and total (*cis* + *trans*) vitamin K_1 content — Normal phase HPLC

Formules infantiles et produits nutritionnels pour adultes — Détermination de la teneur en vitamine K_1 trans *et totale (*cis + trans*) — Chromatographie liquide à haute performance (CLHP) en phase normale*

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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 2015.09: *Trans vitamin* K_1 *in Infant, Pediatric, and Adult Nutritionals, HPLC with Fluorescence Detection.*

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

Infant formula and adult nutritionals — Determination of *trans* and total (*cis* + *trans*) vitamin K_1 content — Normal phase HPLC

1 Scope

This document specifies a method for the quantitative determination of *trans* and total (*cis* + *trans*) vitamin K_1 in infant, pediatric and adult nutritionals using normal phase (NP) high-performance liquid chromatography (HPLC) with post-column reduction and fluorescence detection. The method demonstrated good linearity over a standard range of ~2 µg/l to 80 µg/l *trans* vitamin K_1, and the limit of quantification (LOQ) was estimated to be 0,4 µg/l for standards and 0,09 µg/100 g ready to feed (RTF) for samples assuming 4 grams of sample are diluted to 10 ml.

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

Vitamin K_1 is extracted from products with iso-octane after precipitation of proteins and release of lipids with methanol. Prepared samples are injected onto a silica HPLC column where *cis* and *trans* vitamin K_1 are separated with an iso-octane–isopropanol mobile phase. The column eluent is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate, and acetic acid, and *cis* and *trans* vitamin K_1 are reduced to fluorescent derivatives in a zinc reactor column. The resulting fluorescent compounds are then detected by fluorescence at an excitation wavelength of 245 nm and an emission wavelength of 440 nm.

5 Reagents and materials

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

5.1 Acetic acid, glacial, > 99 %, American Chemical Society (ACS).

5.2 Helium or nitrogen, zero grade or equivalent helium or nitrogen.

5.3 Hexane, HPLC grade.

5.4 Iso-octane (2,2,4-trimethylpentane), HPLC grade.

5.5 Isopropanol (isopropyl alcohol), HPLC grade.

5.6 Methanol, HPLC grade.

5.7 Phytonadione/phylloquinone (vitamin K_1), primary reference standard. Store per label instructions.

5.8 Laboratory or distilled water, with conductivity of 0,067 µS/cm (15 Mohm/cm).

5.9 Ethanol, 95 %, ACS.

5.10 Sodium acetate, anhydrous, ACS.

5.11 Zinc, < 150 µm, 99,995 % or equivalent.

5.12 Zinc chloride, > 97 %, ACS.

6 Standard and solution preparation

CAUTION — Since vitamin K_1 is light-sensitive, all standards shall be prepared, handled, and stored in the dark or under yellow shielded lighting unless otherwise stated. If the standards are transported through or into an area without yellow shielded lighting, they shall be wrapped tightly in foil. All standard solutions shall be prepared using Class A volumetric glassware.

6.1 Mixture with volume fraction of 10 % isopropanol in iso-octane

Add about 70 ml of iso-octane (5.4) to a 100 ml volumetric flask. Add 10 ml isopropanol (5.5) to the volumetric flask and dilute to volume with iso-octane. Expiration is 6 months.

6.2 Mobile phase

Add about 900 ml iso-octane (5.4) to a 1 000 ml volumetric flask. Add 3 ml to 4 ml 10 % isopropanol (6.1) to the volumetric flask and dilute to volume with iso-octane. Expiration is 6 months if stored in tightly stoppered container.

NOTE The isopropanol concentration in the mobile phase can be adjusted slightly until baseline resolution of *cis* and *trans* vitamin K_1 from other peaks present in some samples is achieved, see Figures A.2 and A.3.

6.3 Post-column electrolyte solution

Transfer 0,5 g ± 0,05 g zinc chloride (5.12) and 0,20 g ± 0,02 g sodium acetate anhydrous (5.10) to a 1 000 ml volumetric flask with ethanol (5.9). Add 150 µl ± 15 µl glacial acetic acid (5.1) and dilute to volume with ethanol. Mix solution for about 30 min or until solution is clear and all salts are dissolved. Expiration is 6 months.

6.4 Vitamin K_1 (phytonadione) stock standard solution

6.4.1 Vitamin K_1 (phytonadione) stock standard solution

Accurately weigh to 0,000 01 g about 0,055 00 g vitamin K_1 (phytonadione) (5.7) into a 250 ml volumetric flask. Dissolve standard and dilute to volume with iso-octane (5.4). Store in a refrigerator in a tightly stoppered container protected from light. Expiration is 6 months.

6.4.2 Stock standard concentration (for non USP standard)

To determine the stock standard concentration, evaporate 0,5 ml of vitamin K_1 stock standard under a stream of nitrogen and redissolve the residue in 10,0 ml of hexane. Measure the absorbance of this solution in a 1 cm cell against a reference of hexane at wavelength of 248 nm with a spectrophotometer. Calculate the vitamin K_1 stock standard concentration, ρ_{ss}, in milligrams per litre, using Formula (1):

$$\rho_{\mathrm{ss}} = \frac{A_{248} \times 10\,000 \times 20}{419} \tag{1}$$

where

A_{248} is the absorbance of the solution at 248 nm;

419 is the $A_{1\mathrm{cm}}^{1\%}$ value of vitamin K_1 in hexane at 248 nm;

10 000 is the conversion of $A_{1\mathrm{cm}}^{1\%}$ to milligrams per litre;

20 is the dilution of the stock standard solution.

