INTERNATIONAL STANDARD

ISO 5765-2

IDF 79-2

First edition
2002-09-01

Dried milk, dried ice-mixes and processed cheese — Determination of lactose content —

Part 2: Enzymatic method utilizing the galactose moiety of the lactose

Lait sec, mélanges secs pour crèmes glacées et fromages fondus — Détermination de la teneur en lactose —

Partie 2: Méthode enzymatique par la voie galactose

Reference numbers
ISO 5765-2:2002(E)
IDF 79-2:2002(E)

© ISO and IDF 2002

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS

Not for Resale

PDF disclaimer

This PDF file may contain embedded typefaces. In accordance with Adobe's licensing policy, this file may be printed or viewed but shall not be edited unless the typefaces which are embedded are licensed to and installed on the computer performing the editing. In downloading this file, parties accept therein the responsibility of not infringing Adobe's licensing policy. Neither the ISO Central Secretariat nor the IDF accepts any liability in this area.

Adobe is a trademark of Adobe Systems Incorporated.

Details of the software products used to create this PDF file can be found in the General Info relative to the file; the PDF-creation parameters were optimized for printing. Every care has been taken to ensure that the file is suitable for use by ISO member bodies and IDF national committees. In the unlikely event that a problem relating to it is found, please inform the ISO Central Secretariat at the address given below.

© ISO and IDF 2002

All rights reserved. Unless otherwise specified, no part of this publication may be reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying and microfilm, without permission in writing from either ISO or IDF at the respective address below.

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

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

Printed in Switzerland

--`,,`,-`-`,,`,,`,`,,`---

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS

Not for Resale

Contents

Foreword iv
Foreword v
Introduction vi
1 Scope 1
2 Terms and definitions 1
3 Principle 1
4 Reagents 1
5 Apparatus 3
6 Sampling 4
7 Procedure 4
7.1 Test to check the procedure 4
7.2 Preparation of test sample 4
7.3 Test portion 5
7.4 Reagent blank test 5
7.5 Deproteination 5
7.6 Determination 6
8 Calculation and expression of results 7
8.1 Calculation 7
8.2 Expression of results 8
9 Precision 8
9.1 Interlaboratory trial 8
9.2 Repeatability 8
9.3 Reproducibility 8
10 Test report 9
Annex A (normative) Good Laboratory Practice (GLP) rules for the performance of enzymatic analyses 10
Bibliography 14

© ISO 2002 – All rights reserved

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

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 3.

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 part of ISO 5765|IDF 79 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 5765-2|IDF 79-2 was prepared by Technical Committee ISO/TC 34, *Food Products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

ISO 5765|IDF 79 consists of the following parts, under the general title *Dried milk, dried ice-mixes and processed cheese — Determination of lactose content*:

— *Part 1: Enzymatic method utilizing the glucose moiety of the lactose*

— *Part 2: Enzymatic method utilizing the galactose moiety of the lactose*

Annex A forms a normative part of this part of ISO 5765|IDF 79.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of National Committees casting a vote.

ISO 5765-2|IDF 79-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

ISO 5765|IDF 79 consists of the following parts, under the general title *Dried milk, dried ice-mixes and processed cheese — Determination of lactose content*:

— *Part 1: Enzymatic method utilizing the glucose moiety of the lactose*

— *Part 2: Enzymatic method utilizing the galactose moiety of the lactose*

All work was carried out by the Joint ISO/IDF/AOAC Action Team *Lactose and lactate determination*, of the Standing Committee on *Main components of milk*, under the aegis of its project leader, Mr J. Labrijn (NL).

© ISO 2002 – All rights reserved

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Introduction

This part of ISO 5765|IDF 79 describes the enzymatic method for the determination of lactose utilizing the galactose moiety of the lactose. It is complementary to ISO 5765-1|IDF 79-1 which utilizes the glucose moiety of the lactose.

The choice of whether to use the method described in part 1 or part 2 of ISO 5765|IDF 79 depends from the amount of glucose or galactose present in the sample to be analysed. If the glucose content of a sample is considerably higher than its lactose content, it is recommended to use the method described in this part of ISO 5765|IDF 79. Conversely, for a sample with a considerably higher galactose content than its lactose content, it is recommended to use the method described in ISO 5765-1|IDF 79-1.

