DRAFT INTERNATIONAL STANDARD
ISO/DIS 13904

ISO/TC **34**/SC **10** Secretariat: **ISIRI**

Voting begins on: **2014-12-04** Voting terminates on: **2015-05-04**

Animal feeding stuffs — Determination of tryptophan content

Aliments des animaux — Détermination de la teneur en tryptophane

ICS: 65.120

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7dad79fe-fbb2-4293-8296-a53bfb742794/iso-13904-2016

ISO/CEN PARALLEL PROCESSING

This draft has been developed within the International Organization for Standardization (ISO), and processed under the **ISO lead** mode of collaboration as defined in the Vienna Agreement.

This draft is hereby submitted to the ISO member bodies and to the CEN member bodies for a parallel five month enquiry.

Should this draft be accepted, a final draft, established on the basis of comments received, will be submitted to a parallel two-month approval vote in ISO and formal vote in CEN.

To expedite distribution, this document is circulated as received from the committee secretariat. ISO Central Secretariat work of editing and text composition will be undertaken at publication stage.

THIS DOCUMENT IS A DRAFT CIRCULATED FOR COMMENT AND APPROVAL. IT IS THEREFORE SUBJECT TO CHANGE AND MAY NOT BE REFERRED TO AS AN INTERNATIONAL STANDARD UNTIL PUBLISHED AS SUCH.

IN ADDITION TO THEIR EVALUATION AS BEING ACCEPTABLE FOR INDUSTRIAL, TECHNOLOGICAL, COMMERCIAL AND USER PURPOSES, DRAFT INTERNATIONAL STANDARDS MAY ON OCCASION HAVE TO BE CONSIDERED IN THE LIGHT OF THEIR POTENTIAL TO BECOME STANDARDS TO WHICH REFERENCE MAY BE MADE IN NATIONAL REGULATIONS.

RECIPIENTS OF THIS DRAFT ARE INVITED TO SUBMIT, WITH THEIR COMMENTS, NOTIFICATION OF ANY RELEVANT PATENT RIGHTS OF WHICH THEY ARE AWARE AND TO PROVIDE SUPPORTING DOCUMENTATION.

Reference number
ISO/DIS 13904:2014(E)

© ISO 2014

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7dad79fe-fbb2-4293-8296-a53bfb742794/iso-13904-2016

Copyright notice

This ISO document is a Draft International Standard and is copyright-protected by ISO. Except as permitted under the applicable laws of the user's country, neither this ISO draft nor any extract from it may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, photocopying, recording or otherwise, without prior written permission being secured.

Requests for permission to reproduce should be addressed to either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Reproduction may be subject to royalty payments or a licensing agreement.

Violators may be prosecuted.

© ISO 2014 – All rights reserved

Contents

Page

Foreword iv
1 Scope 1
2 Principle 1
3 Reagents and materials 1
4 Apparatus 3
5 Procedure 4
5.1 Preparation of samples 4
5.2 Determination of free tryptophan (extract) 4
5.3 Determination of total tryptophan (hydrolysates) 5
5.4 HPLC determination 6
6 Calculation of results 6
6.1 Feeding stuffs 6
6.2 Commercial pure products and premixtures containing more than 2 % of tryptophan 7
7 Precision 7
7.1 Interlaboratory test 7
7.2 Repeatability 8
7.3 Reproducibility 8
8 Test report 8
Annex A (informative) Results of an interlaboratory test 9
Annex B (informative) Observations on the method 11
Annex C (informative) Table of sample preparation : example for analyses in pure products and premixtures 12
Annex D Results of an interlaboratory test for tryptophan in pure products and premixtures containing more than 2 % of tryptophan 13
Bibliography 14

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7dad7ae-fb03-4203-8290-a53bfb727794/iso-13904-2016

© ISO 2014 – All rights reserved

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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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 document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 13904 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

This second/third/... edition cancels and replaces the first/second/... edition (), [clause(s) / subclause(s) / table(s) / figure(s) / annex(es)] of which [has / have] been technically revised.