6.5 Vitamin K_1 (phytonadione) intermediate I standard solution

Dilute 1,0 ml vitamin K_1 stock standard (6.4.1) to 100 ml with iso-octane. Prepare from stock standard solution each time new working standards are made.

6.6 Vitamin K_1 (phytonadione) intermediate II standard solution

Dilute 10,0 ml vitamin K_1 intermediate I standard (6.5) to 50 ml with iso-octane. Prepare each time new working standards are made.

6.7 Vitamin K_1 (phytonadione) working standard solutions

Dilute 1,0 ml, 3,0 ml, 6,0 ml, 10,0 ml and 20,0 ml intermediate II standard (6.6) to 100 ml with iso-octane. Store at 2 °C to 8 °C in a refrigerator in tightly closed containers protected from light. Expiration is 3 months.

Transfer working standards to autosampler vials with Pasteur pipets or equivalent glass. Do not pour the standards from the volumetric flasks into vials.

7 Apparatus

7.1 HPLC system.

Two isocratic pumps; autosampler capable of injecting 20 µl; fluorescence detector; instrument degasser (optional), high-pressure mixing tee or T-junction, and post-column reactor column about 20 mm × 4 mm stainless steel e.g. old HPLC column with packing removed or equivalent. The system should be configured as shown in Figure 1.

7.2 Analytical column, silica 150 mm × 3,0 mm, 3 µm, 60 Å, or equivalent.

7.3 Analytical balance, capable of weighing to the nearest 0,000 01 g.

7.4 Beakers, glass, assorted sizes.

7.5 Centrifuge.

7.6 Centrifuge tubes and caps, 50 ml glass tubes with caps lined with polytetrafluoroethylene (PTFE).

7.7 Cylinders, graduated, glass, assorted sizes.

7.8 Gas regulator, compatible with helium or nitrogen.

7.9 Gas sparge, tubing and filtering assembly.

7.10 Magnetic stirrer and stir bar, with rack to hold centrifuge tubes.

7.11 Pipet, disposable glass, Pasteur.

7.12 Pipet, mechanical, variable volume, 0,5 ml to 5 ml and 10 µl to 100 µl.

7.13 Pipet, repeating 5 ml and 25 ml or equivalent.

7.14 Spectrophotometer, capable of measuring absorbance at 248 nm.

7.15 Volumetric flasks, glass, Class A, assorted sizes.

7.16 Volumetric pipets, glass, Class A, assorted sizes.

7.17 Vortex mixers.

7.18 Yellow lights or yellow shields, with cutoff of at least 440 nm.

8 Procedure

8.1 Sample preparation

8.1.1 Liquid samples

For ready-to-feed liquids, mix samples well or homogenize to ensure homogeneity and accurately weigh to 0,001 g, up to 4 g of sample into 50 ml centrifuge tubes. To liquids with sample masses less than 4 g, add enough water (5.8) to the tubes so that the sample mass plus the amount of water added (g or ml) equal about 4 and mix well.

8.1.2 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.

8.1.3 Dry blended/non-homogenous powder samples

For dry blended/non-homogenous powder samples, accurately weigh approximately 25,0 g of powder and add 200 g of water (5.8). Record all masses. Mechanically stir or mix by hand until a homogeneous suspension is obtained. A homogenizer can be used when necessary. Accurately weigh to 0,001 g up to 4 g of homogeneous suspension into 50 ml centrifuge tubes. If less than 4 g of homogeneous suspension are weighed, add enough water to the tubes so that the sample mass plus the amount of water added (g or ml) equal about 4 and mix well.

8.1.4 Wet blended powder samples

For wet blended homogenous powder samples, accurately weigh to 0,000 1 g up to 0,5 g of powder into 50 ml centrifuge tubes. Add 4 ml of water (5.8) and mix well.

8.1.5 Extraction

Add 25 ml ± 2,0 ml methanol (5.6) to each sample just prior to vortexing or stirring. Methanol should not be added to more than two samples consecutively without vortexing or stirring. Cap each centrifuge tube. Vortex each sample for at least 30 s at a rate that causes a vortex within the tube and then allow samples to sit undisturbed for at least 10 min, but no more than 40 min, or add a magnetic stir bar (7.10) to each centrifuge tube, cap tubes and place onto a magnetic stir plate, and stir samples for at least 10 min, but not more than 40 min, at a spin rate that causes a vortex. Begin timing after vortex forms in the tubes.

Quantitatively add 10 ml ± 0,05 ml iso-octane (5.4) to each sample with a volumetric pipet and cap tubes. Iso-octane can be added to all samples before vortexing or stirring any of the samples. Vortex each sample for at least 45 s or stir each sample for at least 45 s at a rate that causes a vortex to form within the tubes. Begin timing after vortex forms in the tubes. Add 5 ml ± 0,5 ml laboratory water (5.8) to each sample and cap tubes. Vortex or shake each sample for at least 20 s or stir each sample for at least 20 s at a spin rate that causes a vortex to form within the sample. Begin timing after vortex forms in the tubes.

Centrifuge the samples until a clean separation of the isooctane and aqueous–methanol layers results. The iso-octane layer should be a clear layer at the top of the centrifuge tube, and the aqueous–methanol layer should be a cloudy layer below the iso-octane layer. In some samples, there may be a small emulsion layer between the iso-octane and aqueous-methanol layers. A good separation of solvent layers can usually be achieved by centrifuging samples for approximately 10 min at 800 relative centrifugal force.

