INTERNATIONAL STANDARD

ISO 12228-1

First edition
2014-07-15

Corrected version
2015-05-15

Determination of individual and total sterols contents — Gas chromatographic method —

Part 1: Animal and vegetable fats and oils

Détermination de la teneur en stérols individuels et totaux — Méthode par chromatographie en phase gazeuse —

Partie 1: Corps gras d'origines animale et végétale

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

Reference number
ISO 12228-1:2014(E)

© ISO 2014

iTeh STANDARD PREVIEW
(standards.iteh.ai)

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

COPYRIGHT PROTECTED DOCUMENT

© ISO 2014, Published in Switzerland

All rights reserved. Unless otherwise specified, no part of this publication may be reproduced or utilized otherwise in any form or by any means, electronic or mechanical, including photocopying, or posting on the internet or an intranet, without prior written permission. Permission can be requested from either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

Contents

Page

Foreword ... **iv**

1 Scope ... **1**

2 Normative references ... **1**

3 Terms and definitions ... **1**

4 Principle ... **1**

5 Reagents ... **2**

6 Apparatus ... **3**

7 Sample ... **3**
- 7.1 Sampling ... 3
- 7.2 Preparation of the test sample ... 3

8 Procedure ... **4**
- 8.1 Preparation of the aluminium oxide column ... 4
- 8.2 Test portion ... 4
- 8.3 Extraction of the unsaponifiable matter ... 4
- 8.4 Thin-layer chromatography ... 4
- 8.5 Isolation of the sterols ... 4
- 8.6 Preparation of sterol trimethylsilyl ethers ... 5
- 8.7 Gas chromatography ... 5

9 Expression of results ... **5**
- 9.1 Identification of sterols ... 5
- 9.2 Composition of sterols ... 5
- 9.3 Determination of the total sterol content ... 6

10 Precision ... **7**
- 10.1 Interlaboratory test ... 7
- 10.2 Repeatability limit, ***r*** ... 7
- 10.3 Reproducibility limit, ***R*** ... 7

11 Test report ... **7**

Annex A (informative) **Figures** ... **8**

Annex B (informative) **Interlaboratory trial** ... **15**

Bibliography ... **24**

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

© 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.

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 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*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This first edition of ISO 12228-1, together with ISO 12228-2, cancels and replaces ISO 12228:1999, which has been technically revised.

ISO 12228 consists of the following parts, under the general title *Determination of individual and total sterols content — Gas chromatographic method*:

— *Part 1: Animal and vegetable fats and oils*

— *Part 2: Olive oils and olive pomace oils*

This corrected version of ISO 12228-1:2014 incorporates the following corrections:

— In Annex B, the data in the tables have been revised and a new table for ß-Sitosterol (Table B.11) has been added.

© ISO 2014 – All rights reserved

Determination of individual and total sterols contents — Gas chromatographic method —

Part 1: Animal and vegetable fats and oils

1 Scope

This part of ISO 12228 specifies a procedure for the gas chromatographic determination of the content and composition of sterols in animal and vegetable fats and oils. However, the determination of the contents and composition of sterols in olive and olive pomace oils is to be carried out using ISO 12228-2.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

iTeh STANDARD PREVIEW
(standards.iteh.ai)

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

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

3 Terms and definitions

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

3.1
composition of sterols
composition of individual sterols in the sample, beginning with cholesterol and ending with Δ7-avenasterol (see Table 1) under the conditions specified in this part of ISO 12228

Note 1 to entry: The composition is expressed as a percentage of all peak areas, normalized to 100 %.

3.2
total sterol content
mass fraction of the sum of all individual sterols, as determined in accordance with the method specified in this part of ISO 12228, beginning with cholesterol and ending with Δ7-avenasterol (see Table 1), divided by the mass of the test portion

Note 1 to entry: The content is expressed in milligrams per kilogram.