For samples with a low content of both glucose and galactose, either method may be used without preference. For samples with a high content of both glucose and galactose, the accuracy of the lactose determination is considerably reduced for both methods.

In heat-treated milk and milk products, a proportion of lactose may have been converted to lactulose. Lactulose cannot be determined by applying the method described in ISO 5765-1|IDF 79-1. If, however, the method in this part of ISO 5765|IDF 79 is applied, the lactulose will partially be determined as lactose. Moreover, in intensively heat-treated milk (e.g. sterilized milk) or milk products, a proportion of the lactose may be bound to protein because of a Maillard reaction. In such cases the bound lactose cannot be determined by the method described either in part of ISO 5765|IDF 79.

Only when the good laboratory practice (GLP) rules for enzymatic analyses have been applied strictly, will reliable results be obtained. The GLP rules are stated in annex A.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Dried milk, dried ice-mixes and processed cheese — Determination of lactose content —

Part 2:
Enzymatic method utilizing the galactose moiety of the lactose

1 Scope

This part of ISO 5765|IDF 79 specifies an enzymatic method for the determination of the lactose content of all types of dried milk, of ice-mixes in dry form in the presence of other carbohydrates and reducing substances, and of processed cheese.

2 Terms and definitions

For the purposes of this part of ISO 5765|IDF 79, the following term and definition applies.

2.1
lactose content
mass fraction of substances determined by the procedure in this part of ISO 5765|IDF 79

NOTE The lactose content is expressed as a percentage by mass.

3 Principle

3.1 A solution or suspension of the test portion is deproteinated to obtain a pure extract.

3.2 The purified extract of the test portion is reacted with the following enzymes and biochemical substances:

a) β-galactosidase, to split the lactose into glucose and galactose;

b) β-galactosidase dehydrogenase in the presence of nicotinamide adenine dinucleotide phosphate (NAD^+) to catalyse the oxidation of galactose into galactonic acid, the NAD^+ being reduced to NADH.

3.3 The amount of NADH is determined from the absorbance of the test solution at 340 nm.

3.4 The lactose content is calculated, which is proportional to the amount of NADH if a correction is made for the galactose present in the test sample at the start of the analysis.

4 Reagents

Use only reagents of recognized analytical grade unless otherwise specified. The water used for the preparation of the enzyme solutions shall be of at least doubly glass-distilled purity. The water used for other purposes shall be glass-distilled or of at least equivalent purity.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Take note of the production and expiry dates of the reagents given by the manufacturer.

If an enzyme suspension is applied with other than the prescribed activity, the volume of the suspension as stated in the pipetting scheme (7.6.1) should be increased or decreased proportionally.

NOTE The reagents described in 4.4 and in 4.6 to 4.8 inclusive may be obtained commercially as a test combination, for example, Boehringer test kit. [1)]

4.1 Potassium hexacyanoferrate(II) solution, $K_4 [Fe(CN)_6]$

Dissolve 3,6 g of potassium hexacyanoferrate(II) trihydrate in water. Dilute with water to 100 ml and mix.

4.2 Zinc sulfate solution, $ZnSO_4$

Dissolve 7,2 g of zinc sulfate heptahydrate in water. Dilute with water to 100 ml and mix.

4.3 Sodium hydroxide solution, c(NaOH) = 0,1 mol/l

Dissolve 4,0 g of sodium hydroxide in water. Dilute with water to 1 000 ml and mix.

4.4 Citrate buffer solution, pH 6,6 ± 0,1

Dissolve 2,8 g of trisodium citrate dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$), 0,042 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), and 0,625 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in about 40 ml of water.

Adjust the pH to 6,6 ± 0,1 at 20 °C with sulfuric acid (2 mol/l) or sodium hydroxide solution (0,1 mol/l). Dilute with water to 50 ml and mix.

This solution may be kept for 3 months if stored in a refrigerator set at between 0 °C and 5 °C.