Do not add ethanol to samples to remove emulsions. Ethanol will change the final dilution volume and affect results.

Remove samples from the centrifuge and inspect each sample to verify that the iso-octane and aqueous–methanol layers are separated. With a glass pipet, carefully rinse down the upper walls of the centrifuge tube with a portion of the iso-octane layer. If the layers become mixed together, centrifuge the sample again. Pipette a portion of the clear iso-octane layer into a labelled autosampler vial and cap the vial.

8.2 HPLC analysis

8.2.1 Instrumental operating conditions

HPLC analytical column mobile phase flow rate: 0,4 ml/min;

Post-column flow rate: 0,4 ml/min;

Injection volume: 20 µl;

Run time: 20 min;

Fluorescence excitation and emission: 245 nm and 440 nm, respectively.

8.2.2 Instrument start-up

The system should be configured as shown in Figure 1.

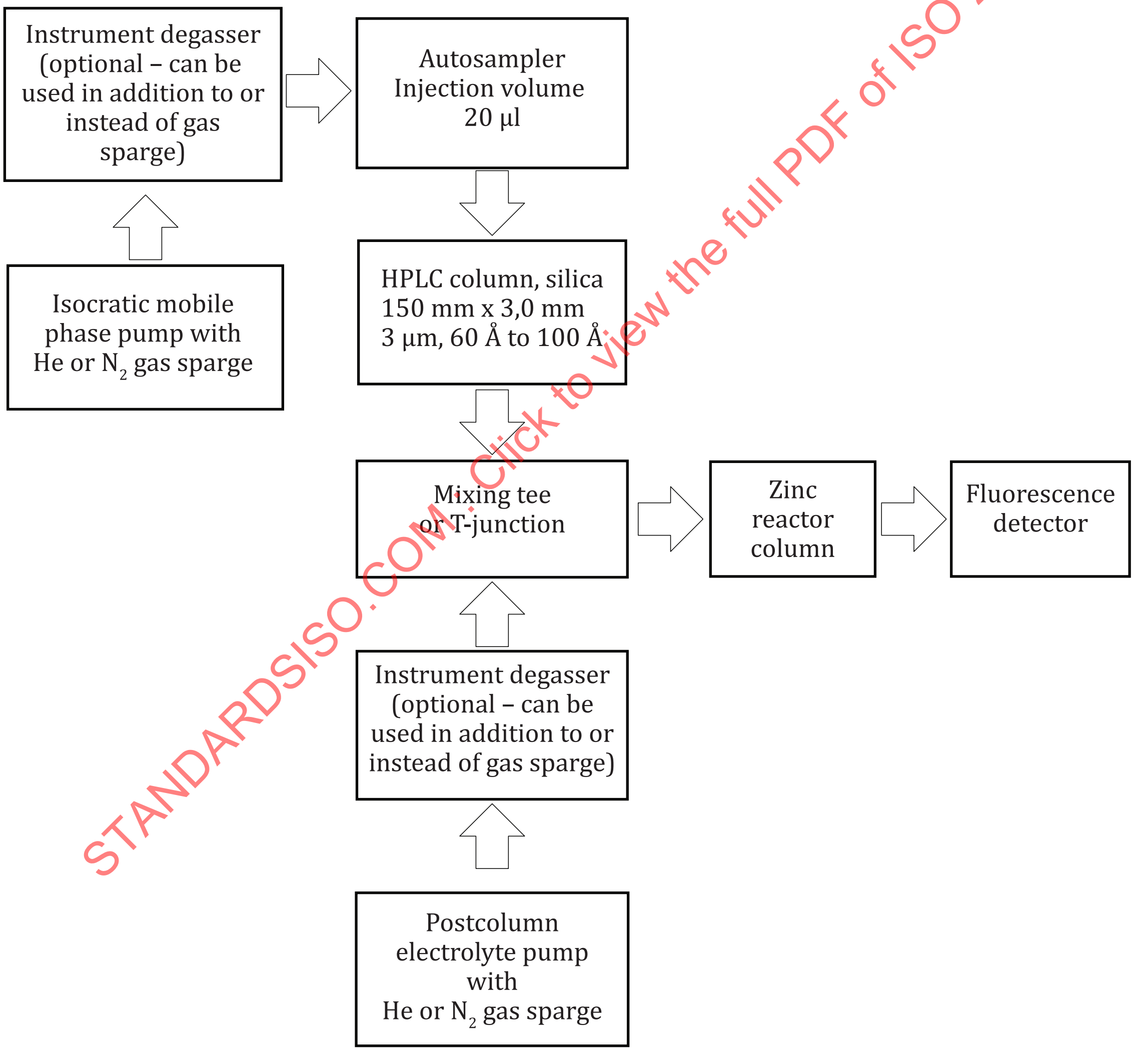


Figure 1 — Vitamin K system configuration

If necessary, remove used zinc and repack the post-column reactor column with fresh zinc (5.11). The zinc reactor column should be repacked whenever the S/N in the lowest standard is too low to accurately integrate the vitamin K_1 peak, linearity requirements ($r^2 \geq 0,999$) cannot be met unless the highest standard is excluded from the curve, peak responses from injections of the same standard drop by more than 7 % and the drop cannot be attributed to other system components, or the system back pressure through the zinc reactor increases significantly and vitamin K_1 peak widths begin to increase.

