COMMISSION REGULATION (EC) No 322/96

of 22 February 1996

laying down detailed rules of application for the public storage of skimmed-milk powder

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Regulation (EEC) No 804/68 of 27 June 1968 on the common organization of the market in milk and milk products [1], as last amended by Commission Regulation (EC) No 2931/95 [2], and in particular Articles 7 (5) and 28 thereof,

Whereas Council Regulation (EEC) No 1014/68 of 20 July 1968 laying down general rules for the public storage of skimmed-milk powder [3], as last amended by Regulation (EEC) No 3577/90 [4], will be repealed with effect from 1 March 1996 by Council Regulation (EC) No 1538/95 [5]; whereas the rules which have not been incorporated into Regulation (EEC) No 804/68 should be included in the detailed rules of application for the public storage of skimmed-milk powder laid down by Commission Regulation (EEC) No 625/78 [6], as last amended by Regulation (EC) No 1802/95 [7]; whereas, in view of the number of adjustments to be made to that Regulation and the many amendments which have already been made to it, it should be redrafted in the interests of clarity and transparency; whereas Regulation (EEC) No 625/78 should therefore be repealed;

Whereas intervention agencies may only buy skimmed-milk powder with a minimum protein content; whereas, furthermore, the buying-in price may vary according to the protein content; whereas, therefore, the analysis method to be used for determining that protein content and the method of calculating the buying-in price should be laid down;

Whereas, in order to ensure that payments are not made incorrectly for skimmed-milk powder bought in, it should be laid down that compliance with all the relevant conditions must be checked and the protein content measured before payment is made;

Whereas a reference method has been developed for the detection of buttermilk; whereas, in order to ensure uniform application of control measures, that analysis method should be used for checking skimmed-milk powder bought in by intervention agencies;

Whereas Council Directive 92/46/EEC of 16 June 1992 laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products [8], as last amended by Directive 94/71/EC [9], lays down that milk with an antimicrobial, substance content exceeding the maximum level authorized by Council Regulation (EEC) No 2377/90 [10], as last amended by Regulation (EC) No 282/96 [11], cannot be used for human consumption; whereas skimmed-milk powder bought in by intervention agencies should be checked for the presence of antimicrobial substances currently used; whereas a method should be laid down for such checks;

Whereas, for the rest, the provisions concerning the public storage of skimmed-milk powder have proven satisfactory and, subject to certain technical adjustments, may therefore remain unchanged;

Whereas the operative events for the agricultural conversion rate applicable to public storage arrangements are laid down by Commission Regulation (EEC) No 1756/93 [12], as last amended by Regulation (EC) No 693/95 [13];

Whereas the measures provided for in this Regulation are in accordance with the opinion of the Management Committee for Milk and Milk Products,

HAS ADOPTED THIS REGULATION:

Article 1

1. Intervention agencies shall buy in only such skimmed-milk powder as meets the definition laid down in Article 7 (1) of Regulation (EEC) No 804/68 and the quality requirements laid down in Annex I hereto, and for which the following conditions have been fulfilled:

(a) quality checks have been carried out in accordance with the analysis methods set out in Annexes I, V, VI and VII hereto on samples taken in accordance with the procedure set out in Annex IV hereto;

[1] OJ No L 148, 28. 6. 1968, p. 13.
[2] OJ No L 307, 20. 12. 1995, p. 10.
[3] OJ No L 173, 22. 7. 1968, p. 4.
[4] OJ No L 353, 17. 12. 1990, p. 23.
[5] OJ No L 148, 30. 6. 1995, p. 17.
[6] OJ No L 84, 31. 3. 1978, p. 19.
[7] OJ No L 174, 26. 7. 1995, p. 27.
[8] OJ No L 268, 14. 1. 1992, p. 1.
[9] OJ No L 368, 31. 12. 1994, p. 33.
[10] OJ No L 224, 18. 8. 1990, p. 1.
[11] OJ No L 37, 15. 2. 1996, p. 9.
[12] OJ No L 161, 2. 7. 1993, p. 48.
[13] OJ No L 71, 31. 3. 1995, p. 52.