4 Principle

A test portion is saponified by boiling under reflux with an ethanolic potassium hydroxide solution. The unsaponifiable matter is isolated by solid-phase extraction on an aluminium oxide column. The aluminium oxide column is used to retain the fatty acid anions; sterols pass through the column. The sterol fraction from the unsaponifiable matter is separated by thin-layer chromatography. The qualitative and quantitative compositions of the sterol fraction are determined by gas chromatography using cholestanol or betulin as the internal standard.

© ISO 2014 – All rights reserved

5 Reagents

WARNING — Attention is drawn to the regulations which specify the handling of hazardous substances. Technical, organizational, and personal safety measures shall be followed.

Use only reagents of recognized analytical grade, unless otherwise stated, and water complying with grade 3 of ISO 3696[1].

5.1 Potassium hydroxide (KOH), ethanolic solution, molar concentration *c*(KOH) approximately 0,5 mol/l.

Dissolve 3 g of potassium hydroxide in 5 ml of water and dilute to 100 ml with ethanol (5.3). The solution should be colourless or straw-coloured.

5.2 Internal standard solution, cholestanol (5α-cholestan-3β-ol) or betulin, volume fraction of 1,0 mg/ml solution in ethanol (see note to 5.10).

NOTE In case of hydrogenated oils, which may contain cholestanol, the use of betulin (peak 17 in Table 1) is recommended.

5.3 Ethanol, of minimum volume fraction φ = 95 %.

5.4 Aluminium oxide, neutral, particle size 0,063 mm to 0,200 mm, activity grade I (water content = 0 %).

iTeh STANDARD PREVIEW

5.5 Diethyl ether, freshly distilled, free from peroxides and residue.

(standards.iteh.ai)

WARNING — Diethyl ether is highly flammable and can form explosive peroxides. Explosive limits in air are 1,7 % to 48 % (volume fraction). Take special precautions when using it. Keep away from heat sources and sunlight.

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

5.6 Silica gel thin-layer chromatography (TLC) plates, commercially available, dimensions 20 cm × 20 cm, thickness of layer 0,25 mm.

5.7 Developing solvent, hexane/diethyl ether.

Volume fraction of each solvent is 50 ml/100 ml.

5.8 Standard solution for thin-layer chromatography, volume fraction of 1,0 mg/ml cholesterol/cholestanol in acetone or 5,0 mg/ml betulin in acetone.

NOTE 1 Cholesterol and cholestanol have the same Rf value (0,35) in TLC while the Rf value for betulin is 0,30 (see Figure A.1).

NOTE 2 In case of hydrogenated oils, which may contain cholestanol, the use of betulin (peak 17 in Table 1) is recommended.

5.9 Spraying reagent, methanol.

5.10 Silylating reagent, prepared by adding 50 µl of 1-methyl imidazole to 1 ml of N-methyl-N-(trimethylsilyl)-hepta-fluorobutyramide (MSHFBA).

NOTE Ready-to-use solutions are commercially available. Other silylation reagents, e.g. bis trimethylsilyl trifluoroacetamide with 1 % trimethylchlorosilane, are also available and can be used when cholestanol is used as internal standard. However for betulin special precautions are taken to ensure that both hydroxyl groups of betulin are silylated. If not, betulin may show two peaks in the chromatogram.

 © ISO 2014 – All rights reserved

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Round-bottomed flasks, of 25 ml and 50 ml capacity, with ground neck.

6.2 Reflux condenser, with ground glass joint to fit a flask (6.1).

6.3 Glass column, with polytetrafluoroethylene (PTFE) stopper, sintered glass filter and reservoir for 100 ml, length 25 cm, internal diameter 1,5 cm.

