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(Acts whose publication is obligatory)

COMMISSION REGULATION (EC) No 213/2001**of 9 January 2001**

laying down detailed rules for the application of Council Regulation (EC) No 1255/1999 as regards methods for the analysis and quality evaluation of milk and milk products and amending Regulations (EC) No 2771/1999 and (EC) No 2799/1999

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

amended so that the annexes to those Regulations concerning analysis methods can be included in this Regulation.

Having regard to the Treaty establishing the European Community,

Having regard to Council Regulation (EC) No 1255/1999 of 17 May 1999 on the common organisation of the market in milk and milk products ⁽¹⁾, and in particular Articles 10 and 15 and Articles 26(3), 29(1) and 31(14) thereof,

Whereas:

(1) Commission Regulations (EEC) No 1216/68 and (EEC) No 3942/92 and (EC) No 86/94, (EC) No 2721/95, (EC) No 1080/96, (EC) No 1081/96, (EC) No 1082/96, (EC) No 1854/96, (EC) No 880/98 and (EC) No 1459/98, the full references of which are given in Annex XXVI to this Regulation, lay down reference and routine methods for the analysis and quality evaluation of milk and milk products and establish the scope and rules for applying those methods. In the interests of clarity and to provide those operating in the sector with a single set of methods and rules for applying them, the abovementioned Regulations should be recast and brought together in a single text. For the same reason, Commission Regulations (EC) No 2771/1999 of 16 December 1999 laying down detailed rules for the application of Council Regulation (EC) No 1255/1999 as regards intervention on the market in butter and cream ⁽²⁾ and (EC) No 2799/1999 of 17 December 1999 laying down detailed rules for applying Regulation (EC) No 1255/1999 as regards the grant of aid for skimmed milk and skimmed-milk powder intended for animal feed and the sale of such skimmed-milk powder ⁽³⁾ should be

(2) The composition and quality requirements for milk and milk products laid down under the arrangements provided for in Regulation (EC) No 1255/1999 must be verified to ensure that they are strictly complied with.

(3) The reference methods for such verifications are often methods published by international organisations such as the CEN, IDF, ISO and AOAC International, which are regularly updated by those organisations. In some cases a Community reference method is laid down, while in other cases no reference method is specified in the Community rules. In order to ensure that reference methods are uniformly applied, a list of reference methods should be drawn up each year and only methods included in the list may be applied.

(4) The use of routine methods should not be ruled out. The conditions for using them should therefore be specified.

(5) Common methods should also be established to ensure uniform practice in evaluating the results of analyses, in sensory evaluation of the products concerned and in re-examining results which have been disputed.

(6) For some analyses, there are currently no internationally accepted reference methods which have been validated and thus no information is available on the between-laboratory-variation of analytical results. Community methods should therefore be laid down, which have been validated according to internationally established rules and are applied as reference methods.

⁽¹⁾ OJ L 160, 26.6.1999, p. 48.

⁽²⁾ OJ L 333, 24.12.1999, p. 11.

⁽³⁾ OJ L 340, 31.12.1999, p. 3.

- (7) Commission Regulation (EC) No 2571/97 of 15 December 1997 on the sale of butter at reduced prices and the granting of aid for cream, butter and concentrated butter for use in the manufacture of pastry products, ice-cream and other foodstuffs ⁽¹⁾, as last amended by Regulation (EC) No 635/2000 ⁽²⁾, provides for the tracing of cream, butter and concentrated butter in certain circumstances in order to ensure the correct end use of these products. Since tracing is important if the scheme is to function properly, and in order to ensure that operators participating in it receive equal treatment, common methods should be established for determining some of these tracers.
- (8) Under Commission Regulation (EEC) No 3143/85 of 11 November 1985 on the sale at reduced prices of intervention butter intended for direct consumption in the form of concentrated butter ⁽³⁾, as last amended by Regulation (EC) No 101/1999 ⁽⁴⁾, Commission Regulation (EEC) No 429/90 of 20 February 1990 on the granting by invitation to tender of an aid for concentrated butter intended for direct consumption in the Community ⁽⁵⁾, as last amended by Regulation (EC) No 124/1999 ⁽⁶⁾, and Regulation (EC) No 2571/97, concentrated butter must have tracers added to it under supervision. Compliance with the requirements for tracing concentrated butter must be strictly enforced to ensure that products are not diverted. A common method for detecting such tracers should therefore be laid down.
- (9) Under Article 9 of Regulation (EC) No 1255/1999, private storage aid may be granted for cheeses made from ewes' milk. A special refund for these same products can be granted under Article 31 of that Regulation. Cheeses made from ewes' milk, goats' milk, buffalos' milk and mixtures of ewes', goats' and buffalos' milk may be imported into the Community under preferential arrangements from certain third countries. In view of the above provisions, appropriate checks are needed to ensure that no cow's milk has been incorporated in the products concerned. A Community reference method should therefore be established for detecting cow's milk, without prejudice to the use of routine methods, provided they comply with certain criteria.
- (10) Under Commission Regulation (EEC) No 2921/90 of 10 October 1990 on the granting of an aid for skimmed milk used for the production of casein and caseinates ⁽⁷⁾, as last amended by Regulation (EC) No 2654/1999 ⁽⁸⁾, the absence of coliforms must be detected.
- The internationally accepted reference method for detecting coliforms in milk and milk products is International Standard IDF 73A: 1985. However, that standard is applicable only in a modified form for detecting coliforms in a certain quantity of product. A Community reference method for detecting coliforms has therefore been established based on the abovementioned standard.
- (11) Council Regulation (EEC) No 2658/87 of 23 July 1987 on the tariff and statistical nomenclature and on the Common Customs Tariff ⁽⁹⁾, as last amended by Regulation (EC) No 254/2000 ⁽¹⁰⁾, provides for different rates of customs duty for compound feedingstuffs falling within tariff heading No 2309, depending on their milk-product content. To ensure that the rules in question are uniformly applied, a generally recognised method for analysing lactose content should be laid down for compulsory use in all Member States.
- (12) Under Regulation (EC) No 1255/1999, butter and skimmed-milk powder intended for intervention or, in the case of skimmed-milk powder, for use as animal feed, must meet certain quality requirements. Reference methods should be laid down to verify that those requirements are met.
- (13) Implementation of some of the methods introduced for the first time in this Regulation will require a period of adaptation. Application of those methods should therefore be deferred.
- (14) The Management Committee for Milk and Milk Products has not delivered an opinion within the time limit set by its chairman,

HAS ADOPTED THIS REGULATION:

CHAPTER I

GENERAL PROVISIONS

Article 1

Scope and field of application

This Regulation lays down the rules for applying the methods for the chemical, physical and microbiological analysis and sensory evaluation of milk and milk products to be used under the arrangements provided for in the common organisation of the market in milk and milk products established by Regulation (EC) No 1255/1999. It also lays down some of those methods.

⁽¹⁾ OJ L 350, 20.12.1997, p. 3.

⁽²⁾ OJ L 76, 25.3.2000, p. 9.

⁽³⁾ OJ L 298, 12.11.1985, p. 9.

⁽⁴⁾ OJ L 11, 16.1.1999, p. 14.

⁽⁵⁾ OJ L 45, 21.2.1990, p. 8.

⁽⁶⁾ OJ L 16, 21.1.1999, p. 19.

⁽⁷⁾ OJ L 279, 11.10.1990, p. 22.

⁽⁸⁾ OJ L 325, 17.12.1999, p. 10.

⁽⁹⁾ OJ L 256, 7.9.1987, p. 1.

⁽¹⁰⁾ OJ L 28, 3.2.2000, p. 16.

*Article 2***List of methods**

1. Annex I to this Regulation contains the list of reference methods applicable to analyses as referred to in Article 1.
2. The Commission shall update the list at least once a year in accordance with the procedure laid down in Article 42 of Regulation (EC) No 1255/1999.

*Article 3***Routine methods**

Routine methods may be used for analyses required by the Community rules provided that they are properly calibrated and regularly checked against the reference method.

The procedure described in Annex II may be applied for checking results obtained by routine methods which are close to the limits specified in the Regulations concerned.

In cases of dispute, the results obtained by the reference method shall be decisive.

*Article 4***Validation of reference methods**

1. Reference methods shall be validated if they meet pre-determined precision criteria concerning the repeatability and reproducibility limit.
2. If a reference method laid down in the Regulations concerned has not been validated, Member States shall set a provisional reproducibility limit.

That limit shall be obtained according to the procedure described in Annex III(b). However, for the first 18 months following the entry into force of this Regulation, the Member States may use an equivalent procedure.

Compliance with the limit shall be checked at least once a year.

3. Where the results of applying validated reference methods or methods with provisional precision figures show that a limit has been exceeded, the analytical results may be evaluated using the method described in Annex IV to determine the critical difference from the limit concerned.

*Article 5***Admissibility of analysis results**

1. Analyses shall be performed in laboratories operating an internal quality control procedure in accordance with that described in Annex V(a) or a procedure of an equivalent standard.

A detailed description of the procedure applied must be available for consultation in the laboratory.

2. Laboratories shall establish their in-house precision within a run for all methods, following:

- (a) the procedure described in Annex V(b), or
- (b) a published, validated procedure with an established repeatability.

Compliance with the reproducibility limit must be checked at least once per year using the procedure described in Annex III(a).

The second subparagraph shall not apply to laboratories that have participated in a proficiency testing scheme during the year.

3. Laboratory reports of the results of the analysis must contain sufficient information for an evaluation of the results to be made in accordance with Annex IV and Annex VIII.

4. Analysis results shall be considered admissible if they have been obtained in accordance with the acceptability criteria described in the internal quality control procedure referred to in paragraph 1 and the in-house precision referred to in paragraph 2.

*Article 6***Sensory evaluation**

1. For butter, the procedures described in Annex VI shall be applied to check the performance of assessors and the reliability of results. The procedure described in Annex VII shall be applied as a reference method for sensory evaluation.

2. For milk and milk products other than butter, the reference method to be used by the Member States for sensory evaluation shall be either IDF standard 99C/1997 or other comparable methods which they shall notify to the Commission.

The procedures described in Annex VI may be used to check the performance of assessors and the reliability of results.

*Article 7***Sampling and disputes over the results of analyses**

1. Duplicate samples must be taken for analyses required under Community rules.
2. The procedure described in Annex VIII shall be used in cases where the results of an analysis are not accepted by the operator.

CHAPTER II

METHODS OF ANALYSIS

Article 8

Water/solids-non-fat/fat content of butter

1. The analysis method described in Annex IX shall be used as the reference method for determining the water content of butter.
2. The analysis method described in Annex X shall be used as the reference method for determining the solids-non-fat content of butter.
3. The analysis method described in Annex XI shall be used as the reference method for determining the fat content of butter.

Article 9

Tracers

1. The analysis method described in Annex XII shall be used as the reference method for determining vanillin in concentrated butter, butter and cream.
2. The analysis method described in Annex XIII shall be used as the reference method for determining the ethyl ester of beta-apo-8' carotenic acid content of concentrated butter and butter.
3. The analysis method described in Annex XIV shall be used as the reference method for determining the β -sitosterol or stigmasterol content of butter and concentrated butter.
4. Concentrated butter, butter and cream have been traced in conformity with the relevant Community rules if the results obtained are in accordance with the specifications of paragraph 8 of the Annexes referred to in paragraphs 1, 2 and 3.

Article 10

Detection of cows' milk casein

1. The reference method of analysis described in Annex XV shall be used to ensure that cheese which must be made exclusively from ewes' milk, goats' milk or buffalos' milk or from a mixture of ewes', goats' and buffalos' milk does not in fact contain cows' milk casein.

Cows' milk casein shall be held to be present if the apparent cows' milk casein content of the sample to be analysed is equal to or higher than the content of the reference sample containing 1 % cows' milk as described in Annex XV.

2. Routine methods for detecting cows' milk casein in cheeses as referred to in paragraph 1 may be used provided

that:

- (a) the detection limit is 0,5 % or lower,
- (b) there are no false-positive results,
- (c) cows' milk casein is detectable with the required sensitivity even after long ripening periods, as may occur in usual commercial conditions.

If the requirement provided for in (b) is not met, any sample giving a positive result must be analysed using the reference method.

If the requirement provided for in (c) is not met for one of the types of cheese referred to in paragraph 1, that cheese must be analysed using the reference method.

Article 11

Detection of coliforms

1. The reference method of analysis described in Annex XVI shall be used to detect the presence of coliforms in butter, skimmed-milk powder, casein and caseinates.
2. Routine methods for detecting coliforms may be used provided that the results obtained are comparable with the results obtained by the reference method described in the said Annex. Routine methods must in particular have an adequate detection limit. False-negative results must not occur. If the occurrence of false-positive results cannot be excluded, any positive result must be confirmed using the reference method.

Article 12

Lactose content

The method for determining the lactose content of products falling within CN code 2309 is described in Annex XVII.

Article 13

Detection of rennet whey

1. The method for detecting rennet whey in skimmed-milk powder intended for public storage is described in Annex XVIII.
2. The method for detecting rennet whey in skimmed-milk powder and mixtures intended for use as animal feed is described in Annex XIX.

Article 14

Detection of buttermilk

The method for detecting buttermilk in skimmed-milk powder is described in Annex XX.

Article 15

Antimicrobial residues

The method for detecting antibiotic and sulphonamide/dapson residues in skimmed-milk powder is described in Annex XXI.

Article 16

Skimmed-milk powder content

The method for determining the skimmed-milk powder content in compound feedingstuffs is described in Annex XXII.

Article 17

Detection of starch

The method for detecting starch in skimmed-milk powder, denatured milk powder and compound feedingstuffs is described in Annex XXIII.

Article 18

Moisture content of acid buttermilk powder

The method for determining the moisture content of acid buttermilk powder intended for use in feedingstuffs is described in Annex XXIV.

Article 19

Detection of foreign fats

The method for detecting foreign fats in milk fats is described in Annex XXV.

CHAPTER III

FINAL PROVISIONS

Article 20

Amendments to Regulation (EC) No 2771/1999

Regulation (EC) No 2771/1999 is amended as follows:

1. The first sentence of Article 4(1) is replaced by the following: 'The competent authorities shall check the quality of butter using the methods described in Annex I and on the basis of samples taken in accordance with the rules set out in Annex IV.'
2. In Annex I, footnote 2 is replaced by the following: 'See Annex I to Regulation (EC) No 213/2001'.
3. Annexes II and III are deleted.
4. In the penultimate sentence of Annex IV.2, the words 'with Annex III' are replaced by 'with Annex VII to Regulation (EC) No 213/2001'.

Article 21

Amendments to Regulation (EC) No 2799/1999

Regulation (EC) No 2799/1999 is amended as follows:

1. Article 20(1), (2), (3) and (4) is replaced by the following:

'1. The skimmed-milk powder content of mixtures and compound feedingstuffs shall be determined by testing each sample at least in duplicate using the analysis method described in Annex XXII to Regulation (EC) No 213/2001, supplemented by the checks provided for in Article 17(3) of this Regulation. Should there be a discrepancy between the results of these checks, the result of the on-the-spot inspection shall be conclusive.

2. The absence of rennet whey shall be proven using the method described in Annex XIX to Regulation (EC) No 213/2001.

3. The starch content of compound feedingstuffs shall be determined by the checks provided for in Article 17(3) of this Regulation, which must be supplemented with the analysis method described in Annex XXIII to Regulation (EC) No 213/2001.

4. The moisture content of acid buttermilk powder shall be determined using the analysis method described in Annex XXIV to Regulation (EC) No 213/2001.'

2. Annexes III, IV, V and VI are deleted.

Article 22

Repeals

Regulations (EEC) No 1216/68, (EEC) No 3942/92, (EC) No 86/94, (EC) No 2721/95, (EC) No 1854/96, (EC) No 1080/96, (EC) No 1081/96, (EC) No 1082/96, (EC) No 880/98 and (EC) No 1459/98 are repealed.

References to the repealed Regulations shall be construed as references to this Regulation.

Article 23

Entry into force

This Regulation shall enter into force on the seventh day following its publication in the *Official Journal of the European Communities*.

However, the methods described in Annexes III, IV.4, V, VI and VIII shall apply 18 months after the entry into force of this Regulation.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 9 January 2001.

For the Commission
Franz FISCHLER
Member of the Commission

ANNEX I

(Article 2)

LIST OF REFERENCE METHODS

Index

Min. = minimum, Max. = maximum, Annex = Annex to quoted Regulation, SNF = solids not fat, FFA = free fatty acids, PV = peroxide value, A = appearance, F = flavour, C = consistency, TBC = total bacterial count, Therm = thermophilic bacterial count, MS = Member State, IDF = International Dairy Federation, ISO = International Standards Organisation, IUPAC = International Union of Pure and Applied Chemistry, ADPI = American Dairy Products Institute, SCM = sweetened condensed milk, EMC = evaporated milk or cream, MSNF = milk solids non fat.

PART A

Commission Regulation	Product	Parameter	Limit	Reference method	Remark
Regulation (EC) No 2771/1999 Public storage (OJ L 333, 24.12.1999, p. 11)	Unsalted butter	Milk fats	Min. 82 %	Annex XI	Note 1 Note 3
		Water	Up to 16 %	Annex IX	
		SNF	Up to 2 %	Annex X	
		FFA (max.)	1.2 mmole/100 g of fat	IDF Standard 6B:1989	
		PV (max.)	0,3 meq. oxygen/1 000 g fat	IDF Standard 74A:1991 (English version)	
		Coliforms	Not detectable in 1 g	Annex XVII	
		Non-milk fat	Not detectable by triglyceride analysis	Annex XXVI	
		Sterol tracers	Not detectable	Annex XIV	
		Other tracers:			
		— vanillin	Not detectable	Annex XII	
		— ethyl ester of carotenic acid	Not detectable	Annex XIII	
Regulation (EC) No 2771/1999 Private storage	Unsalted butter	— triglycerides of enanthic acid	Not detectable	IUPAC 2.301 sub 5	Note 6
		Sensory characteristics	At least 4 out of 5 points for A, G and C	Annex VII	
Regulation (EC) No 2771/1999 Private storage	Salted butter	Water dispersion	At least 4 points	IDF Standard 112A:1989	Note 6
		Milk fats	Min. 82 %	Annex XI	
		Water	Up to 16 %	Annex IX	
Regulation (EC) No 2771/1999 Private storage	Salted butter	Milk fats	Min. 80 %	Annex XI	Note 6
		Water	Up to 16 %	Annex IX	
		Salt	Up to 2 %	IDF Standard 12B:1988	

Commission Regulation	Product	Parameter	Limit	Reference method	Remark
Regulation (EC) No 2571/97 (OJ L 350, 20.12.1997, p. 3)	Unsalted butter	Milk fats Water Tracers: — sterols — vanillin — ethyl ester of carotenic acid — triglycerides of enanthic acid	Min. 82 % Up to 16 %	Annex XI Annex IX Annex XIV Annex XII Annex XIII IUPAC 2.301 sub 5	
Regulation (EC) No 2571/97	Salted butter	Milk fats Water Salt Tracers: — sterols — vanillin — ethyl ester of carotenic acid — triglycerides of enanthic acid	Min. 80 % Up to 16 % Up to 2 %	Annex XI Annex IX IDF Standard 12B:1988 Annex XIV Annex XII Annex XIII IUPAC 2.301 sub 5	
Regulation (EC) No 2571/97	Concentrated butter	Milk fats Moisture and MSNF FFA PV (max.) Non-milk fat Flavour Smell Other Tracers: — sterols — vanillin — ethyl ester of carotenic acid — triglycerides of enanthic acid	Min. 99,8 % Up to 0,2 % Up to 0,35 % (oleic) 0,5 meq. oxygen/1 000 g fat Absence Clean Absence of foreign odours Absence of neutralising agents, anti-oxidants and preservatives	IDF Standard 24:1964 IDF Standard 23A:1988 (moisture) IDF Standard 24:1964 (MSNF) IDF Standard 6B:1989 IDF Standard 74A:1991 (English version) Annex XXV Annex XIV Annex XII Annex XIII IUPAC 2.301 sub 5	Note 1

Commission Regulation	Product	Parameter	Limit	Reference method	Remark
Regulation (EC) No 2571/97	Cream	Fat	35 %	IDF Standard 16C:1987	
		Tracers: — sterols — vanillin — ethyl ester of carotenic acid — triglycerides of enanthic acid		Methods approved by the competent authority Annex XII Methods approved by the competent authority IUPAC 2.301 sub 5	Note 2 Note 2
Regulation (EEC) No 429/90 (OJ L 45, 21.2.1990, p. 8)	Concentrated butter	Milk fats	Min. 96 %	Methods approved by the competent authority	Note 2
		SNF	Up to 2 %	Methods approved by the competent authority	Note 2
		Tracers: — stigmasterol (95 %)	15 g/100 kg of concentrated butter	Annex XIV	
		— stigmasterol (85 %)	17 g/100 kg of concentrated butter	Annex XIV	
		— triglycerides of enanthic acid	1,1 kg/100 kg of concentrated butter	IUPAC 2.301 sub 5	
		— ethyl ester of butyric acid and stigmasterol	See Annex, point 1(c)	Annex XIV	Note 2
		— lecithin (E 322)	Up to 0,5 %	Methods approved by the competent authority	Note 2
		NaCl	Up to 0,75 %	IDF Standard 12B:1988	
		FFA	Up to 0,35 % (oleic)	IDF Standard 6B:1989	
		PV (max.)	Up to 0,5 meq. oxygen/1 000 g fat	IDF Standard 74A:1991 (English version)	Note 1
		Flavour	Clean		
		Smell	Absence of foreign odours		
		Other	Absence of neutralising agents, anti-oxidants and preservatives		
Regulation (EEC) No 2191/81 (OJ L 213, 1.8.1981. p. 20)	Unsalted butter	Milk fats	Min. 82 %	Annex XI	
		Water	Up to 16 %	Annex IX	

Commission Regulation	Product	Parameter	Limit	Reference method	Remark
Regulation (EEC) No 2191/81	Salted butter	Milk fats Water Salt	Min. 80 % Up to 16 % Up to 2 %	Annex XI Annex IX IDF Standard 12B:1988	
Article 9 and Title II of Regulation (EC) No 1255/1999	Cheese made from ewes' and/or goats' milk	Cows' milk	< 1 %	Annex XV	
Regulation (EEC) No 2921/90	Annex I — Acid casein	Water Fat Free acidity	Up to 12,00 % Up to 1,75 % Up to 0,30 % (lactic)	IDF Standard 78C:1991 IDF Standard 127A:1988 IDF Standard 91:1979	
Regulation (EEC) No 2921/90	Annex I — Rennet-casein	Water Fat Ash	Up to 12,00 % Up to 1,00 % Min. 7,50 %	IDF Standard 78C:1991 IDF Standard 127A:1998 IDF Standard 90:1979	
Regulation (EEC) No 2921/90	Annex I — Caseinates	Water Milk protein Fat and ash	Up to 6,00 % Min. 88,00 % Up to 6,00 %	IDF Standard 78C:1991 IDF Standard 92:1979 IDF Standard 127A:1988 IDF Standard 89:1979 or IDF Standard 90:1979	
Regulation (EEC) No 2921/90	Annex II — Acid casein	Water Fat Free acidity TBC (max.) Coliforms Therm. (max.)	Up to 10,00 % Up to 1,50 % Up to 0,20 % (lactic) 30 000 l/g Absence in 0,1 g 5 000 l/g	IDF Standard 78C:1991 IDF Standard 127A:1988 IDF Standard 91:1979 IDF Standard 100B:1991 Annex XVI IDF Standard 100B:1991	Note 3 Note 3 Notes 3 and 4
Regulation (EEC) No 2921/90	Annex II — Rennet-casein	Water Fat Ash TBC (max.) Coliforms Therm. (max.)	Up to 8,00 % Up to 1,00 % Min. 7,50 % 30 000 l/g Absence in 0,1 g 5 000 l/g	IDF Standard 78C:1991 IDF Standard 127A:1988 IDF Standard 90:1979 IDF Standard 100B:1991 Annex XVI IDF Standard 100B:1991	Note 3 Note 3 Notes 3 and 4

Commission Regulation	Product	Parameter	Limit	Reference method	Remark
Regulation (EEC) No 2921/90	Annex II — Caseinates	Water Milk protein Fat and ash TBC (max.) Coliforms Therm. (max.)	Up to 6,00 % Min. 88,00 % Up to 6,00 % 30 000 l/g Absence in 0,1 g 5 000 l/g	IDF Standard 78C:1991 IDF Standard 92:1979 IDF Standard 127A:1988 IDF Standard 89:1979 or 90:1979 IDF Standard 100B:1991 Annex XVI IDF Standard 100B:1991	Note 3 Note 3 Notes 3 and 4
Regulation (EEC) No 2921/90	Annex III — Caseinates	Water Milk protein Fat Lactose Ash TBC (max.) Coliforms Therm. (max.)	Up to 6,00 % Min. 85,00 % Up to 1,50 % Up to 1,00 % Up to 6,50 % 30 000 l/g Absence in 0,1 g 5 000 l/g	IDF Standard 78C:1991 IDF Standard 92:1979 IDF Standard 127A:1988 IDF Standard 106:1982 IDF Standard 89:1979 or 90:1979 IDF Standard 100B:1991 Annex XVI IDF Standard 100B:1991	Note 3 Note 3 Notes 3 and 4
Regulation (EC) No 2799/1999 (OJ L 340, 31.12.1999, p. 3)	Compound feedingstuffs and skimmed-milk powder (SMP) (for use in feeding- stuffs)	Water (acid buttermilk powder) Protein Water (SMP) Fats (SMP) Rennet whey (SMP) Starch (SMP) Water (mixtures) Fats (mixtures) Rennet whey (mixtures) SMP content (of end product) Fats (in end product) Starch (in end product) Copper (in end product)	Up to 5 % 31,4 % (min.) of the non-fat dry matter) Up to 5 % Up to 11 % Absence Absence Up to 5 % of the non-fat dry matter — Absence Min. 50 % Min 2,5 % or 5 % Min 2 % 25 ppm	IDF Standard 20B:1993 IDF Standard 26A:1993 IDF Standard 9C:1987 Annex XIX Annex XXIII IDF Standard 26A:1993 Commission Directive 84/4/EEC (OJ L 15, 18.1.1984, p. 28) Annex XIX Annex XXII Commission Directive 84/4/EEC Annex XXIII Commission Directive 78/633/EEC (OJ L 206 26.7.1987, p. 43)	Note 7 Note 7 Note 8 Note 9

Commission Regulation	Product	Parameter	Limit	Reference method	Remark
Regulation (EC) No 322/96 (OJ L 45, 23.2.96, p. 5)	SMP (spray)	Fat	Up to 1,0 %	IDF Standard 9C:1987	
		Protein	31,4 % (min.) of the non-fat dry matter	IDF Standard 20B:1993	
		Water	Up to 3,5 %	IDF Standard 26A:1993	
		Acidity (N/10 NaOH)	Up to 19,5 ml	IDF Standard 86:1981	
		Lactates	Up to 150 mg/100 g	IDF Standard 69B:1987	
		Phosphate	Negative	ISO Standard 3356:1975	
		Solubility	Up to 0,5 ml at 24 °C	IDF Standard 129A:1988	
		Scorched particles	Disk B min. (15,0 mg)	ADPI:1990	
		TBC	40 000/l g	IDF Standard 100B:1991	Note 3
		Coliforms	Negative/0,1 g	Annex XVI	Note 3
		Buttermilk	Negative	Annex XX	
		Rennet whey	Negative	Annex XVIII	
		Acid whey	Negative	Method approved by the competent authority	Note 2
		Anti-microbial agents		Annex XXI	

PART B

The reference methods listed in Part B may be used for analysing products covered by any of the Regulations listed in column 1.

Commission Regulation	Product	CN code	Parameter	Limit	Reference method	Remark
Regulation (EEC) No 2658/87 (OJ L 256, 7.9.1987, p. 1) Regulation (EC) No 2414/98 (OJ L 299, 10.11.1998, p. 7) Regulation (EC) No 1374/98 (OJ L 185, 30.6.1998, p. 21) Regulation (EC) No 2508/97 (OJ L 345, 16.12.1997, p. 31) Regulation (EC) No 174/1999 (OJ L 20, 27.1.1999, p. 8)	Milk and cream, not concentrated nor containing added sugar or other sweetening matter	0401	Fat (\leq 6 %)	The limits are those specified in the description of the CN code for the specific product, further specified, where applicable, in Commission Regulation (EEC) No 3846/87 (OJ L 366, 24.12.1987, p. 1) Part 9 of the export nomenclature or Regulation (EC) No 1374/98 (OJ L 185, 30.6.1998, p. 21)	IDF Standard 1D:1996	
			Fat ($>$ 6 %)		IDF Standard 16C:1987	

Commission Regulation	Product	CN code	Parameter	Limit	Reference method	Remark
	Milk and cream, concentrated or containing added sugar or other sweetening matter	0402	Fats (liquid form)		IDF Standard 13C:1987	
			Fats (solid form)		IDF Standard 9C:1987	
			Protein		IDF Standard 20B:1993	
			Sucrose (normal content)		IDF Standard 35A:1992	
			Sucrose (low content)		Methods approved by the competent authority	Note 2
			Solids (SCM)		IDF Standard 15B:1991	
			Solids (EMC)		IDF Standard 21B:1987	
			Water (milk and cream powder)		IDF standard 26A:1993	
	Buttermilk, fermented or acidified milk and cream, concentrated or not concentrated, containing added sugar or other sweetening matter	0403	Fat		IDF Standards 1D:1996, 9C:1987, 16C:1987, 22B:1987, 126A:1988	
			Protein		IDF Standard 20B:1993	
			Sucrose (normal content)		IDF Standard 35A:1992	
			Sucrose (low content)		Methods approved by the competent authority	Note 2
			Water (acid buttermilk powder)		Annex XXIV	
			Water (sweet buttermilk powder)		IDF Standard 26A:1993	
			Solids (other products)		Methods approved by the competent authority	
	Whey, whether or not concentrated or containing added sugar or other sweetening matter; products consisting of natural milk constituents	0404	Fat		IDF Standards 9C:1987, 16C:1987, 22B:1987	
			Protein		IDF Standard 20B:1993	
			Sucrose (normal content)		IDF Standard 35A:1992	
			Sucrose (low content)		Methods approved by the competent authority	Note 2
		0404 90	Protein		IDF Standard 20B:1993	
			Water		IDF Standard 26A:1993	
			Solids		IDF Standard 15B:1991	
			(Concentrated products)		IDF Standard 21B:1987	
	Butter and other fats derived from milk; dairy spreads	0405	Fat (if ≤ 85 %)		Annex XI	
			Water		Annex IX	
		Butter	SNF		Annex X	
			NaCl		IDF Standard 12B:1998	
		Butteroil	Fat (if > 99 %)		IDF Standard 24:1964	
			Water (if fat < 99 %)		IDF Standard 23A:1988	

Commission Regulation	Product	CN code	Parameter	Limit	Reference method	Remark
	Cheese and curd	0406	Fat Solids Solids (Ricotta) NaCl Lactose		IDF Standard 5B:1986 IDF Standard 4A:1982 IDF Standard 58:1970 IDF Standard 88A:1988 IDF Standard 79B:1991	
Regulation (EEC) No 2658/87	Compound feedingstuffs	2309	Lactose		Annex XVII	

Notes to list of European Union reference methods

Note 1: Milk fat isolation as described in IDF Standard 6B:1989 (protection from light).

Note 2: No reference method has been established.

Note 3: Sample to be prepared according to IDF Standard 122C:1996 or IDF Standard 73A:1985.

Note 4: incubation for 48 hours at a temperature of 55 °C, care should be taken to prevent the culture medium from drying out.

Note 5: % SNF = % solids – % fat.

Note 6: Butter must correspond to the national quality class of the Member State of production referred to in Annex V to Commission Regulation (EC) No 2771/1999.

Note 7: Commission Directive 84/8/EEC.

Note 8: Commission Regulation (EC) No 1758/94 (OJ L 183, 19.7.1994, p. 14).

Note 9: Commission Directive 78/633/EEC.