(b) the packaging and marking comply with the conditions laid down in Annexes II and III;

(c) it does not contain other products, in particular buttermilk or whey;

(d) it was manufactured during the month preceding the day on which the intervention agency received the offer to sell it or, in the case referred to in the second sentence of point (e) of Annex III, during the four weeks preceding the week during which the offer was received;

(e) its radioactivity levels do not exceed the maximum levels permitted under Community rules.

The applicable levels shall be those laid down in Article 3 of Council Regulation (EEC) No 737/90 (1). The level of radioactive contamination of the product shall be monitored if the situation so requires and only during the period necessary. Where necessary, the duration and scope of the checks shall be determined in accordance with the procedure laid down in Article 30 of Regulation (EEC) No 804/68.

2. For the purposes of this Regulation, the following definitions shall apply:

(a) *buttermilk*: the by-product of butter manufacture obtained after churning of the cream (by batch process or continuous process) and separation of the solid fat;

(b) *whey*: the by-product of cheese or casein manufacture obtained by the action of acids, rennet and/or chemico-physical processes.

3. The minimum quantity offered for sale shall be 20 tonnes. Member States may increase the minimum quantity and/or lay down that the skimmed-milk powder must be offered in whole tonnes.

4. The intervention agency shall register the date on which the offer to sell is received, the corresponding quantities and dates of manufacture and the place at which the skimmed-milk powder is stored.

5. Offers shall only be valid where proof is provided that the seller has constituted a security of ECU 12 per tonne of skimmed-milk powder offered for sale.

For the purposes of this Regulation, the primary requirements whose fulfilment is ensured by the security shall be maintenance of the offer and delivery of the skimmed-milk powder to the storage depot designated by the intervention agency. However, where the checks referred to in paragraph 1 (a) show that the skimmed-milk powder does not conform to the requirements laid down in paragraph 1, the security shall be released for the quantities not yet delivered.

The security shall be constituted in the Member State in which the offer to sell is submitted.

Article 2

1. Undertaking as referred to in Article 7 (1) of Regulation (EEC) No 804/68 shall be approved only if they:

(a) are approved pursuant to Article 10 of Directive 92/46/EEC and have the appropriate technical equipment;

(b) undertake to keep permanent records, in the form determined by the competent agency of each Member State, listing the origin of the raw materials, the quantities of skimmed-milk powder, buttermilk and whey treated, the type of heat-treatment for the skimmed milk, the quantities of products obtained, the packaging and identification of each lot of skimmed-milk powder, buttermilk powder and whey powder and the date on which it left the plant;

(c) agree to submit their production of skimmed-milk powder liable to be offered for intervention to a specific official inspection;

(d) undertake to inform the body responsible for inspection, at least two working days in advance, of their intention to manufacture skimmed-milk powder for public intervention. The Member State may, however, fix a shorter deadline.

2. To ensure compliance with this Regulation, the competent bodies shall carry out unannounced on-the-spot inspections on the basis of the production schedules for skimmed-milk powder for intervention of the plants concerned.

Those inspections must include at least:

— one inspection per period of 28 days of manufacture for intervention with at least one inspection every six months, to examine the records referred to in paragraph 1 (b),

— one inspection every six months, to verify compliance with the conditions for approval referred to in paragraph 1.

3. Approval shall be withdrawn if the prior conditions laid down in paragraph 1 are no longer satisfied. Approval may be regranted at the request of the enterprise concerned after a period of six months, following a thorough inspection.

Where an enterprise is found not to have complied with one of its undertakings as referred to in paragraph 1 (b), (c) and (d), except in cases of *force majeure*, approval shall be suspended for between 1 and 12 months depending on the seriousness of the irregularity.

