INTERNATIONAL STANDARD

**ISO 14565**

First edition
2000-12-01

# Animal feeding stuffs — Determination of vitamin A content — Method using high-performance liquid chromatography

*Aliments des animaux — Détermination de la teneur en vitamine A — Méthode par chromatographie liquide à haute performance*



Reference number
ISO 14565:2000(E)

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Printed in Switzerland

## Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 14565 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Annex A of this International Standard is for information only.

# Animal feeding stuffs — Determination of vitamin A content — Method using high-performance liquid chromatography

## 1 Scope

This International Standard specifies a method for the determination of the total vitamin A (retinol) content of animal feeding stuffs and pet foods using high-performance liquid chromatography. The vitamin A content is the content of all-*trans*-retinyl alcohol and *cis*-isomers determined by the method described in this International Standard, and is expressed in International Units per kilogram (IU/kg).

## 2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative documents referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.



ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*.



ISO 6498, *Animal feeding stuffs — Preparation of test samples*.

## 3 Term and definition

For the purposes of this International Standard, the following term and definition apply.

**3.1**
**vitamin A content**
content of all-*trans*-retinyl alcohol and *cis*-isomers determined in accordance with this International Standard

NOTE The vitamin A content is expressed in International Units per kilogram (IU/kg); 1 IU of vitamin A is equal to 0,300 μg of all-*trans*-retinol.

## 4 Principle

The sample is saponified with ethanolic potassium hydroxide solution and the vitamin A is extracted into light petroleum. The light petroleum is removed by evaporation and the residue is dissolved in 2-propanol. The vitamin A concentration in the 2-propanol extract is determined by reverse-phase liquid chromatography using conditions that give a single peak for all retinol isomers.

## 5 Reagents

Use only reagents of recognized analytical grade.

**5.1 Water**, complying with at least grade 3 in accordance with ISO 3696.

**5.2 Potassium hydroxide solution** (KOH).

Dissolve 500 g of potassium hydroxide in water (4.1) and dilute to 1 litre.

**5.3 Ethanol**, $w(C_2H_5OH)$ = 95 % (by volume), or equivalent industrial methylated spirit.

**5.4 2-Propanol** ($C_3H_7OH$).

**5.5 Light petroleum**, boiling range 40 °C to 60 °C.

The residue on evaporation shall be less than 20 mg/l.

**5.6 Vitamin A standard substances**

**5.6.1 All-*trans*-retinyl acetate**, vitamin A acetate ($C_{22}H_{32}O_2$), 328.5 g/mol, with a purity of at least 90 %.

**5.6.2 All-*trans*-retinol**, vitamin A alcohol ($C_{20}H_{30}O$), 286,5 g/mol, with a purity of at least 90 %

**5.7 Methanol**, HPLC grade.



**5.8 Mobile phase for liquid chromatography**.

Mix together methanol (5.7) and water (4.1) in the proportions 770 + 30 (by volume).



If necessary, filter through a membrane filter (6.6).

**5.9 Sodium sulfate** ($Na_2SO_4$), anhydrous.

**5.10 Sodium ascorbate solution**, $\rho$ = 100 g/l.

**5.11 Inert gas**, e.g. nitrogen.

## 6 Apparatus

Usual laboratory apparatus and, in particular, the following.

**6.1 High-performance liquid chromatograph**, consisting of the following.

**6.1.1 Pump**, set to deliver a constant eluent volume flow rate of 1 ml/min.

**6.1.2 HPLC injection device.**

**6.1.3 Column**, length 250 mm, internal diameter 4,6 mm, packed with a stationary phase consisting of octadecyl ($C_{18}$) groups bonded to silica.

A column with at least 4 000 theoretical plates and a $k'$ value of 0,6, both with respect to all-*trans*-retinol, has been found to be satisfactory. The particle size shall be not smaller than 5 μm and not greater than 10 μm. Other systems may be used provided that a satisfactory separation of vitamin A from other co-extractives is achieved.

**6.1.4 Detector**, allowing the measurement of ultraviolet radiation at 325 nm, and equipped with integrator/data-handling system.