ANNEX II

(Article 3)

CHECKING THE RESULTS OBTAINED BY ROUTINE METHODS WHICH ARE CLOSE TO THE LIMITS SPECIFIED IN THE REGULATIONS FOR COMPOSITION AND QUALITY REQUIREMENTS

If m_o is a limit for composition and quality requirements specified in a Regulation, the decision limit (L) is

$$L = m_o$$

if $R_{\text{Rout}}/R_{\text{Ref}} \leq 1$

R_{Rout} : Reproducibility limit of the routine method

R_{Ref} : Reproducibility limit of the reference method

If m_o is an upper limit and $R_{\text{Rout}}/R_{\text{Ref}} > 1$, this decision limit is obtained using the formula

$$L = m_o - [(R_{\text{Rout}}/R_{\text{Ref}}) - 1] \cdot \text{CrD}_{95}$$

If, under the same conditions, m_o is a lower limit, the decision limit is obtained using the formula

$$L = m_o + [(R_{\text{Rout}}/R_{\text{Ref}}) - 1] \cdot \text{CrD}_{95}$$

where CrD_{95} is the critical difference of the reference method (see Annex IV).

Where m_o is an upper limit, a final result above the decision limit obtained using a routine method must be substituted with a final result obtained using the reference method. This final result must be based on at least the same number of analyses/samples as the final result obtained using the routine method.

Where m_o is a lower limit, the same procedure must be followed for a final result below the decision limit obtained using a routine method.

Remark

The procedure described above can be followed if there are no detectable matrix effects.

Matrix effects can be detected in the following way: for each sample used for calibration, the difference (w_i) between the results obtained by the reference and the routine method is determined.

The standard deviation calculation using the formula

$$s = \sqrt{(\sum w_i^2)/2m}$$

m: Number of samples used for calibration

is compared with the arithmetic mean of the repeatability standard deviation of the reference and the routine method

$$s_r = \sqrt{(s_{r(\text{ref})}^2 + s_{r(\text{rout})}^2)/2}$$

A matrix effect cannot be excluded, if

$$m \bullet s^2/s_r^2 > \text{Chi}_{f;1-\alpha}^2$$

where

f = m (f: number of degrees of freedom)

α = error probability; $\alpha = 0,05$.

In this case, further investigations are necessary before a decision limit can be fixed.

ANNEX III

(Articles 4 and 5)

(a) Procedure for determining compliance with an established reproducibility limit (Chemical analysis)

Compliance with the reproducibility limit is checked by comparing the laboratory results with the results of an experienced laboratory ⁽¹⁾, obtained using an identical sample. A double determination is carried out in both laboratories and the results are evaluated using the formula:

$$CrD_{95}(|\bar{y}_1 - \bar{y}_2|) = \sqrt{R^2 - \frac{r^2}{2}}$$

where:

CrD_{95} : critical difference ($P = 0.95$)

\bar{y}_1 : arithmetic mean of two results obtained in laboratory 1

\bar{y}_2 : arithmetic mean of two results obtained in laboratory 2

R : reproducibility limit: to be determined by interpolation,

r : repeatability limit: if precision varies with level.

If the critical difference is exceeded, another experiment must be performed within the next two months. If the results of the second experiment do not comply with the reproducibility limit, the competent authorities must take the necessary steps.

(b) Procedure for obtaining a provisional reproducibility limit (Chemical analysis)

A provisional reproducibility limit (R_{prov}) is obtained using the following equation:

$$R_{\text{prov}} = \sqrt{(\bar{y}_1 - \bar{y}_2)^2 + \frac{r^2}{2}}$$

where:

\bar{y}_1 : mean of two results obtained in laboratory 1

\bar{y}_2 : mean of two results obtained in laboratory 2 (see Annex IIIa)

r : repeatability limit or provisional repeatability limit.

Remarks:

1. R_{prov} can be used to calculate critical differences (see Annex VI).
2. R_{prov} is fixed at $2r$ if the calculated value for R_{prov} is smaller than $2r$.
3. If the calculated value is larger than $3r$ or greater than twice the R -value predicted from the Horwitz equation (*), then R_{prov} is unacceptably high and cannot be used to calculate the critical difference.
4. R_{prov} should be determined at least once per year on the basis of results obtained in two laboratories (see Annex IV).
5. The mean value of R_{prov} must be used for the calculation of critical differences. The rules given under 2 and 3 apply for the mean value of R_{prov} .

(*) Horwitz equation:

$$RSD_R(\%) = 2^{1-0.5 \log_{10} C}$$

where:

RSD_R : relative standard deviation of reproducibility

C : concentration expressed as a decimal fraction (example: 10 g/100 g = 0,1).

Reference:

Peeler, J.T., Horwitz, W. and Albert, R.
J. Ass. Off. Anal. Chem.
72(5), 784-806 (1989).

⁽¹⁾ The experienced laboratory should generally be one which has participated successfully either in the validation of the test method or in a proficiency test.

The reproducibility limit (R-value) is obtained from the calculated RSD_R -value as follows

$$R = 0.0283 \bar{x} RSD_R$$

\bar{x} : arithmetic mean of the results obtained)

Some calculated RSD_R values (examples)

Concentration	RSD_R (%)
1 g/100 g	4
0,01 g/100 g	8
1 mg/1 000 g	16

A concentration of the analyte of 1 g/100 g will obtain:

$$R = 0.0283 * 1 * 4 = 0,11 \text{ g/100 g.}$$

ANNEX IV

(Article 4)

EVALUATION OF ANALYTICAL RESULTS OBTAINED USING VALIDATED METHODS

If the analytical result shows that a limit has been exceeded, the arithmetic mean of two or more results is calculated. The following procedure is to be followed:

1. In cases where the analytical result represents a single result, a second analysis must be carried out under repeatability conditions. If the two analyses cannot be carried out under repeatability conditions, carry out a further duplicate analysis under repeatability conditions and use those results for the assessment of critical difference compliance.
2. The absolute value of the difference between the arithmetic mean of the results obtained under repeatability conditions and the limit is determined. An absolute value of the difference greater than the critical difference means that the sample which has been analysed does not fulfil the requirements.

The critical difference is determined using the following formula:

$$CrD_{95}(|\bar{y} - m_0|) = \frac{0.84}{\sqrt{2}} \sqrt{R^2 - r^2 \frac{n-1}{n}}$$

where:

\bar{y} : arithmetic mean of the results obtained

m_0 : limit

n : number of analyses/sample

If precision varies with level, it may be necessary to determine r and R by interpolation.

Normally, a final result reported for a sample must show that a limit has been complied with.

Final results

- within the range m_0 and $m_0 + CrD_{95}(|\bar{y} - m_0|)$, if the limit is a maximum;
- within the range m_0 and $m_0 + CrD_{95}(|\bar{y} - m_0|)$, if the limit is a minimum

should therefore occur only exceptionally.

Final results within the ranges mentioned are acceptable only if they occur no more than once for every five samples analysed per consignment. If less than five samples are analysed per consignment, one result within the range mentioned is acceptable. However, the rule that only one result within the ranges mentioned is obtained per five samples analysed must be adhered to if consignments are repeatedly offered by a producer.

3. If the final result x is calculated using a formula of the form $x = y_1 \pm y_2$ (example: water + solids-non-food-fat content of butter to calculate the fat content) where y_1 and y_2 are the final results of a single type of analysis, then the overall repeatability and reproducibility limits r_x and R_x of the final results x are calculated as:

$$r_x = \sqrt{r_1^2 + r_2^2}$$

$$R_x = \sqrt{R_1^2 + R_2^2}$$

where r_1 and r_2 are the repeatability limits and R_1 and R_2 the reproducibility limits of y_1 and y_2 , respectively.

x is compared with the limit m_0 following the rules specified under 1 and 2. The critical difference is determined using the formula:

$$CrD_{95}(|x - m_0|) = \frac{0.84}{\sqrt{2}} \sqrt{R_x^2 - r_x^2 \frac{n-1}{n}}$$

where x is the arithmetic mean of the results x_1 obtained.

4. If the final result is calculated using as formula of the form

$$x = \frac{y_1}{y_2}$$

(example: fat in dry matter content of cheese)

where y_1 and y_2 are the final results of a single type of analysis, then the overall repeatability and reproducibility limits r_x and R_x can be calculated as:

$$r_x = \mu_x \sqrt{r_{s1}^2 + r_{s2}^2}$$

$$R_x = \mu_x \sqrt{R_{s1}^2 + R_{s2}^2}$$

$$\mu_x = \mu_1 | \mu_2$$

μ_1 : limit or target value for y_1 (example: fat)

μ_2 : limit or target value for y_2 (example: dry matter)

$$r_{s1} = \frac{r_1}{\mu_1} \leq 0.15$$

$$r_{s2} = \frac{r_2}{\mu_2} \leq 0.15$$

where

r_1 : repeatability limit, y_1

r_2 : repeatability limit, y_2

$$R_{s1} = \frac{R_1}{\mu_1} \leq 0.15$$

$$R_{s2} = \frac{R_2}{\mu_2} \leq 0.15$$

where

R_1 : reproducibility limit, y_1

R_2 : reproducibility limit, y_2

The procedures for calculating r_x and R_x are applicable only if the relative repeatability and reproducibility limits (r_{s1}^* , r_{s2}^* ; R_{s1}^* ; R_{s2}^*) are smaller than or equal to 0.15.

x is compared with the limit μ_x following the rules specified under 1 and 2. The critical difference is determined using the formula:

$$CrD_{95}(|\bar{x} - \mu_x|) = \frac{0.84}{\sqrt{2}} \sqrt{R_x^2 - r_x^2 \frac{n-1}{n}}$$

Where \bar{x} is the arithmetic mean of the results x obtained in chronological order (*).

(*) Note: If, for example, the results y_{11} , y_{12} , y_{21} and y_{22} , are obtained, the arithmetic mean of y_{11}/y_{21} and y_{12}/y_{22} must be calculated.

ANNEX V
INTERNAL CONTROL

(Article 5)

(a) Internal quality control (IQC) procedure (chemical analysis)

Definition of control material

A material used for the purpose of IQC is subjected to the same, or part of the same, procedure as tested materials.

A control material may be:

- certified reference material,
- in-house reference material,
- material validated by an interlaboratory test,
- fortified material.

Procedure for establishing IQC

The laboratory should introduce IQC following the procedure described in the IUPAC document 'Harmonized Guidelines for Internal Quality Control in analytical laboratories' ⁽¹⁾.

IQC involves including control materials in the analytical sequence or replicating analysis of the test sample. Control materials must be similar in chemical composition to test samples and be adequately stable over the period of interest. It must be demonstrated that they can be suitably divided into identical portions for analysis, and are of concentration of the analyte appropriate to the range of interest.

Control material must be inserted at least once in each analytical run and the value obtained plotted on a control chart in order to measure long-term errors. In addition, the laboratory should periodically demonstrate compliance with repeatability conditions within the run. This may be achieved by duplicate analysis of control and/or test materials. Results of these analyses should be compared with any published repeatability limits and existing data on in-house precision.

Where control materials are used, the values obtained for the between-run analysis of the control material must be plotted on a Shewhart chart (ISO 8258 (1991)) with appropriate control limits. Action limits must be set at

$$x \pm 3s_t$$

where s_t is the total standard deviation,

warning limits at

$$x \pm 2s_t$$

Total standard deviation:

$$s_t = \sqrt{s_b^2 + s_w^2/n}$$

in which:

- s_b : between-run standard deviation
- s_w : within-run standard deviation
- n : number of determinations

In cases where control materials are not used (e.g. because of lack of stability), at least one of the test materials must be analysed in duplicate in each run.

The absolute difference obtained from duplicate analyses within a run (see Annex III) must be plotted. The centre line is $1.128 s_w$, the lower limit is 0, the upper limit (action limit) is $3.686 s_w$, where s_w is the within-run standard deviation.

The control procedure should include materials of low and high levels when the concentration range is large.

⁽¹⁾ M. Thompson and R. Wood: 'Pure and Applied Chemistry' 67 (4), 649-666 (1995).

If the test materials cover a wide range of analyte concentrations, the laboratory should establish the relationship between precision and level. If precision is proportional to level, subsequent control should be based on relative precision (i.e. absolute difference as a percentage of mean value).

An out-of-control condition in the analytical system is signalled if any of the following occur:

- A. the current plot value falls outside the action limits,
- B. the current value and the previous value fall outside the warning limits but within the action limits,
- C. where control materials are used, nine successive values fall on the same side of the mean line.

The laboratory should respond to an out-of-control condition by:

- A. stopping analysis pending diagnostic tests and remedial action, and
- B. rejecting the run of results and re-analysing the test materials.

(b) Procedure for selecting in-house control material and determining 'in-house' precision limits (chemical analysis)

Data on within-laboratory precision may be obtained by replicate analysis of control materials and/or by replicate analysis of test samples.

Laboratories should use the following procedure to establish precision parameters for within-run and between-run variation for subsequent use in constructing control charts. Laboratories may adopt alternative procedures provided that they can adequately demonstrate that reliable precision data have been derived.

1. Selection of control materials

Where it is appropriate for the laboratory to use a control material, data must first be gathered in order to set limits. Where possible, certified reference materials (CRMs) should be used. Candidate control materials should be analysed under repeatability conditions within a run including suitable CRMs with replication and randomisation. Where this approach is not possible, laboratories should seek to participate in proficiency testing and to establish consensus means (assigned values) which may be regarded as a conventional true mean to which a meaningful uncertainty could be attached. Other procedures include assigning true value by formulation or the use of spiked control materials.

In addition, where the laboratory regularly carries out this type of analysis and has already established statistical control, any new control materials (e.g. required because stocks have run out) must be obtained with reference to analyses which are under control using the existing materials.

2. Assignment of limits

Having selected a control material, the laboratory should use it to establish within-run and between-run precision figures.

As a minimum requirement for establishing within-run precision, the control material should be analysed in duplicate on 12 occasions. Duplicate analysis should be performed under repeatability conditions, i.e. same operator, same reagents etc. Duplicate analysis of the control material should be randomised within an analytical run. Each duplicate analysis should be undertaken on a separate day over a period of time so as to reflect reasonable run-to-run variation, taking into account normal variations e.g. reagents, instruments re-calibration and, if appropriate, different analysts.

Note: The use of data which are not fully representative of between-run variation may result in unnecessary replication of analysis because excessively tight limits have been set. Conversely, a laboratory presenting precision data which are too imprecise may be unable to meet prescribed limits in reference methods, could expect to show a poor performance when compared with peer laboratories and may not be producing data which is fit for the intended purpose.

2.1. Determining within-run precision

2.1.1. Within-run precision where a control material is available

Duplicate data (minimum of 12 duplicates) should first be subjected to Cochran's maximum variance test. This involves comparing the squared maximum duplicate range with the sum of square ranges.

$$c = \frac{d^2_{\max}}{\sum_{i=1}^p d_i^2}$$

where

$d_i =$ the difference between duplicates.

The value of Cochran's criterion, C , is compared with tabulated values (ISO 5725 (1994)). Where a value can be classified as a straggler or outlier, the result should be investigated for an explanation, e.g. technical error, computational error, slip in performing the test, analysis of wrong sample. If the explanation of the technical error is such that it proves impossible to replace the suspect result, it should be discarded as a real outlier. When any stragglers or outliers remain that cannot be explained, the stragglers are retained as correct and the statistical outliers are discarded. The laboratory should seek to obtain replacement values.

Once the laboratory is satisfied that data are free from outliers, the within-run standard deviation s_w is obtained as follows:

for each pair x_{i1} , x_{i2} of the p duplicate data, the sum of the duplicates,

$$s_i = x_{i1} + x_{i2}$$

and the difference of the duplicates,

$$d_i = x_{i2} - x_{i1}$$

are computed and summed up to

$$A = \sum_{i=1}^p s_i$$

$$B = \sum_{i=1}^p d_i^2$$

$$C = \sum_{i=1}^p s_i^2$$

An estimate of the within-run standard deviation is

$$s_w = \sqrt{\frac{B}{2p}}$$

The in-house precision limit is $2.8 \cdot s_w$.

If a reference method is used, the in-house precision limit should be compared with the published repeatability limit. The laboratory should comply with the requirement of the reference method. Failure to meet this requirement must be investigated.

The limits set should be regarded as provisional and subject to review.

2.1.2. Within-run precision where a control material is not available

The laboratory may choose to establish within-run precision by duplicate analysis of representative test samples (minimum of 12 duplicate analyses). In cases where it is not possible to use control materials, e.g. because of instability, duplicate data must be accumulated by this method.

Note: It is assumed that the analyses cover a relatively narrow range of values and therefore a single value can be applied to all samples. In cases where the range of results is wider, e.g. over an order of magnitude, and precision is dependent on level, laboratories should investigate use of relative standard deviations.

Data should be subjected to Cochran's test, as in 2.1.1. Once the laboratory is satisfied that data are free from outliers, the within-run standard deviation and in-house precision limit may be obtained as in section 2.1.1.

The within-run standard deviation s_w may be used to construct control charts (see Annex II). The limits set should be regarded as provisional and subject to review.

2.2. Determining between-run precision

Compute the average value ($s_1/2$) for each pair and subject these to Grubbs test (ISO 5725 (1994)). Rejection/acceptance criteria for outliers or stragglers are as described in 2.1.1. The laboratory should seek to obtain a replacement value for any discarded result. Once the laboratory is satisfied that the data are free from outliers, the between-run standard deviation s_b is calculated.

$$s_b = \sqrt{\frac{1}{4(p-1)} \left(C - \frac{p-1}{p} B - \frac{A^2}{p} \right)}$$

or 0 if the expression under the square root sign is negative.

The total standard deviation s_t is used to construct control charts for the average of n determinations (see Annex II). The limits set should be regarded as provisional and subject to review.

3. Review of initial limits

Control limits set as described above must be regarded as initial estimates.

In order to update limits set on the basis of acceptable within-run precision (section 2.1.2), further duplicate data on test samples should be collected. The interval before review will depend on the frequency of analysis. As a guideline, data should be reviewed after a further 10 duplicates have been obtained. All the data should then be subjected to Cochran's test and limits reset on the basis of the new standard deviation figure. Subsequent decisions on the validity of controls limits must be made in the light of further data.

Review of initial data obtained for between-run precision also depends on the frequency of analysis. As a guideline, after a further ten data points have been obtained from analysis of the control material, at a frequency of one analysis per batch, the initial assumptions made on standard deviation and mean should be reviewed.

All the data should be subjected to Grubbs test for outliers. The mean and standard deviation should be recalculated on the basis of the new data.

In addition, at this stage, the laboratory should apply a Cusum chart (BS S700: (1984) and amendment 5480 (1987)) to investigate any problems which may be associated with e.g. ageing of reagents. Any single result falling outside the Cusum 'V-mask' limits must be investigated.

The new limits (mean and standard deviation) must be subject to regular inspection using the Cusum technique. Any indication that the validity of the control material is being brought into question must be thoroughly investigated.

4. Reporting of precision data

Laboratories must send the following information to the competent national authority:

- the method employed,
- the within-run standard deviation s_w , and in-house precision limit,
- the between-run standard deviation s_b ,
- the total standard deviation s_t ,
- the number of analyses involved in obtaining precision data.

ANNEX VI

(Article 6)

EVALUATION OF ASSESSORS AND THE RELIABILITY OF RESULTS IN SENSORY ANALYSES

The following procedures are applicable if scoring methods are used (IDF Standard 99C/1997).

(a) *Determination of the 'repeatability index'*

At least ten samples will be analysed as blind duplicates by an assessor within a period of 12 months. This will usually happen in several sessions. The results for individual product characteristics are evaluated using the following formula:

$$w_i = 1 + \frac{\sum (x_{i1} - x_{i2})^2}{n}$$

where:

w_i : repeatability index

x_{i1} : score for the first evaluation of sample x_i

x_{i2} : score for the second evaluation of sample x_i

n : number of samples

The samples to be evaluated should reflect a broad quality range. w_i should not exceed 1.5 (5-point scales).

(b) *Determining the 'deviation index'*

This index should be used to check whether an assessor uses the same scale for quality evaluation as an experienced group of assessors. The scores obtained by the assessor are compared with the average of the scores obtained by the assessor group.

The following formula is used for the evaluation of results:

$$D_i = 1 + \frac{\sum [(x_{i1} - \bar{x}_{i1})^2 + (x_{i2} - \bar{x}_{i2})^2]}{2n}$$

where:

x_{i1} , x_{i2} : see section (a)

\bar{x}_{i1} , \bar{x}_{i2} : average score of the assessor group for the first and second evaluation respectively of sample x_i

n : number of samples (at least ten per 12 months).

The samples to be evaluated should reflect a broad quality range. D_i should not exceed 1.5 (5-point scales).

Member States must notify any difficulties encountered when applying this procedure.

(c) *Comparison of the results obtained in different regions of a Member State and in different Member States*

Where applicable, a test must be organised at least once per year to compare the results obtained by assessors from different regions. If significant differences are observed, the necessary steps should be taken to identify the reasons and arrive at comparable results.

Member States may organise tests to compare the results obtained by their own assessors and by assessors from neighbouring Member States. Significant differences should lead to an in-depth investigation with the aim of arriving at comparable results.

Member States should notify the Commission of the results of these comparisons.

ANNEX VII

(Article 6)

SENSORY EVALUATION OF BUTTER**1. Scope**

The purpose of this procedure for sensory evaluation of butter is to provide a uniform method applicable in all Member States.

2. Definitions

Sensory evaluation (assessment) means the examination of the attributes of a product by the sense organs.

Panel means a group of selected assessors working, during the assessment, without intercommunication, and without influencing one another.

Scoring means sensory evaluation by a panel, using a numerical scale. A nomenclature of defects must be used.

Grading means a quality classification which is performed on the basis of scoring.

Control documents: documents used to record the individual scores for each attribute and the final grade of the product. (This document may also be used to record chemical composition.)

3. Test room

- 3.1. Precautions must be taken in order that the assessors in the test room are not influenced by external factors.
- 3.2. The test room must be free from foreign odours and easy to clean. The walls must be of a light colour.
- 3.3. The test room and its lighting must be such that the properties of the products to be scored are not affected. The room must be equipped with appropriate temperature control.

4. Selection of assessors

The assessor must be familiar with butter products and be competent to carry out sensory grading. His competence should be assessed on a regular basis (at least once a year) by the competent authority.

5. Requirements for the panel

The number of assessors in the panel should be uneven, the minimum number being three. The majority must be employees of the competent authority or authorised persons not employed by the dairy industry.

A number of factors must be taken into account before evaluation in order to obtain optimal performances from the subjects:

- subjects must not be suffering from an illness which could affect their performance. In such a case, the assessor concerned should be replaced on the panel by another
- subjects must be on time to take part in the evaluation and make sure that they have enough time to make their evaluation
- subjects must not use strong-smelling substances like perfume, after-shave lotion, deodorant, etc. and should avoid eating strong-favoured (e.g. highly spiced) food, etc.
- subjects may not smoke, eat or drink anything other than water during the half hour before the evaluation.

6. Assessment of the value of each attribute

- 6.1. The sensory evaluation is to be carried out in relation to the following three attributes: appearance, consistency and flavour.

Appearance involves the following features: colour, visible purity, mould growth and water dispersion. Water dispersion is tested according to IDF-Standard 112A/1989.

Consistency involves the following features: firmness and spreadability.

Physical methods may be applied for the evaluation of butter consistency. The Commission envisages the future harmonisation of these methods.

Flavour involves the following features: taste and odour.

A significant deviation from the recommended temperature prevents a reliable evaluation of consistency and flavour. The temperature is of paramount importance.

- 6.2. Each attribute has to be sensory evaluated separately. The scoring has to be done according to table 1.
- 6.3. It may be desirable for the assessors to score together, before starting the assessment, one or more reference samples for appearance, consistency and flavour, in order to achieve uniformity.
- 6.4. Scoring for acceptance is as follows:

	Maximum	Required
Appearance	5	4
Consistency	5	4
Flavour	5	4

Where the required score is not obtained, a description of the defect has to be given. The score given by each assessor for each attribute must be recorded in the control document. The product is accepted or rejected on the basis of a majority decision. Cases where differences between the individual scoring for each attribute are wider than adjacent points should not occur frequently (not more than once per 20 samples). Otherwise the competence of the panel should be checked by the panel leader.

7. Supervision

A panel leader who must be an official employee of the competent authority and may be a member of the panel must be generally responsible for the entire procedure. He must record the individual scores for each attribute in the control document and certify whether the product is accepted or rejected.

8. Sampling and preparation of the sample

- 8.1. — It is desirable that the identity of the samples are concealed during the assessment so that any possible bias is avoided.
 - This should be organized by the panel leader prior to the evaluation without the presence of the other panel members.
- 8.2. When the sensory evaluation is carried out at the cold store, the sample is taken using a butter trier. If the sensory evaluation is carried out at another location other than the cold store, then at least a 500 g sample should be taken.
- 8.3. During the evaluation, the butter should have the temperature 10 to 12 °C. Large deviations should be avoided at all cost.

9. Nomenclature

Refer to the appended table 2.

Table 1: Butter scoring

Appearance			Consistency			Flavour + aroma		
Points	No ⁽¹⁾	Remarks	Points (quality class)	No ⁽¹⁾	Remarks	Points (quality class)	No ⁽¹⁾	Remarks
5		Very good ideal type highest quality (equal dry)	5		Very good ideal type highest quality (well spreadable)	5		Very good ideal type highest quality (absolutely pure finest aroma)
4		Good ⁽²⁾ No evident defects	4	17 18	Good ⁽²⁾ hard soft	4		Good ⁽²⁾ No evident defects
3	1 2 3 4 5 6 7 8	Fair (slight defects) loose (free), moisture not uniform, two coloured streaky mottled, marbled speckled oil separation overcoloured weak, open texture	3	14 15 16 17 18	Fair (slight defects) short, brittle, crumbly pasty, doughy, greasy sticky hard soft	3	21 22 25 27 33 34 35	Fair (slight defects) unclear foreign flavour acid cooked flavour, scorched flavour feed flavour coarse, bitter oversalted
2	1 3 4 5 6 10 11 12	Poor (evident defects) loose (free) moisture streaky mottled, marbled speckled oil separation foreign matter mouldy undissolved salt	2	14 15 16 17 18	Poor (evident defects) short, brittle, crumbly pasty, doughy, greasy sticky hard soft	2	21 22 23 25 32 33 34 35 36 38	Poor (evident defects) unclean foreign flavour stale acid oxidized flavour, metallic flavour feed flavour coarse, bitter oversalted musty-flat, putrid chemical flavour
1	1 3 4 5 6 7 9 10 11 12	Very poor (strong defects) loose (free) moisture streaky mottled, marbled, speckled oil separation overcoloured granular foreign matter mouldy undissolved salt	1	14 15 16 17 18	Very poor (strong defects) short, brittle, crumbly pasty, doughy, greasy sticky hard soft	1	22 24 25 26 28 29 30 31 32 34 36 37 38	Very poor (strong defects) foreign flavour cheesy, lactic cheese flavour acid yeasty mouldy flavour rancid oily, fishy tallowy oxidized flavour, metallic flavour coarse, bitter musty-flat, putrid malty chemical flavour

⁽¹⁾ Table 2.⁽²⁾ The defects mentioned under 'good' are only very small deviations from the ideal type.

Table 2: Table of butter defects*I. Appearance*

1. loose (free), moisture
2. not uniform, two coloured
3. streaky
4. mottled, marbled
5. speckled
6. oil separation
7. overcoloured
8. weak (open texture)
9. granular
10. foreign matter
11. mouldy
12. undissolved salt

II. Consistency

14. short, brittle, crumbly
15. pasty, doughy, greasy
16. sticky
17. hard
18. soft

III. Flavour & Aroma

20. without flavour
21. unclean ⁽¹⁾
22. foreign flavour
23. stale
24. cheesy, lactic cheese flavour
25. acid
26. yeasty
27. (a) cooked flavour
(b) scorched flavour
28. mouldy flavour
29. rancid
30. oily, fishy
31. tallowy
32. (a) oxidized flavour
(b) metallic flavour
33. feed flavour
34. coarse, bitter
35. oversalted
36. musty-flat, putrid
37. malty
38. chemical flavour

⁽¹⁾ This designation should be used as seldom as possible and only when the defect cannot be described more accurately.

ANNEX VIII

(Article 7)

PROCEDURE APPLICABLE WHEN THE RESULTS OF AN ANALYSIS ARE DISPUTED (chemical analysis)

1. A further analysis may be carried out at the request of the operator, within seven working days of notification of the results of the first analysis, provided that sealed duplicate samples of the product are available and have been stored appropriately with the competent authorities.
2. The competent authority will send these samples to a second laboratory, at the request and expense of the operator. This laboratory must be authorised to carry out official analyses and must have a proven competence for the analyses under question. This competence should be documented by a successful participation in collaborative studies, proficiency tests or interlaboratory comparisons. The second laboratory must use the reference method. The results obtained by the two laboratories must be evaluated as follows:

(a) *Where both laboratories meet the repeatability requirement and the reproducibility requirement*

The arithmetic mean of the test results obtained by both laboratories is reported as the final result. This final result is evaluated taking the critical difference into consideration, using the following formula:

$$CrD_{95}(|\bar{y} - m_o|) = \frac{0.84}{\sqrt{2}} \sqrt{R^2 - r^2 \left(1 - \frac{1}{2n_1} - \frac{1}{2n_2}\right)}$$

where

\bar{y} : arithmetic mean of all results obtained by both laboratories

m_o : limit

R: reproducibility

r: repeatability

n_1 : number of results obtained by laboratory 1

n_2 : number of results obtained by laboratory 2.

Note: If the final result is calculated using the formulae

$$x = y_1 \pm y_2 \text{ or } x = y_1/y_2$$

(see Annex IV(3) and (4), respectively), R_x^2 and r_x^2 should be inserted into the formulae instead of R^2 and r^2 .

(b) *Where both laboratories meet the repeatability requirement but not the reproducibility requirement*

If the second analysis confirms the first, the quantity under analysis should be rejected as non-compliant. Otherwise, the quantity should be accepted.

(c) *Where only one laboratory meets the repeatability requirement*

The final result of the laboratory meeting the repeatability requirement is to be used to decide whether to accept the quantity under analysis.

(d) *Where neither laboratory meets the repeatability requirement but the reproducibility requirement is met*

(a) applies.

(e) *Where neither laboratory meets either the repeatability requirement or the reproducibility requirement*

The quantity under analysis should be accepted if the results obtained by one laboratory lead to this conclusion.

(f) *Where the results have been obtained using non-validated methods*

The quantity under analysis should be accepted if the results obtained by one laboratory lead to this conclusion.

3. The results of the second analysis must be notified by the competent authority to the operator as soon as possible. The costs of the second analysis are to be borne by the operator if the quantity under analysis is rejected.
4. If the operator can prove, within five working days of sampling, that the sampling procedure was not carried out correctly, sampling must be repeated where possible. If sampling cannot be repeated, the quantity under analysis must be accepted.

ANNEX IX

(Article 8)

DETERMINATION OF THE WATER CONTENT OF BUTTER**1. Scope and field of application**

This reference method specifies a method for the determination of the water content of butter.

2. Reference

IDF standard 50 C: 1995 — Milk and milk products — Methods of sampling

3. Definition

Water content of butter: the loss of mass after completion of the heating process specified in this standard. It is expressed in grams per 100 grams.

4. Principle

Evaporation of the water from a test portion in the presence of pumice at a temperature of 102 °C in a drying oven.

5. Apparatus and materials

Usual laboratory apparatus, and in particular:

- 5.1. Analytical balance, sensitivity 1 mg.
- 5.2. Desiccator provided with efficient drying agent (for example, freshly dried silica gel with hygroscopic indicator).
- 5.3. Drying oven, ventilated, thermostatically controlled, operating at 102 ± 2 °C throughout the total working space.
- 5.4. Glass, porcelain or non-corrosive metal dishes, height of about 20 mm, diameter 60 to 80 mm.
- 5.5. Pumice stone, granulated, washed with a diameter of 0,8-10 mm.

6. Sampling

See IDF 50 C: 1995

7. Procedure**7.1. Preparation of the test sample**

Warm the laboratory sample in the closed glass or suitable plastic container, which should be from one-half to two-thirds full, to a temperature at which the sample will be soft enough to facilitate thorough mixing to a homogeneous state (either by a mechanical shaker or by hand). The temperature of mixing should normally not exceed 35 °C. Cool the sample to ambient temperature. As soon as possible after cooling open the sample container and stir briefly (not longer than 10 seconds) with a suitable device, for example a spoon or spatula, before weighing.

7.2. Determination of the water content

7.2.1. Place approximately 10 g of pumice in the dish (5.4).

7.2.2. Dry the dish with the pumice in the oven (5.3) at 102 ± 2 °C for at least one hour.

Note: the drying periods mentioned in 7.2.2, 7.2.5 and 7.2.7 start when the temperature of the oven reaches 102 ± 2 °C.