4.5 Phosphate (KH_2PO_4) buffer solution, pH 8,6 ± 0,1

Dissolve 16,6 g of potassium dihydrogen phosphate in about 80 ml of water. Adjust the pH to 8,6 ± 0,1 at 20 °C with sodium hydroxide solution (1 mol/l). Dilute with water to 100 ml and mix.

This solution may be kept for 8 weeks if stored in a refrigerator set at between 0 °C and 5 °C.

4.6 NAD^+/citrate buffer solution

Dissolve 25 mg of nicotinamide adenine dinucleotide ($C_{21}H_{27}N_7O_{17}P_2$; approximately 98 % assay) in 5 ml of citrate buffer solution (4.4).

This solution may be kept for 3 weeks if stored in a refrigerator set at between 0 °C and 5 °C.

4.7 β-Galactosidase suspension (from *Escherichia coli*), suspension in 3,2 mol/l ammonium sulfate solution, of pH 6 ± 0,1.

The activity of the suspension of β-galactosidase (EC 3.2.1.23) [5] shall be at least 60 units/ml (lactose as substrate, at 25 °C). The suspension may be kept for about 12 months if stored in a refrigerator set at between 0 °C and 5 °C. When using the suspension, the vessel containing the suspension shall be kept immersed in crushed ice.

NOTE A β-galactosidase suspension which contains not more than 0,001 % each of β-fructosidase, α-galactosidase, glucose dehydrogenase, α-glucosidase and NADH-oxidase, calculated in terms of the specific activity of β-galactosidase, has been found to be suitable.

---

1) Boehringer test kit is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 5765|IDF 79 and does not constitute an endorsement by ISO or IDF of this product.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

4.8 β-Galactose dehydrogenase suspension (from *Pseudomonas fluorescens*), suspension in 3,2 mol/l of ammonium sulfate solution, of pH 6 ± 0,1.

The activity of the suspension of β-galactosidase dehydrogenase (EC 1.1.1.48) [5] shall be at least 8 units/ml.

The suspension may be kept for at least 6 months if stored in a refrigerator set at between 0 °C and 5 °C. When using the suspension, the vessel containing the suspension shall be kept immersed in crushed ice.

NOTE A β-galactosidase dehydrogenase suspension which contains not more than 0,01 % each of alcohol dehydrogenase and β-galactosidase, not more than 0,1 % of NADH-oxidase and glucose-reacting enzymes, and not more than 0,5 % of lactate dehydrogenase has been found to be suitable.

4.9 Lactose standard solution, $c(C_{12}H_{22}O_{11} \cdot H_2O)$ = 0,8 mg/ml.

Before use, dry the lactose monohydrate to a constant mass in a drying oven set at 87 °C.

Dissolve 400 mg of dried lactose monohydrate in water. Dilute with water to 500 ml and mix. The solution may be kept for 2 days if stored in a refrigerator set at between 0 °C and 5 °C. Warm the solution to about 20 °C just before use.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to the nearest 1 mg and a readability to 0,1 mg.

5.2 Glass beakers, of capacities 50 ml, 100 ml and 250 ml.

5.3 Graduated pipettes, capable of delivering 5 ml and 10 ml, graduated in 0,1 ml divisions.

5.4 Pipettes, capable of delivering 10 ml, 5 ml, 1 ml, 0,2 ml, and 0,05 ml.

5.5 One-mark volumetric flasks, of capacity 100 ml.

5.6 Filter paper, medium grade, of diameter about 15 cm.

5.7 Filter funnels, of diameter about 7 cm.

5.8 Spectrometer, suitable for measuring at 340 nm, equipped with cells of optical path length 1 cm.

5.9 Plastic paddles, suitable for mixing the sample/enzyme mixture in the spectrometer cells.

5.10 Glass rods, of diameter approximately 6 mm and length 150 mm, for macerating the sample.

5.11 Water bath, capable of being maintained at between 20 °C and 25 °C, with rack suitable for holding the spectrometer cells (5.8) (optional; see 7.6).

NOTE Incubation of the cells in the water bath is only necessary if the room temperature is below 20 °C.

5.12 Blending apparatus, suitable for preparing suspensions of test portions of processed cheese (e.g. Ultra Turrax[2)]).