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7da279fe-fbb2-4293-8296-a53bfb742794/iso-13904-2016

© ISO 2014 – All rights reserved

Animal feeding stuffs — Determination of tryptophan content

1 Scope

This International Standard describes a method for determination of the total and free tryptophan (Trp) content in feeding stuffs (e.g. complete and complementary feeds, supplementary feeds, raw materials, ingredients, and concentrates) and determination of free tryptophan in commercial pure substances and premixtures containing more than 2 % of tryptophan.

It does not distinguish between D- and L-forms.

2 Principle

For the determination of the total tryptophan, the sample is hydrolysed under alkaline conditions with saturated barium hydroxide solution and heated to 110 °C for 20 h. After hydrolysis, an internal standard is added.

For the determination of free tryptophan, the sample is extracted under mild acidic conditions in the presence of an internal standard. For commercial pure substances and premixtures containing more than 2 % of tryptophan, it is possible to add the internal standard after the extraction because if it is added before, a large volume of a large is necessary.

The tryptophan and the internal standard in the hydrolysate or in the extract are determined by reversed phase C_{18} HPLC with fluorescence detection.

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7dad79fe-fbb2-4293-8296-a53bfb742794/iso-13904-2016

3 Reagents and materials

Use only reagents of recognized analytical grade, unless otherwise specified.

3.1 Double-distilled water, or water of equivalent purity (conductivity < 10 µS/cm).

3.2 Standard substance and control substance: tryptophan (purity/content w ≥ 99 %) dried under vacuum over phosphorus pentoxide. The product is considered as 100 % pure.

NOTE The control of the purity of the standard substance can be performed by measuring the absorbance of a solution of tryptophan at 280 nm. Prepare a solution of about 5 mg/l in HCl 10-3 N from a stock solution and measure the OD at 280 nm versus HCl 10-3 N. Then, the concentration of tryptophan is: C = OD/5630 * 10exp06 where:

— 5630 is the molar extinction coefficient of tryptophan in water à 280 nm;

— C is expressed in µmole/ l.

The standard substance purity is then: $(C/C_0)*100$ where C_0 is the theoretical concentration of the diluted, expressed in µmole/l, about 25 µmole/l.

The control of the purity is performed every 6 months of use; it shall be ≥ 99 %.

3.3 Internal standard substance: α-methyltryptophan (purity/content w ≥ 99 %), dried under vacuum over phosphorus pentoxide.

© ISO 2014 – All rights reserved

3.4 Barium hydroxide octahydrate.

Care should be taken not to expose the $Ba(OH)_2 \cdot 8H_2O$ excessively to air in order to avoid formation of $BaCO_3$, which could disturb the determination (see observation in B.3).

3.5 Sodium hydroxide.

3.6 Orthophosphoric acid, w = 85 %.

3.7 Concentrated hydrochloric acid, ρ_{20} = 1,19 g/ml.

3.8 Methanol, HPLC grade.

3.9 Light petroleum, boiling range 40 °C to 60 °C.

3.10 Sodium hydroxide solution, c = 1 mol/l.

Dissolve 40,0 g of NaOH (3.5) in water (3.1) and make up to 1 l with water (3.1).

3.11 Hydrochloric acid, c = 6 mol/l.

Take 492 ml of HCl (3.7) and make up to 1 l with water (3.1).

3.12 Hydrochloric acid, c = 1 mol/l.

Take 82 ml of HCl (3.7) and make up to 1 l with water (3.1).

3.13 Hydrochloric acid, c = 0,1 mol/l.

Take 8,2 ml of HCl (3.7) and make up to 1 l with water (3.1).

3.14 Orthophosphoric acid, c = 0,5 mol/l.

Take 34 ml of orthophosphoric acid (3.6) and make up to 1 l with water (3.1).

3.15 Concentrated tryptophan solutions (3.2), c = 0,50 g/l.

In a 500 ml volumetric flask, dissolve 0,25 g of tryptophan (3.2) (weighed to the nearest 0,1 mg) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at approximately - 18 °C for a maximum of four weeks.

3.16 Concentrated internal standard solution, c = 0,54 g/l.

In a 500 ml volumetric flask, dissolve 0,27 g of α-methyltryptophan (3.3) (weighed to the nearest 0,1 mg) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at approximately - 18 °C for a maximum of four weeks.