7.2.3. Allow the dish to cool in the desiccator (5.2) to the temperature of the balance room and weigh to the nearest 1 mg.

- 7.2.4. Weigh into the dish, to the nearest 1 mg, a test portion of approximately 5 g of the test sample.
- 7.2.5. Place the dish in the oven at 102 ± 2 °C and leave it for three hours.
- 7.2.6. Allow the dish to cool in the desiccator to the temperature of the balance room and weigh to the nearest 1 mg.
- 7.2.7. Repeat the drying process for additional periods of one hour, cooling and weighing each time as specified in 7.2.6 until constant mass (mass change not exceeding 1 mg) is reached.

In the event of an increase in mass, take for calculation the lowest mass recorded.

8. Expression of results

8.1. Method of calculation and formula

Calculate the water content, W, as a percentage by mass using the following formula:

$$W = \frac{m_1 - m_2}{m_1 - m_0} \times 100$$

where

m_0 is the mass in grams of the dish with the pumice (7.2.3)

m_1 is the mass in grams, of the test portion, dish and pumice before drying (7.2.4)

m_2 is the mass in grams, of the test portion, dish and pumice after drying (7.2.7)

Report the result to the first decimal place.

8.2. Repeatability

The absolute difference between the results of two single determinations, carried out simultaneously or in rapid succession by the same operator under the same conditions on identical test material, shall not exceed 0,2 %.

8.3. Reproducibility

The absolute difference between two single and independent results, obtained by two operators working in different laboratories on identical test material, shall not exceed 0,3 %.

9. Test report

The test report shall specify the method used and the results obtained. It shall also mention all operating details not specified in this international standard or regarded as optional, together with details of any incidents which may have influenced the results. The test report shall include all information necessary for the complete identification of the sample.

ANNEX X

(Article 8)

BUTTER: DETERMINATION OF SOLIDS-NON-FAT CONTENT**1. Scope and field of application**

This standard specifies a method for the determination of the solids-non-fat content of butter.

2. References

IDF Standard 50 C: 1995 — Milk and Milk products — Methods of sampling

3. Definitions

Solids-non-fat content of butter: The percentage by mass of substances as determined by the procedure specified. It is expressed in grams per 100 grams.

4. Principle

Evaporation of water from a known mass of butter, extraction of the fat with light petroleum and weighing of the residue.

5. Reagent

Light petroleum with a boiling range between 30 and 60 °C. The reagent shall not leave more than 1 mg of residue after evaporation of 100 ml.

6. Apparatus and materials

- 6.1. Analytical balance sensitivity 1 mg.
- 6.2. Desiccator provided with efficient drying agent (for example, freshly dried silica gel with hygroscopic indicator).
- 6.3. Drying oven, ventilated, thermostatically controlled, operating at 102 ± 2 °C throughout the total working space.
- 6.4. Glass, porcelain or non-corrosive metal dishes, with a spout height of about 20 mm, diameter 60 to 80 mm, provided with a glass stirring rod.
- 6.5. Filter crucible, sintered glass, pore diameter 16 to 40 µm, with suction flask.

7. Sampling

See IDF standard 50 C: 1995

8. Procedure**8.1. Preparation of the test sample:**

Warm the laboratory sample in the closed glass or suitable plastic container, which should be from one-half to two-thirds full, to a temperature at which the sample will be soft enough to facilitate thorough mixing to a homogeneous state (either by a mechanical shaker or by hand). The temperature of mixing should normally not exceed 35 °C. Cool the sample to ambient temperature. As soon as possible after cooling open the sample container and stir briefly (not longer than 10 seconds) with a suitable device, for example a spoon or spatula, before weighing.

8.2. Determination

- 8.2.1. Dry the dish with the rod (6.4) and the crucible (6.5) in the oven (6.3) for 1 hour. Allow these objects to cool in the desiccator and weigh them together (i.e. dish, rod and crucible) to the nearest 1 mg (m_0).

Notes: — As a rule, a cooling time of 45 minutes is sufficient,

— It is important that the same combination of dish, rod and crucible is used for each test portion if more than one test portion is being analysed in the batch.

- 8.2.2. Remove the crucible, record the weight of the dish and rod together, to the nearest 1 mg (m_1).

- 8.2.3. Weigh into the dish, to the nearest 1 mg, a test portion of about 5 g of the test sample (8.1) (m_2).

- 8.2.4. Place the dish (containing the rod and the butter) in the oven at $102 \pm 2^\circ\text{C}$ and leave it over night.
- 8.2.5. Allow the dish (8.2.3) to cool to room temperature.
- 8.2.6. Add 15 ml of warm (approximately 25°C) light petroleum to the dish and detach as much as possible of the sediment adhering to the dish using the glass rod. Transfer the solvent into the crucible and allow it to filter in the suction flask.
- 8.2.7. Carry out operation 8.2.6 four additional times. If there are no traces of fat on the surface of the dish, quantitatively transfer, during the fourth washing, as much as possible of the sediment into the crucible. Otherwise, repeat operation 8.2.6 till complete elimination of all traces of fat.
- 8.2.8. Wash the sediment in the crucible with 25 ml of warm light petroleum.
- 8.2.9. Dry the dish and the glass rod, and the crucible together in the oven at $102 \pm 2^\circ\text{C}$ for 30 minutes.
- 8.2.10. Allow to cool in the desiccator to room temperature and weigh to the nearest 1 mg.
- 8.2.11. Repeat operations 8.2.9 and 8.2.10 until constant mass (mass change not exceeding 1 mg) is reached for the dish, rod and crucible together (m_3).

9. Expression of results

9.1. Calculation of solids-non-fat content

Calculate the solids-non-fat content SNF, as a percentage by mass, using the following formula:

$$\text{SNF} = \frac{m_3 - m_0}{m_2 - m_1} \times 100$$

where

m_0 is the mass, in grams, of the empty dish with the glass rod and the crucible (8.2.1)

m_1 is the mass, in grams, of the empty dish with the glass rod (8.2.2)

m_2 is the mass, in grams, of the test portion and dish with the glass rod (8.2.3)

m_3 is the final mass, in grams, in the dish with the rod and the crucible containing sediment (8.2.11)

Report the result to the first decimal place.

9.2. Repeatability

The absolute difference between the results of two single determinations, carried out simultaneously or in rapid succession by the same operator under the same conditions on identical test material, shall not exceed 0,1 %.

9.3. Reproducibility

The absolute difference between two single and independent results, obtained by two operators working in different laboratories on identical test material, shall not exceed 0,2 %.

10. Test report

The test report shall specify the method used and the results obtained. It shall also mention all operating details not specified in this international standard or regarded as optional, together with details of any incidents which may have influenced the results. The test report shall include all information necessary for the complete identification of the sample.

Note:

If salted butter is analysed, added salt is determined as solids-non-fat. For the determination of the milk solids-non-fat content, the content of added salt has to be subtracted from the solids-non-fat content. The calculated precision figures for the determination of milk solids-non-fat are:

Repeatability: $r = 0,104 \%$

Reproducibility: $R = 0,206 \%$

It can be concluded that the precision figures obtained for the solids-non-fat determination are valid for the determination of the milk solids-non-fat content.

ANNEX XI

(Article 8)

DETERMINATION OF THE FAT CONTENT OF BUTTER

The fat content is obtained indirectly by determination of the water content and the solids-non-fat content according to Annex IX and Annex X, respectively. The percentage, by mass, of fat is equal to

$$100 - (W + \text{SNF})$$

where

W: is the percentage, by mass, of water

SNF: is the percentage, by mass, of solids-non-fat

The calculated precision figures for the determination of the fat content are:

Repeatability: $r = 0,22 \%$

Reproducibility: $R = 0,36 \%$.

ANNEX XII

(Article 9)

DETERMINING THE VANILLIN CONTENT IN CONCENTRATED BUTTER, BUTTER OR CREAM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**1. Scope and field of application**

The method describes a procedure for the quantitative determination of vanillin in concentrated butter, butter or cream.

2. Principle

Extraction of a known quantity of sample with a mixture of isopropanol/ethanol/acetonitrile (1:1:2). Precipitation of the majority of fat by cooling between -15°C and -20°C , followed by centrifugation.

After dilution with water, determination of the vanillin content by high-performance liquid chromatography (HPLC).

3. Apparatus

Usual laboratory apparatus and, in particular, the following:

- 3.1. freezer, operating in the temperature range -15°C to -20°C ;
- 3.2. syringes, disposable of 2 ml capacity;
- 3.3. membrane microfilters of $0,45\text{ }\mu\text{m}$ pore size, resistant to a solution containing 5 % extraction solution (4.4);
- 3.4. liquid chromatography system consisting of a pump (flow of $1,0\text{ ml/min}$), an injector ($20\text{ }\mu\text{l}$ injection, automatic or manual), an UV detector (operated at 306 nm , $0,01\text{ AU}$ full scale), a recorder or integrator and a column thermostat operating at 25°C ;
- 3.5. analytical column ($250\text{ mm} \times 4,6\text{ mm ID}$) packed with LiChrospher RP 18 (Merck, $5\text{ }\mu\text{m}$) or equivalent;
- 3.6. guard column (ca. $20\text{ mm} \times 3\text{ mm ID}$) dry-packed with Perisorb RP 18 ($30\text{ to }40\text{ }\mu\text{m}$) or equivalent.

4. Reagents

All reagents used must be of recognised analytical quality.

- 4.1. Isopropanol
- 4.2. Ethanol 96 % (v/v)
- 4.3. Acetonitrile
- 4.4. Extraction solution

Mix isopropanol (4.1), ethanol (4.2) and acetonitrile (4.3) in the ratio of 1:1:2 (v/v).

- 4.5. Vanillin (4-hydroxy-3-methoxybenzaldehyde)

- 4.5.1. Vanillin stock solution (= $500\text{ }\mu\text{g/ml}$)

Weigh accurately to $0,1\text{ mg}$ about 50 mg (CM mg) vanillin (4.5) in a 100 ml volumetric flask, add 25 ml extraction solution (4.4) and make up with water.

- 4.5.2. Vanillin standard solution (= $10\text{ }\mu\text{g/ml}$).

Pipette 5 ml of the vanillin stock solution (4.5.1) into a volumetric flask of 250 ml and make up with water.

- 4.6. Methanol, HPLC quality
- 4.7. Acetic acid, glacial
- 4.8. Water, HPLC quality

4.9. HPLC mobile phase

Mix 300 ml methanol (4.6) with about 500 ml water (4.8) and 20 ml acetic acid (4.7) in a volumetric flask of 1 000 ml and make up with water (4.8). Filter through 0,45 µm filter (3.3).

5. Procedure

5.1. Preparation of the test sample

5.1.1. Butter

Heat the sample until melting starts. Avoid local overheating above 40 °C. When the sample becomes sufficiently plastic, homogenise it by shaking. Stir the butter for 15 s before taking a sample. Weigh, to the nearest 1 mg, about 5 g (SM g) of butter into a 100 ml volumetric flask.

5.1.2. Concentrated butter

Immediately before sampling place the container, with concentrated butter, into an oven at 40 to 50 °C until it is melted completely. Mix the sample by swirling or stirring, avoiding formation of air bubbles by too vigorous stirring. Weigh, to the nearest 1 mg, about 4 g (SM g) of concentrated butter into a 100 ml volumetric flask.

5.1.3. Cream

Heat the sample in a waterbath or incubator at a temperature of 35 to 40 °C. Distribute the fat homogeneously by swirling and, if necessary, by stirring. Cool the sample quickly to 20 ± 2 °C. The sample should look homogenous, otherwise the procedure should be repeated. Weigh, to the nearest 1 mg, about 10 g (SM g) of cream into a 100 ml volumetric flask.

5.2. Preparation of the test solution

Add about 75 ml extraction solution (4.4) to the test portion (5.1.1, 5.1.2 or 5.1.3), stir, or shake vigorously, for about 15 minutes and make up with extraction solution (4.4). Transfer about 10 ml of this extract to a test tube fitted with stopper. Place the test tube in the freezer (3.1) and allow it to stand for about 30 minutes. Centrifuge the cold extract for 5 minutes at approximately 2 000 rpm and decant immediately. Allow the decanted solution to cool to room temperature. Pipette 5 ml of the decanted solution into a 100 ml volumetric flask and make up with water. Filter an aliquot through a membrane microfilter (3.3). The filtrate is ready for determination by HPLC.

5.3. Calibration

Pipette 5 ml of the vanillin standard solution (4.5.2) into a 100 ml volumetric flask. Add 5 ml extraction solution (4.4) and make up to the mark with water. This solution contains 0,5 µg/ml of vanillin.

5.4. Determination by HPLC

Allow the chromatographic system to stabilise for about 30 minutes. Inject the standard solution (5.3). Repeat this until the difference in peak area or peak height between two successive injections is less than 2 %. Under the conditions described the retention time of vanillin is about 9 minutes. Analyse the standard solution (5.3) in duplicate by injecting 20 µl. Inject 20 µl of the test solutions (5.2). Determine the area or height of the vanillin peak obtained. Repeat the duplicate injection of the standard solution (5.3) after 10 injections of test samples (5.2).

6. Calculation of the results

Calculate the average peak area (or height) (AC), of the vanillin peaks associated with the bracketing duplicate injections for each batch of test solutions (four areas or heights in total).

Calculate the response factor (R):

$$R = AC/CM$$

where CM is the mass of vanillin in mg (4.5.1).

The content (mg/kg) of vanillin (C) in the test sample is given by:

$$C = \frac{AS \times 20 \times 0,96}{SM \times R}$$

where:

AS = peak area of the vanillin peak of the test sample

SM = mass of test sample in g (5.1.1, 5.1.2 or 5.1.3).

Where cream is analysed for vanillin, the tracer concentration has to be expressed as mg tracer/kg milk fat. This is done by multiplying C by 100/f. f is the fat content of the cream in percent (m/m).

20 = factor which takes into account the dilutions of the standard and the test sample

0,96 = correction factor for the fat content in first dilution of the test sample

Note: Instead of peak area, peak heights can be used (see 8.3).

7. Accuracy of the method

7.1. Repeatability (r)

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 16 mg/kg.

7.2. Reproducibility (R)

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 27 mg/kg.

8. Tolerance limits

8.1. Three samples must be taken from the traced product in order to check homogeneity.

8.2. Tracer obtained either from vanilla or from synthetic vanillin:

8.2.1. The incorporation rate for 4-hydroxy-3-methoxybenzaldehyde is 250 g per tonne of concentrated butter or butter. Where cream is traced, the incorporation rate is 250 g per tonne of milk fat.

8.2.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (critical difference for a 95 % probability level (DCr_{95}) taken into consideration):

- 221,0 mg/kg (95 % of the minimum incorporation rate),
- 159,0 mg/kg (70 % of the minimum incorporation rate).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation between 221,0 mg/kg and 159,0 mg/kg.

8.3. Tracer obtained exclusively from vanilla beans or integral extracts thereof:

8.3.1. The incorporation rate for 4-hydroxy-3-methoxybenzaldehyde is 100 g per tonne of concentrated butter or butter. Where cream is traced, the incorporation rate is 100 g per tonne of milk fat.

8.3.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (critical difference for a 95 % probability level (DCr_{95}) taken into consideration):

- 79,0 mg/kg (95 % of the minimum incorporation rate),
- 54,0 mg/kg (70 % of the minimum incorporation rate).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation between 79,0 mg/kg and 54,0 mg/kg.

9. Notes

9.1. Repeatability r is the value below which the absolute difference between two single test results obtained with the same method on identical test material, under the same conditions (same apparatus, same laboratory, and in a short interval of time), may be expected to lie with a specified probability; in the absence of other indications, the probability is 95 %.

- 9.2. Reproducibility R is the value below which the absolute difference between two single test results obtained with the same method on identical test material, under different conditions (different operators, different apparatus, different laboratories and/or different time), may be expected to lie with a specified probability; in the absence of other indications, the probability is 95 %.
- 9.3. Recovery of added vanillin at a level of 250 mg/kg butteroil varies from 97,0 to 103,8. The average content found was 99,9 % with a standard deviation of 2,7 %.
- 9.4. The standard solution contains 5 % extraction solution to compensate for peak broadening caused by the presence of 5 % of the extraction solution of the test samples. This enables quantification by peak height.
- 9.5. The analysis is based on a linear calibration line with a zero intercept.

By using appropriate dilutions of the standard solution (4.5.2), the linearity should be checked the first time the analysis is carried out and then at regular intervals and after changes in or repair of the HPLC equipment.

ANNEX XIII

(Article 9)

DETERMINING THE ETHYL ESTER OF BETA-APO-8'-CAROTENIC ACID IN CONCENTRATED BUTTER AND BUTTER BY SPECTROMETRY**1. Scope and field of application**

The method describes a procedure for the quantitative determination of the ethyl ester of beta-apo-8'-carotenic acid (apo-carotenic ester) in concentrated butter and butter. The apo-carotenic ester is the sum of all substances present in an extract of samples obtained under the conditions described in the method which absorb light at 440 nm.

2. Principle

The butterfat is dissolved in light petroleum and the absorbance measured at 440 nm. The apo-carotenic ester content is determined by reference to an external standard.

3. Apparatus

- 3.1. Pipettes — graduated, of capacity 0.25, 0.50, 0.75 and 1.0 ml
- 3.2. Spectrophotometer — suitable for use at 440 nm (and 447-449 nm) and equipped with cells of optical path length 1 cm
- 3.3. Volumetric flasks, 20 ml and 100 ml
- 3.4. Analytical balance, sensitivity 0.1 mg.

4. Reagents

All reagents must be of recognised analytical grade.

4.1. Apo-carotenic ester suspension (approximately 20 %)**4.1.1. Establish the content of the suspension as follows:**

Weigh about 400 mg in a volumetric flask (100 ml), dissolve in 20 ml chloroform (4.4) and make up the volume with cyclohexane (4.5). Dilute 5 ml of this solution to 100 ml with cyclohexane (solution A). Dilute 5 ml of solution A to 100 ml with cyclohexane. Measure the absorbance at 447-449 nm (measure the maximum against cyclohexane as a blank using cells with 1 cm optical path length).

$$\text{Apo-carotenic ester content (\%)} = \frac{A_{\max} \cdot 40\,000}{A \cdot 2\,550}$$

A_{\max} = absorbance of the measuring solution at the maximum

A = weight of sample (g)

2 550 = reference A (1 %, 1 cm) value

The purity of the suspension is P (%).

Note: Apo-carotenic ester suspension is sensitive to air, heat and light. In the unopened, original container (sealed under nitrogen) and in a cool place it can be stored for about 12 months. After opening the contents should be used within a short period.

4.1.2. Apo-carotenic ester standard solution, approx. 0.2 mg/ml

Weigh to the nearest 0.1 mg about 0.100 g of apo-carotenic ester suspension (4.1.1) (Wg), dissolve in petroleum spirit (4.2), transfer quantitatively into a volumetric flask of capacity 100 ml, and make up to the mark with petroleum spirit.

This solution contains (W.P)/10 mg/ml of apo-carotenic ester.

Note: The solution must be stored in a cool place in the dark. Discard unused solution after one month.

- 4.2. Petroleum spirit (40 — 60 °C).
- 4.3. Sodium sulphate, anhydrous, granular, previously dried at 102 °C for two hours.
- 4.4. Chloroform.
- 4.5. Cyclohexane.

5. Procedure

5.1. Preparation of the test sample

5.1.1. Concentrated butter

Melt the sample in an oven at approximately 45 °C.

5.1.2. Butter

Melt the sample in an oven at approximately 45 °C and filter a representative portion through a filter containing about 10 g of anhydrous sodium sulphate (4.3) in an environment shielded from strong natural and artificial light and maintained at 45°C. Collect a suitable amount of butterfat.

5.2. Determination

Weigh, to the nearest 1 mg approximately 1 g of concentrated butter (or extracted butterfat (5.1.2)), (Mg). Transfer quantitatively to a 20 ml (Vml) volumetric flask using petroleum spirit (4.2), make up to the mark and mix thoroughly.

Transfer an aliquot to a 1 cm cell and measure the absorbance at 440 nm, against a petroleum spirit blank. Obtain the concentration of apo-carotenic ester in the solution by reference to the calibration graph (C µ/ml).

5.3. Calibration graph

Pipette 0, 0.25, 0.5, 0.75 and 1.0 ml of apo-carotenic ester standard solution (4.1.2) into five 100 ml volumetric flasks. Dilute to volume with petroleum spirit (4.2) and mix.

The approximate concentrations of the solutions range from 0 to 2 µg/ml and are calculated accurately by reference to the concentration of the standard solution (4.1.2) (W.P)/10 mg/ml. Measure the absorbances at 440 nm against a petroleum spirit (4.2) blank.

Plot the values of absorbance on the y axis against apo-carotenic ester concentration on the x axis.

6. Calculation of the results

6.1. Apo-carotenic ester content, expressed as mg/kg product, is given by:

Concentrated butter: $(C.V)/M$

Butter: $0.82 (C.V)M$

where:

C= apo-carotenic ester content, µg/ml, read from the calibration graph (5.3)

V = volume (ml) of the test solution (5.2)

M= mass (g) of the test portion (5.2)

0.82 = a correction factor for the butterfat content of butter.

7. Accuracy of the method

7.1. Repeatability

7.1.1. Butter analysis

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 1.4 mg/kg.

7.1.2. Concentrated butter analysis

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 1.6 mg/kg.

7.2. Reproducibility

7.2.1. Butter analysis

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 4.7 mg/kg.

7.2.2. Concentrated butter analysis

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 5.3 mg/kg.

7.3. Source of precision data

The precision data were determined from an experiment conducted in 1995 involving 11 laboratories and 12 traced samples (six blind duplicates) for butter and 12 traced (six blind duplicates) for concentrated butter.

8. Tolerance limits

8.1. Three samples must be taken from the traced product in order to check on the correct tracing of the product.

8.2. Butter

8.2.1. The incorporation rate for butter, taking into account background absorbance, is 22 mg/kg.

8.2.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits [critical difference for a 95 % probability level (DCr_{95}) taken into consideration]:

- 18.0 mg/kg (95 % of the minimum incorporation rate),
- 13.0 mg/kg (70 % of the minimum incorporation rate).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation between 18.0 mg/kg and 13.0 mg/kg.

8.3. Concentrated butter

8.3.1. The incorporation rate for concentrated butter, taking into account background absorbance, is 24 mg/kg.

8.3.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits [critical difference for a 95 % probability level (DCr_{95}) taken into consideration]:

- 20.0 mg/kg (95 % of the minimum incorporation rate),
- 14.0 mg/kg (70 % of the minimum incorporation rate).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation between 20.0 mg/kg and 14.0 mg/kg.

ANNEX XIV

(Article 9)

**DETERMINING SITOSTEROL OR STIGMASTEROL IN BUTTER OR CONCENTRATED BUTTER BY
CAPILLARY-COLUMN GAS CHROMATOGRAPHY****1. SCOPE AND FIELD OF APPLICATION**

The method describes a procedure for the quantitative determination of sitosterol or stigmasterol in butter and concentrated butter. Sitosterol is taken to be the sum of β -sitosterol and 22 dihydro- β -sitosterol, other sitosterols are assumed to be insignificant.

2. PRINCIPLE

The butter or concentrated butter is saponified with potassium hydroxide in ethanolic solution and the unsaponifiables are extracted with diethyl ether.

The sterols are transformed into trimethyl-silyl ethers and are analysed by capillary-column gas chromatography with reference to an internal standard/betulin.

3. APPARATUS

- 3.1. 150 ml saponification flask fitted with a reflux condenser having ground-glass joints.
- 3.2. 500 ml separating funnels.
- 3.3. 250 ml flasks.
- 3.4. Pressure equalising funnels, 250 ml or similar, to collect waste diethyl ether.
- 3.5. Glass column, 350 mm \times 20 mm² fitted with sintered glass plug.
- 3.6. Waterbath or isomantle.
- 3.7. Reaction vials, 2 ml.
- 3.8. Gas chromatograph suitable for use with a capillary column, provided with a splitting system consisting of:
 - 3.8.1. a thermostatic chamber for columns capable of maintaining the desired temperature with an accuracy of ± 1 °C;
 - 3.8.2. a temperature-adjustable vaporisation unit;
 - 3.8.3. a flame ionisation detector and converter-amplifier;
 - 3.8.4. an integrator-recorder suitable for use with the converter-amplifier (3.8.3).
- 3.9. A fused-silica capillary column entirely coated with BP1 or equivalent in a uniform thickness 0,25 μ m; the column must be capable of resolving trimethyl-silyl derivatives of lanosterol and sitosterol. A BP1, length 12 m, internal diameter 0,2 mm, is suitable.
- 3.10. A 1 μ l gas chromatography microsyringe with hardened needle.

4. REAGENTS

All reagents must be of recognised analytical grade. The water used must be distilled water or water of at least equivalent purity.

- 4.1. Ethanol, at least 95 % pure.
- 4.2. Potassium hydroxide, 60 % solution, dissolve 600 g potassium hydroxide (minimum 85 %) in water and make up to one litre with water.
- 4.3. Betulin of at least 99 % purity.
 - 4.3.1. Solutions of betulin in diethyl ether (4.4).
 - 4.3.1.1. The concentration of betulin solution used for sitosterol determination should be 1,0 mg/ml.
 - 4.3.1.2. The concentration of betulin solution used for stigmasterol determination should be 0,4 mg/ml.

- 4.4. Diethyl ether, analytical purity (free from peroxides or residue).
- 4.5. Sodium sulphate, anhydrous, granular, previously dried at 102 °C for two hours.
- 4.6. Silylating reagent, for example TRI-SIL (available from Pierce Chemical Co, Cat No 49001) or equivalent (Important: TRI-SIL is inflammable, toxic, corrosive and possibly carcinogenic. Laboratory personnel must be familiar with TRI-SIL safety data and take the appropriate precautions.)
- 4.7. Lanosterol.
- 4.8. Sitosterol, of known purity not less than 90 % pure (P).

Note 1: The purity of standard materials used for calibration must be determined using the method of normalisation. Assume that all sterols present in the sample are represented on the chromatogram, the total area of the peaks represents 100 % of the sterol constituents and that the sterols give the same detector response. Linearity of the system must be validated over the concentration ranges of interest.

- 4.8.1. Sitosterol standard solution — prepare a solution containing, to the nearest 0,001 mg/ml, approximately 0,5 mg/ml (W_1) sitosterol (4.8) in diethyl ether (4.4).
- 4.9. Stigmasterol, of known purity not less than 90 % pure (P).
- 4.9.1. Stigmasterol standard solution — prepare a solution containing, to the nearest 0,001 mg/m, approximately 0,2 mg/ml (W_1) stigmasterol (4.9) in diethyl ether (4.4).
- 4.10. Resolution test mixture. Prepare a solution containing 0,05 mg/ml lanosterol (4.7) and 0,5 mg/ml sitosterol (4.8) in diethyl ether (4.4).

5. METHOD

- 5.1. Preparation of standard solutions for chromatography. The internal standard solution (4.3.1) must be added to the appropriate sterol standard solution at the same time as it is added to the saponified sample (see 5.2.2).
- 5.1.1. Sitosterol standard chromatographic solution: transfer 1 ml of sitosterol standard solution (4.8.1) to each of two reaction vials (3.7) and remove the diethyl ether with a stream of nitrogen. Add 1 ml of internal solution (4.3.1.1) and remove the diethyl ether with a stream of nitrogen.
- 5.1.2. Stigmasterol standard chromatographic solution: transfer 1 ml of stigmasterol standard solution (4.9.1) to each of two reaction vials (3.7) and remove the diethyl ether with a stream of nitrogen. Add 1 ml of internal standard solution (4.3.1.2) and remove the diethyl ether with a stream of nitrogen.

5.2. *Preparation of the unsaponifiables*

- 5.2.1. Melt the butter sample at a temperature not exceeding 35 °C, mix the sample thoroughly by stirring.

Weigh, to the nearest 1 mg, approximately 1 g of butter (W_2) or concentrated butter (W_2) into a 150 ml flask (3.1). Add 50 ml ethanol (4.1) and 10 ml potassium hydroxide solution (4.2). Fit the reflux condenser and heat at approximately 75 °C for 30 minutes. Detach the condenser and cool the flask to approximately ambient temperature.

- 5.2.2. Add 1,0 ml of internal standard solution (4.3.1.1) to the flask if sitosterol is to be determined, or (4.3.1.2) if stigmasterol is to be determined. Mix thoroughly. Transfer the contents of the flask quantitatively into a 500 ml separating funnel (3.2), washing the flask in turn with 50 ml water and 250 ml diethyl ether (4.4). Shake the separating funnel vigorously for two minutes and allow the phases to separate. Run off the lower aqueous layer and wash the ether layer by shaking with four successive 100 ml aliquots of water.

Note 2: To avoid formation of an emulsion, it is essential that the first two water washes are carried out gently (10 inversions). The third wash can be shaken vigorously for 30 seconds. If an emulsion is formed it can be destroyed by the addition of 5-10 ml of ethanol. If ethanol is added it is essential to carry out a further two vigorous water washes.

- 5.2.3. Pass the clear, soap-free ether layer through a glass column (3.5) containing 30 g anhydrous sodium sulphate (4.5). Collect the ether in a 250 ml flask (3.3). Add one anti-bumping granule and evaporate to near dryness in a waterbath or isomantle, taking care to collect the waste solvents.

Note 3: If sample extracts are taken to complete dryness at too high a temperature sterol losses may occur.

5.3. Preparation of trimethyl silyl ethers

- 5.3.1. Transfer the ether solution remaining in the flask to a 2 ml reaction vial (3.7) with 2 ml diethyl ether and remove the ether using a stream of nitrogen. Wash the flask with two further 2 ml aliquots of diethyl ether, transferring to the vial and removing the ether with nitrogen each time.
- 5.3.2. Silylate the sample by addition of 1 ml TRI-SIL (4.6). Close the vial and shake vigorously to dissolve. If dissolution is incomplete, warm to 65-70 °C. Allow to stand for at least five minutes before injecting into the gas-chromatograph. Silylate standards in the same way as samples. Silylate the resolution test mixture (4.10) in the same way as samples.

Note 4: Silylation must be effected in a water-free environment. Incomplete silylation of betulin is indicated by a second peak close to that of betulin.

The presence of ethanol at the silylation stage will interfere with silylation. This may result from inadequate washing at the extraction stage. If this problem persists, a fifth wash may be introduced at the extraction stage, shaking vigorously for 30 seconds.

5.4. Gas-chromatographic analysis

5.4.1. Choice of operating conditions

Set up the gas-chromatograph according to the manufacturer's instructions.

The guideline operating conditions are as follows:

- column temperature: 265 °C
- injector temperature: 265 °C
- detector temperature: 300 °C
- carrier gas flow rate: 0,6 ml/min.
- hydrogen pressure: 84 kPa
- air pressure: 155 kPa
- sample split: 10:1 to 50:1; the split ratio must be optimised in accordance with the manufacturer's instructions and linearity of detector response, then validated over the concentration range of interest.

Note 5: It is especially important that the injection liner is regularly cleared.

- amount of substance injected: 1 µl of TMSE solution.

Allow the system to equilibrate and obtain a satisfactory stable response before commencing any analysis.

These conditions must be varied in the light of column and gas-chromatograph characteristics so as to obtain chromatograms which meet the following requirements:

- the sitosterol peak must be adequately resolved from lanosterol. Figure 1 shows a typical chromatogram which should be obtained from a silylated resolution test mixture (4.10),
- the relative retention times of the following sterols should be approximately:
 - cholesterol: 1.0
 - stigmasterol: 1.3
 - sitosterol: 1.5
 - betulin: 2.5
- the retention time for betulin should be approximately 24 minutes.

5.4.2. Analytical procedure

Inject 1 µl of silylated standard solution (stigmasterol or sitosterol) and adjust the integrator calibration parameters.

Inject a further 1 µl of silylated standard solution to determine the response factors with reference to betulin.

Inject 1 µl of silylated sample solution and measure peak areas. Each chromatographic run must be bracketed by an injection of standards.

As a guide, six injections of sample should be included in each bracketed run.

Note 6: Integration of the stigmasterol peak should include any tailing as defined by points 1, 2 and 3 in Figure 2b.

Integration of the sitosterol peak should include the area of the 22 dihydro- β -sitosterol (stigmastanol) peak which elutes immediately after sitosterol (see Figure 3b) when evaluating total sitosterol.