To repack the zinc reactor column, remove the hex nuts and retainers from both ends of the column and force the used zinc out of the column with a thin wire or similar apparatus. Flush the zinc reactor column with ethanol to remove residual zinc. Replace the hex nut and retainer on one end of the zinc reactor column. Carefully transfer a small amount of fresh zinc powder to the reactor column with a spatula, and press down on the zinc in the column with an old HPLC piston or similar apparatus to pack it tightly. Continue adding zinc and pressing it down until the level of zinc is even with the top of the column. After the reactor column is full, replace the second retainer and hex nut. The more tightly zinc is packed into the reactor column, the more symmetrical the vitamin K_1 peaks will be.

When using a helium or nitrogen, sparge the mobile phase and post-column electrolyte solutions by bubbling helium or nitrogen through them at a flow rate just fast enough to cause small ripples on the surface of the mobile phase and post-column solutions. To maximize the life of the zinc reactor column, sparge the mobile phase and post-column electrolyte solution for at least 30 min before connecting the zinc reactor column if mobile phase and post-column solutions are flowing, or do not pump mobile phase and post-column electrolyte solutions through the zinc reactor column until at least 30 mins after sparging begins. Once the mobile phase and post-column electrolyte solutions have been sparged, allow the column and post-column reactor to equilibrate with mobile phase flowing at 0,4 ml/min and post-column electrolyte solution flowing at 0,4 ml/min for at least 30 min prior to the first injection if the zinc reactor has been used for previous analyses, or for several hours if the zinc post-column reactor has been freshly packed. Once the mobile phase and post-column solutions have been sparged, reduce the helium or nitrogen flow rate so that only a small stream of helium or nitrogen bubbles are visible in the mobile phase and post-column solutions and there is minimal disturbance to the surface of these solutions. Bubble helium or nitrogen very slowly through the mobile phase and post-column electrolyte solutions continuously throughout the entire run. Once the run has started, do not adjust the helium or nitrogen flow rate.

When using an instrument degasser, allow the column and post-column reactor to equilibrate with mobile phase flowing at 0,4 ml/min and post-column electrolyte solution flowing at 0,4 ml/min for at least 30 min prior to the first injection.

Allow the fluorescence detector lamp to warm up 30 min prior to the first injection.

When the mobile phase and post-column electrolyte solution are continuously sparged with helium or nitrogen or flow through an instrument degasser throughout a run, it is not necessary to pack the post-column reactor with zinc at the beginning of every run. It should be possible to analyse hundreds of extracts before the zinc reactor column shall be repacked.

8.3 HPLC of standards and samples

Inject the most concentrated standard (approximately 80 µg/l) and observe the response on the fluorescence detector. If necessary, adjust the detector gain and sensitivity settings so that the standard response is within the range of the detector. Once the detector settings have been determined, inject the most concentrated standard 3 to 4 times and note the peak areas. If the system is equilibrated, the RSD of the standard peak areas should be ≤ 2 %, and the peak areas should not steadily increase or decrease by more than 4 % from the first injection to the third or fourth injection. If the RSD is > 2 %, locate the source of the imprecision and correct it before beginning the sample analysis. If peak areas steadily increase or decrease by more than 4 %, the system is not equilibrated and shall be allowed to equilibrate longer. Once the system has reached equilibrium and the injection precision is ≤ 2 % RSD, inject a set of standards, sample extracts, and another set of standards. Every set of sample extracts shall be bracketed by standards.

8.4 Instrument shut-down

8.4.1 Short-term shut-down

After analysing a set of sample extracts, simultaneously turn off the flow on the mobile phase and post-column electrolyte solution pumps. Remove the helium or nitrogen sparge lines from the mobile phase and post-column electrolyte solutions and turn off the helium or nitrogen. Turn off the fluorescence detector lamp.

8.4.2 Long-term shut-down

Remove the helium or nitrogen sparge lines from the mobile phase and post-column electrolyte solution and turn off the helium or nitrogen. Turn off the fluorescence detector lamp. Remove and cap the zinc reactor column. Flush the post-column pump and fluorescence detector cell with reagent alcohol to remove residual zinc chloride, sodium acetate, and acetic acid.

9 Calculations

The vitamin K_1 concentrations of samples analysed on the HPLC system are determined by comparison of peak areas from samples of known weight with the peak areas of standards of known concentration. Because the *cis* and *trans* vitamin K_1 retention times may shift slightly during a run, peak areas shall be used to quantitate vitamin K_1.

Calculate the standard concentrations using Formula (2):

$$\rho_{\text{ws}} = \frac{m \times b \times (V_1 \times V_2 \times V_3) \times 10^9}{(D_1 \times D_2 \times D_3 \times D_4)} \tag{2}$$

where

ρ_{ws} is the working standard concentration in µg/l;

m is the mass of the standard, in g;

b is the purity of the standard, in mg/mg;

V_1 is the aliquot of stock solution, in ml;

V_2 is the aliquot of intermediate I solution, in ml;

V_3 is the aliquot of intermediate standard II solution, in ml;

10^9 is conversion factor from g/ml to µg/l;

D_1 is the dilution volume of the stock solution, in ml;

D_2 is the dilution volume of the intermediate I solution, in ml;

D_3 is the dilution volume of the intermediate II solution, in ml;

D_4 is the dilution volume of the working standard solution, in ml.