5.13 Drying oven, thermostatically controlled, capable of maintaining a temperature of 87 °C ± 2 °C.

5.14 Grinding or grating device, capable of grinding or grating cheese and of being easily cleaned.

---

2) Ultra Turrax is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 5765|IDF 79 and does not constitute an endorsement by ISO or IDF of this product.

© ISO 2002 – All rights reserved

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

6 Sampling

Sampling is not part of the method specified in this part of ISO 5765|IDF 79. A recommended sampling method is given in ISO 707.

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

7 Procedure

7.1 Test to check the procedure

7.1.1 Carry out the following test to check the recovery of lactose if one or more of the following conditions apply:

a) if using a new batch of the NAD^+/citrate buffer solution (4.6), the β-galactosidase suspension (4.7) or the β-galactosidase dehydrogenase suspension (4.8),

b) if the NAD^+/citrate buffer solution (4.6) and/or the β-galactosidase suspension (4.7) and/or the β-galactosidase dehydrogenase suspension (4.8) have been stored in a refrigerator for more than 2 weeks without being used;

c) if restarting the analytical work after a period of analytical inactivity;

d) if circumstances justify carrying out such a test.

7.1.2 Pipette 5,0 ml and 10,0 ml respectively of the lactose standard solution (4.9) in each of two 100 ml volumetric flasks (5.5). Add about 50 ml of water to each flask. Proceed as specified in 7.5 and 7.6.

7.1.3 Calculate the lactose monohydrate content of the lactose standard solution (4.9) according to equation (3) (see 8.1), but using the following values:

V_3 is the volume of the lactose standard solution (4.9), V_3 = 500 ml;

V_4 is the volume of the lactose standard solution used (7.1.2), V_4 = 5 ml and 10 ml respectively;

V_5 is the total volume of the diluted lactose standard solution (7.1.2), V_5 = 100 ml.

7.1.4 Taking into account the purity of the lactose monohydrate, the recovery obtained for both dilutions (7.1.2) shall be within the range 100 % ± 2 %.

If the recovery is not within this range, check the reagents, the operating technique, the accuracy of the pipettes and the condition of the spectrometer. Take the required action to obtain appropriate results. Repeat the test to check the procedure until satisfactory test results are obtained.

7.2 Preparation of test sample

7.2.1 Dried milk and dried ice-mixes

Transfer the test sample to a container, provided with an airtight lid, of capacity about twice the volume of the sample. Close the container immediately. Mix the sample thoroughly by repeatedly shaking and inverting the container.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

7.2.2 Processed cheese

Remove the rind, smear or mouldy layer of the cheese so as to provide a representative test sample of the cheese as it is usually consumed. Grind or grate the test sample using an appropriate grinding or grating device (5.14). Mix the ground or grated mass quickly and, if possible, grind or grate a second time. Again mix thoroughly. If the test sample cannot be ground or grated, mix it thoroughly by intensive stirring and kneading.

If delay is unavoidable, transfer the test sample to a container, provided with an airtight lid, of capacity about twice the volume of the sample to await analyses. Close the container immediately. Take all precautions to ensure proper preservation of the test sample and to prevent condensation of moisture on the inside surface of the container.

As soon as possible after grinding, grating or stirring and kneading or after the forced delay, transfer the test sample to a 250 ml glass beaker (5.2). Add the same amount of water and form a thorough suspension of the mixture with the blending apparatus (5.12).

7.3 Test portion

Weigh, to the nearest 1 mg, 1 g or more (see below) of the test sample (7.2.1) or test sample suspension (7.2.2) in a 100 ml beaker (5.2). Dissolve or suspend the test portion in at least 20 ml of water preheated to between 40 °C and 50 °C, using a glass rod (5.10) or blending apparatus (5.12) respectively. Transfer the contents of the beaker quantitatively to a 100 ml volumetric flask (5.5). Dilute with water to approximately 60 ml and mix.