6. CALCULATION OF RESULTS

- 6.1. Determine the area of the sterol peaks and betulin peaks in both standards bracketing a batch, and calculate R_1 :

$$R_1 = \frac{\text{average area of the sterol peak in the standard}}{\text{average area of the betulin peak in the standard}}$$

Determine the area of the sterol peak (stigmasterol and sitosterol) and betulin peak in the sample and calculate R_2 :

$$R_2 = \frac{\text{area of the sterol peak in the sample}}{\text{area of the betulin peak in the sample}}$$

W_1 = sterol content of the standard (mg) contained in 1 ml of standard solution (4.8.1 or 4.9.1)

W_2 = weight of sample (g) (5.2.1)

P = purity of standard sterol (4.8 or 4.9)

$$\text{Sterol content of the sample (mg/kg)} = \frac{R_2}{R_1} \times \frac{W_1}{W_2} \times P \times 10$$

7. ACCURACY OF THE METHOD

7.1. Butter

7.1.1. Repeatability

7.1.1.1. Stigmasterol

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 19,3 mg/kg.

7.1.1.2. Sitosterol

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 23,0 mg/kg.

7.1.2. Reproducibility

7.1.2.1. Stigmasterol

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 31,9 mg/kg.

7.1.2.2. Sitosterol

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 8,7 % relative to the mean of the determination.

7.1.3. Source of precision data

The precision data were determined from an experiment conducted in 1992 involving eight laboratories and six samples (three blind duplicates) for stigmasterol and six samples (three blind duplicates) for sitosterol.

7.2. Concentrated butter

7.2.1. Repeatability

7.2.1.1. Stigmasterol

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 10,2 mg/kg.

7.2.1.2. Sitosterol

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material, may not exceed 3,6 % relative to the mean of the determinations.

7.2.2. Reproducibility

7.2.2.1. Stigmasterol

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 25,3 mg/kg.

7.2.2.2. Sitosterol

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material, may not exceed 8,9 % relative to the mean of the determinations.

7.2.3. Source of precision data

The precision data were determined from an experiment conducted in 1991 involving nine laboratories and six samples (three blind duplicates) for stigmasterol and six samples (three blind duplicates) for sitosterol.

8. TOLERANCE LIMITS

8.1. Three samples must be taken from the traced product in order to check on the correct tracing of the product.

8.2. Butter

8.2.1. Stigmasterol

8.2.1.1. The incorporation rate for stigmasterol is 150 g of at least 95 % pure stigmasterol per tonne of butter, i.e. 142,5 mg/kg, or 170 g of at least 85 % pure stigmasterol per tonne of butter, i.e. 144,5 mg/kg.

8.2.1.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (critical difference for a 95 % probability level (DCr_{95}) taken into consideration):

- 116,0 mg/kg (95 % of the minimum incorporation rate for 95 % pure stigmasterol),
- 118,0 mg/kg (95 % of the minimum incorporation rate for 85 % pure stigmasterol),
- 81,0 mg/kg (70 % of the minimum incorporation rate for 95 % pure stigmasterol),
- 82,0 mg/kg (70 % of the minimum incorporation rate for 85 % pure stigmasterol).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation respectively between 116,0 mg/kg and 81,0 mg/kg or 118,0 mg/kg and 82,0 mg/kg.

8.2.2. Sitosterol

8.2.2.1. The incorporation rate for sitosterol is 600 g of at least 90 % pure sitosterol per tonne of butter, i.e. 540 mg/kg.

8.2.2.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (critical difference for a 95 % probability level (DCr_{95}) taken into consideration):

- 486,0 mg/kg (95 % of the minimum incorporation rate for 90 % pure sitosterol),
- 358,0 mg/kg (70 % of the minimum incorporation rate for 90 % pure sitosterol).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation between 486,0 mg/kg and 358,0 mg/kg.

8.3. Concentrated butter

8.3.1. Stigmasterol

8.3.1.1. The incorporation rate for stigmasterol is 150 g of at least 95 % pure stigmasterol per tonne of concentrated butter, i.e. 142,5 mg/kg; or 170 g of at least 85 % pure stigmasterol per tonne of concentrated butter, i.e. 144,5 mg/kg.

8.3.1.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (critical difference for a 95 % probability level (DCr_{95}) taken into consideration):

- 120,0 mg/kg (95 % of the minimum incorporation rate for 95 % pure stigmasterol),
- 122,0 mg/kg (95 % of the minimum incorporation rate for 85 % pure stigmasterol),
- 84,0 mg/kg (70 % of the minimum incorporation rate for 95 % pure stigmasterol),
- 86,0 mg/kg (70 % of the minimum incorporation rate for 85 % pure stigmasterol).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation respectively between 120,0 mg/kg and 84,0 mg/kg or 122,0 mg/kg and 86,0 mg/kg.

8.3.2. Sitosterol

8.3.2.1. The incorporation rate for sitosterol is 600 g of at least 90 % pure sitosterol per tonne of concentrated butter, i.e. 540 mg/kg.

8.3.2.2. The results for the three samples obtained from the analysis of the product are used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (critical difference for a 95 % probability level (DCr_{95}) taken into consideration):

- 486,0 mg/kg (95 % of the minimum incorporation rate for 90 % pure sitosterol),
- 358,0 mg/kg (70 % of the minimum incorporation rate for 90 % pure sitosterol).

The tracer concentration in the sample giving the lowest result is used in conjunction with interpolation between 486,0 mg/kg and 358,0 mg/kg.

Figure 1

Chromatogram of resolution test mixture.

Complete resolution is preferable, i.e. the peak trace for lanosterol should return to baseline before leaving for the sitosterol peak although incomplete resolution is tolerable.

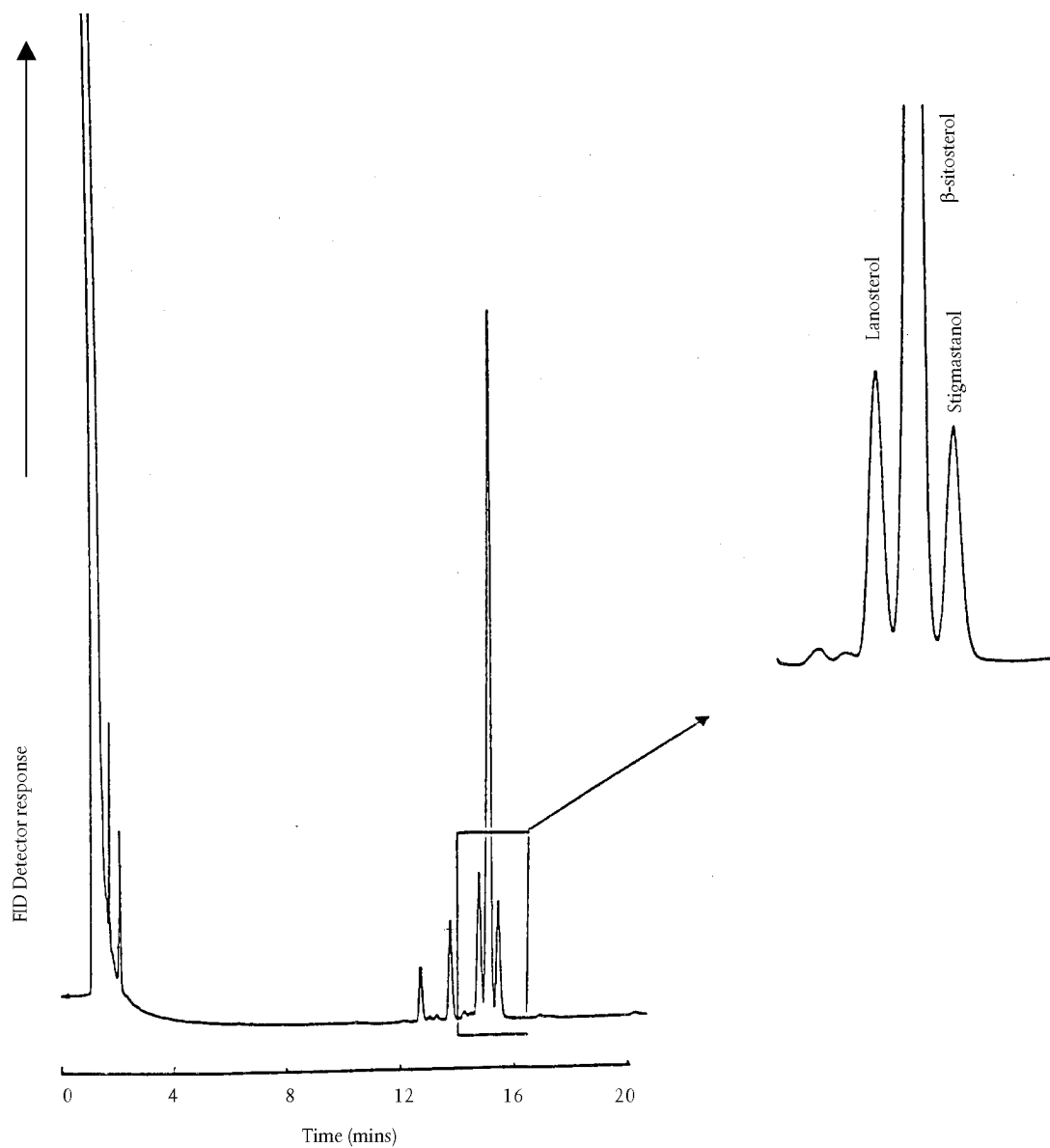


Figure 2a
Stigmasterol standard

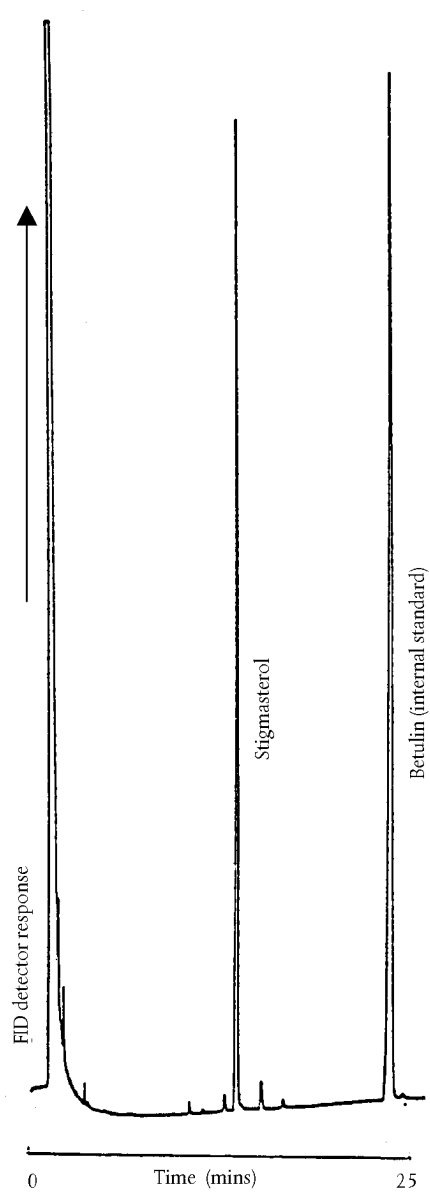
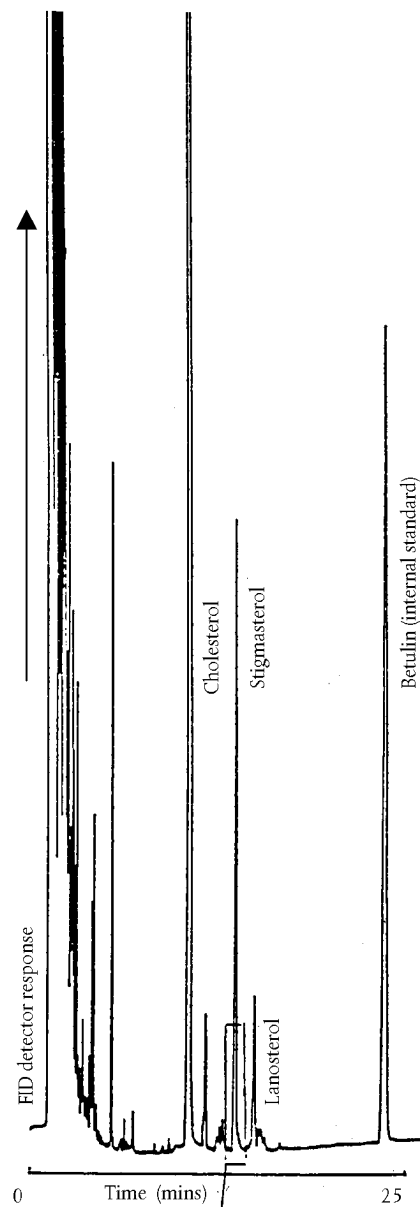


Figure 2b
Butter sample denatured with stigmasterol



Note: Integration of the stigmasterol peak should include any tailing as defined by points 1, 2 and 3.

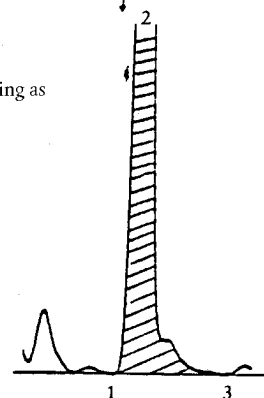
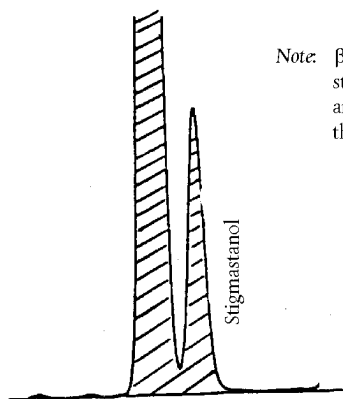
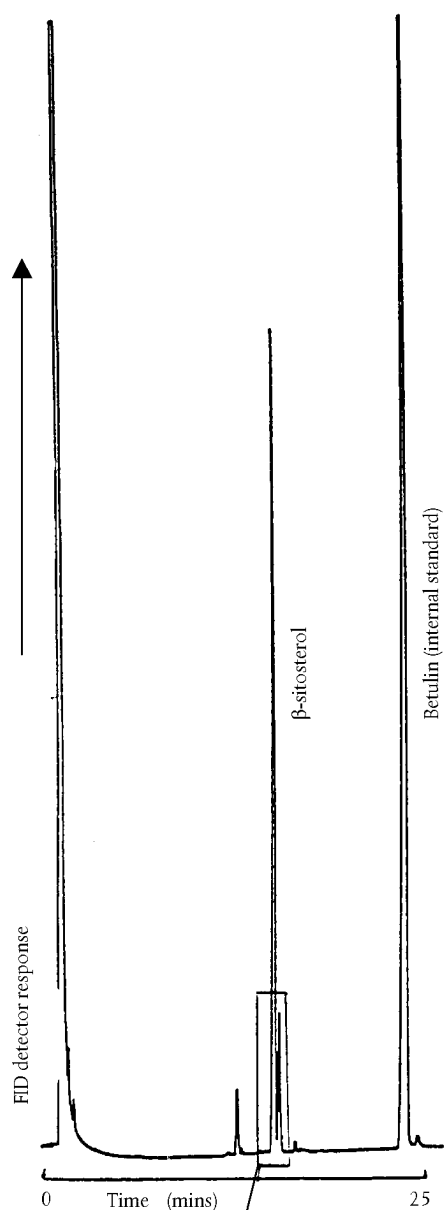
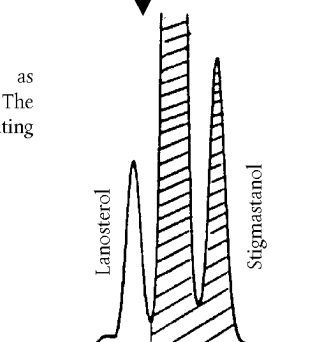
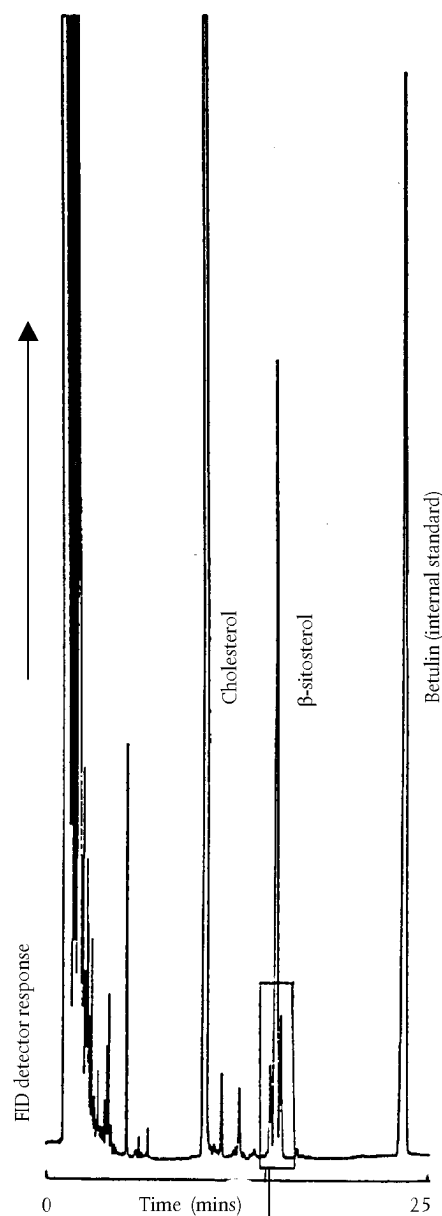


Figure 3a
Sitosterol standard



Note: β -sitosterol often contains an impurity (identified as stigmastanol) which elutes immediately after β -sitosterol. The areas of these two peaks should be summed when evaluating the total β -sitosterol present.

Figure 3b
Butter sample
denatured with β -Sitosterol



ANNEX XV

(Article 10)

REFERENCE METHOD FOR THE DETECTION OF COWS' MILK AND CASEINATE IN CHEESES FROM EWES' MILK, GOATS' MILK OR BUFFALOS' MILK OR MIXTURES OF EWES', GOATS' AND BUFFALOS' MILK**1. Scope**

Detection of cows' milk and caseinate in cheeses made from ewes' milk, goats' milk, buffalos' milk or mixtures of ewes', goats' and buffalos' milk by isoelectric focusing of γ -caseins after plasminolysis.

2. Field of application

The method is suitable for sensitive and specific detection of native and heat-treated cows' milk and caseinate in fresh and ripened cheeses made from ewes' milk, goats' milk, buffalos' milk or mixtures of ewes', goats' and buffalos' milk. It is not suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates.

3. Principle of the method

- 3.1. Isolation of caseins from cheese and the reference standards.
- 3.2. Dissolving of the isolated caseins and submitting to plasmin (EC.3.4.21.7) cleavage.
- 3.3. Isoelectric focusing of plasmin-treated caseins in the presence of urea and staining of proteins.
- 3.4. Evaluation of stained γ_3 and γ_2 -casein patterns (evidence of cows' milk) by comparison of the pattern obtained from the sample with those obtained in the same gel from the reference standards containing 0 % and 1 % cows' milk.

4. Reagents

Unless otherwise indicated, analytical grade chemicals must be used. Water must be double-distilled or of equivalent purity.

Note: The following details apply to laboratory prepared polyacrylamide gels containing urea, of dimensions $265 \times 125 \times 0,25$ mm. Where other sizes and types of gel are used, the separation conditions may have to be adjusted.

Isoelectric focusing**4.1. Reagents for production of the urea containing polyacrylamide gels****4.1.1. Stock gel solution**

Dissolve:

4,85 g acrylamide

0,15 g N, N'-methylene-bis-acrylamide (BIS)

48,05 g urea

15,00 g glycerol (87 % w/w),

in water and make up to 100 ml and store in a brown glass bottle in the refrigerator.

Note: A commercially available preblended acrylamide/BIS solution can be used in preference to the quoted fixed weights of the neurotoxic acrylamides. Where such a solution contains 30 % w/v acrylamide and 0,8 % w/v BIS, a volume of 16,2 ml must be used for the formulation instead of the fixed weights. The shelf life of the stock solution is a maximum of 10 days; if its conductivity is more than 5 μ S, de-ionize by stirring with 2 g Amberlite MB-3 for 30 minutes, then filter through a 0,45 μ m membrane.

4.1.2. Gel solution

Prepare a gel solution by mixing additives and ampholytes with the stock gel solution (see 4.1.1).

9,0 ml stock solution

24 mg β -alanine

500 μ l ampholyte pH 3,5 — 9,5 ⁽¹⁾

250 μ l ampholyte pH 5-7 ⁽¹⁾

250 μ l ampholyte pH 6-8 ⁽¹⁾.

Mix the gel solution and de-gas for two to three minutes in an ultrasonic bath or in vacuo.

Note: Prepare the gel solution immediately prior to pouring it (see 6.2).

4.1.3. Catalyst solutions

4.1.3.1. N, N, N' N' — tetramethylethylenediamine (Temed).

4.1.3.2. 40 % w/v ammonium persulphate (PER):

Dissolve 800 mg PER in water and make up to 2 ml.

Note: Always use freshly prepared PER solution.

4.2. Contact fluid

Kerosene or liquid paraffin

4.3. Anode solution

Dissolve 5,77 g phosphoric acid (85 % w/w) in water and dilute to 100 ml.

4.4. Cathode solution

Dissolve 2,00 g sodium hydroxide in water and dilute to 100 ml with water.

Sample preparation

4.5. Reagents for protein isolation

4.5.1. Dilute acetic acid (25,0 ml of glacial acetic acid made up to 100 ml with water)

4.5.2. Dichloromethane

4.5.3. Acetone

4.6. Protein dissolving buffer

Dissolve

5,75 g glycerol (87 % w/w)

24,03 g urea

250 mg dithiothreitol,

in water and make up to 50 ml

Note: Store in a refrigerator, maximum shelf-life one week.

4.7. Reagents for plasmin cleavage of caseins

4.7.1. Ammonium carbonate buffer

Titrate a 0,2 mol/l ammonium hydrogencarbonate solution (1,58 g/100 ml water) containing 0,05 mol/l ethylenediaminetetraacetic acid (EDTA, 1,46 g/100 ml with a 0,2 mol/l ammonium carbonate solution (1,92 g/100 ml water) containing 0,05 mol/l EDTA to pH 8.

4.7.2. Bovine plasmin (EC. 3.4.21.7), activity at least 5 U/ml.

4.7.3. ϵ -Aminocaproic acid solution for enzyme inhibition

Dissolve 2,624 g ϵ -aminocaproic acid (6 amino-n-hexanoic acid) in 100 ml of 40 % (v/v) ethanol.

⁽¹⁾ The products Ampholine® pH 3,5-9,5 (Pharmacia) and Resolyte® pH 5-7 and pH 6-8 (BDH, Merck) have proved particularly suitable for obtaining the required separation of γ -caseins.

4.8. Standards

- 4.8.1. Certified reference standards of a mixture of renneted ewes' and goats' skimmed milk containing 0 % and 1 % of cows' milk are available from the Commission's Institute for Reference Materials and Measurements, B-2440 Geel, Belgium.

- 4.8.2. Preparation of laboratory interim-standards of buffalos' renneted milk containing 0 % and 1 % of cows' milk

Skimmed milk is prepared by centrifuging of either buffalo or bovine raw bulk milk at 37 °C at 2 500 g for 20 minutes. After cooling the tube and contents rapidly to 6 to 8 °C, the upper fat layer is removed completely. For the preparation of the 1 % standard add 5,00 ml of bovine skimmed milk to a 495 ml of buffalos' skimmed milk in a 1 l beaker, adjust the pH to 6,4 by the addition of dilute lactic acid (10 % w/v). Adjust the temperature to 35 °C and add 100 µl of calf rennet (rennet activity 1: 10 000, c. 3 000 U/ml), stir for 1 minute and then leave the beaker covered with an aluminium foil at 35 °C for one hour to allow formation of the curd. After the curd has formed, the whole renneted milk is freeze-dried without prior homogenization or draining of the whey. After freeze-drying it is finely ground to produce a homogeneous powder. For the preparation of the 0 % standard, carry out the same procedure using genuine buffalo skimmed milk. The standards must be stored at - 20 °C.

Note: It is advisable to check the purity of the buffalos' milk by isoelectric focusing of the plasmin-treated caseins before preparation of the standards.

Reagents for protein staining

4.9. Fixative

Dissolve 150 g trichloroacetic acid in water and make up to 1 000 ml.

4.10. Destaining solution

Dilute 500 ml methanol and 200 ml glacial acetic acid to 2 000 ml with distilled water.

Note: Prepare the destaining solution fresh every day; it can be prepared by mixing equal volumes of stock solutions of 50 % (v/v) methanol and 20 % (v/v) glacial acetic acid.

4.11. Staining solutions

4.11.1. Staining solution (stock solution 1)

Dissolve 3,0 g Coomassie Brilliant Blue G-250 (C.I. 42655) in 1 000 ml 90 % (v/v) methanol using a magnetic stirrer (approximately 45 minutes), filter through two medium-speed folded filters.

4.11.2. Staining solution (stock solution 2)

Dissolve 5,0 g copper sulphate pentahydrate in 1 000 ml 20 % (v/v) acetic acid.

4.11.3. Staining solution (working solution)

Mix together 125 ml of each of the stock solutions (4.11.1, 4.11.2) immediately prior to staining.

Note: The staining solution should be prepared on the day that it is used.

5. Equipment

- 5.1. Glass plates (265 × 125 × 4 mm); rubber roller (width 15 cm); levelling table
- 5.2. Gel carrier sheet (265 × 125 mm).
- 5.3. Covering sheet (280 × 125 mm). Stick on strip of adhesive tape (280 × 6 × 0,25 mm) to each long edge (see Figure 1).
- 5.4. Electrofocusing chamber with cooling plate (e.g. 265 × 125 mm) and suitable power supply (≥ 2,5 kV) or automatic electrophoresis device.
- 5.5. Circulation cryostat, thermostatically controlled at 12 ± 0,5 °C.
- 5.6. Centrifuge, adjustable to 3 000 g.
- 5.7. Electrode strips (≥ 265 mm long).

- 5.8. Plastic dropping bottles for the anode and cathode solutions.
- 5.9. Sample applicators (10 × 5 mm, viscose or low protein-adsorption filter paper).
- 5.10. Stainless steel scissors, scalpels and tweezers.
- 5.11. Stainless steel or glass staining and destaining dishes (e.g. 280 × 150 mm instrument trays).
- 5.12. Adjustable rod homogenizer (10 mm shaft diameter), rpm range 8 000 to 20 000.
- 5.13. Magnetic stirrer.
- 5.14. Ultrasonic bath.
- 5.15. Film welder.
- 5.16. 25 µl micropipettes.
- 5.17. Vacuum concentrator or freeze-dryer.
- 5.18. Thermostatically controlled water bath adjustable to 35 and 40 ± 1 °C with shaker.
- 5.19. Densitometer equipment reading at $\lambda = 634$ nm.

6. Procedure

6.1. Sample preparation

6.1.1. Isolation of caseins

Weigh the amount equivalent to 5 g dry mass of cheese or the reference standards into a 100 ml centrifuge tube, add 60 distilled water and homogenize with a rod homogenizer (8 000 to 10 000 rpm). Adjust to pH 4,6 with dil. acetic acid (4.5.1) and centrifuge (5 minutes, 3 000 g). Decant the fat and whey, homogenize the residue at 20 000 rpm in 40 ml distilled water adjusted to pH 4,5 with dil. acetic acid (4.5.1), add 20 ml dichloromethane (4.5.2), homogenize again and centrifuge (5 minutes, 3 000 g). Remove the casein layer that lies between the aqueous and organic phases (see Figure 2) with a spatula and decant off both phases. Rehomogenize the casein in 40 ml distilled water (see above) and 20 ml dichloromethane (4.5.2) and centrifuge. Repeat this procedure until both extraction phases are colourless (two to three times). Homogenize the protein residue with 50 ml acetone (4.5.3) and filter through a medium-speed folded filter paper. Wash the residue on the filter with two separate 25 ml portions of acetone each time and allow to dry in the air or a stream of nitrogen, then pulverize finely in a mortar.

Note: Dry casein isolates should be kept at -20 °C.

6.1.2. Plasmin cleavage of β -caseins to intensify γ -caseins

Disperse 25 mg of isolated caseins (6.1.1) in 0,5 ml ammonium carbonate buffer (4.7.1) and homogenize for 20 minutes by e.g. using ultrasonic treatment. Heat to 40 °C and add 10 µl plasmin (4.7.2), mix and incubate for one hour at 40 °C with continuous shaking. To inhibit the enzyme add 20 µl ϵ -aminopropionic acid solution (4.7.3), then add 200 mg of solid urea and 2 mg of dithiothreitol.

Note: To obtain more symmetry in the focused casein bands it is advisable to freeze-dry the solution after adding the ϵ -aminopropionic acid and then dissolving the residues in 0,5 ml protein dissolving buffer (4.6).

6.2. Preparation of the urea containing polyacrylamide gels

With the aid of a few drops of water roll the gel carrier sheet (5.2) onto a glass plate (5.1), removing any extraneous water with paper towel or tissue. Roll the cover sheet (5.3) with spacers (0,25 mm) onto another glass plate in the same way. Lay the plate horizontally on a levelling table.

Add 10 µl Temed (4.1.3.1) to the prepared and de-aerated gel solution (4.1.2), stir and add 10 µl PER-solution (4.1.3.2), mix thoroughly and immediately pour out evenly onto the centre of the cover sheet. Place one edge of the gel carrier plate (sheet side down) on the cover sheet plate and lower it slowly so that a gel film forms between the sheets and spreads out regularly and free of bubbles (Figure 3). Carefully lower the gel carrier plate completely using a thin spatula and place three more glass plates on top of it to act as weights. After polymerization is complete (about 60 minutes) remove the gel polymerized onto the gel carrier sheet along with the cover sheet by tipping the glass plates. Clean the reverse of the carrier sheet carefully to remove gel residues and urea. Weld the gel sandwich into a film tube and store in a refrigerator (maximum six weeks).

Note: The cover sheet with the spacers can be re-used. The polyacrylamide gel can be cut to smaller sizes, recommended when there are few samples or if an automatic electrophoresis device is used (two gels, size 4,5 × 5 cm).

6.3. *Isoelectric focusing*

Set the cooling thermostat to 12 °C. Wipe off the reverse of the gel carrier sheet with kerosene, then drip a few drops of kerosene (4.2) onto the centre of the cooling block. Then roll the gel sandwich, carrier side down, onto it, taking care to avoid bubbles. Wipe off any excess kerosene and remove the cover sheet. Soak the electrode strips with the electrode solutions (4.3, 4.4), cut to gel length and place in the positions provided (distance of electrodes 9,5 cm).

Conditions for isoelectric focusing:

6.3.1. Gel size 265 × 125 × 0,25 mm

Step	Time (min.)	Voltage (V)	Current (mA)	Power (W)	Volt-hours (Vh)
1. Pre-focusing	30	maximum 2 500	maximum 15	constant 4	c. 300
2. Sample focusing ⁽¹⁾	60	maximum 2 500	maximum 15	constant 4	c. 1 000
3. Final focusing	60	maximum 2 500	maximum 5	maximum 20	c. 3 000
	40	maximum 2 500	maximum 6	maximum 20	c. 3 000
	30	maximum 2 500	maximum 7	maximum 25	c. 3 000

⁽¹⁾ Sample application: After pre-focusing (step 1), pipette 18 µl of the sample and standard solutions onto the sample applicators (10 × 5 mm), place them on the gel at 1 mm intervals from each other and 5 mm longitudinally from the anode and press lightly. Carry out focusing using the above conditions, carefully removing the sample applicators after the 60 minutes of sample focusing.

Note: If thickness or width of the gels are changed, the values for current and power have to be suitably adjusted (e.g. double the values for electric current and power if a 265 × 125 × 0,5 mm gel is used).

6.3.2. Example of a voltage programme for an automatic electrophoresis device (2 gels of 5,0 × 4,5 cm), electrodes without strips applied directly to the gel.

Step	Voltage	Current	Power	Temp.	Volt-hours
1. Pre-focusing	1 000 V	10,0 mA	3,5 W	8 °C	85 Vh
2. Sample focusing	250 V	5,0 mA	2,5 W	8 °C	30 Vh
3. Focusing	1 200 V	10,0 mA	3,5 W	8 °C	80 Vh
4. Focusing	1 500 V	5,0 mA	7,0 W	8 °C	570 Vh

Place sample applicator in step 2 at 0 Vh.

Remove sample applicator in step 2 at 30 Vh.

6.4. *Protein staining*

6.4.1. Protein fixation

Remove the electrode strips immediately after turning off the power and put the gel immediately into a staining/destaining dish filled with 200 ml fixative (4.9); leave for 15 minutes, shaking continuously.