If using sources of reference standard other than USP, the stock standard concentration shall be determined spectrophotometrically following the instructions in 6.4.2, and Formula (3) should be used to calculate working standard concentrations.

$$\rho_{ws} = \frac{(\rho_{ss} \times V_1 \times V_2 \times V_3) \times 10^3}{(D_2 \times D_3 \times D_4)} \qquad (3)$$

where ρ_{ss} is the stock standard concentration, in mg/l.

Peak areas are measured with a data system. Before calculating concentrations, review all chromatograms to make sure that *cis* and *trans* vitamin K_1 are baseline separated and that there are no interfering peaks. Vitamin K_1 concentrations cannot be calculated for any samples with interfering peaks or poor separation between the *cis* and *trans* isomers (see Figures A.1, A.2 and A.3).

Check the integration of the *cis* and *trans* vitamin K_1 peaks. *Cis* vitamin K_1 elutes 1 min to 3 min before *trans* vitamin K_1 depending on the analytical column used. If the peak areas of the same standards injected before and after a set of samples have changed by more than 7 %, the system was not equilibrated and the data are not acceptable.

Calculate the *trans* vitamin K_1 standard concentration using Formula (4):

$$T = \frac{A_2}{(A_1 + A_2)} \qquad (4)$$

where

T is the *trans* vitamin K_1 fraction;

A_1 is the *cis* vitamin K_1 peak area;

A_2 is the *trans* vitamin K_1 peak area.

The *trans* vitamin K_1 fraction is calculated for all standard injections, and the calculated *trans* vitamin K_1 fraction for all standard injections is averaged together (T_a) and used to calculate the *trans* vitamin K_1 concentration of all working standards.

Calculate the *trans* vitamin K_1 working standard concentration using Formula (5):

$$\rho_{st} = \rho_{ws} \times T_a \qquad (5)$$

where

ρ_{st} is the concentration of *trans* vitamin K_1 in the working standard, in µg/l;

ρ_{ws} is the working standard concentration, in µg/l;

T_a is the average standard *trans* vitamin K_1 fraction.

For each working standard concentration, average the *trans* peak areas from each two consecutive sets of standard injections. Prepare a standard curve by performing a linear least-squares regression on *trans* concentration versus averaged *trans* peak areas. A standard curve shall have an r^2 of 0,999 or better to be acceptable.

Calculate the *trans* vitamin K$_1$ mass fraction in a sample using Formula (6):

$$w_{tr} = \frac{\rho_c \times 10{,}0 \times m_r}{m_s \times m_p} \tag{6}$$

where

w_{tr} is the mass fraction of *trans* vitamin K$_1$, in µg/kg;

ρ_c is the mass concentration of *trans* vitamin K$_1$ in the injected sample extract determined from the standard curve, in µg/l;

10,0 is the dilution volume of the sample, in ml;

m_r is the final dilution mass of a product reconstitution, in g (if necessary);

m_s is the sample size, in g;

m_p is the mass of product that is reconstituted, in g (if necessary).

If a total vitamin K$_1$ mass fraction is required, total vitamin K$_1$ can be calculated with Formula (7), from the *trans* concentration determined in Formula (6) and the *cis* and *trans* peak areas for the sample using Formula (4).

$$w_{tot} = \frac{w_{tr}}{T_{sample}} \tag{7}$$

10 Precision

10.1 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.

The results of the laboratory comparison between EN 14148[6] and this document are given in Annex C.

10.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in not more than 5 % of the cases. The values of *r* are given in Table 1.

10.3 Reproducibility

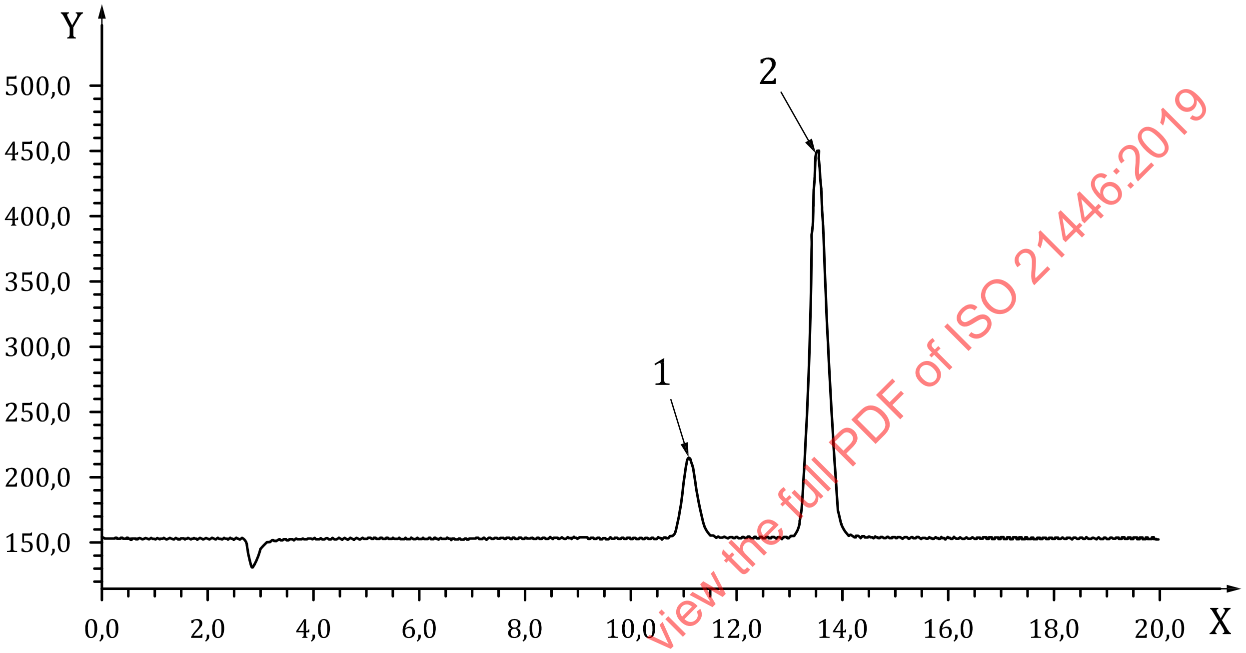
The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit *R* in not more than 5 % of the cases. The values of *R* are given in Table 1.