**6.2 UV (or UV-Visible) spectrometer**, capable of measuring absorbance at the wavelengths defined in 9.6, equipped with quartz cells of 10 mm path length.

**6.3 Boiling water bath**.

**6.4 Rotary vacuum evaporator**, with water bath at 40 °C.

**6.5 Extraction apparatus** (see Figure 1) consisting of the following:

— a cylinder of 1 litre capacity fitted with a ground glass neck and stopper;

— a ground glass joint, fitting the cylinder and equipped with an adjustable tube passing through the centre; and

— a side-arm.

The adjustable tube should have a U-shaped lower end and a jet at the opposite end so that the upper liquid layer in the cylinder may be transferred to a separating funnel of 1 litre capacity.

Other extraction equipment such as conical flasks and separating funnels may be used in place of the apparatus shown in Figure 1, provided that satisfactory recoveries of vitamin A are achieved.

**6.6 Membrane filter**, 0,45 µm pore size, for filtration of mobile phase (5.8) and sample test solutions.



## 7 Sampling



It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [4].

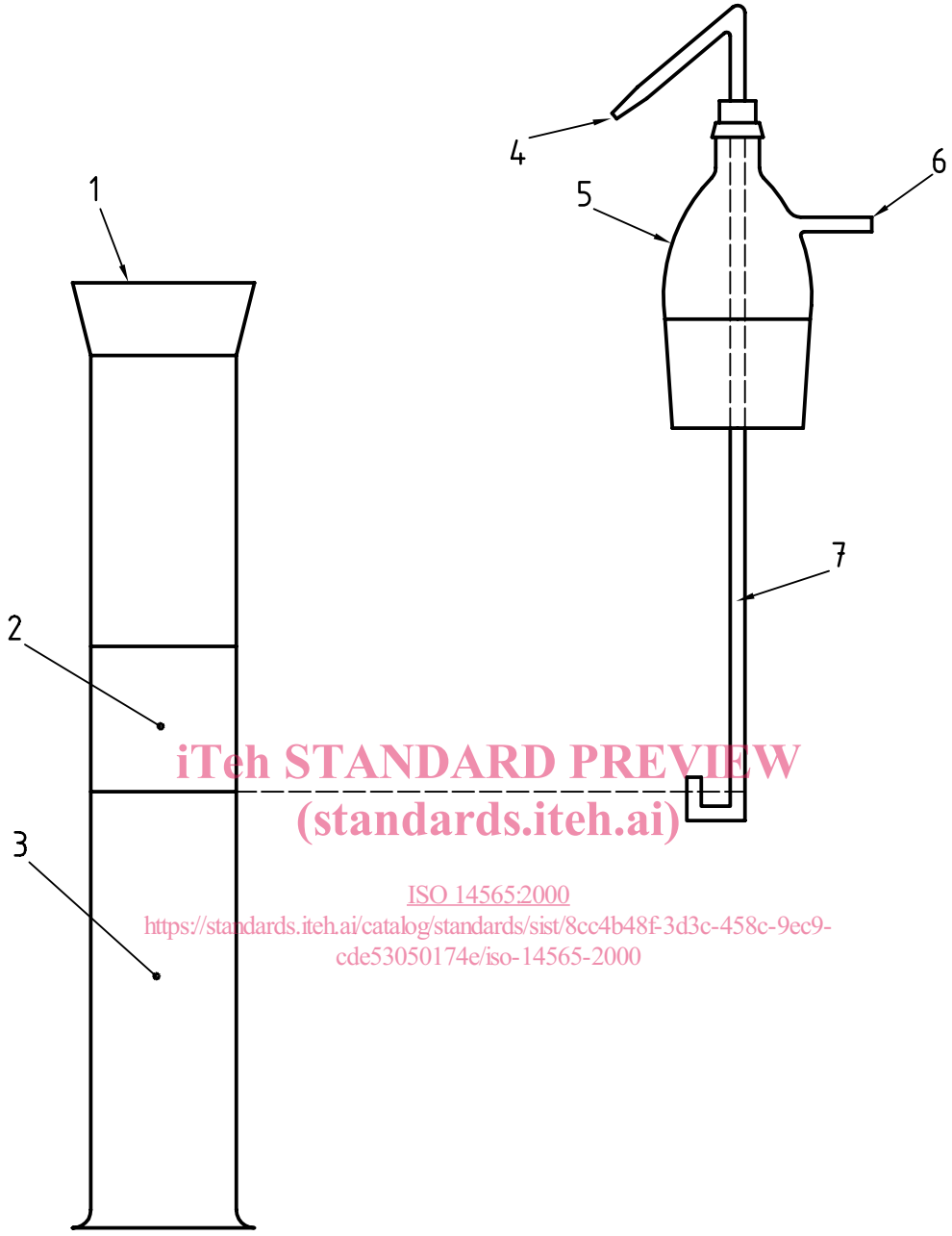
Store the sample in such a way that deterioration and change in its composition are prevented.