6.4.2. Washing and staining the gel plate

Thoroughly drain off the fixative and wash the gel plate twice for 30 seconds each time with 100 ml destaining solution (4.10). Pour off the destaining solution and fill the dish with 250 ml staining solution (4.11.3); allow to stain for 45 minutes with gentle shaking.

6.4.3. Destaining the gel plate

Pour off the staining solution, wash the gel plate twice using a 100 ml destaining solution (4.10) each time, then shake with 200 ml destaining solution for 15 minutes and repeat the destaining step at least two or three times until the background is clear and uncoloured. Then rinse the gel plate with distilled water (2×2 minutes) and dry in the air (2 to 3 hours) or with a hairdryer (10 to 15 minutes).

Note 1: Carry out fixing, washing, staining and destaining at 20 °C. Do not use elevated temperatures.

Note 2: If more sensitive silver staining (e.g. Silver Staining Kit, Protein, Pharmacia Biotech, Code No 17-1150-01) is preferred, plasmin-treated casein samples have to be diluted to 5 mg/ml.

7. Evaluation

Evaluation is performed by comparing the protein patterns of the unknown sample with reference standards on the same gel. Detection of cows' milk in cheeses from ewes' milk, goats' milk and buffalos' milk and mixtures of ewes', goats' and buffalos' milk is done via the γ_3 - and γ_2 -caseins, whose isoelectric points range between pH 6,5 and pH 7,5 (Figures 4 a, b, Figure 5). The detection limit is less than 0,5 %.

7.1. Visual estimation

For visual evaluation of the amount of bovine milk it is advisable to adjust the concentrations of samples and standards to obtain the same level of intensity of the ovine, caprine and/or buffalo γ_2 - and γ_3 -caseins (see ' γ_2 E,G,B' and ' γ_3 E,G,B' in Figures 4 a, b and Figure 5). After which the amount of bovine milk (less than, equal to or greater than 1 %) in the unknown sample can be judged directly by comparing the intensity of the bovine γ_3 - and γ_2 -caseins (see ' γ_3 C' and ' γ_2 C' in Figures 4 a, b and Figure 5) to those of the 0 % and 1 % reference standards (ewe, goat) or, laboratory interim-standards (buffalo).

7.2. Densitometric estimation

If available, apply densitometry (5.19) for the determination of the peak area ratio of bovine to ovine, caprine and/or buffalo γ_2 - and γ_3 -caseins (see Figure 5). Compare this value to γ_2 - and γ_3 -casein peak area ratio of the 1 % reference standard (ewe, goat) or laboratory interim-standard (buffalo) analysed on the same gel.

Note: The method is operating satisfactorily, if there is a clear positive signal for both bovine γ_2 - and γ_3 -caseins in the 1 % reference standard but not in the 0 % reference standard. If not, optimize the procedure following the details of the method precisely.

A sample is judged as being positive, if both bovine γ_2 - and γ_3 -caseins or the corresponding peak area ratios are equal to or greater than the level of the 1 % reference standard.

8. References

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3. Krause I., Berner I., Klostermeyer H.: *Sensitive detection of cow milk in ewe and goat milk and cheese by carrier ampholyte — and carrier ampholyte/immobilized pH gradient — isoelectric focusing of γ -caseins using plasmin as signal amplifier.* in: *Electrophoresis-Forum 89* (B. J. Radola, ed.) pp 389-393, Bode-Verlag, München (1989).
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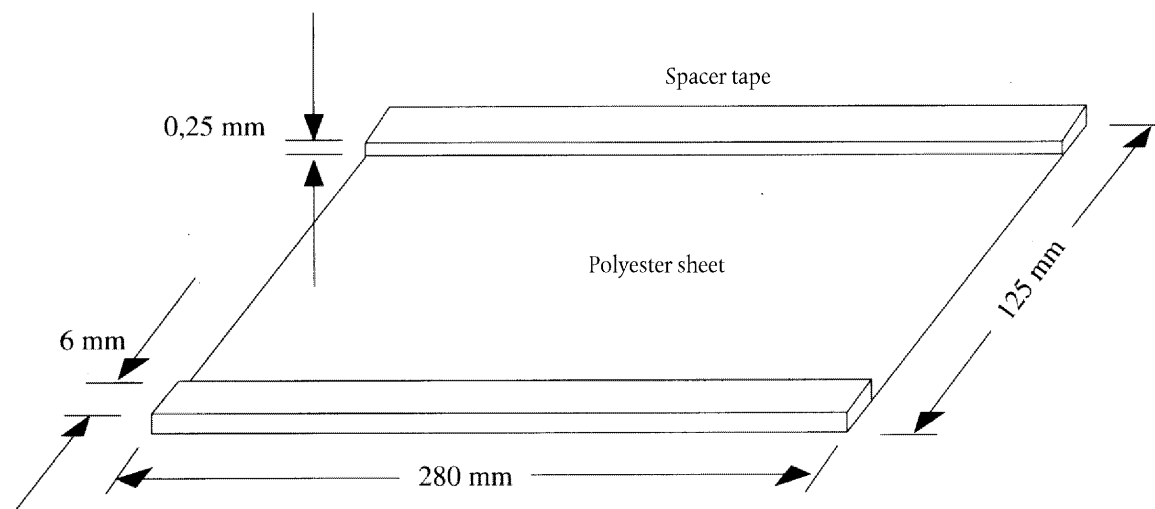


Figure 1: Schematic drawing of the covering sheet

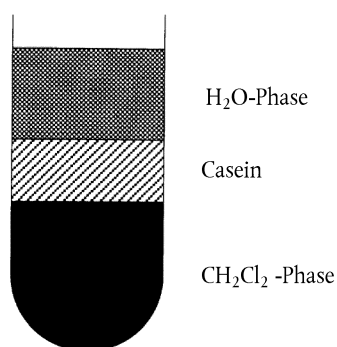


Figure 2: Casein layer floating between aqueous and organic phases after centrifugation

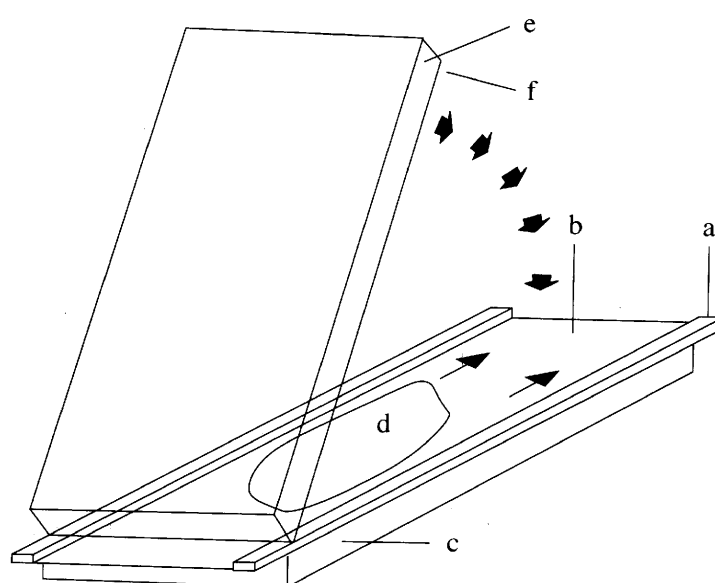


Figure 3: Flapping technique for casting of ultrathin polyacrylamide gels

a = spacer tape (0.25 mm); b = covering sheet (5.3); c, e = glass plates (5.1); d = gel solution (4.1.2); f = gel carrier sheet (5.2)

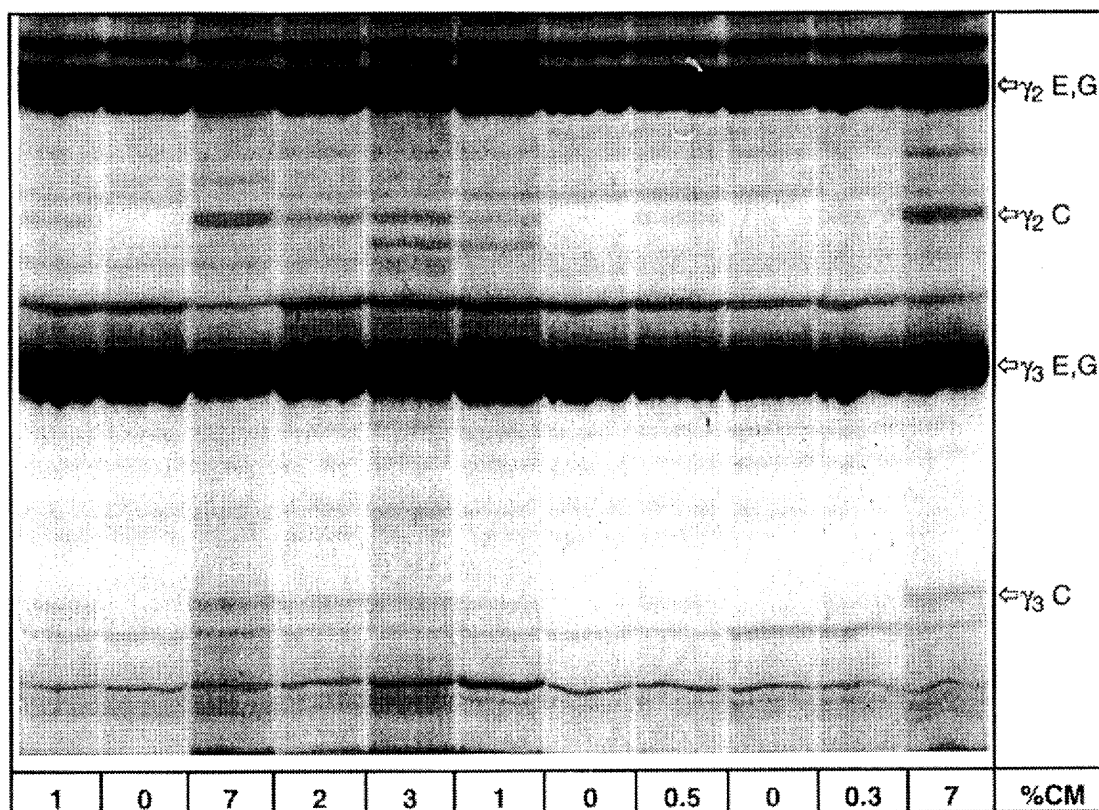


Figure 4a: Isoelectric focusing of plasmin-treated caseins from ewes' and goats' milk cheese containing different amounts of cows' milk.

% CM = percentage of cows' milk, C = cow, E = ewe, G = goat

Upper half of the IEF gel is shown.

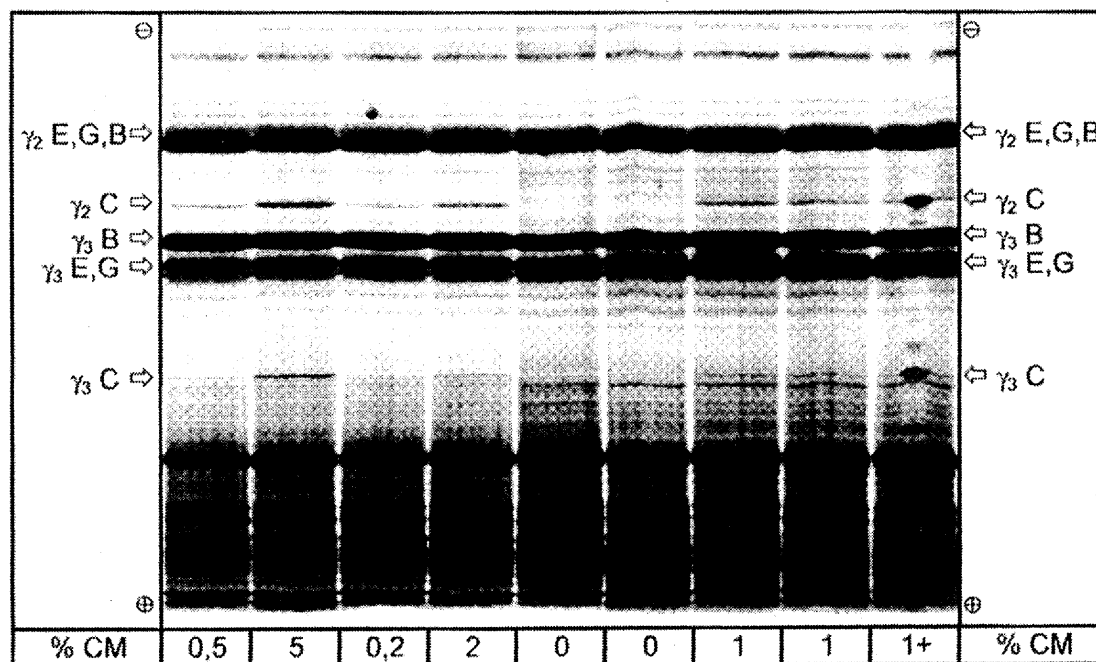


Figure 4b: Isoelectric focusing of plasmin treated caseins from cheese made from mixtures of ewes', goats' and buffalos' milk containing different amounts of cows' milk.

% CM = percentage of cows' milk; 1+ = sample containing 1 % of cows' milk and spiked with pure bovine casein at the middle of the track. C = cow, E = ewe, G = goat, B = buffalo.

Total separation distance of the IEF gel is shown.

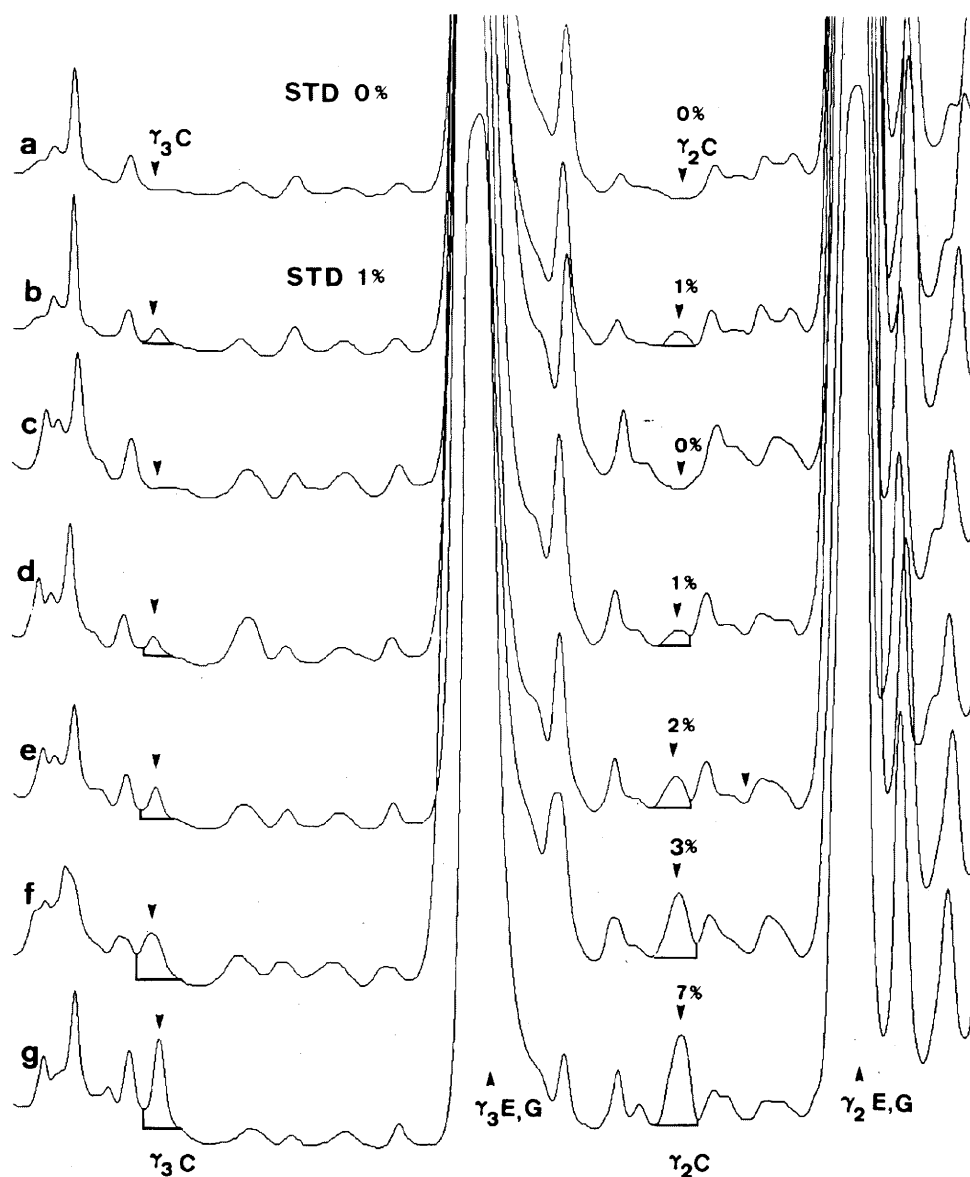


Figure 5: Surperposition of densitograms of standards (STD) and cheese samples made from a mixture of ewes' and goats' milk after isoelectric focusing.

a,b = standards containing 0 and 1 % of cows' milk; c-g = cheese samples containing 0, 1, 2, 3 and 7 % of cows' milk. C = cow, E = ewe, G = goat.

Upper half of the IEF gel was scanned at $\lambda = 634$ nm.

ANNEX XVI

(Article 11)

REFERENCE METHOD FOR DETECTING COLIFORMS IN BUTTER, SKIMMED-MILK POWDER, CASEIN AND CASEINATES

Samples corresponding to 1 g butter are inoculated into the culture medium, if butter is tested for the presence of coliforms.

Where skimmed-milk powder or casein/caseinates is tested for the presence of coliforms, 0,1 g samples are inoculated into the culture medium.

IDF Standard 73A: 1985, method B, is applied with the following modifications:

- 1) Sample preparation is according to IDF Standard 122B:1992. For acid casein, the sample preparation procedure described in IDF Standard 73A:1985 may be used alternatively.
- 2) Only tubes inoculated with 1 g samples (butter) or 0,1 g samples (skimmed-milk powder, casein/caseinates) respectively, are incubated and evaluated. No decimal dilutions are made.

Evaluation of the results

Three negative results:	Requirement fulfilled
Two or three positive results:	Requirement not fulfilled
Two negative results:	Two further samples should be analysed, weighing 1 g (butter) and 0,1 g (skimmed-milk powder, casein/caseinates). If the last two results are negative, the requirement is fulfilled; otherwise the requirement is not fulfilled.

Remark

Coliform content: 1/10 g for butter; 1/g for skimmed-milk powder, casein/caseinates, on average.

Results indicating that the requirement is fulfilled are obtained with a probability of 93 %.

Coliform content: 1/g for butter; 1/0,1 g for skimmed milk powder or casein/caseinates on average.

Results indicating that the requirement is not fulfilled are obtained with a probability of 91 %.

(Assumption: Poisson distribution)

ANNEX XVII

(Article 12)

METHOD FOR DETERMINING THE LACTOSE CONTENT OF PRODUCTS FALLING WITHIN CN CODE 2309 ⁽¹⁾

PART I

1. Field of application

The method should be applied in cases where the lactose content exceeds 0,5 %.

2. Principle

Dissolve sugars in water. Allow the yeast (*Saccharomyces cerevisiae*) to act; this will leave the lactose intact. Determine the lactose content of that solution, by the Luff-Schoorl method, after clarification and filtering.

3. Reagents

Sodium thiosulphate 0,1 N

Indicator: starch solution. Add a mixture of 5 g of soluble starch (10 mg of mercuric iodide may be added as a preservative agent) and 30 ml of water to 1 litre of boiling water; keep the mixture boiling for three minutes; leave to cool.

Potassium iodide solution AR at 30 % (w/v).

Sulphuric acid solution 6 N

Luff-Schoorl reagent:

(a) Dissolve 25 g of iron-free copper II sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in 100 ml water

(b) Dissolve 50 g of citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) in 50 ml of water

(c) Dissolve 143,8 g of anhydrous sodium carbonate (Na_2CO_3) in about 300 ml of hot water and allow to cool.

Add (b) to (c), shaking gently, then add (a). Make up to 1 litre, allow to stand overnight and then filter. Check the normality of the reagent thus obtained (Cu 0.1 N, Na_2CO_3 2 N). The pH must be approximately 9.4.

Carrez I solution: dissolve 23,8 g Zn ($\text{C}_2\text{H}_3\text{O}_2$)₂·2H₂O and 3 g glacial acetic acid in water and make up to 100 ml.

Carrez II: dissolve 10,6 g de $\text{K}_4\text{F}_2(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ in water and make up to 100 ml.

Grains of pumice stone, treated when boiling with hydrochloric acid, washed with water and dried. Suspension of *Saccharomyces cerevisiae*: 25 g of fresh yeast in 100 ml of water (do not keep for more than one week in a refrigerator).

4. Procedure

Weigh, to the nearest 1 mg, 1 g of the sample for analysis; place this in a calibrated 100 ml flask. Add 25 to 30 ml of water. Place the flask in a boiling waterbath for thirty minutes, then cool to approximately 35 °C.

Add 5 ml of the yeast suspension ⁽²⁾ and shake. Leave the calibrated flask and its contents in a waterbath at a temperature of 38 to 40 °C for two hours.

After fermentation, cool to a temperature of approximately 20 °C. Add 2,5 ml of the Carrez I solution and shake for thirty seconds; then add 2,5 ml of the Carrez II solution and shake again for thirty seconds. Make up to 100 ml with water, mix and filter. Pipette a quantity of the filtrate of not more than 25 ml, preferably containing 40 to 80 mg lactose; if necessary, make up to 25 ml with water and determine the anhydrous lactose content by the Luff-Schoorl method.

Carry out complete blank test with yeast only.

⁽¹⁾ Regulation (EEC) No 222/88.

⁽²⁾ For products containing more than 40 % fermentable sugar, increase the quantity of suspension.

PART II

1. Determining the lactose content by the Luff-Schoorl method

Pipette 25 ml of Luff-Schoorl reagent and place this in an Erlenmeyer flask of 300 ml; add 25 ml of the clarified solution measured exactly.

After adding two grains of pumice stone, heat, shaking by hand over a naked flame of average height and bring the liquid to the boil for approximately two minutes. Immediately place the Erlenmeyer flask on a wire gauze with an asbestos screen, under which a flame has previously been lit. This is so regulated that the Erlenmeyer flask is heated solely at the bottom; then fit a reflux condenser. From that moment, boil for ten minutes exactly. Cool immediately in cold water and after approximately five minutes test as follows:

Add to the liquid 10 ml of potassium iodide and immediately afterwards, but with caution (since considerable foaming may occur), 25 ml of sulphuric acid 6 N.

The test with sodium thiosulphate until a dull yellow colour appears and towards the end of the test add the starch indicator.

Carry out the same test with a mixture of precisely 25 ml of Luff-Schoorl reagent and 25 ml of water, after adding 10 ml of potassium iodide and 25 ml of sulphuric acid 6 N, this time without bringing to the boil.

Using the following table, establish the amount in mg of lactose corresponding to the difference between the results of the two tests (expressed in ml of sodium thiosulphate 0,1 N).

TABLE

Table for 25 ml de Luff-Schoorl reagent
(see conditions in the text)

1. Sodium thiosulphate 0,1 N

2. Lactose $C_{12}H_{22}O_{11}$

1	2		1	2	
ml	mg	Difference	ml	mg	Difference
1	3,6	3,7	12	44,6	3,8
2	7,3	3,7	13	48,4	3,8
3	11,0	3,7	14	52,2	3,8
4	14,7	3,7	15	56,0	3,9
5	18,4	3,7	16	59,9	3,9
6	22,1	3,7	17	63,8	3,9
7	25,8	3,7	18	67,7	4,0
8	29,5	3,7	19	71,7	4,0
9	33,2	3,8	20	75,7	4,1
10	37,0	3,8	21	79,8	4,1
11	40,8	3,8	22	83,9	4,1
		3,8	23	88,0	

ANNEX XVIII

(Article 13)

DETECTION OF RENNET WHEY IN SKIMMED-MILK POWDER FOR PUBLIC STORAGE BY DETERMINATION OF GLYCOMACROPEPTIDES HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**1. Scope and field of application**

This method allows detection of rennet whey in skimmed-milk powder intended for public storage by determination of the glycomacropeptides.

2. Reference

International Standard ISO 707 — Milk and Milk Products — Methods of sampling, conforming to the guidelines contained in Annex I(2)(c) last paragraph.

3. Definition

Glycomacropeptide content of skimmed-milk powder: the content of substances determined by the method set out below, expressed as a percentage by mass.

4. Principle

- Reconstitution of the skimmed-milk powder, removal of fat and proteins with trichloroacetic acid, followed by centrifugation;
- Determination of the quantity of glycomacropeptides (GMP) in the supernatant by high-performance liquid chromatography (HPLC);
- Evaluation of the result obtained for the samples by reference to standard samples consisting of skimmed-milk powder with or without the addition of a known percentage of whey powder.

5. Reagents

All reagents must be of recognised analytical grade. The water used must be distilled water or water of at least equivalent purity.

5.1. Trichloroacetic acid solution

Dissolve 240 g of trichloroacetic acid (Cl_3CCOOH) in water and make up to 1 000 ml.

5.2. Eluent solution, pH 6,0

Dissolve 1,74 g of dipotassium hydrogen phosphate (K_2HPO_4), 12,37 g of potassium dihydrogen phosphate (KH_2PO_4) and 21,41 g of sodium sulphate (Na_2SO_4) in about 700 ml of water. Adjust, if necessary, to pH 6,0, using a solution of phosphoric acid or potassium hydroxide.

Make up to 1 000 ml with water and homogenise.

Filter the eluent solution, prior to use, through a membrane filter with a 0,45 μm pore diameter.

5.3. Flushing solvent

Mix one volume acetonitrile (CH_3CN) with nine volumes water. Filter the mixture prior to use through a membrane filter with a 0,45 μm pore diameter.

Note: Any other flushing solvent with a bactericidal effect which does not impair the columns' resolution efficiency may be used.

5.4. Standard samples

5.4.1. Skimmed-milk powder meeting the requirements of this Resolution (i.e. [0]).

5.4.2. The same skimmed-milk powder adulterated with 5 % (m/m) rennet-type whey powder of standard composition (i.e. [5]).

6. Apparatus

- 6.1. Analytical balance.
- 6.2. Centrifuge capable of attaining a centrifugal force of 2 200 g, fitted with stoppered centrifuge tubes of about 25 ml capacity.
- 6.3. Mechanical shaker.
- 6.4. Magnetic stirrer.
- 6.5. Glass funnels, diameter about 7 cm.
- 6.6. Filter papers, medium filtration, diameter about 12,5 cm.
- 6.7. Glass filtration equipment with 0,45 µm pore diameter membrane filter.
- 6.8. Graduated pipettes allowing delivery of 10 ml (ISO 648, Class A, or ISO/R 835).
- 6.9. Thermostatic waterbath, set at $25 \pm 0,5$ °C.
- 6.10. HPLC equipment, consisting of:
 - 6.10.1. Pump.
 - 6.10.2. Injector, hand or automatic, with a 15 to 30 µl capacity.
 - 6.10.3. Two TSK 2 000-SW columns in series (length 30 cm, internal diameter 0,75 cm) or equivalent columns and a precolumn (3 cm × 0,3 cm) packed with I 125 or material of equivalent effectiveness.
 - 6.10.4. Thermostatic column oven, set at 35 ± 1 °C.
 - 6.10.5. Variable wavelength UV detector, permitting measurements at 205 nm with a sensitivity of 0,008 A.
 - 6.10.6. Integrator capable of valley-to-valley integration.

Note: Working with columns kept at room temperature is possible, but their power of resolution is slightly lower. In that case, the temperature should vary by less than ± 5 °C in any one range of analyses.

7. Sampling

- 7.1. International Standard ISO 707 — 'Milk and Milk Products — Methods of sampling', conforming to the guidelines contained in Annex I(2)(c) last paragraph.
- 7.2. Store the sample in conditions which preclude any deterioration or change in composition.

8. Procedure

8.1. Preparation of the test sample

Transfer the milk powder into a container with a capacity of about twice the volume of the powder, fitted with an airtight lid. Close the container immediately. Mix the milk powder well by means of repeated inversion of the container.

8.2. Test portion

Weight 2,000 + 0,001 g of test sample into a centrifuge tube (6.2).

8.3. Removal of fat and proteins

- 8.3.1. Add 20 g of warm water (50 °C) to the test portion. Dissolve the powder by shaking for five minutes using a mechanical shaker (6.3). Cool the tube to 25 °C.
- 8.3.2. Add 10,0 ml of the trichloroacetic acid solution (5.1) in two minutes, while stirring with the aid of the magnetic stirrer (6.4). Place the tube in a waterbath (6.9) and leave for 60 minutes.
- 8.3.3. Centrifuge (6.2) for 10 minutes at 2 200 g, or filter through paper (6.6), discarding the first 5 ml of filtrate.

8.4. *Chromatographic determination*

- 8.4.1. Inject 15 to 30 µl of accurately measured supernatant or filtrate (8.3.3) into the HPLC apparatus (6.10) operating at a flow rate of 1,0 ml of eluent solution (5.2) per minute.

Notes:

1. Keep the eluent solution (5.2) at 85 °C throughout the chromatographic analysis in order to keep the eluent degassed and to prevent bacterial growth. Any precaution with a similar effect may be used.
2. Rinse the columns with water during each interruption. Never leave the eluent solution in them (5.2).

Prior to any interruption of more than 24 hours, rinse the columns with water then wash them with solution (5.3) for at least three hours at a flow rate of 0,2 ml per minute.

- 8.4.2. The results of chromatographic analysis of the test sample [E] are obtained in the form of chromatogram in which each peak is identified by its retention time RT as follows:

Peak II: The second peak of the chromatogram which an RT of about 12,5 minutes.

Peak III: The third peak of the chromatogram, corresponding to the GMP, with an RT of $15,5 \pm 1,0$ minutes.

Peak IV: The fourth peak of the chromatogram with an RT of about 17,5 minutes.

The quality of the columns can effect the retention times of the individual peaks.

The integrator (6.10.6) automatically calculates the area A of each peak:

A_{II} : area of peak II,

A_{III} : area of peak III,

A_{IV} : area of peak IV.

It is essential to examine the appearance of each chromatogram prior to quantitative interpretation, in order to detect any abnormalities due either to malfunctioning of the apparatus or the columns, or to the origin and nature of the sample analysed.

If in doubt, repeat the analysis.

8.5. *Calibration*

- 8.5.1. Apply exactly the procedure described from point 8.2 to point 8.4.2 to the standard samples (5.4).

Use freshly prepared solutions, because GMP degrade in an 8 % trichloroacetic environment. The loss is estimated at 0,2 % per hour at 30 °C.

- 8.5.2. Prior to chromatographic determination of the samples, condition the columns by repeatedly injecting the standard sample (5.4.2) in solution (8.5.1) until the area and retention time of the peak corresponding to the GMP are constant.

- 8.5.3. Determine the response factors R by injecting the same volume of filtrates (8.5.1) as used for the samples.

9. **Expression of results**9.1. *Method of calculation and formulae*

- 9.1.1. Calculation of the response factors R:

$$\text{Peak II:} \quad R_{II} = \frac{100}{A_{II}[0]}$$

$$\text{Peak IV:} \quad R_{IV} = \frac{100}{A_{IV}[0]}$$

where:

R_{II} and R_{IV} = the response factors of peaks II and IV respectively,

$A_{II}[0]$ and $A_{IV}[0]$ = the areas of peaks II and IV respectively of the standard sample [0] obtained in 8.5.3.

$$\text{Peak III:} \quad R_{III} = \frac{W}{A_{III}[5] - A_{III}[0]}$$

where:

R_{III} = the response factor of peak III,

$A_{III}[0]$ and $A_{III}[5]$ = the areas of peak III in standard samples [0] and [5] respectively obtained in 8.5.3,

W = the quantity of whey in standard sample [5], i.e. 5.

9.1.2. Calculation of the relative area of the peaks in the sample [E]

$$S_{II} [E] = R_{II} \times A_{II} [E]$$

$$S_{III} [E] = R_{III} \times A_{III} [E]$$

$$S_{IV} [E] = R_{IV} \times A_{IV} [E]$$

where:

$S_{II} [E]$, $S_{III} [E]$, $S_{IV} [E]$ = the relative areas of peaks II, III and IV respectively in the sample [E],

$A_{II} [E]$, $A_{III} [E]$, $A_{IV} [E]$ = the areas of peaks II, III and IV respectively in the sample [E] obtained in 8.4.2,

R_{II} , R_{III} , R_{IV} = the response factors calculated in 9.1.1.