3.17 Calibration standard solutions of tryptophan and internal standard.

3.17.1 Calibration standard solution for the analysis of tryptophan in feeding stuffs (c = 0,010 g/l)

Take 2,00 ml of the concentrated tryptophan solution (3.15) and 2,00 ml of concentrated internal standard solution (α-methyltryptophan) (3.16). Dilute with water (3.1) and methanol (3.8) to approximately the same volume and to approximately the same concentration of methanol (10 % to 30 %) as the finished hydrolysate.

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7dad79fe-4b2-4403-8296-a53bfb742794/iso-13904-2016

© ISO 2014 – All rights reserved

This solution shall be prepared freshly before use.

Protect from direct sunlight during preparation.

3.17.2 Calibration standard solution of tryptophan for the analysis of tryptophan in commercial pure substances and premixtures containing more than 2 % of tryptophan (c = 0,010 g/l)

Take 2,00 ml of the concentrated tryptophan solution (3.15) and 2,00 ml of concentrated internal standard solution (α-methyltryptophan) (3.16).

Dilute it with hydrochloric acid 0,1 mol/l (3.13) in a 100 ml volumetric flask. Fill up to the mark.

This solution shall be prepared freshly before use

Protect from direct sunlight during preparation

NOTE For verification of the calibration standard solution it is possible to use a control solution of tryptophan. This control solution is prepared and analysed as it is described for the calibration standard solution but shall come from another manufacturer than the standard substance (3.2). The recovery of tryptophan in the control solution sample shall between 99 % to 101 %.

3.18 Ethanolamine > 98 %.

3.19 1,1,1-Trichloro-2-methyl-2-propanol solution.

Add 1 g of 1,1,1-trichloro-2-methyl-2-propanol to 100 ml of methanol (3.8).

3.20 Mobile phase for HPLC.

Dissolve 3,00 g of acetic acid in 900 ml of water (3.1) and add 50,0 ml of 1,1,1-trichloro-2-methyl-2-propanol solution (3.19). Adjust the pH to 5,00 using ethanolamine (3.18). Make up to 1 000 ml with water (3.1).

iTeh STANDARD PREVIEW
(standards.iteh.ai)
Full standard:
https://standards.iteh.ai/catalog/standards/sist/7dad79fe-fbb2-4a03-8296-a531bb72794/iso-13904-2016

4 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.1 HPLC equipment with a spectrofluorimetric detector.

4.2 Liquid chromatographic column, 125 mm × 4 mm, with C_{18}, 3 μm packing, or equivalent.

4.3 pH-meter.

4.4 Polypropylene flask, of capacity 125 ml, with wide neck and screw cap.

4.5 Membrane filter consisting of cellulose acetate (0,45 μm or 0,22 μm pore size).

4.6 Autoclave, capable of being maintained at (110 ± 2) °C, [(140 ± 10) kPa (1,4 ± 0,1) bar].

A pressure-tight covered dish that may be put into a drying oven adjustable to (110 ± 2) °C may be used.

4.7 Mechanical shaker or **magnetic stirrer**.

4.8 Vortex mixer

4.9 Glassware – filters

4.10 Graduated Erlen meyers flasks: 200 ml, 250 ml, 600ml

© ISO 2014 – All rights reserved

4.**11** **Volumetric** : 100 ml, 500 ml, 1000 ml (all class A).

5 Procedure

5.1 Preparation of samples

5.1.1 Feeding stuffs

Grind the sample to pass through a 0,5 mm sieve. Samples high in moisture shall be either air-dried at a temperature not exceeding 50 °C or freeze-dried prior to grinding. Samples with high fat content shall be extracted with light petroleum (3.9) prior to grinding.

5.1.2 Commercial pure substances and premixtures containing more than 2 % of tryptophan

Grind the sample to pass through to a 0,25 mm sieve and homogenize it well.