Consider the following facts when determining the mass of the test portion to be taken:

- the test portion should be representative of the complete test sample;
- the content of lactose in the spectrometer cell should preferably be between 5 µg and 100 µg;
- the absorbance (A_2) of the solution in the spectrometer cell, for galactose in the test sample (see 8.1), should lie between 0,1 and 0,4;
- if the mass fraction of lactose in the sample is less than 0,2 %, more than 1 g of test portion will be required. In that case, the volume of fat, protein and other substances precipitated in 7.5.1 can have a significant influence on the volume of the solution (see V_3 in 8.1).

7.4 Reagent blank test

Carry out a blank test in duplicate. Proceed as specified in 7.5 and 7.6 by using all reagents but omitting the test portion.

7.5 Deproteination

7.5.1 Add, in the following order, to the test solution or suspension (7.3) in the 100 ml one-mark volumetric flask:

- 5,0 ml of potassium hexacyanoferrate(II) solution (4.1),
- 5,0 ml of zinc sulfate solution (4.2), and
- 10,0 ml of sodium hydroxide solution (4.3), while swirling thoroughly after each addition.

Dilute with water to 100 ml mark and mix thoroughly.

Allow the mixture to stand for 30 min. Do not remix the contents of the volumetric flask prior to filtration.

7.5.2 Filter the supernatant liquid through a filter paper (5.6), discarding the first fraction of the filtrate.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

7.6 Determination

7.6.1 Scheme for procedure

Carry out the determination according to the scheme in Table 1, taking care to bring the NAD+/citrate buffer solution (4.4), the phosphate buffer solution (4.5) and the water to room temperature (20 °C to 25 °C) just before use.

Table 1 — Determination scheme

	Sample or standard test for		**Reagent blank test for**	
	lactose and galactose	**galactose**	**lactose and galactose**	**galactose**
Pipette into the spectrometer cells:				
NAD+/citrate buffer solution (4.4)	0,20 ml	0,20 ml	0,20 ml	0,20 ml
β-Galactosidase suspension (4.7)	0,05 ml	—	0,05 ml	—
Water	—	0,05 ml	—	0,05 ml
Sample or standard filtrate (7.5.2)	1,00 ml	1,00 ml	—	—
Reagent blank filtrate (7.5.2)	—	—	1,00 ml	1,00 ml
Mix the contents of the spectrometric cells, using the plastic paddles (5.9), and incubate for 15 min at a temperature above 20 °C, using the water bath (5.11), if necessary.				
Then add to the spectrometer cells using a pipette (5.4):				
Phosphate buffer solution (4.5)	1,00 ml	1,00 ml	1,00 ml	1,00 ml
Water	1,00 ml	1,00 ml	1,00 ml	1,00 ml
Mix the contents of the spectrometric cells. Two minutes after mixing, measure the absorbance, A_0, of the solution in each cell against air at a wavelength of 340 nm.				
Then add the following to the spectrometer cells using a pipette (5.4):				
β-Galactosidase dehydrogenase suspension (4.8)	0,05 ml	0,05 ml	0,05 ml	0,05 ml
Mix the contents of the spectrometric cells and incubate for 15 min at a temperature above 20 °C, using the water bath (5.11), if necessary. Measure the absorbance, A_{15}, of the solution in each cell against air.				
After a further 5 min, measure the absorbance of each of the solutions again. If the reaction has not stopped, continue to read the absorbance of each of the solutions at 5-min intervals until the absorbance increase remains constant over successive 5-min intervals.				

7.6.2 Calculation of the absorbance

7.6.2.1 If no increase of the absorbance has occurred after 15 min of incubation, calculate the absorbance, A, of each cell content to be used for the calculation (see 8.1) using the following equation:

$$A = A_t - A_0 \quad (1)$$

where

A_0 is the numerical value of the absorbance, measured before the addition of the β-galactosidase dehydrogenase suspension;

A_t is the numerical value of the absorbance, measured after the incubation time of 15 min.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

7.6.2.2 If the reaction has not stopped after 15 min, calculate the absorbance, A, of each cell content to be used for the calculation (see 8.1) using the following equation:

$$A = (A_t - A_0) - \frac{t}{5}(A_t - A_{t-5}) \quad (2)$$

where

A_0 is the numerical value of the absorbance, measured before the addition of the β-galactosidase dehydrogenase suspension;

A_t is the numerical value of the absorbance, measured after an incubation time of t min;

$A_{(t-5)}$ is the numerical value of the absorbance, measured after an incubation time of (t – 5) min.