9.1.3. Calculation of the relative retention time of peak III in sample [E]:

$$RRT_{III}[E] = \frac{RT_{III}[E]}{RT_{III}[5]}$$

where:

$RRT_{III} [E]$ = the relative retention time of peak III in sample [E],

$RT_{III} [E]$ = the retention time of peak III in sample [E] obtained in 8.4.2,

$RT_{III} [5]$ = the retention time of peak III in control sample [5] obtained in 8.5.3.

9.1.4. Experiments have shown that there is a linear relation between the relative retention time of peak III, i.e. $RRT_{III} [E]$ and the percentage of whey powder added up to 10 %.

— The $RRT_{III} [E]$ is < 1,000 when the whey content is > 5 %;

— The $RRT_{III} [E]$ is \geq 1,000 when the whey content is \leq 5 %.

The uncertainty allowed for the values of RRT_{III} is \pm 0,002.

Normally the value of $RRT_{III} [0]$ deviates little from 1,034. Depending on the condition of the columns, the value may approach 1,000, but it must always be greater.

9.2. Calculation of the percentage of rennet whey powder in the sample:

$$W = S_{III}[E] - [1,3 + (S_{III}[0] - 0,9)]$$

where:

W = the percentage m/m of rennet whey in the sample [E];

$S_{III} [E]$ = the relative area of peak III of test sample [E] obtained as in 9.1.2;

1,3 represents the relative average area of peak III expressed in grams of rennet whey per 100 g determined in non-adulterated skimmed-milk powder of various origins. This figure was obtained experimentally;

$S_{III} [0]$ represents the relative area of peak III which is equal to $R_{III} \times A_{III} [0]$. These values are obtained in 9.1.1 and 8.5.3 respectively;

$(S_{III} [0] - 0,9)$ represents the correction to be made to the relative average area 1,3 when $S_{III} [0]$ is not equal to 0,9. Experimentally the relative average area of peak III of the control sample [0] is 0,9.

9.3. Accuracy of the procedure

9.3.1. Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst using the same apparatus on identical test material shall not exceed 0,2 % m/m.

9.3.2. Reproducibility

The difference between two single and independent results, obtained in two different laboratories on identical test material shall not exceed 0,4 % m/m.

9.4. Interpretation

- 9.4.1. Assume the absence of whey if the relative area of peak III, S_{III} [E] expressed in grams of rennet whey per 100 g of the product is $\leq 2,0 + (S_{III} [0] - 0,9)$

where

2,0 is the maximum value allowed for the relative area of peak III taking into account the relative area of peak III, i.e. 1,3, the uncertainty due to variations in the composition of skimmed-milk powder and the reproducibility of the method (9.3.2),

$(S_{III} [0] - 0,9)$ is the correction to be made when the area $S_{III} [0]$ is different from 0,9 (see point 9.2)

- 9.4.2. If the relative area of peak III, S_{III} [E] is $> 2,0 + (S_{III} [0] - 0,9)$ and the relative area of peak II, S_{II} [E] ≤ 160 , determine the rennet whey content as indicated in point 9.2.

- 9.4.3. If the relative area of peak III, S_{III} [E] is $> 2,0 + (S_{III} [0] - 0,9)$ and the relative area of peak II, S_{II} [E] ≤ 160 , determine the total protein content (P %); then examine graphs 1 and 2.

- 9.4.3.1. The data obtained after analysis of samples of unadulterated skimmed-milk powders with a high total protein content have been assembled in graphs 1 and 2.

The continuous line represents the linear regression, the coefficients of which are calculated by the least squares method.

The dashed straight line fixes the upper limit of the relative area of peak III with a probability of not being exceeded in 90 % of cases.

The equations for the dashed straight lines of graphs 1 and 2 are:

$$S_{III} = 0,376 P \% - 10,7 \quad (\text{graph 1}),$$

$$S_{III} = 0,0123 S_{II} [E] + 0,93 \quad (\text{graph 2}),$$

respectively where:

S_{III} is the relative area of peak III calculated either according to total protein content or according to the relative area of peak S_{II} [E],

P % is the total protein content expressed as a percentage, by weight,

$(S_{II} [E])$ is the relative area of sample calculated in point 9.1.2.

These equations are equivalent to the figure of 1,3 mentioned in point 9.2.

The discrepancy (T_1 and T_2) between the relative area S_{III} [E] found and the relative area S_{III} is given by means of the following:

$$T_1 = S_{III}[E] - [(0,376 P \% - 10,7) + (S_{III}[0] - 0,9)]$$

$$T_2 = S_{III}[E] - [(0,0123 S_{II}[E] + 0,93) + (S_{III}[0] - 0,9)]$$

- 9.4.3.2. If T_1 and/or T_2 are zero or less, the presence of rennet whey cannot be determined.

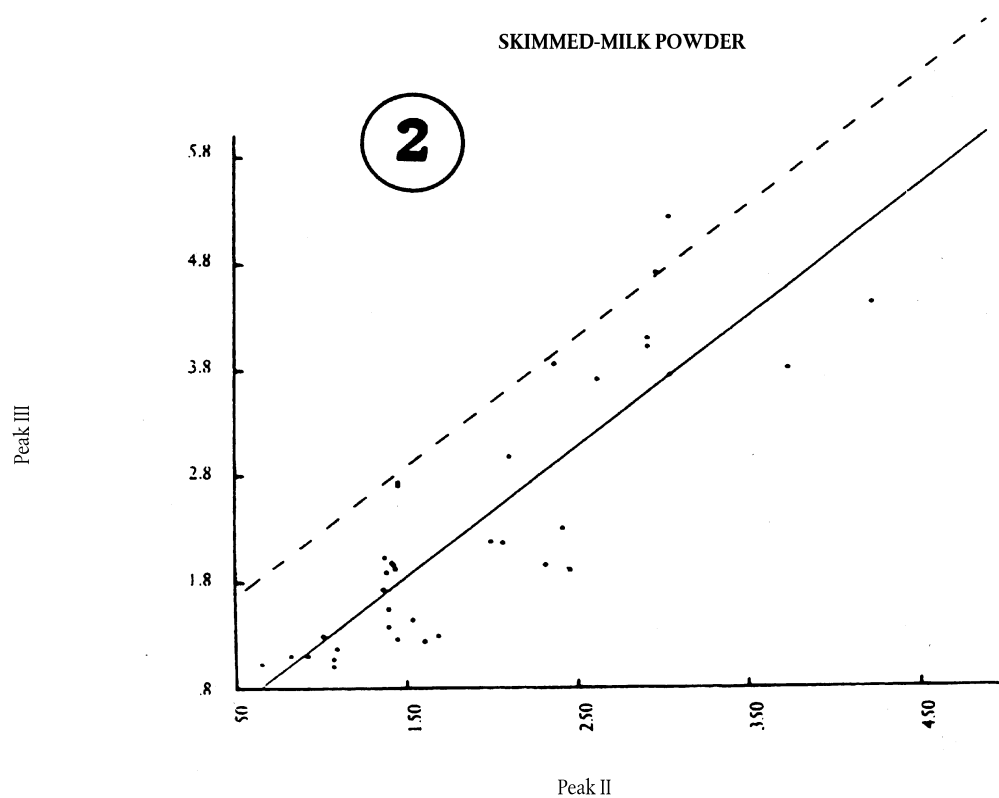
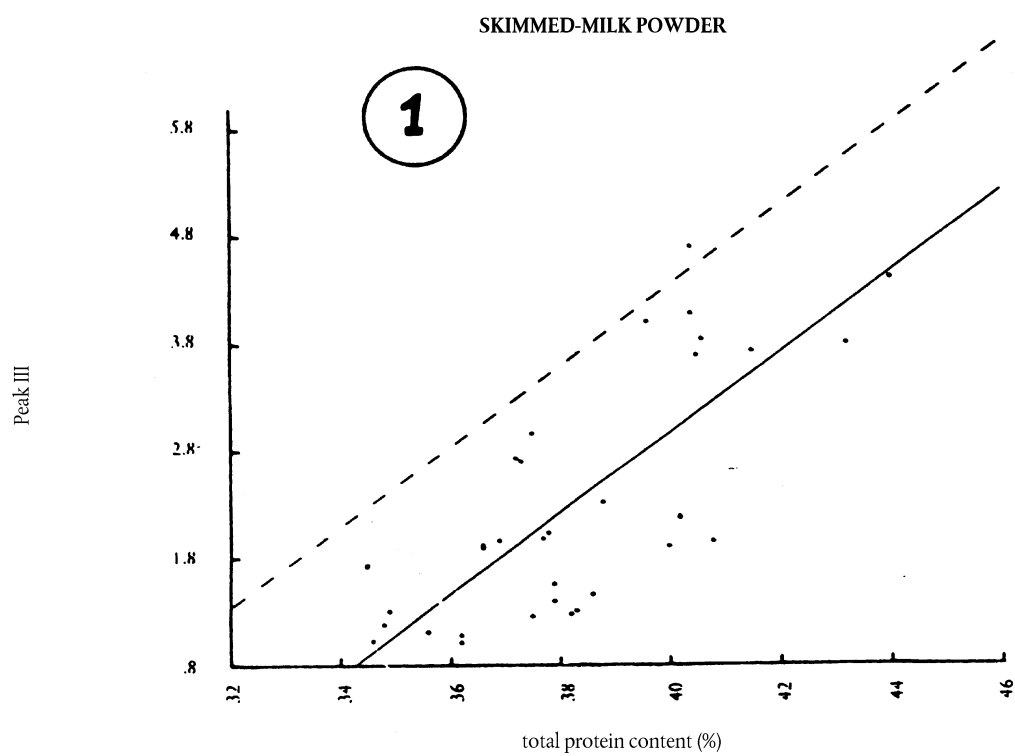
If T_1 and T_2 exceed zero, rennet whey is present.

The rennet whey content is calculated according to the following formula:

$$W = T_2 + 0,91$$

where:

0,91 is the distance on the vertical axis between the continuous and dotted straight lines.



ANNEX XIX

(Article 13)

DETERMINING RENNET WHEY SOLIDS IN SKIMMED-MILK POWDER AND THE MIXTURES REFERRED TO IN REGULATION (EC) No 2799/1999**1. Purpose: Detecting the addition of rennet whey solids to:**

- (a) skimmed-milk powder as defined in Article 2 of Regulation (EC) No 2799/1999, and
- (b) mixtures as defined in Article 4 of Regulation (EC) No 2799/1999

2. References: International standard ISO 707**3. Definition**

The content of rennet whey solids is defined as the percentage by mass as determined by the procedure described.

4. Principle

Glycomacropeptide A content is determined in accordance with Annex XVIII. Samples giving positive results are analysed for glycomacropeptide A by a reversed-phase high-performance liquid chromatography procedure (HPLC procedure). Evaluation of the result is obtained by reference to standard samples consisting of skimmed-milk powder with and without a known percentage of whey powder. Results higher than 1 % (m/m) show that rennet whey solids are present.

5. Reagents

All reagents must be of recognised analytical grade. The water used must be distilled water or water of at least equivalent purity. Acetonitrile should be of spectroscopic or HPLC quality.

Reagents needed for the procedure are described in Annex XVIII to this Regulation.

Reagents for reversed phase HPLC.

5.1. Trichloroacetic acid solution

Dissolve 240 g of trichloroacetic acid (CCl_3COOH) in water and make up to 1 000 ml.

5.2. Eluents A and B

Eluent A: 150 ml of acetonitrile (CH_3CN), 20 ml of isopropanol ($\text{CH}_3\text{CHOHCH}_3$), and 1,00 ml of trifluoroacetic acid (TFA, CF_3COOH) are placed in a 1 000 ml volumetric flask. Make up to 1 000 ml with water. Eluent B: 550 ml of acetonitrile, 20 ml of isopropanol and 1,00 ml of TFA are placed in a 1 000 ml volumetric flask. Make up to 1 000 ml with water. Filter the eluent solution, prior to use, through a membrane filter with a 0,45 μm pore diameter.

5.3. Conservation of the column

After the analyses the column is flushed with eluent B (via a gradient) and subsequently flushed with acetonitrile (via a gradient for 30 minutes). The column is stored in acetonitrile.

5.4. Standard samples

- 5.4.1. Skimmed-milk powder meeting the requirements for public storage (i. e. (0)).
- 5.4.2. The same skimmed-milk powder adulterated with 5 % (m/m) rennet-type whey powder of standard composition (i.e. (5)).
- 5.4.3. The same skimmed-milk powder adulterated with 50 % (m/m) rennet-type whey powder of standard composition (i.e. (50))⁽¹⁾.

6. Apparatus

Apparatus needed for the procedure described is described in Annex XVIII to this Regulation.

6.1.1. Analytical balance.

- 6.2. Centrifuge capable of attaining a centrifugal force of 2 200 g, fitted with stoppered centrifuge tubes of about 50 ml capacity.

⁽¹⁾ Rennet-type whey powder of standard composition and also the adulterated skimmed-milk powder are available from NIZO, Kerkweg 2, PO Box 20 — NL-6710 BA Ede. However, powders giving equivalent results to the NIZO powders may also be used.

- 6.3. Mechanical shaker with a provision to shake at 50 °C.
- 6.4. Magnetic stirrer.
- 6.5. Glass funnels, diameter about 7 cm.
- 6.6. Filter papers, medium filtration, diameter about 12,5 cm.
- 6.7. Glass filtration equipment with 0,45 µm pore diameter membrane filter.
- 6.8. Graduated pipettes, allowing delivery of 10 ml (ISO 648, Class A, or ISO/R 835), or a system capable of delivering 10,0 ml in two minutes.
- 6.9. Thermostatic waterbath, set at $25 \pm 0,5$ °C.
- 6.10. HPLC equipment, consisting of:
 - 6.10.1. Binary gradient pumping system.
 - 6.10.2. Injector, hand or automatic, with a 100 µl capacity.
 - 6.10.3. Dupont Protein Plus column (length 25 cm, 0,46 cm internal diameter) or an equivalent wide-pore silica based reversed-phase column.
 - 6.10.4. Thermostatic column oven, set at 35 ± 1 °C.
 - 6.10.5. Variable wavelength UV detector, permitting measurements at 210 nm (if necessary, a higher wavelength up to 220 nm may be used) with a sensitivity of 0,02 Å.
 - 6.10.6. Integrator capable of valley-to-valley integration.

Note

Operation of the column at room temperature is possible, provided that the room temperature does not fluctuate more than 1 °C, otherwise too much variation in the retention time of GMP_A takes place.

7. Sampling

- 7.1. Samples must be taken in accordance with the procedure laid down in International Standard ISO 707. However, Member States may use another method of sampling provided that it complies with the principles of the abovementioned standard.
- 7.2. Store the sample in conditions which preclude any deterioration or change in composition.

8. Procedure

8.1. Preparation of the test sample

Transfer the milk powder into a container with a capacity of about twice the volume of the powder, fitted with an airtight lid. Close the container immediately. Mix the milk powder well by means of repeated inversion of the container.

8.2. Test portion

Weigh $2,00 \pm 0,001$ g of test sample into a centrifuge tube (6.2) or suitable stoppered flask (50 ml).

8.3. Removal of fat and proteins

- 8.3.1. Add 20,0 g of warm water (50 °C) to the test portion. Dissolve the powder by shaking for five minutes or 30 minutes in the case of acid buttermilk using a mechanical shaker (6.3). Place the tube into the waterbath (6.9) and allow to equilibrate to 25 °C.
- 8.3.2. Add 10,0 ml of the trichloroacetic acid solution at 25 °C (5.1) constantly over two minutes, while stirring vigorously with the aid of the magnetic stirrer (6.4). Place the tube in a waterbath (6.9) and leave for 60 minutes.
- 8.3.3. Centrifuge (6.2) 2,200 g for 10 minutes, or filter through paper (6.6), discarding the first 5 ml of filtrate.

8.4. Chromatographic determination

- 8.4.1. Perform HPLC-analysis as described in Annex XVIII. If a negative result is obtained, the sample analysed does not contain rennet-whey solids in detectable amounts. If the result is positive, the reversed-phase HPLC procedure described below must be applied. The presence of acid buttermilk powder may give rise to false-positive results. The reversed-phase HPLC method excludes this possibility.

- 8.4.2. Before the reversed phase HPLC-analysis is carried out, the gradient conditions should be optimised. A retention time of 26 ± 2 minutes for GMPA is optimal for gradient systems with a dead volume of about 6 ml (volume from the point where the solvents come together to the volume of the injector loop, inclusive). Gradient systems with a lower dead volume (e.g. 2 ml) should use 22 minutes as an optimal retention time.

Take solutions of the standard samples (5.4) without and with 50 °6 rennet whey.

Inject 100 µl of supernatant or filtrate (8.3.3) into the HPLC apparatus operating at the scouting gradient conditions given in Table 1.

Table 1

Scouting gradient conditions for optimisation of the chromatography

Time (minutes)	Flow (ml/minutes)	% A	% B	Curve
Init	1,0	90	10	*
27	1,0	60	40	lin
32	1,0	10	90	lin
37	1,0	10	90	lin
42	1,0	90	10	lin

Comparison of the two chromatograms should reveal the location of the (peak of GMP_A).

Using the formula given below, the initial solvent composition to be used for the normal gradient (see 8.4.3) can be calculated

$$\begin{aligned} \% B &= 10 - 2,5 + (13,5 + (RT_{GMPA} - 26)/6) * 30/27 \\ \% B &= 7,5 + (13,5 + (RT_{GMPA} - 26)/6) * 1,11 \end{aligned}$$

Where:

- RT_{GMPA}: retention time of GMP_A in the scouting gradient
 10: the initial % B of the scouting gradient
 2,5: % B at midpoint minus % B at initial in the normal gradient
 13,5: midpoint time of the scouting gradient
 26: required retention time of GMP_A
 6: ratio of slopes of the scouting and normal gradient
 30: % B at initial minus % B at 27 minutes in the scouting gradient
 27: run-time of the scouting gradient.

- 8.4.3. Take solutions of the test samples

Inject 100 µl of accurately measured supernatant or filtrate (8.3.3) into the HPLC apparatus operating at a flow rate of 1,0 ml of eluent solution (5.2) per minute.

The composition of the eluent of the start of the analysis is obtained from 8.4.2. It is normally close to A:B = 76:24 (5.2). Immediately after the injection a linear gradient is started, which results in a 5 % higher percentage of B after 27 minutes. Subsequently a linear gradient is started, which brings the eluent composition to 90 % B in five minutes. This composition is maintained for five minutes, after which the composition is changed, via a linear gradient in five minutes to the initial composition. Depending on the internal volume of the pumping system, the next injection can be made 15 minutes after reaching the initial conditions.

Remarks

1. The retention time of the glycomacropptide should be $26 \pm$ two minutes. This can be achieved by varying the initial and end conditions of the first gradient. However, the difference in the % B for the initial and end conditions of the first gradient must remain 5 % B.
2. The eluents should be degassed sufficiently and should also remain degassed. This is essential for proper functioning of the gradient pumping system. The standard deviation for the retention time of the GMP peak should be smaller than 0,1 minutes (n = 10).
3. Every five samples the reference sample (5) should be injected and used to calculate a new response factor R. (9.1.1).

- 8.4.4. The results of the chromatographic analysis of the test sample (E) are obtained in the form of a chromatogram in which the GMP peak is identified by its retention time of about 26 minutes.

The integrator (6.40.6) automatically calculates the peak height H of the GMP peak. The baseline location should be checked in every chromatogram. The analysis or the integration should be repeated if the baseline was incorrectly located.

It is essential to examine the appearance of each chromatogram prior to quantitative interpretation, in order to detect any abnormalities due either to malfunctioning of the apparatus or the column, or to the origin and nature of the sample analysed. If in doubt, repeat the analysis.

8.5. Calibration

- 8.5.1. Apply exactly the procedure described from point 8.2 to point 8.4.4 to the standard samples (5.4.1 to 5.4.2). Use freshly prepared solutions, because GMP degrades in an 8 % trichloroacetic acid environment at room temperature. At 4 °C the solution remains stable for 24 hours. In the case of long series of analyses the use of a cooled sample tray in the automatic injector is desirable.

Note

8.4.2. may be omitted if the %B at initial conditions is known from previous analyses.

The chromatogram of the reference sample (5) should be analogous to Figure. 1. In this figure the GMP_A peak is preceded by two small peaks. It is essential to obtain a similar separation.

- 8.5.2. Prior to chromatographic determination of the samples inject 100 µl of the standard sample without rennet whey (0) (5.4.1).

The chromatogram should not show a peak at the retention time of the GMP_A peak.

- 8.5.3. Determine the response factors R by injecting the same volume of filtrate (8.5.1) as used for the samples.

9. Expression of results

9.1. Method of calculation and formulae

9.1.1. Calculation of the response factor R:

GMP peak: $R = W/H$

Where:

R = the response factor of the GMP peak

H = the height of the GMP peak

W = the quantity of whey in the standard sample (5).

9.2. Calculation of the percentage of rennet whey powder in the sample

$W(E) = R \times H(E)$

Where:

W(E) = the percentage (m/m) of rennet whey in the sample (E).

R = the response factor of the GMP peak (9.1.1)

H(E) = the height of the GMP peak of the sample (E)

If W(E) is greater than 1 % and the difference between the retention time and that of the standard sample (5) is smaller than 0,2 minutes then rennet whey solids are present.

9.3. Accuracy of the procedure

9.3.1. Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst using the same apparatus on identical test material shall not exceed 0,2 % m/m.

9.3.2. Reproducibility

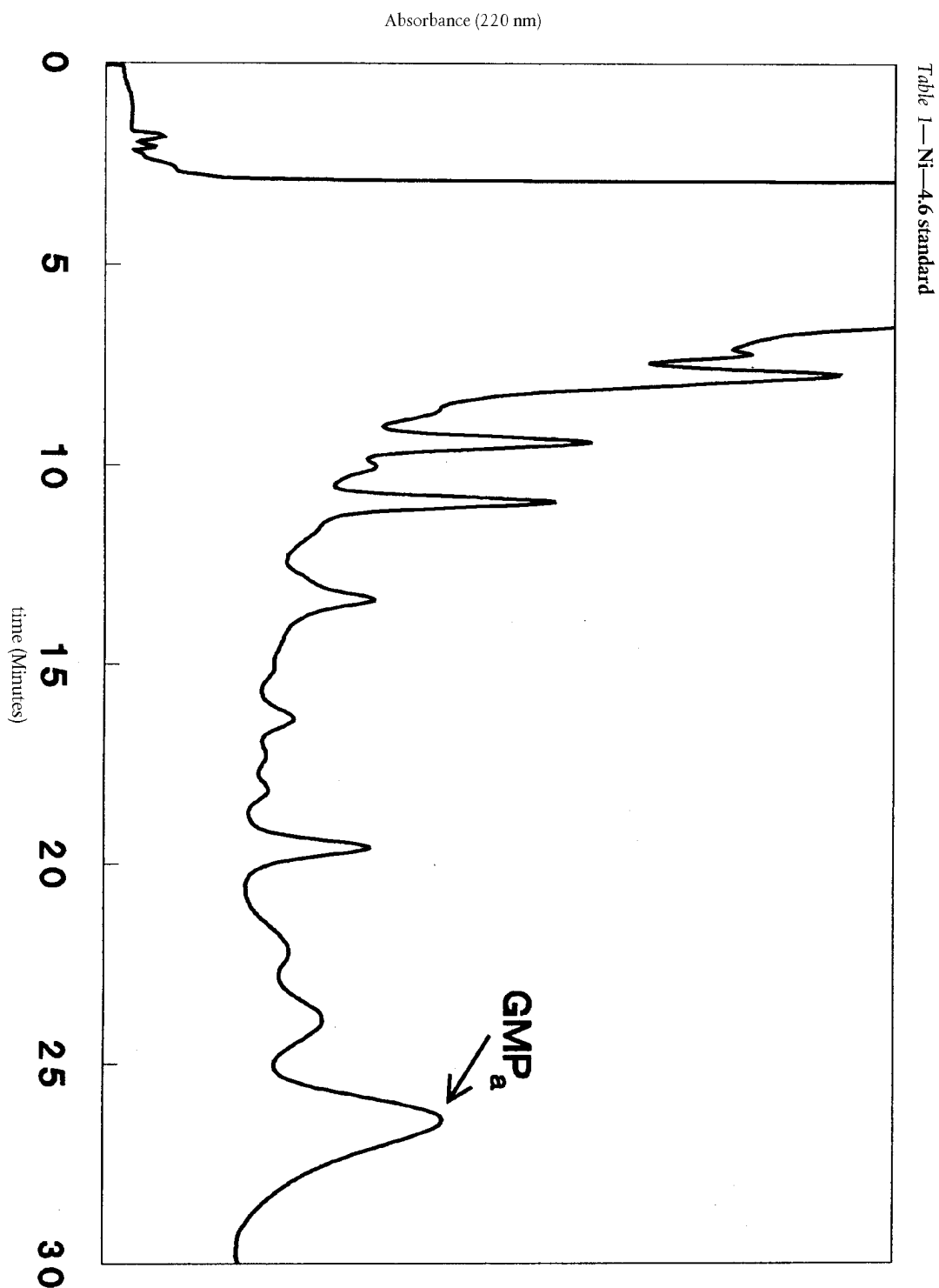
Not yet determined.

9.3.3. Linearity

From 0 to 16 % of rennet whey a linear relationship should be obtained with a coefficient of correlation $> 0,99$.

9.4. Interpretation

- 9.4.1. Whey is considered to be present if the result obtained in 9.2 is higher than 1 % m/m and the retention time of the GMP peak differs less than 0,2 minutes from that of the standard sample (5). The 1 % limit is fixed in agreement with the provisions of points 9.2 and 9.4.1 of Annex V to Regulation (EEC) No 625/78.



ANNEX XX

(Article 14)

SKIMMED-MILK POWDER: QUANTITATIVE DETERMINATION OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE**Method: reversed-phase HPLC****1. Purpose and field of application**

The method describes a procedure for the quantitative determination of phosphatidylserine (PS) and phosphatidylethanolamine (PE) in skimmed-milk powder (SMP) and is suitable for detecting buttermilk solids in SMP.

2. Definition

PS + PE content: the mass fraction of substance determined using the procedure here specified. The result is expressed as milligrams of phosphatidylethanolamine dipalmitoyl (PEDP) per 100 g powder.

3. Principle

Extraction of aminophospholipids by methanol from reconstituted milk powder. Determination of PS and PE as o-phthalaldehyde (OPA) derivatives by reversed-phase (RP) HPLC and fluorescence detection. Quantification of PS and PE content in the test sample by reference to a standard sample containing a known amount of PEDP.

4. Reagents

All reagents must be of recognised analytical grade. Water must be distilled or of at least equivalent purity unless otherwise specified.

4.1. *Standard material: PEDP, at least 99 % pure*

Note: Standard material must be stored at -18°C .

4.2. *Reagents for standard sample and test sample preparation*

4.2.1. HPLC-grade methanol

4.2.2. HPLC-grade chloroform

4.2.3. Tryptamine-monohydrochloride

4.3. *Reagents for o-phthalaldehyde derivatisation*

4.3.1. Sodium hydroxide, 12 M water solution

4.3.2. Boric acid, 0,4 M water solution adjusted to pH 10,0 with sodium hydroxide (4.3.1)

4.3.3. 2-mercaptoethanol

4.3.4. o-phthalaldehyde (OPA)

4.4. *HPLC elution solvents*

Elution solvents must be prepared using HPLC-grade reagents.

4.4.1. HPLC-grade water

4.4.2. Methanol of fluorimetric tested purity

4.4.3. Tetrahydrofuran

4.4.4. Sodium dihydrogen phosphate

4.4.5. Sodium acetate

4.4.6. Acetic acid

5. Apparatus

5.1. Analytical balance

5.2. Beakers, 25 and 100 ml capacity

5.3. Pipettes, delivering 1 and 10 ml

5.4. Magnetic stirrer

- 5.5. Graduated pipettes, delivering 0,2, 0,5 and 5 ml
- 5.6. Volumetric flasks, 10, 50 and 100 ml capacity
- 5.7. Syringes, 20 and 100 µl capacity
- 5.8. Ultrasonic bath
- 5.9. Centrifuge operating at $27\,000 \times g$
- 5.10. Glass vials, about 5 ml capacity
- 5.11. Graduated cylinder, 25 ml capacity
- 5.12. pH-meter
- 5.13. HPLC equipment
 - 5.13.1. Gradient pumping system, capable of operating at 1,0 ml/min at 200 bar
 - 5.13.2. Autosampler with derivatisation capability
 - 5.13.3. Column heater set at 30 °C
 - 5.13.4. Fluorescence detector set at 330 nm excitation wavelength and 440 nm emission wavelength
 - 5.13.5. Integrator or data processing software capable of peak area measurement
 - 5.13.6. A Lichrosphere — 100 column (250 × 4,6 mm) or an equivalent column packed with octadecylsilane (C 18), 5 µm particle size

6. Sampling

Sampling must be carried out in accordance with IDF Standard 50B:1985.

7. Procedure

7.1. Preparation of the internal standard solution

Weigh $30,0 \pm 0,1$ mg of tryptamine-monohydrochloride (4.2.3) into a 100 ml volumetric flask (5.6) and make up to the mark with methanol (4.2.1). Pipette 1 ml (5.3) of this solution into a 10 ml volumetric flask (5.6) and make up to the mark with methanol (4.2.1) in order to obtain a 0,15 mM tryptamine concentration.

7.2. Preparation of the test sample solution

Weigh $1,000 \pm 0,001$ g of the SMP sample into a 25 ml beaker (5.2). Add 10 ml of distilled water at 40 °C by a pipette (5.3) and stir with a magnetic stirrer (5.4) for 30 minutes in order to dissolve any lumps. Pipette 0,2 ml (5.5) of the reconstituted milk into a 10 ml volumetric flask (5.6), add 100 µl of the 0,15 mM tryptamine solution (7.1) using a syringe (5.7) and make up to the volume with methanol (4.2.1). Mix carefully by inversion and sonicate (5.8) for 15 min. Centrifuge (5.9) at $27\,000 \times g$ for 10 minutes and collect the supernatant in a glass vial (5.10).

Note: Test sample solution should be stored at 4 °C until the HPLC analysis is performed.

7.3. Preparation of the external standard solution

Weigh 55,4 mg PEDP (4.1) into a 50 ml volumetric flask (5.6) and add about 25 ml of chloroform (4.2.2) using a graduated cylinder (5.11). Heat the stoppered flask to 50 °C and mix carefully till the PEDP dissolves. Cool the flask to 20 °C, make up to the volume with methanol (4.2.1) and mix by inversion. Pipette 1 ml (5.3) of this solution into a 100 ml volumetric flask (5.6) and make up to the volume with methanol (4.2.1). Pipette 1 ml (5.3) of this solution into a 10 ml volumetric flask (5.6), add 100 µl (5.7) of 0,15 mM tryptamine solution (7.1) and make up to the volume with methanol (4.2.1). Mix by inversion.

Note: Reference sample solution should be stored at 4 °C until the HPLC analysis is performed.

7.4. Preparation of the derivatising reagent

Weigh $25,0 \pm 0,1$ mg of OPA (4.3.4) into a 10 ml volumetric flask (5.6), add 0,5 ml (5.5) of methanol (4.2.1) and mix carefully to dissolve the OPA. Make up to the mark with boric acid solution (4.3.2) and add 20 µl of 2-mercaptoethanol (4.3.3) by syringe (5.7).

Note: The derivatising reagent should be stored at 4 °C in a dark vial and is stable for one week.

7.5. Determination by HPLC

7.5.1. Elution solvents (4.4)

Solvent A:

Solution of 0,3 mM sodium dihydrogen phosphate and 3 mM sodium acetate solution (adjusted to pH 6,5 with acetic acid); methanol: tetrahydrofuran = 558:440:2 (v/v/v)

Solvent B:

methanol

7.5.2. Suggested eluting gradient:

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)
Initial	40	60	0
0,1	40	60	0,1
5,0	40	60	0,1
6,0	40	60	1,0
6,5	40	60	1,0
9,0	36	64	1,0
10,0	20	80	1,0
11,5	16	84	1,0
12,0	16	84	1,0
16,0	10	90	1,0
19,0	0	100	1,0
20,0	0	100	1,0
21,0	40	60	1,0
29,0	40	60	1,0
30,0	40	60	0

Note: The eluting gradient may require slight modification in order to achieve the resolution shown in figure 1.