Table 1 — Precision data

Sample	$\overline{x}$ µg/100 g RTF	r µg/100 g RTF	R µg/100 g RTF
Precision data for *trans* vitamin K_1			
IF powder milk protein based	6,16	0,370	0,881
IF powder soy based	6,19	0,377	0,842
IF powder partially hydrolysed milk based	7,74	0,648	0,841
IF powder partially hydrolysed soy based	8,24	0,526	1,04
AN RTF high fat	9,28	0,581	2,19
AN RTF high protein	7,00	1,40	3,16
Child formula powder milk based	3,61	0,312	0,595
IF powder stage 1 milk based	7,24	0,575	0,935
IF RTF milk based	6,78	0,676	1,01
IF RTF milk based placebo	1,86	0,332	0,55
SRM 1849a (results in µg /kg powder)	1 077	42,1	111
Child formula powder milk based	4,46	0,294	0,545
AN powder low fat	2,91	0,081 2	0,265
Infant elemental powder	8,67	0,774	2,33
IF powder FOS/GOS based	3,96	0,244	0,495
Child formula powder milk based placebo	1,37	0,078 1	0,250
Infant elemental powder placebo	1,41	0,180	0,319
AN RTF high protein placebo	2,85	0,140	0,446
AN RTF high fat placebo	2,85	0,385	0,476
Precision data for total (*cis* + *trans*) vitamin K_1			
IF powder milk protein based	7,09	0,398	0,886
IF powder soy based	7,12	0,631	0,923
IF powder partially hydrolysed milk based	8,72	0,675	0,894
IF powder partially hydrolysed soy based	9,31	0,594	1,03
AN RTF high fat	10,7	0,606	2,45
AN RTF high protein	8,12	1,56	3,81
Child formula powder milk based	3,93	0,446	0,698
IF powder stage 1 milk based	7,97	0,572	0,853
IF RTF milk based	7,94	0,771	1,08
IF RTF milk based placebo	2,15	0,339	0,688
SRM 1849a (results in µg/kg powder)	1 154	58,5	95,3
Child formula powder milk based	4,76	0,319	0,573
AN powder low fat	3,15	0,093 4	0,270
Infant elemental powder	9,32	0,928	2,39
IF powder FOS/GOS based	4,53	0,332	0,509
Child formula powder milk based placebo	1,46	0,086 6	0,239
Infant elemental powder placebo	1,51	0,203	0,321
Adult nutritional RTF high protein placebo	3,16	0,191	0,444
Adult nutritional RTF high fat placebo	3,13	0,399	0,530
Key RTF: ready to feed, IF: infant formula, AN: adult nutritional			

Annex A
(informative)