6.4 Rotary evaporator, attached to a vacuum pump and water bath maintained at 40 °C.

6.5 Developing tank, made of glass, with a ground glass lid, suitable for use with plates of dimensions 20 cm × 20 cm.

6.6 Microsyringe, to deliver 100 µl.

6.7 Oven, maintained at 105 °C ± 3 °C.

6.8 Desiccator, containing an efficient desiccant, for storing the plates.

6.9 Reaction vials, of 0,3 ml to (1,0 to 1,5) ml capacity, with screw caps and PTFE-lined seals, for preparation of sterol derivatives.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

6.10 Gas chromatograph, for capillary columns, with split injector, flame ionization detector and suitable recorder.

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

6.11 Capillary column, made of fused silica or glass, length 25 m to 60 m, internal diameter 0,2 mm to 0,25 mm, stationary phase SE-54 (or equivalent non-polar phase with a temperature limit of at least 280 °C to 300 °C); film thickness about 0,1 µm.

NOTE A better resolution of the peaks is obtained with a film thickness of 0,1 µm.

6.12 Microsyringe for gas chromatography, for injecting volumes of 1 µl.

6.13 Analytical balance, capable of weighing to the nearest 0,001 g and displaying 0,000 1 g.

7 Sample

7.1 Sampling

Sampling is not part of the method specified in this part of ISO 12228. A recommended sampling method is given in ISO 5555[1].

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

7.2 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

© ISO 2014 – All rights reserved

8 Procedure

8.1 Preparation of the aluminium oxide column

Suspend 10 g of aluminium oxide (5.4) in 20 ml of ethanol (5.3) and pour the slurry into the glass column (6.3). Allow the aluminium oxide to settle and let the solvent run out of the column until the level of the solvent reaches the top level of the aluminium oxide layer.

8.2 Test portion

Weigh, to the nearest 1 mg, about 250 mg of the test sample into a 25 ml flask (6.1), proceed with 8.3.

For fats and oils with a low sterol content (for example less than 2 000 mg per kilogram) or for other reasons, proceed using a threefold amount of the test sample. Adjust reagents and apparatus accordingly.

8.3 Extraction of the unsaponifiable matter

Add exactly 1,00 ml of internal standard solution (5.2) to the test portion (8.2). Add 5 ml of ethanolic potassium hydroxide solution (5.1) and a few anti-bumping granules. Attach the reflux condenser (6.2) to the flask and keep the contents gently boiling for 15 min. Stop heating. Immediately dilute the contents of the flask while still hot with 5 ml of ethanol (5.3) and swirl or shake to homogenize.

Pipette 5 ml of this solution onto the prepared aluminium oxide column (8.1). Collect the eluate in a 50 ml round-bottom flask (6.1) and allow the column to run off until the solvent level has reached the top level of the aluminium oxide layer. Elute the unsaponifiable matter first with 5 ml of ethanol (5.3) and then with 30 ml of diethyl ether (5.5), with a flow rate of about 2 ml/min. Remove the solvents from the flask by means of the rotary evaporator (6.4).

iTeh STANDARD PREVIEW
(standards.iteh.ai)

WARNING — The aluminium oxide column is essential for this procedure. It shall not be replaced by silica or other columns or by solvent extraction.

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

8.4 Thin-layer chromatography

Dissolve the unsaponifiable matter obtained in 8.3 in a small amount (approximately 0,5 ml) of diethyl ether (5.5). Apply the solution as a line at a distance of 2 cm from the lower edge onto a TLC plate (5.6) using the microsyringe (6.6). Leave a gap of at least 3 cm from each side edge of the plate. Apply a spot of 5 µl of the TLC standard solution (5.8) at 1,5 cm from the edge. Fill the developing tank (6.5) with about 100 ml of developing solvent (5.7). Place the plate into the tank and develop it until the solvent reaches the upper edge. Remove the plate from the tank and allow the solvent to evaporate in a fume cupboard.

NOTE Quantitative transfer of the material (8.3) to the TLC plate is not necessary in this step. Automatic apparatus for applying streaks may be used. Saturation of the chamber is not required.

8.5 Isolation of the sterols

Spray the plates with methanol (5.9) until the sterol (and betulin) zones appear white on a translucent (darker) background. The cholestanol is part of the Δ5-sterol zone (see Figure A.1). Mark the zones at the height of the standard spots 2 mm above and 4 mm below the visible zones (see Figure A.1). Scratch off this part of the layer completely using a spatula and quantitatively collect the silica in a small beaker.