Column temperature: 30 °C.

7.5.3. Injection volume: 50 µl derivatising reagent and 50 µl sample solution.

7.5.4. Column equilibration

Starting up the system on a daily basis, flush the column with 100 % solvent B for 15 minutes, then set at A : B = 40 : 60 and equilibrate at 1 ml/min for 15 minutes. Perform a blank run by injecting methanol (4.2.1).

Note: Before long-term storage flush the column with methanol : chloroform = 80 : 20 (v/v) for 30 minutes.

7.5.5. Determination of the PS + PE content in the test sample

7.5.6. Perform the sequence of the chromatographic analyses keeping constant the run-to-run time in order to obtain constant retention times. Inject the external standard solution (7.3) every 5-10 test sample solutions in order to evaluate the response factor.

Note: The column must be cleaned by flushing with 100 % solvent B (7.5.1) for at least 30 minutes every 20-25 runs.

7.6. Integration mode

7.6.1. PEDP peak

PEDP is eluted as a single peak. Determine the peak area by valley-to-valley integration.

7.6.2. Tryptamine peak

Tryptamine is eluted as a single peak (Figure 1). Determine the peak area by valley-to-valley integration.

7.6.3. PS and PE peaks groups

Under the described conditions (Figure 1), PS elutes as two main partially unresolved peaks preceded by a minor peak. PE elutes as three main partially unresolved peaks. Determine the whole area of each peak cluster setting the baseline as reported in Figure 1.

8. Calculation and expression of results

PS and PE content in the test sample shall be calculated as follows:

$$C = 55,36 \times \frac{A_2}{A_1} \times \frac{T_1}{T_2}$$

where:

- C = PS or PE content (mg/100 g powder) in the test sample
A₁ = PEDP peak area of the standard sample solution (7.3)
A₂ = PS or PE peak area of the test sample solution (7.2)
T₁ = Tryptamine peak area of the standard sample solution (7.3)
T₂ = Tryptamine peak area of the test sample solution (7.2).

9. Precision

Note: The values for repeatability were calculated according to the IDF International Standard ⁽¹⁾. The provisional reproducibility limit was calculated according to the procedure defined in Annex III(b) hereto.

9.1. Repeatability

The relative standard derivation of the repeatability, which expresses the variability of independent analytical results obtained by the same operator using the same apparatus under the same conditions on the same test sample and in a short interval of time, should not exceed 2 % relative. If two determinations are obtained under these conditions, the relative difference between the two results should not be greater than 6 % of the arithmetic mean of the results.

9.2. Reproducibility

If two determinations are obtained by operators in different laboratories using different apparatus under different conditions for the analysis on the same test sample, the relative difference between the two results should not be greater than 11 % of the arithmetic mean of the results.

10. References

- 10.1. Resmini P., Pellegrino L., Hogenboom J.A., Sadini V., Rampilli M., 'Detection of buttermilk solids in skimmilk powder by HPLC quantification of aminophospholipids.' Sci. Tecn. Latt.-Cas., 39,395 (1988).

⁽¹⁾ International IDF Standard 135B/1991. Milk and milk products. Precision characteristics of analytical methods. Outline of collaborative study procedure.

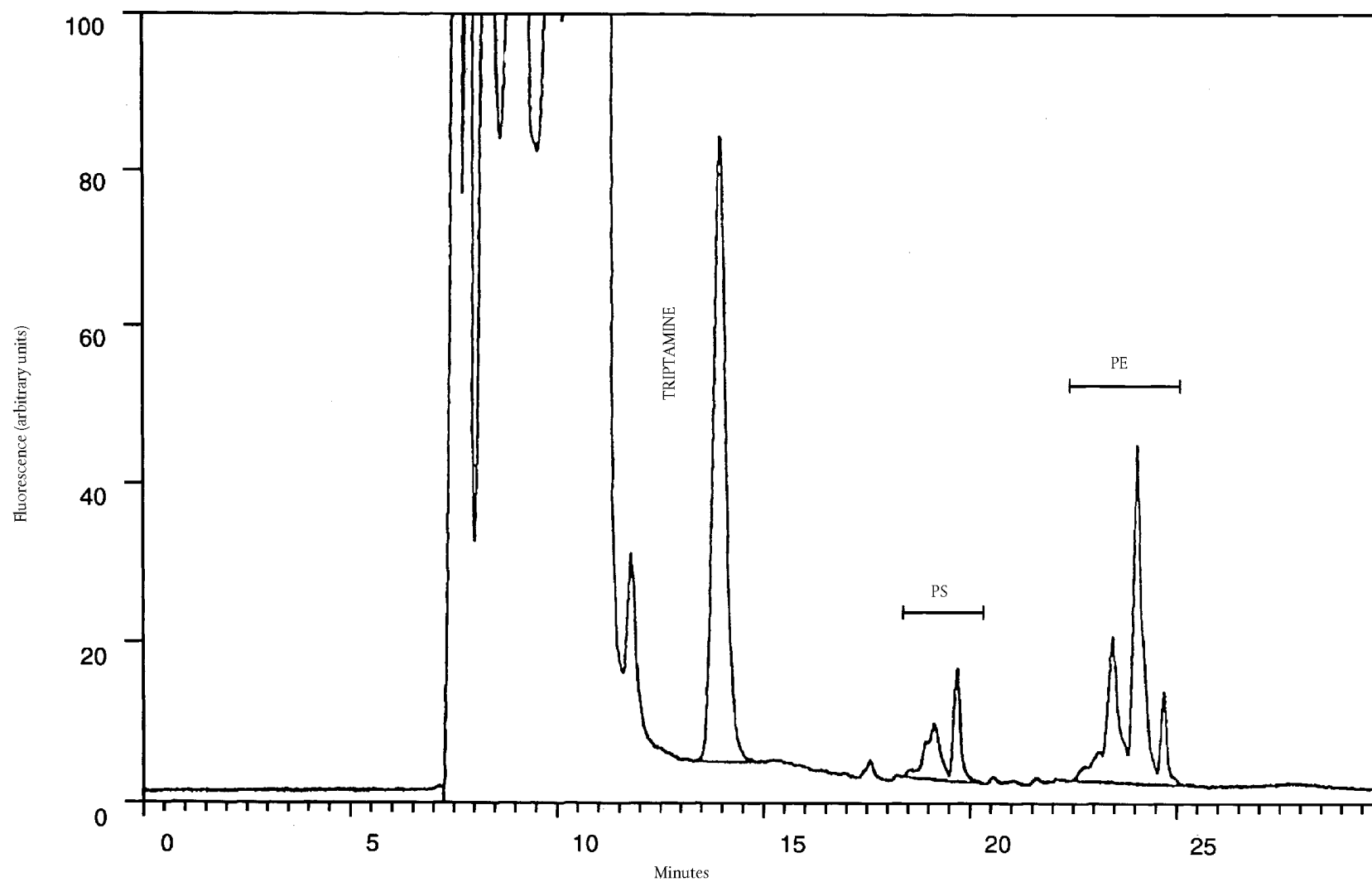


Figure 1: HPLC pattern of OPA-derivatives of phosphatidylserine (PS) and phosphatidylethanolamine (PE) in methanol extract of reconstituted skim-milk powder. Integration mode for the peaks of PS, PE and tryptamine (internal standard) is reported.

ANNEX XXI

(Article 15)

DETECTION OF ANTIBIOTIC AND SULFONAMIDE/DAPSON RESIDUES IN SKIMMED MILK POWDER

A microbial inhibitor screening test using *Bacillus stearothermophilus* var. *calidolactis* C953 as test micro-organism and being sufficiently sensitive to detect 4 µg Benzylpenicillin per milk and 100 µg sulfadimidine per milk shall be used. Commercial tests kits are available and can be used if they have the required sensitivity for Benzylpenicillin and sulfadimidine ⁽¹⁾.

For the test, reconstituted skimmed milk powder (1 g powder + 9 ml aqua dest) is used. The test is carried out as described in IDF — Bulletin No 258/1991, section 1, Chapter 2, or according to the instructions of the test kit manufacturer.

Positive results are to be interpreted as follows:

1. Repeat the test adding penicillinase to the test system:
Positive result: Inhibiting substance cannot be identified by this procedure.
Negative result: Inhibiting substance is a β-lactam antibiotic.
2. Repeat the test adding p-amino benzoic acid to the test system:
Positive result: Inhibiting substance cannot be identified by this procedure.
Negative result: Inhibiting substance is a sulfonamide/dapson.
3. Repeat the test adding penicillinase + p-amino benzoic acid to the test system:
Positive result: Inhibiting substance cannot be identified by this procedure.
Negative result: Inhibiting substances are a β-lactam antibiotic and a sulfonamide/dapson.

⁽¹⁾ *Important notice:* False-positive results may be obtained, when skimmed milk powder is analysed. It is important, therefore, to verify that the test system used does not yield false-positive results.

ANNEX XXII

(Article 16)

QUANTITATIVE DETERMINATION OF SKIMMED-MILK POWDER IN COMPOUND FEEDINGSTUFFS BY ENZYMATIC COAGULATION OF PARA-CASEIN**1. Purpose**

Quantitative determination of skimmed-milk powder in compound feedingstuffs by enzymatic coagulation of para-casein.

2. Scope

This method applies to compound feedingstuffs containing at least 10 % skimmed-milk powder; large quantities of buttermilk and/or of certain non-milk proteins may lead to interferences.

3. Principle of the method

- 3.1. Dissolving of casein contained in the compound feedingstuff by extraction with sodium citrate solution.
- 3.2. Adjustment of the calcium ion concentration to the required level to precipitate para-casein; by addition of rennet para-casein is obtained from casein.
- 3.3. The nitrogen content of the para-casein precipitate is determined by the Kjeldahl method as described by IDF standard 20A 1986; the quantity of skimmed-milk powder is calculated on the basis of a minimum casein content of 27,5 % (see 9.1).

4. Reagents

The reagents used must be of analytical grade. The water used must be distilled water or water of equivalent purity. With the exception of the rennet (4.5), all the reagents and solutions must be free of nitrogenous substances.

- 4.1. Trisodium citrate, dihydrate (1 % w/v solution).
- 4.2. Calcium chloride (2M solution). Weigh 20,018 g of CaCO_3 (analytical grade) in a porcelain vessel of suitable size (150 to 200 ml) or in a beaker. Cover with distilled water and transfer onto a boiling water bath. Add slowly 50 to 60 ml of HCl solution (conc. HCl:water = 1:1) to solubilise the carbonate completely. Keep on the boiling water bath until the CaCl_2 is dried, to eliminate the HCl which has not reacted. Transfer with distilled water to a 100 ml measuring flask and dilute to the mark. Measure the pH value, which must be not lower than 4,0. Store the solution in a refrigerator.
- 4.3. 0,1 N sodium hydroxide.
- 4.4. 0,1 N hydrochloric acid.
- 4.5. Liquid calf rennet (standard strength of 1:10 000). Store in a refrigerator at 4 to 6 °C.
- 4.6. Reagents for the quantitative determination of nitrogen according to the Kjeldahl method as described by IDF standard 20A 1986.

5. Apparatus

Common laboratory apparatus, including:

- 5.1. Mortar or homogeniser
- 5.2. Analytical balance
- 5.3. Bench-top centrifuge (2 000 to 3 000 rpm) with 50 ml tubes
- 5.4. Magnetic stirrer with (10 to 15 mm) followers
- 5.5. 150 to 200 ml beakers
- 5.6. 250 and 500 ml flasks
- 5.7. Glass funnels of 60 to 80 mm diameter
- 5.8. Fast-filtering ashless filters of diameter 150 mm (S.S. 589², S.S. 595 1/2)
- 5.9. Pipettes of various nominal volume

- 5.10. Thermostatically controlled water bath at 37 °C
- 5.11. pH meter
- 5.12. Kjeldahl digestion and distillation assembly with fittings
- 5.13. 25 ml graduated burette
- 5.14. Plastic wash bottle for distilled water
- 5.15. Stainless steel spatulas
- 5.16. Thermometers
- 5.17. Temperature-controlled drying oven.

6. Procedure

- 6.1. Preparation of the sample.

Grind in the mortar or homogenise in the mill 10 to 20 g of the sample to obtain a homogeneous mixture.

- 6.2. Dissolving of milk powder and separation of the insoluble residue.

6.2.1. Weigh $1,000 \pm 0,002$ g of well-homogenised compound feedingstuff (6.1) directly into a 50 ml centrifuge tube. Add 30 ml of trisodium citrate solution (4.1) previously heated to 45 °C. Mix with the aid of the magnetic stirrer for at least five minutes.

6.2.2. Centrifuge at 500 g (2 000 to 3 000 rpm) for 10 minutes and decant the clear aqueous supernatant into a 150 to 200 ml beaker, taking care that no loose material on the bottom goes over.

6.2.3. Carry out two further extractions on the residue, according to the same procedure, adding the extracts to the first one.

6.2.4. If a layer of oil forms at the surface, cool in the refrigerator until the fat solidifies and remove the solid layer with a spatula.

- 6.3. Coagulation of casein with the enzymes of rennet.

6.3.1. While stirring continuously, add dropwise 3,4 ml of a saturated solution of calcium chloride (4.2) to the total aqueous extract (about 100 ml). Adjust the pH to 6,4-6,5 with solutions of NaOH (4.3) or HCl (4.4). Place in the thermostatically-controlled water bath at 37 °C for 15 to 20 minutes to obtain saline balance. It becomes more evident by the formation of a light turbidity.

6.3.2. Transfer the liquid into one (or two) centrifuge tubes and centrifuge at 2 000 g for 10 minutes in order to remove the precipitated material. Transfer the supernatant, without washing the sediment, into one (or two) centrifuge tubes.

6.3.3. Bring the temperature of the supernatant back to 37 °C. While stirring the extract, add, dropwise, 0,5 ml of the liquid rennet (4.5). Coagulation occurs in one or two minutes.

6.3.4. Return the sample to the water bath and leave at a temperature of 37 °C for 15 minutes. Remove the sample from the bath and break the coagulum by stirring. Centrifuge at 2 000 g for 10 minutes. Filter the supernatant through a suitable filter paper ⁽¹⁾ (Whatman No 541 or equivalent) and retain the filter paper. Wash the precipitate in the centrifuge tube with 50 ml of water at approximately 35 °C by stirring the precipitate.

Centrifuge again at 2 000 g for 10 minutes. Filter the supernatant through the filter paper retained previously.

- 6.4. Determination of casein nitrogen.

6.4.1. After washing, transfer quantitatively the precipitate to the filter paper retained from 6.3.4 using distilled water. Transfer the filter paper to the Kjeldahl flask. Determine the nitrogen by the Kjeldahl method as described by IDF standard 20A 1986.

7. Blank test

7.1. A blank test shall be made regularly using an ashless filter paper (5.8) moistened with a mixture of 90 ml (4.1) sodium citrate solution, 1 ml saturated solution of calcium chloride (4.2), 0,5 ml of liquid rennet (4.5), and washed with 3×15 ml of distilled water before mineralisation by the Kjeldahl method as described at IDF standard 20A 1986.

7.2. The volume of acid used for the blank test must be subtracted from the volume of acid (4.4) used for titration of the sample.

⁽¹⁾ As fast filtering ashless paper should be used.

8. Control test

- 8.1. To test the abovementioned procedure and reagents, make a determination on a standard compound feedingstuff with a known skimmed-milk powder content as established by collaborative study. The average result of a duplicate determination should not differ by more than 1 % from that of the collaborative study.

9. Expression of results

- 9.1. The percentage of skimmed-milk powder in the compound feedingstuff is calculated by the following formula:

$$\% \text{ MMP} = \frac{\left(\frac{N \times 6,38}{27,5} \times 100 \right) - 2,81}{0,908}$$

where N is the percentage of para-casein nitrogen; 27,5 is the factor for converting determined casein into the percentage of skimmed-milk powder; 2,81 and 0,908 are correction factors obtained from regression analysis.

10. Accuracy of the method**10.1. Repeatability**

In at least 95 % of the cases studied, duplicate analysis of the same sample by the same operator in the same laboratory must give differences in the results equivalent to no greater than 2,3 g of skimmed-milk powder in 100 g of compound feedingstuff.

10.2. Reproducibility

In at least 95 % of the cases studied, the same sample analysed by two laboratories, must give differences in the results no greater than 6,5 g of skimmed-milk powder in 100 g of compound feedingstuff.

11. Tolerance limit

The CrD_{95} value (critical difference; 95 % confidence limit) is calculated using the formula (ISO 5725):

$$\text{CrD}_{95} = \frac{1}{\sqrt{2}} \sqrt{R^2 - r^2 \left(\frac{n-1}{n} \right)}$$

(R: reproducibility; r: repeatability)

Double determination: $\text{CrD}_{95} = 4,5 \text{ g}$

Where the result of the chemical analysis differs from the declared content of skimmed-milk powder by not more than 4,5 g (double determination) the consignment of compound feedingstuff is deemed to comply with this provision of the Regulation.

12. Observations

- 12.1. The addition of large percentages of certain non-milk proteins and especially of soya proteins, when heated together with skimmed-milk powder, may lead to too high results due to co-precipitation with the para-casein of milk.
- 12.2. Addition of buttermilk may lead to somewhat low figures, due to the fact that only the non-fat portion is determined. Addition of certain acid buttermilk may give considerably low figures, due to incomplete dissolving in the citrate solution.
- 12.3. Lecithin additions of 0,5 % or more may also lead to low results.
- 12.4. Incorporation of high-heat skimmed-milk powder may lead to too high figures due to the co-precipitation of certain whey proteins with the para-casein of milk.
-

ANNEX XXIII

(Article 17)

**QUALITATIVE DETERMINATION OF STARCH IN SKIMMED-MILK POWDER, DENATURED MILK POWDER
AND COMPOUND FEEDINGSTUFFS****1. Scope**

This method is for the detection of starch which is issued as a tracer in denatured milk powders.

Limit of detection of the method is approximately 0,05 g of starch per 100 g of sample.

2. Principle

The reaction is based on the one used in iodometry:

- fixation by the colloids of the free iodine in aqueous solution,
- absorption by the starch micelles and by colour formation.

3. Reagents**3.1. Iodine solution**

- iodine: 1 g,
- potassium iodine: 2 g,
- distilled water: 100 ml.

4. Apparatus

- 4.1. Analytical balance
- 4.2. Water bath
- 4.3. Test tubes, 25 mm × 200 mm

5. Procedure

Weight 1 g of the sample and transfer it into the test tube (4.3).

Add 20 ml of distilled water and shake in order to disperse the sample.

Place in the boiling water bath (4.2) and leave for 5 minutes.

Remove from the bath and cool to room temperature.

Add 0,5 ml of the iodine solution (3.1), shake and observe the resulting colour.

6. Expression of results

A blue colouration indicates the presence of native starch in the sample.

When the sample contains modified starch the colour may not be blue.

7. Remarks

The colour, the intensity of the colour and the microscopic appearance of the starch, will vary depending on the origin of native starch (e.g. maize or potato) and the type of modified starch present in the sample.

In the presence of modified starches the colour produced turns violet, red or brown, according to the degree of modification of the crystalline structure of native starch.

ANNEX XXIV

(Article 18)

DETERMINATION OF MOISTURE IN ACID BUTTERMILK POWDER**1. Scope**

To determine the moisture content of acid buttermilk powder intended for animal feedingstuffs.

2. Principle

The sample is dried under vacuum. The loss of mass is determined by weighing.

3. Apparatus

- 3.1. Analytical balance
- 3.2. Dry containers of non-corrodible metal or of glass with lids ensuring airtight closure; working surface allowing the test sample to be spread at about 0,3 g/cm².
- 3.3. Adjustable electrically heated vacuum oven fitted with an oil pump and either a mechanism for introducing hot dried air or a drying agent (e.g. calcium oxide).
- 3.4. Desiccator with an efficient drying agent.
- 3.5. Drying oven ventilated, thermostatically controlled, at 102 ± 2 °C.

4. Procedure

Heat a container (3.2) with its lid in the oven (3.5) for at least one hour. Place the lid on the container, immediately transfer to a desiccator (3.4) allow to cool to room temperature and weigh to the nearest 0,5 mg.

Weigh a container (3.2) with its lid to the nearest 0,5 mg. Weight in the weighted container, to the nearest 1 mg, about 5 g of the sample and spread evenly. Place the container without its lid, in the vacuum oven (3.3) preheated to 83 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible.

Bring the pressure up to 100 Torr (13,3 kPa) and leave to dry for four hours at this pressure, either in a current of hot, dry air or using a drying agent (about 300 g for 20 samples). In the latter instance, disconnect the vacuum pump when the prescribed pressure has been reached. Reckon drying time from the moment when the oven temperature returns to 83 °C. Carefully bring the oven back to atmospheric pressure. Open the oven, place the lid on the container immediately, remove the container from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.4) and weigh to the nearest 1 mg. Dry for an additional 30 minutes in the vacuum oven (3.3) at 83 °C and reweigh. The difference between the two weighings must not exceed 0,1 % of moisture.

5. Calculation

$$(E - m) \cdot \frac{100}{E}$$

where

E = initial mass, in grammes of the test sample,

m = mass, in grammes, of the dry test sample.

6. Precision**6.1. Repeatability limit**

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material shall not exceed 0,4 g water/100 g acid buttermilk powder.

6.2. *Reproducibility limit*

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material shall not exceed 0,6 g water/100 g acid buttermilk powder.

6.3. *Source of precision data*

The precision data were determined from an experiment conducted in 1995 involving eight laboratories and 12 samples (6 blind duplicates).

ANNEX XXV

(Article 19)

REFERENCE METHOD FOR THE DETECTION OF FOREIGN FATS IN MILK FAT BY GAS CHROMATOGRAPHIC ANALYSIS OF TRIGLYCERIDES — REVISION 1**1. Scope and field of application**

This standard lays down a method for the detection of foreign fats, of both vegetable fats and animal fats such as beef tallow and lard in milk fat of milk products using gas chromatographic analysis of triglycerides.

Using defined triglyceride formulae vegetable and animal fats are qualitatively and quantitatively detected in pure milk fat irrespective of feeding or lactation conditions.

Note 1: Although butyric acid (C4) occurring exclusively in milk fats enables quantitative estimations of low to mean amounts of milk fat in vegetable fats to be made, qualitative and quantitative information can hardly be provided in the range of an addition of up to at least 20 % (weight %) foreign fat to pure milk fat because of the large variation of C4 ranging approximately between 3,5 to 4,5 % (weight %).

Note 2: Quantitative results can practically only be obtained by triglyceride analyses, because the sterol content of the vegetable fats is different as a function of production and treatment conditions.

2. Definition

Foreign fats in milk fat: foreign fats as defined in this standard are all vegetable and animal fats except milk fat.

3. Principle of the method

After extraction of the milk fat a stock solution is prepared. From this solution the triglycerides (total carbon numbers) are determined gas chromatographically on packed columns. By inserting the weight % of the fat molecules of different size (C24 — C54 — only even numbers) in the triglyceride formula the foreign fats are either qualitatively detected or quantitatively determined.

Note: Observing the evaluation described here capillary gas chromatography can be used, if it is guaranteed that comparable results are achieved⁽¹⁾.

4. Reagents

Analysis-grade chemicals must be used.

- 4.1. Carrier gas: nitrogen, purity degree $\geq 99,996\%$.
- 4.2. Standard triglycerides⁽²⁾, saturated as well as cholesterol for standardizing a standard milk fat according to section 6.5.4.
- 4.3. Methanol, water-free.
- 4.4. n-Hexane
- 4.5. n-Heptane
- 4.6. Toluene
- 4.7. Dimethylchlorosilane solution: 50 ml dimethylchlorosilane are dissolved in 283 ml toluene.
- 4.8. Combustible gas: hydrogen and synthetic air
- 4.9. Stationary phase, 3-% OV-1 on 125/150 μm (100/120 mesh) Gas ChromQ.⁽³⁾
- 4.10. 10 % cocoa butter solution

5. Instruments

Normal laboratory apparatus and particularly the following:

- 5.1. High temperature gas chromatograph suited for temperatures of at least 400 — 450 °C, equipped with a flame ionization detector (FID) and constant mass flow controller for the carrier gas. Combustion gas: 30 ml/min H₂, 270 ml/min synthetic air.

⁽¹⁾ Suited methods have already been described, see D. Precht and J. Molkentin: Quantitative triglyceride analysis using short capillary columns, Chrompack News 4, 16-17 (1993).

⁽²⁾ Suitable products are commercially available.

⁽³⁾ Trade names such as, e.g. Extrelut, Gas ChromQ, Chrompack are examples for suited products available in the specialised trade. This information shall serve the purpose of easy handling of the standard by the user and does not represent a request of the product. The indication of grain was transferred to the SI-unit μm according to BS 410:1988 'British Standard Specification for test sieves'.

Given the high carrier gas flow, the flame jet should be particularly large.

Note: Because of the high temperatures occurring during triglyceride analyses the glass inserts in the FID or in the injector system must be frequently cleaned.

The gas chromatograph must be equipped with septa, withstanding high temperatures, which can be frequently used and exhibit generally a very low degree of 'bleeding'

Note: Suited are Chromblue (tm) septa (Chrompack).

The septa must be exchanged at regular intervals, e.g. after roughly 100 injections or as soon as the resolution deteriorates (see figure 4).

5.2. *Chromatography column*

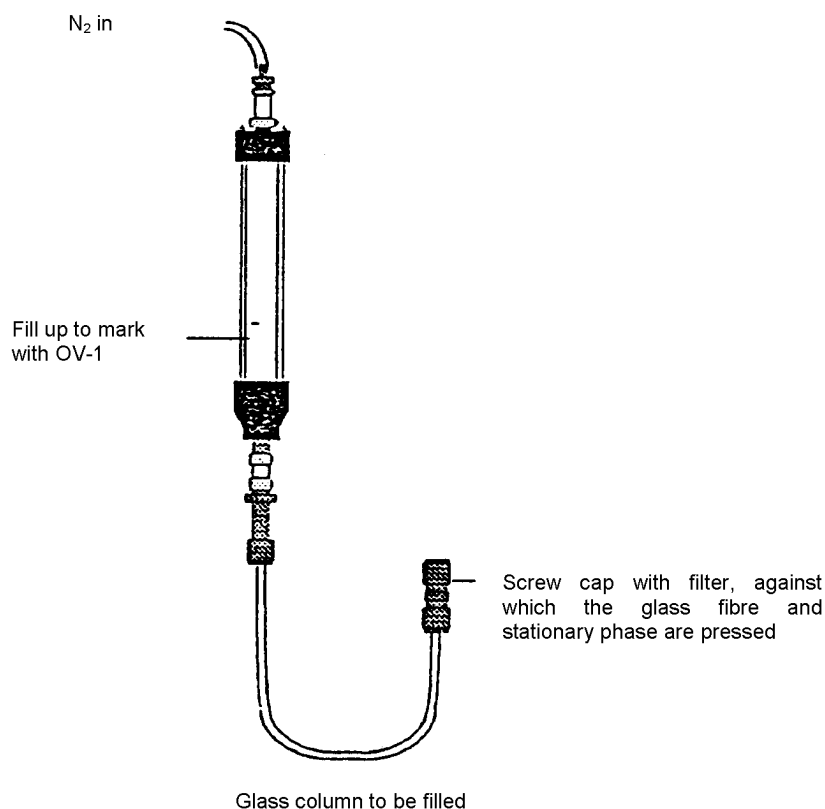
U-shaped glass column (inside diameter 2 mm, 500 mm in length), which is first silanized according to section 6.1 with dimethylchlorosilane in order to deactivate the glass surface.

Note: Suited are also somewhat longer (80 — 200 mm in length) packed columns. With them a slightly better reproducibility of the results can be achieved. On the other hand, the stationary phase exhibits occasionally fractures after operation, which may lead, in turn, to worse quantitative results. Further, the FID flame is easily extinguished as a result of the required extremely high carrier gas flow of 75 to 85 ml/min.

5.3. Arrangement for filling the column (see figure 1)

Figure 1

Filling of the column



- 5.3.1. Plastic column with screwed-on end caps, provided with a mark up to which the required quantity of stationary phase can be filled
- 5.3.2. Fine sieve (mesh size approximately 100 µm) with screw cap, suited for sealing the glass column according to figure 1.
- 5.3.3. Deactivated, silanized glass wool
- 5.3.4. Vibrator for uniform distribution of the stationary phase during filling
- 5.4. 1 to 3 ml Extrelut column⁽¹⁾ with silica gel. This column can alternatively be used for the extraction for obtaining milk fat.

⁽¹⁾ See footnote 3 on page 86.

- 5.5. Graphite seal 6,4 mm (1/4") with 6 mm bore
- 5.6. Devices for silanizing the glass surface of the column according to section 6.1.
 - 5.6.1. Woulff bottle
 - 5.6.2. Water suction pump
- 5.7. Water bath, adjustable to $(50 \pm 2) ^\circ\text{C}$
- 5.8. Drying cabinet, adjustable to $(50 \pm 2) ^\circ\text{C}$ and $(100 \pm 2) ^\circ\text{C}$
- 5.9. Microlitre pipette
- 5.10. 5 ml graduated pipette for dosing 1,5 ml methanol
- 5.11. 50 ml round-bottomed flask
- 5.12. Erlenmeyer flask, nominal volume 50 ml
- 5.13. Funnel
- 5.14. Fine-pored filter
- 5.15. Rotary evaporator
- 5.16. Ampoules, nominal volume 1 ml, sealable with an aluminium cap, with a septum in the interior
- 5.17. Injection syringe, the plunger of the syringe used must not reach into the tip of the needle.

Note: Such syringes allow a better reproducibility of the results to be obtained.

In order to avoid deterioration of the septum, the tip of the needle should be checked at regular intervals (e.g. with a stereomicroscope).

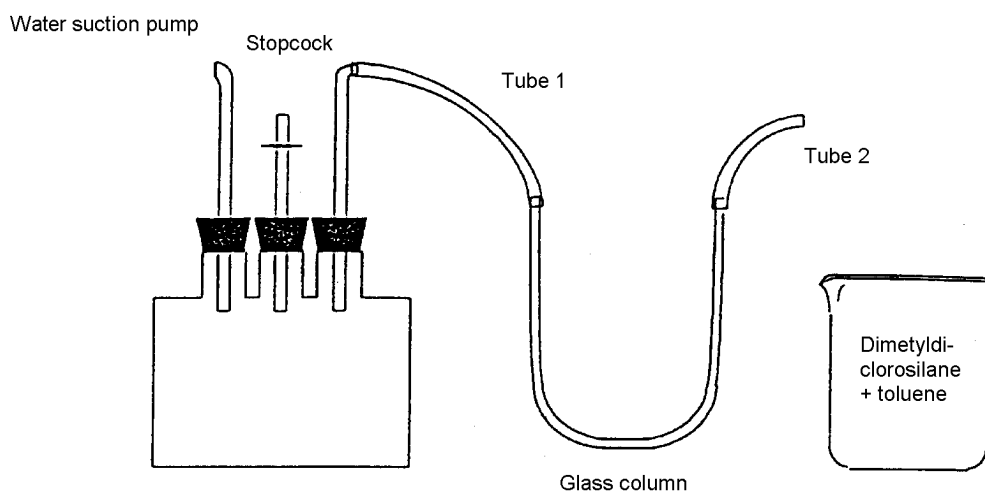
6. Procedure

6.1. Preparation of the column (silanization)

After connecting the Woulff bottle, as shown in figure 2, with the water suction pump tube 2 is dipped into the solution according to section 4.7. By closing the stopcock the column is filled; subsequently the two tubes are removed.

Figure 2

Arrangement for silanisation



The column is fixed on a stand and completely filled with the dimethyldichlorosilane solution by means of a pipette.

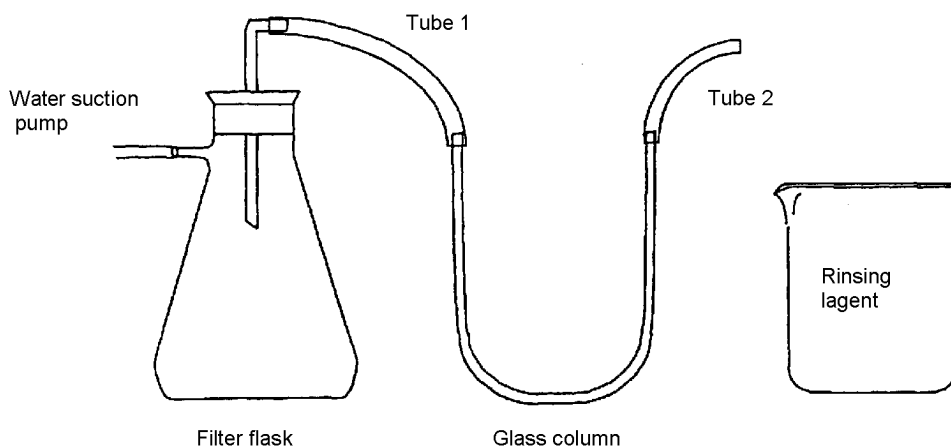
After 20-30 min the Woulff bottle is replaced by a filter flask and the column emptied by connecting it with the water suction pump (see figure 3).