## 8 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

Just prior to starting the analysis, grind a portion of the well-mixed laboratory sample so that it passes through a sieve with 1 mm apertures. Mix thoroughly.

Homogenize canned pet foods. Mince semi-moist pet foods to pass through a plate with 4 mm apertures.






**Key**

1 Cylinder, of capacity 1 litre, with ground-glass neck
2 Light petroleum layer
3 Aqueous layer + saponified feed
4 Jet
5 Bottle, of capacity 1 litre, with ground-glass joint
6 Side-arm
7 Adjustable tub

**Figure 1 — Example of extraction apparatus**

# 9 Procedure

## 9.1 General

Because of the sensitivity of vitamin A to UV radiation and air, perform all operations away from natural and strong fluorescent light and as rapidly as is consistent with accurate working. Use amber glassware where possible. Complete each assay within one working day. Carry out the saponification and extraction of the all-*trans*-retinyl acetate standard and the feeding stuff samples at the same time.

## 9.2 Saponification

Weigh, to the nearest 0,1 g, approximately 50 g of the prepared test sample (see clause 8) into a 1 litre conical flask.

Add 200 ml of ethanol (5.3). Swirl the flask contents to disperse the sample.

Add 2 ml of sodium ascorbate (5.10) and 50 ml of potassium hydroxide solution (5.2) and mix by swirling.

Fit a reflux condenser to the flask and immerse the flask in the boiling water bath (6.3).

Allow the contents of the flask to reflux for 60 min, swirling occasionally.

Remove and cool the flask to room temperature as rapidly as possible under a stream of cold water.

## 9.3 Extraction of vitamin A (retinol)



Transfer the contents of the flask to the extraction cylinder (see 6.5).

Rinse the flask with two 25 ml portions of ethanol or industrial methylated spirit (5.3) and transfer the rinsings to the cylinder.



Repeat the rinsing of the flask with two 125 ml portions of light petroleum (5.5) and one 250 ml portion of water (4.1), each time transferring the rinsings to the cylinder.

Stopper the cylinder and shake well for 1 min, taking care to release any pressure from time to time.

Cool the cylinder under a stream of cold water while waiting for the two liquid phases to separate, before removing the stopper.

When the layers have separated, remove the stopper, wash the sides of the stopper with a few millilitres of light petroleum (5.5) and insert the adjustable tube (see 6.5), positioning the lower open end so that it is just above the level of the interface.

By application of a slight pressure of inert gas (5.11) to the side-arm tube, transfer the upper, light petroleum layer to a 1 litre separating funnel (see 6.5).

Add 125 ml of light petroleum (5.5) to the cylinder, stopper and shake well for 1 min.

Allow the layers to separate and transfer the upper layer to the separating funnel using the adjustable tube (see 6.5) as before.

Again, add 125 ml of light petroleum (5.5) to the cylinder, stopper and shake well for 1 min.

Again, allow the layers to separate and transfer the upper layer to the separating funnel using the adjustable tube as before.

Wash the combined light petroleum extracts with four 100 ml portions of water using at first only gentle inversion then only gentle shaking in order to keep emulsion formation to a minimum.