NOTE 1 The wider margin at the lower edge of the visible zones (4 mm vs. 2 mm at the upper edge) is a precaution to avoid partial loss of betulin in this step. Sunflower seed oil may show three bands (Δ5-sterols, Δ7-sterols and betulin).

NOTE 2 Betulin, if used as internal standard, appears slightly below the sterol zone (see Figure A.1).

Add 0,5 ml of ethanol to the collected silica gel. Extract the silica gel in the beaker three times with 5 ml of diethyl ether (5.5) and filter into a flask (6.1). Reduce the combined ether extracts to about 1 ml in

 © ISO 2014 – All rights reserved

the rotary evaporator (6.4) and transfer the remaining solution into the reaction vial (6.9). Blow off the solvent in the reaction vial with a stream of nitrogen.

8.6 Preparation of sterol trimethylsilyl ethers

Add 100 µl of the silylation reagent (5.10) to the reaction vial (6.9) containing the isolated sterols. Seal and heat the vial for 15 min in the oven set at (105 ± 3) °C. Allow the reaction vial to cool to room temperature and inject the solution directly into the gas chromatograph (6.10).

8.7 Gas chromatography

Optimize the temperature programme and the carrier gas flow rate so that chromatograms similar to Figures A.2 to A.7 are obtained. Test the separation with silylated sterol fractions obtained from known oils, as shown in Figures A.2 to A.7.

NOTE 1 The following parameters were tested and found useful (see chromatograms in Annex A): GC column: SE-54, 50 m length, 0,25 mm internal diameter, 0,10 µm film thickness; carrier gas H_2, carrier gas flow rate 36 cm/s, split 1:20, detector/injector 320° C, temperature programme 245 °C to 265 °C at 5 °C/min, 40 min isothermal at 265 °C; injection volume 1 µl. Capillary columns of equivalent quality can be used.

NOTE 2 A standard solution containing cholesterol, campesterol, stigmasterol, and sitosterol may be used to check the retention times. Use a blank run to test for possible contamination (e.g. cholesterol) from solvents, glass walls, filter, fingerprints, etc.

9 Expression of results

iTeh STANDARD PREVIEW
(standards.iteh.ai)

9.1 Identification of sterols

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e550b0-0f30-41d0-9958-0f079e6974b0/iso-12228-1-2014

To identify the sterols present in the test sample, determine the relative retention times (RRT) by dividing the retention time (RT) of the sterol in question by the RT of cholesterol and/or betulin. Table 1 shows the RRT of the various sterols corresponding to cholesterol (RRT_C) and betulin (RRT_B), with SE-54 stationary phase.

NOTE The RRT in Table 1 (determined under the conditions of note 1 in 8.7) are mentioned as an aid for the identification of the individual sterols only, and to illustrate the elution sequence (cf. also Figure A.1). The actual RRT found may deviate slightly from the RRT given in Table 1 because the RRT depends on the experimental conditions (type and length of GLC column, temperature programme, and quality of stationary phase).

9.2 Composition of sterols

Calculate the mass fraction w_i, of the individual sterol i, in g/100 g (percent), according to the following equation:

$$w_i = \frac{A_i}{\sum A} \cdot 100 \quad (1)$$

where

A_i is the area of the peak of sterol i;

$\sum A$ is the sum of the peak areas of all sterols (peaks 1, 3 to 16 or 1 to 16, if betulin is used).