Example chromatograms



Key

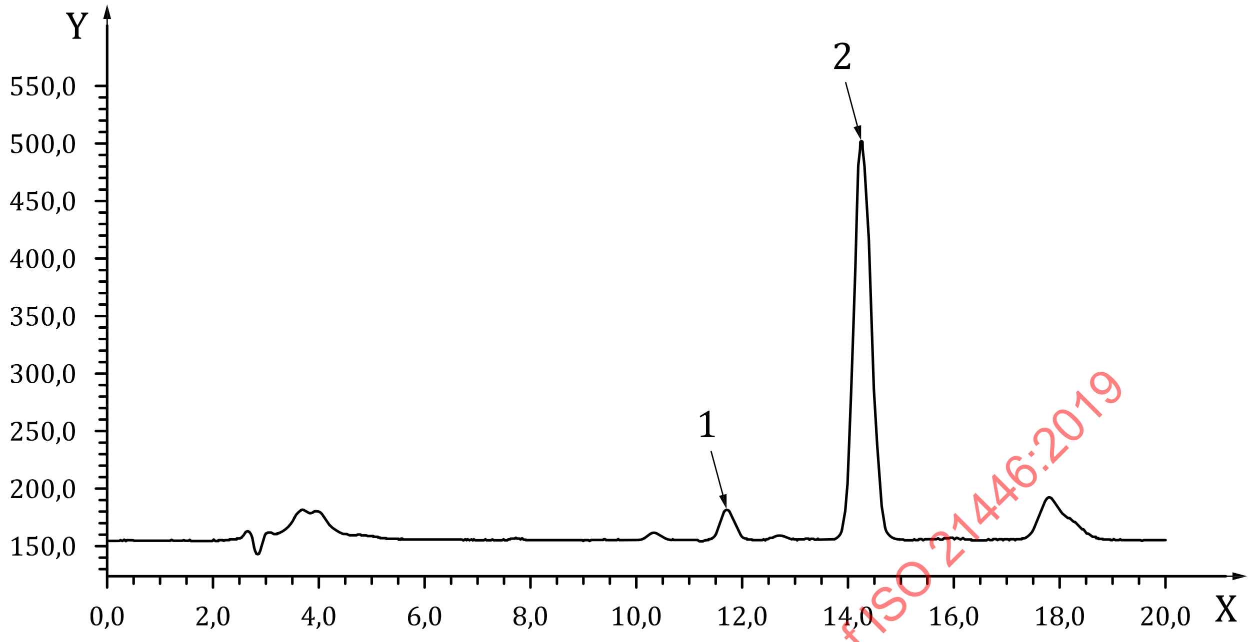
X time, in min

Y fluorescence, arbitrary units

1 *cis* vitamin K_1, 11,1 min

2 *trans* vitamin K_1, 13,5 min

Figure A.1 — Chromatogram of vitamin K_1 standard



Key

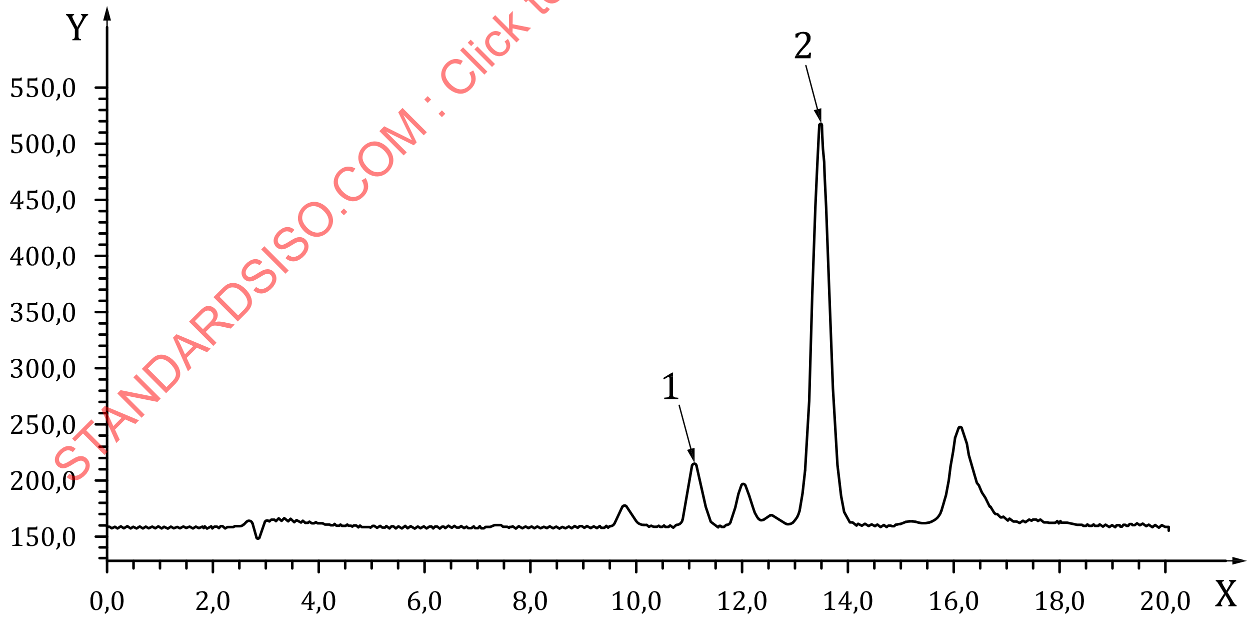
X time, in min

Y fluorescence, arbitrary units

1 *cis* vitamin K_1, 11,7 min

2 *trans* vitamin K_1, 14,3 min

Figure A.2 — Chromatogram of SRM 1849a



Key

X time, in min

Y fluorescence, arbitrary units

1 *cis* vitamin K_1, 11,1 min

2 *trans* vitamin K_1, 13,5 min

Figure A.3 — Chromatogram of an adult nutritional

Annex B
(informative)

Precision data

The data given in Table B.1 were obtained in an interlaboratory study and published in 2018[1], in accordance with ISO 5725-2[2] and the AOAC-IUPAC Harmonized Protocol for collaborative study procedures, to asses precision characteristics of a method of analysis[3]. The study was performed based on requirements given in Reference [4].

Table B.1 — Precision data for *trans* vitamin K_1

Sample	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[e]	**6**[f]	**7**[g]	**8**[h]	**9**[i]	**10**[j]
Year of interlaboratory test	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017
Number of laboratories	8	8	8	8	8	8	8	8	8	8
Number of non-compliant laboratories	0	0	0	0	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	8	8	8	8	8	8	8	8	8	8
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0
Number of accepted results	16	16	15	16	16	16	16	16	16	16
Mean value, $\bar{x}$, µg/100 g RTF[i]	6,16	6,19	7,74	8,24	9,28	7,00	3,61	7,24	6,78	1,86
Repeatability standard deviation, s_r, µg/100 g RTF	0,132	0,135	0,231	0,188	0,208	0,501	0,111	0,205	0,241	0,119
Reproducibility standard deviation, s_R, µg/100 g RTF	0,314	0,301	0,300	0,372	0,781	1,13	0,213	0,334	0,361	0,198
Coefficient of variation of repeatability, $C_{V,r}$, %	2,15	2,18	2,98	2,28	2,24	7,16	3,08	2,83	3,56	6,37
Coefficient of variation of reproducibility, $C_{V,R}$, %	5,11	4,86	3,87	4,52	8,42	16,1	5,88	4,61	5,33	10,6
Repeatability limit, r [$r = 2{,}8 \times s_r$], µg/100 g RTF	0,370	0,377	0,648	0,526	0,581	1,40	0,312	0,575	0,676	0,332
Reproducibility limit, R [$R = 2{,}8 \times s_R$], µg/100 g RTF	0,881	0,842	0,841	1,04	2,19	3,16	0,595	0,935	1,01	0,555
HorRat value, according to Reference [5]	0,210	0,200	0,165	0,194	0,368	0,676	0,220	0,194	0,222	0,365