The Member State may decide not to impose the said suspension where it is found that the irregularity was not committed deliberately or as a result of serious negligence and it is of minor importance with regard to the effectiveness of the inspection provided for in paragraph 2.

(1) OJ No L 82, 29. 3. 1990, p. 1.

4. A report shall be drawn up on the inspections carried out pursuant to paragraphs 2 and 3 specifying:

— the date of inspection,

— its duration,

— the operations carried out.

The inspection report must be signed by the inspector responsible.

5. Member States shall inform the Commission of the measures taken with regard to the inspections provided for in paragraphs 2 and 3 within one month of their adoption.

Article 3

The body responsible for ensuring compliance with the provisions of Article 1 (1) shall record the results of the analysis of each lot offered, specifying each of the characteristics of the product listed in paragraph 1 of Annex I; the results shall then be entered on a certificate to be issued on request, in particular in the case of trade between Member States and export.

Article 4

1. After checking the offer, the intervention agency shall immediately issue a dated and numbered delivery order showing;

(a) the quantity to be delivered;

(b) the final date for delivery of the skimmed-milk powder;

(c) the storage depot to which it must be delivered.

2. The skimmed-milk powder must be delivered within 28 days of the day of receipt of the offer to sell referred to in Article 1 (1) (d). Delivery may be in several consignments.

3. For the purposes of this Regulation, the skimmed-milk powder shall be deemed to be taken over by the intervention agency on the day when it enters the storage depot but no earlier than the day following the day of issue of the delivery order referred to in paragraph 1.

4. Payment for the skimmed-milk powder bought in by the interventiion agency shall be made between the 120th day and the 140th day following the date on which it is taken over by the intervention agency, provided that it is found to comply with the requirements laid down in Article 1.

5. The buying-in price for the skimmed-milk powder shall be calculated as follows:

— where the protein content of the non-fat dry matter, determined in accordance with the method described in Annex I, is equal to or more than 35,6 %, the buying-in price shall be equal to the intervention price,

— where that protein content is equal to or more than 31,4 % but less than 35,6 %, the buying-in price shall be equal to the intervention price less an amount calculated as follows: intervention price × [(0,356 − protein content) × 1,75].

6. In making the offer the seller shall undertake, in the event of the inspection showing that the skimmed-milk powder does not comply with the requirements laid down in Article 1 (1):

— to take back the goods in question,

— to pay the storage costs for the quantities concerned from the date they are taken over to the date of their removal from storage.

Such storage costs shall be payable at a flat rate per tonne as follows:

(a) ECU 21 for fixed costs;

(b) ECU 0,10 per day of storage for warehousing costs.

The amounts shall be credited to the account of the European Agricultural Guidance and Guarantee Fund (EAGGF), Guarantee Section.

Article 5

1. The storage depots referred to in the fourth subparagraph of Article 7 (1) of Regulation (EEC) No 804/68 must:

(a) be dry, well maintained and free of vermin;

(b) be free of extraneous odours;

(c) permit good ventilation;

(d) be of sufficient capacity and have equipment relevant to that capacity.

The risks of storing the skimmed-milk powder shall be covered by insurance in the form of either a contractual obligation on storers or a comprehensive coverage of the liability borne by the intervention agency. The Member States may also act as their own insurers.

2. The intervention agencies shall require that the skimmed-milk powder be placed and kept in storage on pallets and in easily identifiable and readily accessible lots.

3. The competent body responsible for inspection shall also carry out checks on the presence of the products in store as provided for in Article 4 of Commission Regulation (EEC) No 618/90 [1].

Article 6

1. The intervention agency shall choose the available storage depot nearest to the place where the skimmed-milk powder is stored.

[1] OJ No L 67, 15. 3. 1990, p. 21.