7.6.3 Verification

If the absorbance A exceeds 0,500, repeat the procedure specified in 7.6.1 and 7.6.2, using an appropriate aqueous dilution of the filtrate (7.5.2).

8 Calculation and expression of results

8.1 Calculation

The lactose content, w_L, is given by the following equation:

$$w_L = \frac{[(A_1 - A_3) - (A_2 - A_4)] \times M_r}{K \times l} \times \frac{V_1 \times V_3 \times V_5}{V_2 \times V_4} \times \frac{100}{m}$$

where

w_L is the lactose content, expressed as a percentage by mass;

A_1 is the numerical value of the absorbance (calculated in 7.6.2) of the sample or standard test for lactose and galactose;

A_2 is the numerical value of the absorbance (calculated in 7.6.2) of the sample or standard test for galactose;

A_3 is the numerical value of the absorbance (calculated in 7.6.2) of the reagent blank test for lactose and galactose;

A_4 is the numerical value of the absorbance (calculated in 7.6.2) of the reagent blank test for galactose;

M_r is the relative molecular mass of lactose; for anhydrous lactose, M_r = 342,30 and for lactose monohydrate, M_r = 360,31;

K is the molar absorption coefficient of NADH at 340 nm (i.e. $K = 6{,}3 \times 10^6$ cm²/mol);

l is the numerical value of theoretical path length of the spectrometer cells in centimetres (1 cm);

V_1 is the total volume of liquid in the spectrometer cell (7.6.1), in millilitres (3,30 ml);

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS Not for Resale

V_2 is the volume of the filtrate (7.5.2) or its dilution (7.6.3) added to the spectrometer cell (1,00 ml), in millilitres;

V_3 is the volume of the solution prepared in 7.5.1 (100 ml), in millilitres;

V_4 is the volume of the filtrate (7.5.2) taken for dilution (7.6.3) as appropriate, in millilitres;

V_5 is the volume to which the test solution was diluted (7.6.3) as appropriate, in millilitres;

m is the mass of the test portion (7.3) , in grams;

If a negative value is found for ($A_2 - A_4$), take it into account.

NOTE If the mass of the test sample is greater than 1 g, V_3 may be calculated using the equation:

$$V_3 = 100 - P$$

where P is the volume of the precipitate, in millilitres. P can be calculated from the approximate composition of the test sample using the following formula: P = 1,1 × (grams fat) + 0,73 × (grams protein) + 0,65 × (grams starch) + 0,55 × (grams insoluble ash).

8.2 Expression of results

Report the result to three significant figures.

9 Precision

9.1 Interlaboratory trial

Details of the interlaboratory test on the precision of the method have been published (see reference [6]).

The values for repeatability and reproducibility derived from this interlaboratory test were determined in accordance with ISO 5725-2. The values obtained may not be applicable to concentration ranges and matrices other than those given.

NOTE IDF 135 provides specific guidance for interlaboratory tests on methods of analysis and milk products. It is based on ISO 5725.

9.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than:

— for dried milk and dried ice-mixes: 3 % of the arithmetic mean of the test result;

— for processed cheese: 6 % of the arithmetic mean of the test result.

9.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than:

— for dried milk and dried ice-mixes: 6 % of the arithmetic mean of the test results;

— for processed cheese: 14 % of the arithmetic mean of the test results.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

10 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, with reference to this part of ISO 5765|IDF 79;

d) all operational details not specified in this part of ISO 5765|IDF 79, 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.

© ISO 2002 – All rights reserved

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Annex A
(normative)

Good Laboratory Practice (GLP) rules for the performance of enzymatic analyses

A.1 Introduction

Good Laboratory Practice rules for enzymatic analyses are often less well known than those for other chemical analyses.

It is advisable to pay more attention to such rules in order to obtain results with a satisfactory accuracy and precision.

Therefore, before the analyses read the GLP rules described below conscientiously.

A.2 Reagents

A.2.1 Use only enzymes of the prescribed grade (specific activity, concentration, contaminants with enzymatic activities, solvent).