Table B.1 *(continued)*

Sample	**11**[k]	**12**[l]	**13**[m]	**14**[n]	**15**[o]	**16**[p]	**17**[q]	**18**[r]	**19**[s]	
Year of interlaboratory test	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	
Number of laboratories	8	8	8	8	8	8	8	8	8	
Number of non-compliant laboratories	0	0	0	0	0	0	0	0	0	
Number of laboratories retained after eliminating outliers	8	8	8	8	8	8	8	8	8	
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	
Number of accepted results	15	16	16	16	16	16	15	16	15	
Mean value, $\bar{x}$, µg/100 g RTF[i]	1 077	4,46	2,91	8,67	3,96	1,37	1,41	2,85	2,85	
Repeatability standard deviation, s_r, µg/100 g RTF	15,0	0,105	0,029 0	0,276	0,087 3	0,027 9	0,064 2	0,050 2	0,138	
Reproducibility standard deviation, s_R, µg/100 g RTF	39,5	0,195	0,094 8	0,834	0,177	0,089 3	0,114	0,159	0,170	
Coefficient of variation of repeatability, $C_{V,r}$, %	1,39	2,35	0,995	3,19	2,20	2,03	4,55	1,76	4,81	
Coefficient of variation of reproducibility, $C_{V,R}$, %	3,66	4,36	3,25	9,62	4,46	6,50	8,07	5,58	5,94	
Repeatability limit, r [$r = 2{,}8 \times s_r$], µg/100 g RTF	42,1	0,294	0,081 2	0,774	0,244	0,078 1	0,180	0,140	0,385	
Reproducibility limit, R [$R = 2{,}8 \times s_R$], µg/100 g RTF	111	0,545	0,265	2,33	0,495	0,250	0,319	0,446	0,476	
HorRat value, according to Reference [5]	0,231	0,121	0,084 5	0,294	0,121	0,151	0,188	0,144	0,154	

Key

RTF: ready to feed

NOTE The results are expressed as µg trans vitamin K_1 per 100 g RTF.

[a] Infant formula powder milk protein based. [b] Infant formula powder soy based. [c] Infant formula powder partially hydrolysed milk based. [d] Infant formula powder partially hydrolysed soy based. [e] Adult nutritional RTF high fat. [f] Adult nutritional RTF high protein. [g] Child formula powder milk based. [h] Infant formula powder stage 1 milk based. [i] Infant formula RTF milk based. [j] Infant formula RTF milk based placebo. [k] SRM 1849a (results in µg /kg powder). [l] Child formula powder milk based. [m] Adult nutritional powder low fat. [n] Infant elemental powder. [o] Infant formula powder FOS/GOS based. [p] Child formula powder milk based placebo. [q] Infant elemental powder placebo. [r] Adult nutritional RTF high protein placebo. [s] Adult nutritional RTF high fat placebo.

Table B.2 — Precision data for total (*cis* + *trans*) vitamin K_1

Sample	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[e]	**6**[f]	**7**[g]	**8**[h]	**9**[i]	**10**[j]
Year of interlaboratory test	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017	2016 to 2017
Number of laboratories	8	8	8	8	8	8	8	8	8	8
Number of non-compliant laboratories	0	0	0	0	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	8	8	8	8	8	8	8	8	8	8
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0
Number of accepted results	16	16	15	16	16	16	16	16	16	16
Mean value, $\bar{x}$, µg/100 g RTF[i]	7,09	7,12	8,72	9,31	10,7	8,12	3,93	7,97	7,94	2,15
Repeatability standard deviation, s_r, µg/100 g RTF	0,142	0,225	0,241	0,212	0,216	0,558	0,159	0,204	0,275	0,121
Reproducibility standard deviation, s_R, µg/100 g RTF	0,316	0,330	0,319	0,367	0,873	1,36	0,249	0,305	0,384	0,246
Coefficient of variation of repeatability, $C_{V,r}$, %	2,00	3,16	2,76	2,28	2,02	6,87	4,05	2,56	3,47	5,64
Coefficient of variation of reproducibility, $C_{V,R}$, %	4,46	4,63	3,66	3,94	8,16	16,7	6,34	3,82	4,84	11,4
Repeatability limit, r [$r = 2,8 \times s_r$], µg/100 g RTF	0,398	0,631	0,675	0,594	0,606	1,56	0,446	0,572	0,771	0,339
Reproducibility limit, R [$R = 2,8 \times s_R$], µg/100 g RTF	0,886	0,923	0,894	1,03	2,45	3,81	0,698	0,853	1,08	0,688
HorRat value, according to Reference [5]	0,187	0,195	0,158	0,172	0,365	0,717	0,244	0,163	0,207	0,401