© ISO 2014 – All rights reserved

Table 1 — Gas chromatograhic peak identification of individual sterols and betulin by RRT (SE-54 stationary phase)<Tbl_large></Tbl_large>

Peak No	Common names of sterols	Systematic names of sterols	RRT$_C$	RRT$_B$
1	Cholesterol	Cholest-5-en-3β-ol	1,00	0,44
2	Cholestanol	5α-Cholestan-3β-ol	1,02	0,45
3	Brassicasterol	[24S]-24-Methyl cholesta-5,22-dien-3β-ol	1,09	0,48
4	24-Methylene cholesterol	24-Methylene cholesta-5,24-dien-3β-ol	1,21	0,53
5	Campesterol	[24R]-24-Methyl cholest-5-en-3β-ol	1,23	0,54
6	Campestanol	[24R]-24-Methyl cholestan-3β-ol	1,25	0,55
7	Stigmasterol	[24S]-24-Ethyl cholesta-5,22-dien-3β-ol	1,31	0,57
8	Δ7-Campesterol	[24R]-24-Methyl cholest-7-en-3β-ol	1,38	0,59
9	Δ5,23-Stigmastadienol	[24R,S]-24-Ethyl cholesta-5,23-dien-3β-ol	1,40	0,60
10	Clerosterol	[24S]-24-Ethyl cholesta-5,25-dien-3β-ol	1,42	0,62
11	Sitosterol	[24R]-24-Ethyl cholest-5-en-3β-ol	1,47	0,64
12	Sitostanol	[24R]-24-Ethyl cholestan-3β-ol	1,50	0,65
13	Δ5-Avenasterol	[24Z]-24(28)-Ethylidene cholest-5-en-3β-ol	1,52	0,66
14	Δ5,24-Stigmastadienol	[24R,S]-24-Ethyl cholesta-5,24-dien-3β-ol	1,59	0,69
15	Δ7-Stigmastenol	[24R,S]-24-Ethyl cholest-7-en-3β-ol	1,65	0,72
16	Δ7-Avenasterol	[24Z]-24(28)-Ethylidene cholest-7-en-3β-ol	1,70	0,74
X	(Erythrodiol)		2,03	0,88
Y	(Uvaol)		2,17	0,95
17	Betulin	Lup-20[29]-ene-3β,28-diol	2,30	1,00

RRT$_C$: relative retention time based on cholesterol = 1,00

RRT$_B$: relative retention time based on betulin = 1,00

NOTE Sitosterol may coelute together with α-spinasterol and Δ7,22,25-stigmastatrienol. [24R]-24-Ethyl cholesta-7,25(27)-dien-3β-ol is present in sterols of sunflower and pumpkin seed oil and may coelute with peak 14 (Δ5,24- stigmastadienol).

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b0/iso-12228-1-2014

9.3 Determination of the total sterol content

For the purposes of this method, it is assumed that the response factors of all sterols and of betulin are equal.

NOTE In several tests silylated sterols and silylated betulin in equal amounts gave the same detector response using an FID detector under these conditions.

 © ISO 2014 – All rights reserved

Calculate the total sterol content w, in milligrams per kilogram of fat, according to Formula (2):

$$w = \frac{\sum (A) \cdot m_{IS} \cdot 1\,000}{A_{IS} \cdot m} \tag{2}$$

where

m_{IS} is the mass of the internal standard (cholestanol) in milligrams;

$\sum$ (A) is the sum of the peak areas of all sterols (peaks 1, 3 to 16 or 1 to 16, if betulin is used);

A_{IS} is the peak area of the internal standard;

m is the mass of the test sample, in grams.

For calculation of the total sterol content, consider all peaks of sterols beginning with cholesterol and ending with Δ7-avenasterol (peak 16), but without erythrodiol and uvaol (peaks X and Y).

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

10.2 Repeatability limit, *r*

ISO 12228-1:2014
https://standards.iteh.ai/catalog/standards/sist/80e55fc8-9130-41d8-9958-0f079a8974b9/iso-12228-1-2014

The repeatability limit (r) is the value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be with a probability of 95 %.

Repeatability conditions are conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.

10.3 Reproducibility limit, *R*

The reproducibility limit (R) is the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95 %.