5.2 Determination of free tryptophan (extract)

5.2.1 Feeding stuffs

Weigh, to the nearest 1 mg, an appropriate amount (1 g to 5 g) of the prepared sample (5.1.1) into a conical flask. Add 100,0 ml of hydrochloric acid, (3.13) and 5,00 ml of concentrated internal standard solution (3.16). Shake or mix for 60 min using a mechanical shaker or a magnetic stirrer (4.7). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a beaker. Add 5 ml of orthophosphoric acid (3.14). Adjust the pH to 3,0 using sodium hydroxide (3.10). Add sufficient methanol (3.8) to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water (3.1) to a volume necessary for the chromatography [approximately the same volume as the calibration standard solution (3.17.1)].

Filter a few millilitres of the solution through a 0,45 μm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to 5.4.

Protect the standard solution and extracts against direct sunlight. If it is not possible to analyse the hydrolysates the same day, they may be stored at 5 °C for a maximum of three days

5.2.2 Commercial pure substances and premixtures containing more than 2 % of tryptophan

Weigh to the nearest of 0,1 mg, 0,5 g to 5 g of well homogenized sample (5.1.2), depending on the expected concentration of tryptophan in the sample (in accordance with Table 1) into a 1000 ml volumetric flask.

 © ISO 2014 – All rights reserved

Table 1 — Sample weight according to the expected value of free Tryptophan

Expected free Trp %	Sample weight in 1000 ml g
2 -10	5
10 - 20	2,5
20 - 40	1,25
40 - 60	0,8
60 - 80	0,6
80 - 100	0,5

Fill up to volume with 0,1 mol/l hydrochloric acid (3.13).

The mixture is stirred during 30 min on a mechanical shaker or a magnetic stirrer (4.7). Allow the particles to settle.

Transfer an aliquot of 2 ml of clear solution into a 100 ml volumetric flask. Add 2 ml of the concentrated internal standard (3.16).

Fill up to the mark with 0,1 mol/l hydrochloric acid (3.13).

This diluted test solution should have a tryptophan concentration as close as possible as the tryptophan concentration in the calibration standard solution (3.17.2) and the internal standard concentration of 0,001 g/l shall be similar to the one of the calibration standard solution (3.17.2). Refer to Annex A for example of samples preparation.

Filter a few millilitres of the solution through a 0,45 μm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to 5.4.

Protect the standard solution and extracts against direct sunlight. If it is not possible to do the analyses the same day, then the extracts shall be stored below 5 °C for not more than 3 days.

5.3 Determination of total tryptophan (hydrolysates)

Weigh, to the nearest 0,2 mg, from 0,1 g to 1 g of the prepared sample (5.1.1) into the polypropylene flask (4.4). The weighed test portion should have a nitrogen content of about 10 mg. Add 8,4 g of barium hydroxide octahydrate (3.4) and 10 ml of water (3.1). Mix on a vortex mixer (4.8) or magnetic stirrer (4.7). Leave the Teflon-coated magnet in the mixture. Wash down the walls of the vessel with 4 ml of water (3.1). Put on the screw cap and close the flask loosely. Transfer to an autoclave (4.6) which contains boiling water, and steam for 30 min to 60 min. Close the autoclave and autoclave at (110 ± 2) °C for 20 h.

Before opening the autoclave, reduce the temperature to just under 100 °C. In order to avoid crystallization of $Ba(OH)_2 \cdot 8H_2O$, add to the warm mixture 30 ml of water (3.1) which is at room temperature. Shake or stir gently. Add 2,00 ml of concentrated internal standard solution (α-methyltryptophan) (3.16). Cool the vessel in a water/ice bath for 15 min.

Then, add 5 ml of orthophosphoric acid (3.14). Keep the vessel in the cooling bath and neutralize with 6 mol/l HCl (3.11) whilst stirring and adjust the pH to 3,0 using 1 mol/l HCl (3.12). Add sufficient methanol to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water (3.1) to the defined volume necessary for the chromatography (for example 100 ml). The addition of methanol should not cause precipitation.

Filter a few millilitres of the solution through a 0,45 μm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to 5.4.

iTeh STANDARD PREVIEW (standards.iteh.ai) Full standard: https://standards.iteh.ai/catalog/standards/sist/7dad79fe-f0b8-4293-82f5-a5359b42794/iso-13904-2016

© ISO 2014 – All rights reserved