A.2.2 Use only coenzymes of the prescribed grade (purity grade, salt or acid form, contaminants).

A.2.3 All reagents other than the enzymes and the coenzymes shall be of analytical grade.

A.2.4 The water for the preparation of the enzyme solutions and the other reagents shall be doubly glass-distilled.

A.2.5 The water for the preparation of the sample solutions shall be glass-distilled or de-ionized.

A.2.6 Store the reagents and enzyme suspensions/solutions according to the instructions (usually between 2 °C and 8 °C).

A.2.7 Do not freeze enzyme suspensions.

A.2.8 When the expiry date of a reagent has been exceeded, either discard the reagent or check the efficiency of this reagent by examining standard solutions with varying amounts of analyte. The absorbances obtained shall be proportional to the concentrations.

A.2.9 Buffer solutions taken from the refrigerator shall be warmed up to room temperature before being added to the assay mixture.

A.3 Photometric and spectrometric cells

A.3.1 Use glass or plastic cells with an optical path length of 1 cm.

NOTE Plastic cells have the following advantages over glass cells:

- they are cheap (disposable);
- greater numbers of analyses may be run;
- within one batch, plastic cells agree very well regarding the absorbance.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

A.3.2 Whenever a new batch of cells is put into use, check the optical path length of the cells against that of a precision cell (e.g. quartz cell), as follows:

Fill the precision cell and plastic cells with water and measure the absorbance (A_1) of each cell against air. Fill the cells, after rinsing, with a solution of NADH (approximately 0,15 mg/ml) and again measure the absorbance (A_2) against air.

For both the precision cell and the plastic cells, calculate ($A_2 - A_1$). If the differences ($A_2 - A_1$) between the two types of cells shall not deviate significantly. If the differences ($A_2 - A_1$) exceed 0,5 % of the net absorbance measurement for the precision cell, calculate the average percentage difference and take this into account for the path length, l, in the calculation (see 8.1)

A.3.3 Always use clean and unscratched cells. Dry or clean the optical sides of the cells with a soft tissue only.

A.3.4 Do not measure the absorbance of the sample test cell against that of the blank test cell, since no information will be obtained about the order of magnitude of the absorbance of the blank test itself. Measure the absorbance of both the sample and the blank test cell against air and calculate the difference.

A.3.5 Do not measure the absorbance of a sample or blank test cell against an empty cell (because of light diffusion).

A.3.6 Mix the contents of a cell with a plastic paddle or by sealing the cell with parafilm and gently swirling.

A.3.7 Remove air bubbles from the walls of the cells using a paddle. Avoid scratching the optical side of the cell.

A.3.8 Always use the same kind of cells for the measurement of the sample test and the blank test.

A.3.9 Always place cells in the same position and orientation in the cell holder. For this purpose, mark one optical side of the cell.

A.4 Photometers and spectrometers

A.4.1 Use a spectrometer (bandwidth $\leqslant$ 10 nm), provided with an interference filter (bandwidth $\leqslant$ 10 nm) or a spectrum line photometer, equipped with a mercury vapor lamp. Measurements, carried out using a spectrometer or filter photometer, shall be made at the absorption maximum of NADH or NADPH, i.e. 340 nm; those carried out using a spectral line photometer with a mercury vapour lamp shall be made at 365 nm or 334 nm.

NOTE The molar absorption coefficients of NADH and NADPH measured at 334 nm, 340 nm and 365 nm are as follows.

— NADH and NADPH at 334 nm (Hg): $6{,}18 \times 10^6$ cm^2/mol;

— NADH and NADPH at 340 nm: $6{,}3 \times 10^6$ cm^2/mol;

— NADPH at 365 nm (Hg): $3{,}5 \times 10^6$ cm^2/mol;

— NADH at 365 nm (Hg): $3{,}4 \times 10^6$ cm^2/mol.