However, provided that the choice of another storage depot does not result in additional storage costs, it may:

(a) choose another depot situated within the distance referred to in paragraph 2;

(b) choose a storage depot situated beyond that distance if the resuting expenditure, inclusive of storage and transport costs, is less. In that case, the intervention agency shall notify the Commission of its choice forthwith.

2. The maximum distance referred to in the third subparagraph of Article 7 (1) of Regulation (EEC) No 804/68 shall be 350 km. Beyond that distance, the additional transport costs borne by the intervention agency shall be ECU 0,05 per tonne and per kilometre.

However, where the intervention agency buying in the skimmed-milk powder is in a Member State other than that in whose territory it is stored, no account shall be taken, for calculating the maxium distance referred to in subparagraph 1, of the distance between the store of the seller and the border of the Member State of the purchasing intervention agency.

Article 7

At the time of removal from storage, the intervention agency shall, in the case of delivery ex-storage depot, make the skimmed-milk powder available:

— at the storage depot loaded on to the means of transport, where this is a lorry or a railway wagon, but not stowed,

— at the exit of the storage depot in the case of other means of transport, in particular containers.

Article 8

Regulation (EEC) No 625/78 is hereby repealed.

References to Regulation (EEC) No 625/78, shall be understood as being to this Regulation.

Article 9

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

It shall apply from 1 March 1996.

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

Done at Brussels, 22 February 1996.

For the Commission

Franz FISCHLER

Member of the Commission

ANNEX I

QUALITY OF SKIMMED-MILK POWDER

1. **Characteristics:**

(a) protein content:	31,4 % minimum of the non-fat dry matter
(b) Fat content:	1,00 % maximum
(c) Water content:	3,5 % maximum
(d) Titratable acidity in ml of decinormal sodium hydroxide solution:	19,5 maximum
(e) Lactate content:	150 mg/100 g maximum
(f) Additives:	None
(g) Phosphatase test:	Negative, i.e. equal to or less than 4 micrograms of phenol per gram of constituted milk
(h) Solubility index:	0,5 ml maximum (24 °C)
(i) Burnt-particles index:	15,0 mg maximum, i.e. disc B minimum
(j) Micro-organism content:	40 000 per gram maximum
(k) Detection of coliforms:	Negative in 0,1 g
(l) Detection of buttermilk:	Negative
(m) Detection of whey:	Negative
(n) Taste and smell:	Clean
(o) Appearance:	White or slightly yellowish colour, free from impurities and coloured particles
(p) Antimicrobial substances	Negative (1)

2. **Control methods**

(a) Without prejudice to the relative measures concerning the harmonization of analytical methods, the followng reference methods are obligatory for the application of this Regulation:

— protein dosage (N × 6,38):	International Standard FIL 20B: 1993
— fat content:	International Standard FIL 9C: 1987
— water content:	International Standard FIL 26A: 1993
— acidity:	International Standard FIL 86: 1981
— lactate content:	International Standard FIL 69B: 1987
— phosphatase test:	ISO Standard 3356: 1975
— solubility index:	International Standard FIL 129A: 1988
— burnt particles index:	ADPI Standard Methods 1990
— micro-organism count:	International Standard FIL 100B: 1991
— detection of coliforms:	International Standard FIL 73A: 1985

(b) as regards the detection of:

— rennet whey:	determination of glycomacropeptides by high-performance liquid chromatography in accordance with the method described in Annex V
— acid whey:	tests laid down by the Member States
— buttermilk:	the absence of buttermilk can be established either by an on-the-spot inspection of the production plant carried out without prior notice at least once a week, or by a laboratory analysis of the end product, in accordance with the method described in Annex VI, indicating a maximum of 69,31 mg of PEDP per 100 g
— antimicrobial substances:	in accordance with the method described in Annex VII.

(c) Samples are to be taken in accordance with the procedure laid down by International Standard ISO 707; however, Member States may use another method of sampling provided that that method complies with the principles laid down by the abovementioned Standard.