Reproducibility conditions are conditions where independent test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment within short intervals of time.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

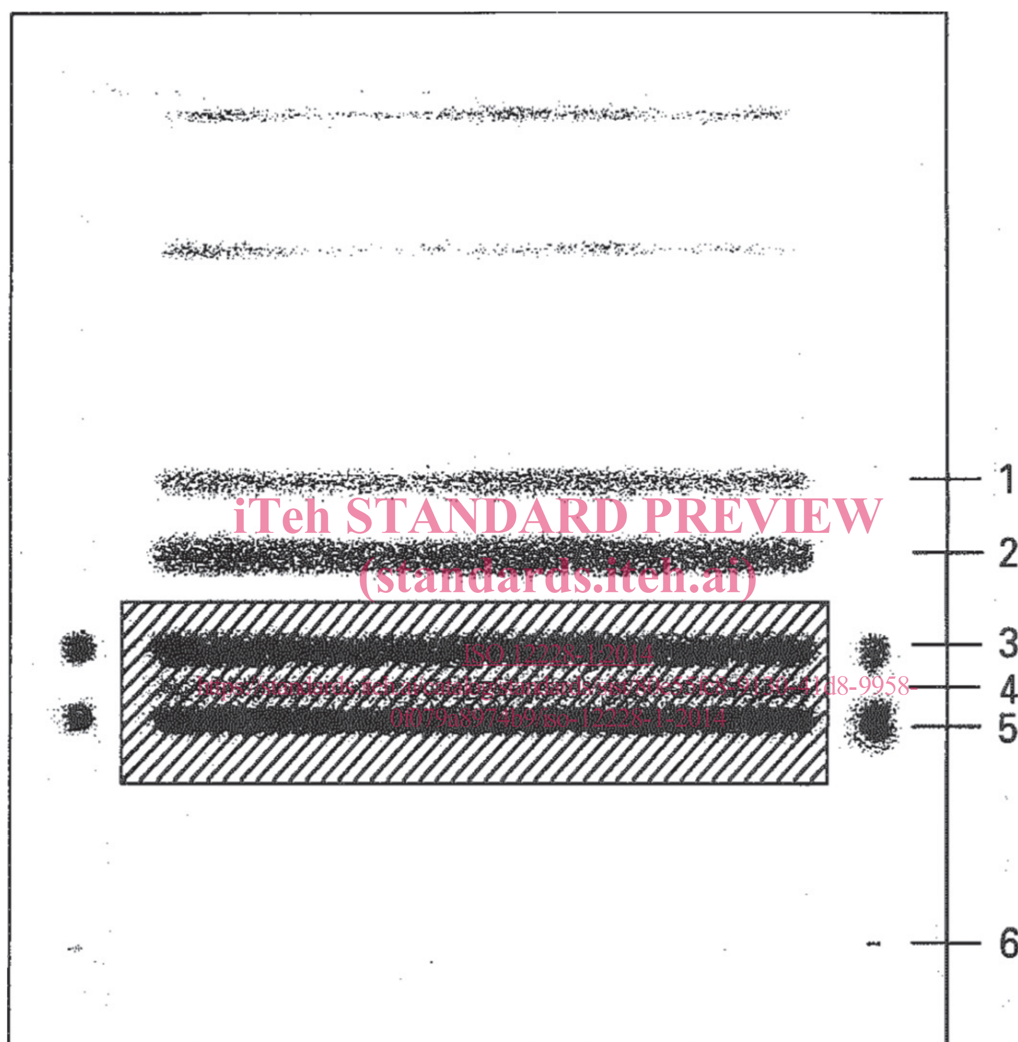
c) the test method used, together with reference to this part of ISO 12228 (i.e. ISO 12228-1);

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained; or if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Figures



iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 12228-1:2014

Key

1 triterpenes

2 methyl sterols

3 cholestanol and Δ5-sterols

4 Δ7-sterols

5 betulin

6 start

NOTE Zones appear white on a transparent background. The hatched area is scratched off; note wider margin at the bottom (4 mm vs. 2 mm at the top). Rf values of the bands: betulin 0,30; Δ7-sterols 0,33; Δ5-sterols 0,35; methyl sterols 0,45; triterpenes 0,53.

Figure A.1 — TLC isolation of sterols from unsaponifiable matter (steps 8.3 and 8.4)

 © ISO 2014 – All rights reserved