A.4.2 A linear relationship up to an absorbance of 2,0 shall exist between the absorbance and the concentration of NADH or NADPH. Check this as follows.

a) Pipette 2,00 ml of distilled water into a cell. Measure the absorbance A_0 against air.

b) Pipette 0,10 ml of NADH solution (0,5 mg/ml) into the cell and mix. Measure the absorbance A_1.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Calculate the reduced absorbance, A_{rn}, using the following equation:

$$A_{r1} = (A_1 - A_0) \times \frac{2,1}{3,5}$$

where

A_1 is the numerical value of the absorbance obtained with the measurement of the NADH solution (b);

A_0 is the numerical value of the absorbance obtained with the measurement of the water (a).

c) Repeat the procedure described in point b) above 14 times.

After each pair of measurements, calculate the reduced absorbance, A_{rn}, with the following equation:

$$A_{rn} = (A_n - A_0) \times \frac{V}{3,5}$$

where

A_n is the numerical value of the absorbance obtained at measurement n;

V is the volume of the cell contents at measurement n.

d) For each measurement, plot the volume of NADH solution present in the cell against the corresponding reduced absorbances. A straight line shall be obtained connecting the obtained intersection points.

A.5 Automatic pipettes and other dispensers

A.5.1 Use automatic pipettes and other dispensers in accordance with the manufacturer's instructions.

A.5.2 Use the appropriate tips for each pipette.

A.5.3 Check the specifications of volume and repeatability of automatic pipettes and other dispensers periodically (e.g. monthly) as follows:

a) weigh a glass beaker with water at time t;

b) pipette or dispense one measure of water into the beaker and weigh exactly at $t + 1$ min after the first weighing;

c) repeat nine times the pipetting or dispensing procedure as described in point b);

d) weigh, without pipetting or dispensing, the beaker at the moments $t + 11$ min, $t + 12$ min, $t + 13$ min, $t + 14$ min and $t + 15$ min; calculate from these weighings the evaporation loss per minute;

e) calculate the volume and repeatability of the pipette or dispenser, taking into account the loss of water by evaporation.

A.5.4 The volume of some automatic pipettes can be affected by heat transfer from the palm of the hand during prolonged use.

Check this phenomenon by the procedure described in A.5.3 and avoid the use of such pipettes.

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

A.5.5 Just before use, rinse the tip of the pipette several times with the solution/suspension to be delivered. For each sample solution, use a new pipette tip.

A.5.6 Pipette the water, buffer, enzyme, coenzyme and sample solution — inserting the tip as low as possible — in different corners of the cell.

NOTE Small amounts of enzyme solution/suspension (10 µl to 50 µl) can be pipetted onto the paddle, brought into the cell and mixed with the cell contents by the paddle.

A.5.7 Avoid contamination.

A.6 Additional information

A.6.1 Check for possible interferences and for gross errors by determining the absorbances of two solutions with different concentrations of analyte. The absorbances obtained shall be proportional to the concentration of the analyte.

A.6.2 Use a standard to check the enzymatic reaction(s). This standard shall be considered as a working standard.

NOTE Reference materials having a certified purity can be obtained from organizations such as the National Institute of Standards and Technology (formerly the National Bureau of Standards) or the European Community Bureau of Reference (BCR).

A.6.3 Carry out a recovery test in the presence of the sample solution. The amount of analyte added shall be about the same as that already present in the sample solution.

A.6.4 Use one plastic paddle per cell or use each paddle once only.

NOTE The amount of liquid remaining on the paddle can be considered negligible.

© ISO 2002 — All rights reserved

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Bibliography

[1] ISO 707, *Milk and milk products — Guidance on sampling*

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[4] IDF 135B:1991, *Milk and milk products — Precision characteristics of analytical methods — Outline of collaborative study procedure*

[5] Comité de Nomenclature de l'Union Internationale de Biochimie, *Recommandations en matière de Nomenclature des Enzymes.* Academic Press, New York, 1984

[6] Interlaboratory Collaborative Studies, second series. *Bulletin International Dairy Federation*, **285**, 1993, annex A, annex B and annex C

© ISO 2002 – All rights reserved
Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS
Not for Resale

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS Not for Resale

ICS 67.100.10; 67.100.30; 67.100.40

Price based on 14 pages

© ISO and IDF 2002 – All rights reserved

Copyright International Organization for Standardization
Provided by IHS under license with ISO
No reproduction or networking permitted without license from IHS Not for Resale