6.2. Filling of the column

This is followed by successive rinsing using 75 ml toluene and 50 ml methanol; then the emptied column is dried in the drying cabinet at 100 °C for approximately 30 minutes.

Figure 3

Arrangement for rinsing



For filling of the column the arrangement as represented in figure 1 is used. The stationary phase according to 4.9 is filled into the plastic column up to the mark. The lower end of the glass column to be filled is sealed with an approximately 1 cm long plug of glass wool, which had been silanized before, and which is pressed in using a steel rod. Then the end of the column is closed with the sieve according to section 5.3.2.

The column is filled under pressure (3 bar, with N₂) with the stationary phase. To obtain a uniform, continuous and firm packing, a vibrator is moved up and down the glass column during filling.

After filling a solid plug of silanized glass wool is pressed into the other end of the packed column, the protruding ends are cut off and the plug pressed into the column a few millimetres with a spatula.

6.3. Preparation of the samples

For sample preparation one of the three following methods is used:

6.3.1. Isolation of the milk fat from butter

5 to 10 g of butter is melted in a suitable vessel in a water bath according to section 5.7 at 50 °C.

A 50 ml Erlenmeyer flask and a funnel with inserted filter according to section 5.14 are heated in the drying cabinet to 50 °C. The fat layer of the molten butter sample is filtered using the preheated device.

Such a milk fat is almost phospholipid-free.

6.3.2. Extraction of the fat fraction according to the Röse-Gottlieb method

Extraction is done either according to IDF Standard 1 C: 1987, 16C: 1987, 116A: 1987 or 22B: 1987.

With such a milk fat phospholipids allow a cholesterol peak to be obtained which is increased by approximately 0,1 %.

The triglyceride spectrum standardized to 100 with the cholesterol is thereby influenced only to a negligible extent.

6.3.3. Extraction from milk using silica gel columns

0,7 ml of a milk sample tempered to 20 °C are applied to a 1 to 3 ml Extrelut column with a microlitre pipette according to section 5.4 and allowed to distribute uniformly on the silica gel for approximately five minutes.

For denaturing the protein-lipid complexes 1,5 ml of methanol is added by pipette. Subsequently the sample is extracted with 20 ml n-hexane. The n-hexane is slowly added in small amounts and the solvent draining off collected in a 50 ml round-bottomed flask that had been dried to a constant, known weight.

After extraction led the column drain until empty.

From the eluate the solvents are distilled off on a rotatory evaporator at a water bath temperature of 40 to 50 °C.

The flask is dried and the fat yield determined by weighing.

Note: Fat extractions according to Gerber, Weibull-Berntrop, Schmid-Bondzynski-Ratzlaff or isolation of milk fat using detergents (BDI method) are not suited for triglyceride analysis, because with these methods more or less large quantities of partial glycerides or phospholipids can pass into the fat phase.

6.4. *Preparation of the sample solution*

For gas chromatography a 5 % solution of the fat in n-heptane obtained according to section 6.3 is used. For preparing this sample solution corresponding amounts of the sample material obtained according to the sections 6.3.1 and 6.3.2 are weighed and dissolved in corresponding amounts of n-heptane.

With sample preparation according to section 6.3.3 the amount of n-heptane to be added to the sample material in the flask is calculated on the basis of weighing and the remainder dissolved in it.

Approximately 1 ml of the sample solution is filled into an ampule according to section 5.16.

6.5. *Chromatographic triglyceride determination*

With the high temperatures of up to 350 °C for eluting the long-chain triglycerides C52-C56 an increase in baseline occurs easily, particularly if the columns have not been adequately conditioned in the beginning. This rise in baseline at high temperatures can be avoided completely by either combining two columns or baseline subtraction.

With the compensating mode or operation with single columns, as well as for the glass inserts in the injector and in the detector the graphite seals according to section 5.5 have to be used.

6.5.1. *Baseline correction*

To avoid baseline rising one of the four methods is used:

6.5.1.1. *Combination of columns*

Two packed columns are used in compensating mode.

6.5.1.2. *Baseline correction by the gas chromatograph*

By application of a run by the gas chromatograph without injection of a fat solution and subsequent subtraction of the stored baseline rising of the baseline can be avoided.

6.5.1.3. *Baseline correction by integration software*

By application of a run by the integration system without injection of a fat solution and subsequent subtraction of the stored baseline rising of the baseline can be avoided.

6.5.1.4. *Baseline correction by adequate conditioning*

With adequate initial conditioning of the column and approximately 20 injections with milk fat solutions baseline rising at high temperatures is frequently so low that baseline corrections are not necessary.

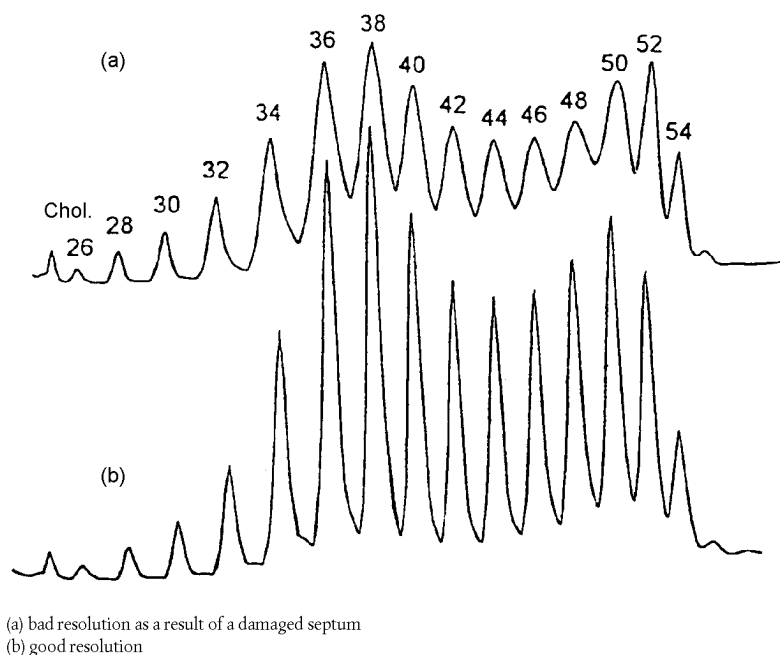
6.5.2. *Injection technique*

To avoid discrimination effects the 'hot injection' technique is applied to achieve better quantitative results with the high-boiling triglyceride components. Here, the fat solution is drawn up in the syringe and the cold needle of the syringe warmed up prior to injection for approximately three seconds in the injector head. Then, the syringe content is rapidly injected.

Note: With this injection technique the risk of fractionation phenomena inside the syringe or the injection block is reduced. 'On-column' direct injection in the upper, extended heated part of the column is not applied, because the fragments of the septum, which accumulate here, as well as contaminations can be easily eliminated with the used technique by regularly changing an injector insert without dismounting the column.

Bending of the tip of the needle caused by touching the bottom of the sample beaker (even if it is hardly visible to the eye) must be absolutely avoided in order not to damage the septum.

Figure 4

Triglyceride chromatogram of a milk fat sample**6.5.3. Conditioning of a packed column**

During steps (a) to (c) the top of the column is not connected to the detector to avoid contamination.

The columns filled according to section 6.2 are conditioned as follows:

- (a) 15 min 40 ml/min N_2 -flow at 50 °C;
- (b) Heating with 1 K/min up to 355 °C at 10 ml N_2 /min;
- (c) Holding for 12 to 15 h at 355 °C;
- (d) two injections of 1 μ l of the cocoa butter solution according to section 4.10 and respective temperature program;
- (e) 20 injections of 0,5 μ l of a milk fat solution for two to three days according to section 6.4.

Note: Cocoa butter consists almost exclusively of high-boiling C50 to C56 triglycerides. Injection with cocoa butter serves the purpose of special conditioning in this long-chain range. With the high-boiling triglycerides C50 to C54 partly response factors of up to approximately 1,20 may occur. Normally, with repeated injection of a milk fat solution a reduction of the initially high response factors for C50 to C54 has to be expected. With triglycerides with low acyl-c number the factors approximate 1. Three pairs, respectively, of the columns filled according to section 6.2 are prepared. The conditioned pairs are checked, respectively, with a milk fat analysis for routine testing.

The pair with the best quantitative results (response factors almost 1) is used in the following. With response factors > 1,20 the column is not used.

6.5.4. Calibration

For calibration the response factors of the corresponding triglycerides, as well as of cholesterol of a milk fat (standardized fat) should be determined using the standardized triglycerides (at least the saturated triglycerides C24, C30, C36, C42, C48 and C54, as well as cholesterol; better still additionally C50 and C52). Intermediate response factors can be found by mathematical interpolation.

Using the standardized fat two to three calibrations have to be performed every day. If almost identical results are obtained, well reproducible quantitative results are achieved with triglyceride analysis of the samples.

The standardized milk fat has a stock life of several months at a storage temperature of maximally -18 °C and can, thus, be used as a standard.

Note: The response factor of each constituent may also be determined using a standardised fat with a certified triglyceride composition, such as CRM 519 (anhydrous milk fat) obtainable from the Institute for Reference Materials and Measurements, Geel, Belgium.

6.5.5. Temperature programme, carrier gas and other conditions for triglyceride analysis

Temperature programme: initial column temperature 210 °C, hold for one minute, then program at 6 °C/min to 350 °C and hold at final temperature for five minutes.

Detector- and injector temperature: 370° C, respectively.

Note: Detector, injector, and oven temperatures (initial temperature) should be maintained at a constant level (also overnight, during weekends and holidays).

Carrier gas: nitrogen, flow rate 40 ml/min.

Note: If 80 cm columns are used, the flow must be at least 75 ml/min N₂. The carrier gas flow must be constantly maintained (also overnight, as well as during weekends and holidays). The exact carrier gas flow should be adjusted in a manner that independent of column length C54 is eluted at 341 °C.

Duration of analysis : 29,3 minutes.

Injection volume : 0,5 µl.

Note: The syringe has to be rinsed several times with pure heptane after each injection.

FID conditions: according to section 5.1

Note: The flame ionization detector is ignited, respectively, at the beginning of each working day.

7. Integration, evaluation and control of the measuring conditions

Triglycerides with odd acyl-c number ($2n + 1$) are combined with the preceding even-numbered triglyceride ($2n$). The less reproducible low C56 contents are not taken into account. The remaining triglycerides (peak area) in the chromatogram, including cholesterol (peak near to C24) are multiplied by the respective response factors of the standard fat (last calibration) and altogether normalized to 100. Besides free cholesterol the triglycerides C24, C26, C28, C30, C32, C34, C36, C38, C40, C42, C44, C46, C48, C50, C52 and C54 are, thus, evaluated. Results are given in weight % (g/100 g).

Evaluation of the chromatogram peaks should be done with an integrator, with which the baseline can be plotted. Reintegration with optimized integration parameters should be possible.

Figures 5 and 6 demonstrate two examples of triglyceride chromatograms. Figure 5 shows a chromatogram which can be well evaluated, whilst figure 6 represents a sporadic error in the C50 to C54 range, the baseline running incorrectly compared with figure 5. Such typical errors can be detected with a high degree of certainty and avoided only by use of an integrator with which the baseline is plotted.

Figure 5

Easy-to-evaluate triglyceride chromatogram of a milk fat with baseline drawn in

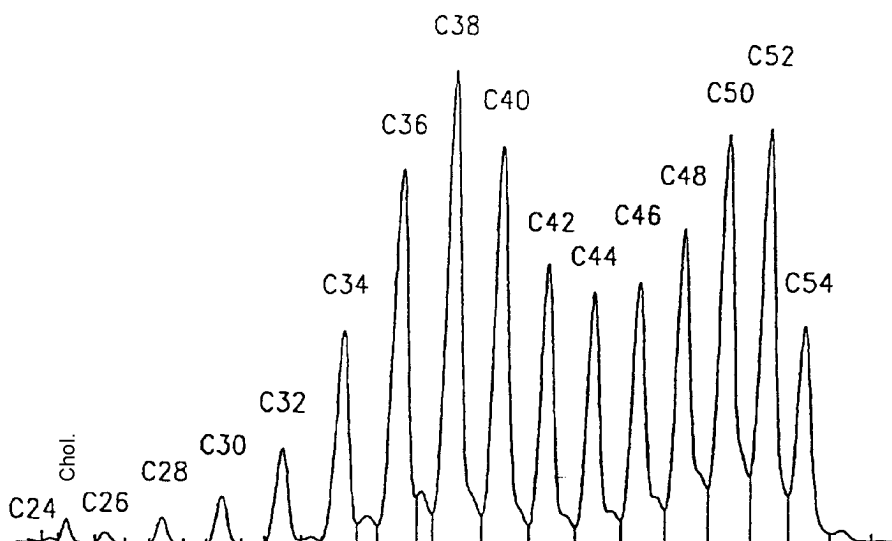
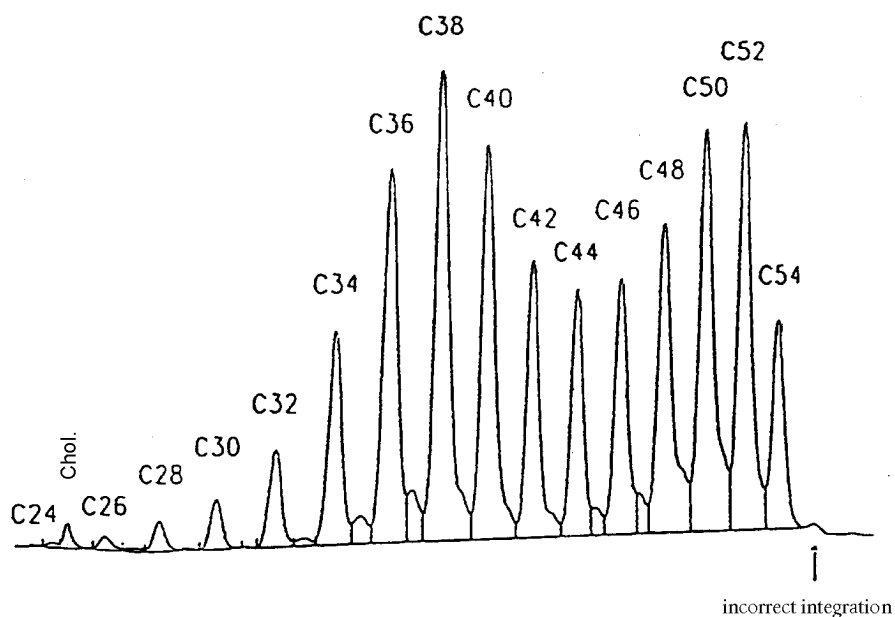


Figure 6

Wrongly integrated chromatogram of milk fat

To control measuring conditions, the relative standard deviations (RSD: coefficient of variation $\times 100$) given in Table 1 for the different triglycerides may be used. They were calculated from 19 consecutive analyses of the same milk fat.

Table 1

Relative standard deviations (RSD) of triglyceride contents (n=19)

Triglyceride	RSD (%)
C24	10,00
C26	2,69
C28	3,03
C30	1,76
C32	1,03
C34	0,79
C36	0,25
C38	0,42
C40	0,20
C42	0,26
C44	0,34
C46	0,37
C48	0,53
C50	0,38
C52	0,54
C54	0,60

If the RSDs are considerably higher than the values in Table 1, the chromatographic conditions are not appropriate and the septa or the carrier gas flow rate must be verified. Further, small particles of the septum may have formed deposits on the glass wool at the entrance of the column or the column might have become unsuited for use as a result of ageing, temperature influences, etc. (see Figure 3).

Note: The values given in Table 1 are not mandatory, but are indicative for quality control purposes. However, if higher RSD values are accepted, the repeatability and reproducibility limits given in point 11 must nevertheless be complied with.

8. Qualitative foreign fat detection

For the detection of foreign fats triglyceride formulae (Table 2) with limits S (Table 3) have been developed, in which the S-values of pure milk fats can fluctuate. If these limits are transgressed, the presence of a foreign fat can be assumed.

The most sensitive formula for the detection of lard addition is, e.g.

$$6,5125 \cdot C_{26} + 1,2052 \cdot C_{32} + 1,7336 \cdot C_{34} + 1,7557 \cdot C_{36} + 2,2325 \cdot C_{42} + 2,8006 \cdot C_{46} + 2,5432 \cdot C_{52} + 0,9892 \cdot C_{54} = S$$

Note: Using 755 different milk fat samples a 99 % confidence range of $S = 97,96 - 102,04$ was established for pure milk fat samples with a standard deviation for all S-values $SD = 0,39897$.

Starting from the triglyceride composition of an unknown fat sample such a formula allows, without using a computer, to verify in a simple manner whether the sum of the triglyceride contents stated here with the corresponding factors falls outside the range of $97,96 - 102,04$ and one has most probably to do with foreign fat addition.

For detecting different foreign fats Table 2 shows different triglyceride formulae. For the detection of the foreign fats soybean oil, sunflower oil, olive oil, rape-seed oil, linseed oil, wheat germ oil, maize germ oil, cotton seed oil, and hydrogenized fish oil, for the vegetable fats coconut- and palm kernel fat, as well as for palm oil and beef tallow a common formula can be used, respectively.

Since the triglyceride composition of the foreign fats is also subjected to fluctuations, up to four different, experimentally measured foreign fat triglyceride data of the same type were used. (With the same foreign fat types the least favourable limit has been considered, respectively (see Table 4)).

With the following 'Total formula' similarly good results can be obtained for all foreign fats:

$$- 2,7575 \cdot C_{26} + 6,4077 \cdot C_{28} + 5,5437 \cdot C_{30} - 15,3247 \cdot C_{32} + 6,2600 \cdot C_{34} + 8,0108 \cdot C_{40} - 5,0336 \cdot C_{42} + 0,6356 \cdot C_{44} + 6,0171 \cdot C_{46} = S$$

Calculations for the detection of any foreign fat combination in milk fat have shown that, e.g., although with the formula for lard given in Table 2 the limit for this foreign fat is low, namely 2,7 %, other fats such as coconut fat, palm oil or palm kernel fat with detection limits of 26,8, 12,5 and 19,3 %, respectively, can, with this formula, only be detected if extremely high amounts have been added to milk fat. This applies also to other formulae in Table 2.

Table 2

Triglyceride formulae for detecting various foreign fats in milk fat, indicating the standard deviations SD for S

Formula for soybean, sunflower, olive, rape-seed, linseed, wheat germ, maize germ, cotton seed and fish oil
$2,0983 \cdot C_{30} + 0,7288 \cdot C_{34} + 0,6927 \cdot C_{36} + 0,6353 \cdot C_{38} + 3,7452 \cdot C_{40} - 1,2929 \cdot C_{42} + 1,3544 \cdot C_{44} + 1,7013 \cdot C_{46} + 2,5283 \cdot C_{50} = S$; SD = 0,38157
Formula for coconut and palm kernel fat
$3,7453 \cdot C_{32} + 1,1134 \cdot C_{36} + 1,3648 \cdot C_{38} + 2,1544 \cdot C_{42} + 0,4273 \cdot C_{44} + 0,5809 \cdot C_{46} + 1,1226 \cdot C_{48} + 1,0306 \cdot C_{50} + 0,9953 \cdot C_{52} + 1,2396 \cdot C_{54} = S$; SD = 0,11323
Formula for palm oil and beef tallow
$3,6644 \cdot C_{28} + 5,2297 \cdot C_{30} - 12,5073 \cdot C_{32} + 4,4285 \cdot C_{34} - 0,2010 \cdot C_{36} + 1,2791 \cdot C_{38} + 6,7433 \cdot C_{40} - 4,2714 \cdot C_{42} + 6,3739 \cdot C_{46} = S$; SD = 0,81094
Formula for lard
$6,5125 \cdot C_{26} + 1,2052 \cdot C_{32} + 1,7336 \cdot C_{34} + 1,7557 \cdot C_{36} + 2,2325 \cdot C_{42} + 2,8006 \cdot C_{46} + 2,5432 \cdot C_{52} + 0,9892 \cdot C_{54} = S$; SD = 0,39897

Therefore for checking an unknown fat sample all formulae given in Table 2 and the Total formula (2) must be used, if the sample is likely to be a mixture of milk fat and one of the 14 different foreign fats or a combination of these foreign fats. If, by inserting the triglyceride of a fat sample to be analysed an S-value is obtained, which falls outside the ranges of Table 3 of only one of the five formulae, then the sample is most likely a modified milk fat. Detection of a foreign fat in milk fat by means of one of the four formulae in Table 2 does not allow conclusions to be drawn on the type of the foreign fat admixture.

Table 3

S-Limits for milk fats

Formula for detection of	S-range
Soybean, sunflower, olive, rape-seed, linseed, wheat germ, maize germ, cotton, fish oil	98,05 — 101,95
Coconut and palm kernel fat	99,42 — 100,58
Palm oil and beef tallow	95,90 — 104,10
Lard	97,96 — 102,04
Total formula	95,68 — 104,32

In Table 4 the detection limits for the different foreign fats with a 99 % confidence are given. The first column shows the minimal detection limits for the best milk fat formulae in Table 2. In the second column the detection limits for the total formula are given. Although the limits are somewhat higher, only this formula is necessary to detect a little bit higher amounts of foreign fats. With all formulae also combinations of the different foreign fats can be detected. The ranges of variation of the triglycerides of different foreign fats of one type have no considerable influence on the detection limits.

Table 4

99 % limits of detection by addition of foreign fat to milk fat in %

	Individual formula	Total formula
Soybean oil	2,1	4,4
Sunflower oil	2,3	4,8
Olive oil	2,4	4,7
Coconut fat	3,5	4,3
Palm oil	4,4	4,7
Palm kernel fat	4,6	5,9
Rape-seed oil	2,0	4,4
Linseed oil	2,0	4,0
What germ oil	2,7	6,4
Maize germ oil	2,2	4,5
Cotton seed oil	3,3	4,4
Lard	2,7	4,7
Beef tallow	5,2	5,4
Hydrogenised fish oil	5,4	6,1

Note: The S-ranges are calculated in that way, that a foreign fat is only assumed, if the limits of the individual formula are exceeded (see Table 4).

9. Quantitative foreign fat determination

In order to obtain quantitative information on the foreign fat concentration in a milk fat sample, the following formula is used

$$X (\%) = 100 \cdot | (100 - S) / (100 - S_F) |,$$

X being the quantity of an unknown foreign fat or foreign fat mixture in an unknown milk fat. S results from addition of an unknown foreign fat by inserting the triglycerides of the foreign fat/milk fat mixture in the above total triglyceride formula. If an unknown foreign fat is added to milk fat, the mean S-value of the different foreign fats for the Total formula is chosen for S_F ; this mean S-value is obtained by inserting the triglyceride data of the pure foreign fats in this formula and by calculating a mean value ($S_F = 7,46$). Good quantitative results concerning any foreign fat additions are also obtained using the palm oil/beef tallow formula (Table 2) and a mean S_F -value of 10,57.

With known foreign fat types the following S_F -values must be inserted in the above formula and the respective foreign fat formula from Table 2 has to be chosen:

Table 5

S_F-values of various foreign fats

Foreign fat	S _F
Soybean oil	8,18
Sunflower oil	9,43
Olive oil	12,75
Coconut fat	118,13
Palm oil	7,55
Palm kernel oil	112,32
Rape-seed oil	3,30
Linseed oil	4,44
Wheat germ oil	27,45
Maize germ oil	9,29
Cotton seed oil	41,18
Lard	177,55
Beef tallow	17,56
Fish oil	64,12

10. Range of application of the detection method

The described method applies to bulk milks and is based on the representativeness of the milk fat samples.

Highly specific detection would be possible, if, for a representative number of milk fats, formulae as described above were derived for different countries.

There could be particularly suited possibilities of detection obtained, if in the different countries formulae, as have been described here, were set up of a representative number of milk fats. In this case, the use of complex computer programmes is not required, if the triglyceride combinations used in Table 2 are applied and the factors redetermined by using the method of least squares.

By applying the S-ranges as shown in Table 3 the formulae are, under particular feeding conditions as, for instance, underfeeding or feeding of cows with feed yeast or Ca-soaps, generally applicable. Only in the case of extreme feeding conditions (e.g. high uptake of pure feed oils, high administration of Ca-soaps combined with feed fat etc.) the formulae partly indicate a modified milk fat.

Note: Fractionated milk fats are generally recognized as unmodified milk fat, if a modification is assumed only, when the limits are exceeded. Only with fractionated milk fats with unusual milk fat composition, as it is, e.g., the case with a hard fraction, obtained with fractionation by physical methods at high temperatures of approximately 30 °C with low yields of a few percent or with fractionation with overcritical CO₂, the formulae indicate a modification.

Milk fat fractionation may, however, be detected using other procedures e.g. Differential-Scanning-Calorimetry.

11. Accuracy of the method

Determined using milk fat on the basis of the formulae from Table 2 and the S-ranges in Table 3.

11.1. Repeatability

As difference of the S-values of two determinations carried out within the shortest feasible time interval by one operator using the same procedure and identical sample material under the same conditions (same person, same instruments/same device, same laboratory):

Table 6

Repeatability limits (r) for the different formulae

Formula for detection of	r
Soyabean, sunflower, olive, rape-seed, linseed, wheat germ, maize germ, cotton, fish oil	0,67
Coconut and palm kernel fat	0,12
Palm oil and beef tallow	1,20
Lard	0,58
Total formula	1,49

11.2. *Reproducibility*

As difference of the S-values of two determinations carried out by operators in different laboratories, according to the same procedure using identical sample material under different conditions (different person, different instruments) at different times.

Table 7

Reproducibility limits (R) for the different formulae

Formula for detection of	R
Soybean, sunflower, olive, rape-seed, linseed, wheat germ, maize germ, cotton, fish oil	1,08
Coconut and palm kernel fat	0,40
Palm oil and beef tallow	1,81
Lard	0,60
Total formula	2,07

11.3. *Critical difference*

With the repeatability (r) and the reproducibility limits (R) the critical differences for all S-ranges of Table 3 can be calculated (duplicate analyses). The respective values are given in Table 8.

Table 8

Critical differences for all triglyceride formulae

Formula for detection of	range
Soybean, sunflower, olive, rape-seed, linseed, wheat germ, maize germ, cotton, fish oil	97,43 — 102,57
Coconut and palm kernel fat	99,14 — 100,86
Palm oil and beef tallow	94,91 — 105,09
Lard	97,65 — 102,35
Total formula	94,58 — 105,42

11.4. *Acceptability of results*

All calibrated with two rounded decimals calculated triglyceride contents of C24, C26, C28 to C54 as well as cholesterol must be exactly normalized to 100.

The results of the duplicate analysis is used as a check on the repeatability. If the absolute difference between the two S-results for all five triglyceride formulae do not transgress the repeatability limits r in Table 6, then the repeatability requirement is met.

For control of optimal gas chromatographic conditions and especially of the quality of the column it should be guaranteed that with 10 repetition runs the difference of the maximum and minimum S-values of all five triglyceride formulae do not transgress the range $x \cdot r$, with $x = 1,58$ (for 10 runs, see literature (16)), and the repeatability limits r for the different formulae in Table 6.

12. **Quoted standards**

DIN 10 336: 1994	Nachweis and Bestimmung von Fremdfetten in Milchfett anhand einer gaschromatographischen Triglyceridanalyse
IDF Standard 1 C: 1987	Milk. Determination of Fat Content — Röse Gottlieb Gravimetric Method
IDF Standard 16C: 1987	Cream. Determination of Fat Content — Röse Gottlieb Gravimetric Method
IDF Standard 116A: 1987	Milk-Based Edible Ices and Ice Mixes. Determination of Fat Content — Röse Gottlieb Gravimetric Method
IDF Standard 22B: 1987	Skimmed Milk, Whey & Buttermilk. Determination of Fat Content — Röse Gottlieb Gravimetric Method.

13. **References**

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2. Commission of the European Communities: *Control of butter fat purity of 100 different samples of different feeding periods from 11 EEC countries*; Doc. No VI/4577/93.
3. Commission of the European Communities: *Consideration of results from the first, second, third, fourth, fifth and sixth EEC collaborative trial: Determination of triglycerides in milk fat*; Doc. No VI/2644/91, VI/8.11.91, VI/1919/92, VI/3842/92, VI/5317/92, VI/4604/93.
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ANNEX XXVI

LIST OF THE REGULATIONS REFERRED TO IN THE FIRST RECITAL

- Commission Regulation (EEC) No 1216/68 of 9 August 1968 laying down the method for determining the lactose content of compound feeding-stuffs imported from third countries ⁽¹⁾, as amended by Commission Regulation (EEC) No 222/88 of 22 December 1987 amending certain measures on the application of the common market organisation in the milk and milk products sector following the introduction of the combined nomenclature ⁽²⁾;
- Commission Regulation (EEC) No 3942/92 of 22 December 1992 establishing a reference method for the determination of sitosterol and stigmasterol in butteroil ⁽³⁾, as last amended by Commission Regulation (EC) No 175/1999 amending Regulations (EEC) No 3942/92, (EC) No 86/94, (EC) No 1082/96 and (EC) No 1459/98 establishing reference methods for the determination of certain tracers in butter, butteroil and cream ⁽⁴⁾;
- Commission Regulation (EC) No 86/94 of 19 January 1994 establishing a reference method for the determination of sitosterol and stigmasterol in butter ⁽⁵⁾, as amended by Regulation (EC) No 175/1999;
- Commission Regulation (EC) No 2721/95 of 24 November 1995 establishing rules for the application of reference and routine methods for the analysis and quality evaluation of milk and milk products under the common market organisation ⁽⁶⁾;
- Commission Regulation (EC) No 1080/96 of 14 June 1996 establishing a reference method for the detection of coliforms in butter, skimmed milk powder and casein/caseinates ⁽⁷⁾;
- Commission Regulation (EC) No 1081/96 of 14 June 1996 establishing a reference method for the detection of cows' milk and caseinate in cheeses made from ewes' milk, goats' milk or buffalos' milk or mixtures of ewes', goats' and buffalos' milk and repealing Regulation (EEC) No 690/92 ⁽⁸⁾;
- Commission Regulation (EC) No 1082/96 of 14 June 1996 establishing a reference method for the determination of the ethyl ester of beta-apo-8' carotenic acid in concentrated butter and butter ⁽⁹⁾, as amended by Regulation (EC) No 175/1999;
- Commission Regulation (EC) No 1854/96 of 26 September 1996 establishing a list of reference methods to be applied for the analysis and quality evaluation of milk and milk products under the common market organisation ⁽¹⁰⁾, as amended by Regulation (EC) No 881/1999 ⁽¹¹⁾;
- Commission Regulation (EC) No 880/98 of 24 April 1998 establishing reference methods for the determination of the water, solids-non-fat and fat content of butter ⁽¹²⁾;
- Commission Regulation (EC) No 1459/98 of 8 July 1998 establishing a reference method for the determination of vanillin in concentrated butter, butter or cream ⁽¹³⁾, as amended by Regulation (EC) No 175/1999.

⁽¹⁾ OJ L 198, 10.8.1968, p. 13.

⁽²⁾ OJ L 28, 1.2.1988, p. 1.

⁽³⁾ OJ L 399, 31.12.1992, p. 29.

⁽⁴⁾ OJ L 20, 27.1.1999, p. 22.

⁽⁵⁾ OJ L 17, 20.1.1994, p. 7.

⁽⁶⁾ OJ L 283, 25.11.1995, p. 7.

⁽⁷⁾ OJ L 142, 15.6.1996, p. 13.

⁽⁸⁾ OJ L 142, 15.6.1996, p. 15.

⁽⁹⁾ OJ L 142, 15.6.1996, p. 26.

⁽¹⁰⁾ OJ L 246, 27.9.1996, p. 5.

⁽¹¹⁾ OJ L 111, 29.4.1999, p. 24.

⁽¹²⁾ OJ L 124, 25.4.1998, p. 16.

⁽¹³⁾ OJ L 193, 9.7.1998, p. 16.