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(1) Raw milk used for the manufacture of skimmed-milk powder must satisfy the requirements laid down in Part D of Chapter III of Annex A to Directive 92/46/EEC.

ANNEX II

PACKAGING

1. **Packaging**

Packaging shall be new, dry and intact with a net content of 25 kg and made up in one of the following ways:

(a) four kraft paper bags of a strength of at least 70 g/m^2,

one interposed tar-lined bag of a strength of at least 140 g/m^2,

one polyethylene inner bag at least 0,08 mm thick, welded or double bound;

(b) one kraft paper bag of a strength of a least 70 g/m^2,

one kraft paper bag with a polyethylene lining, of a strength of at least 80 g plus 15 g/m^2,

three kraft paper bags of a strength of at least 70 g/m^2,

one polyethylene inner bag at least 0,08 mm thick, welded or double bound;

(c) one kraft paper outer bag of a strength of at least 85 g/m^2,

one kraft paper bag with a polyethylene lining, of a strength of at least 70 g plus 15 g/m^2,

two kraft paper bags of a strength of at least 70 g/m^2,

one polyethylene inner bag at least 0,12 mm thick, welded or double bound;

(d) one outer semi-stretchable kraft paper bag of a strength of at least 95 g/m^2,

one semi-stretchable kraft paper bag with a polyethylene lining, of a strength of at least 95 g plus 15 g/m^2,

one semi-stretchable kraft paper bag of a strength of at least 85 g/m^2,

one polyethylene inner bag at least 0,12 mm thick, welded or double bound.

2. **Filling**

When filling, the powder should be well pressed down. Loose powder must on no account be allowed to penetrate between the various layers.

3. **Test procedure**

The packaging, in accordance with point 1, must have a tensile energy absorption average of 420 J/m^2 for a minimum of three layers of paper according to the ISO 1924-2-1985 method.

ANNEX III

MARKING

The packaging must be marked with at least the following, where appropriate in code:

(a) the description in one of the official languages of the Community: 'Spray skimmed-milk powder';

(b) net weight;

(c) production batch number;

(d) authorization number identifying the plant and Member State of manufacture;

(e) date of manufacture. Where the skimmed-milk powder is stored in silos, the date of manufacture is replaced by the week of manufacture.

ANNEX IV

SAMPLING AND ANALYSIS OF SKIMMED-MILK POWDER OFFERED FOR INTERVENTION

1. Number of packages to be selected for sampling checks:
   — offers containing up to 800 25-kg bags: at least eight,
   — offers containing more than 800 25-kg bags: at least eight, plus one for each additional 800 bags or fraction thereof.
2. Weight of sample: samples of at least 200 g are to be taken from each package.
3. Grouping of samples: no more than nine samples are to be combined in a global sample.
4. Analysis of samples: each global sample is to undergo an analysis to verify all the quality characteristics laid down in Annex I.
5. Guidelines to be followed in the event of sample failure:
   (a) where a composite sample shows a defect with regard to one parameter, the quantity from which the sample came is rejected;
   (b) where a composite sample shows a defect with regard to more than one parameter, the quantity from which the sample came is rejected and samples are taken from the remaining quantities from the same plant; the analysis of those samples shall be decisive. In such cases:
      — the number of samples laid down in point 1 is doubled,
      — where a composite sample shows a defect with regard to one or more parameters, the quantity from which the sample came is rejected.

ANNEX V

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 recognized analytical grade. The water used must be distilled water or water of at least equivalent purity.

5.1. *Tricloroacetic 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 homogenize.

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 Regulation (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 ± 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 in 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 appartus (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 ± 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 analyzed.

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:

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

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

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.

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

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} × A_{II} [E]

S_{III} [E] = R_{III} × A_{III} [E]

S_{IV} [E] = R_{IV} × 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 ≤ 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} × 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:

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

$S_{III} = 0{,}0123\ S_{II}\ [E] + 0{,}93$ (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.

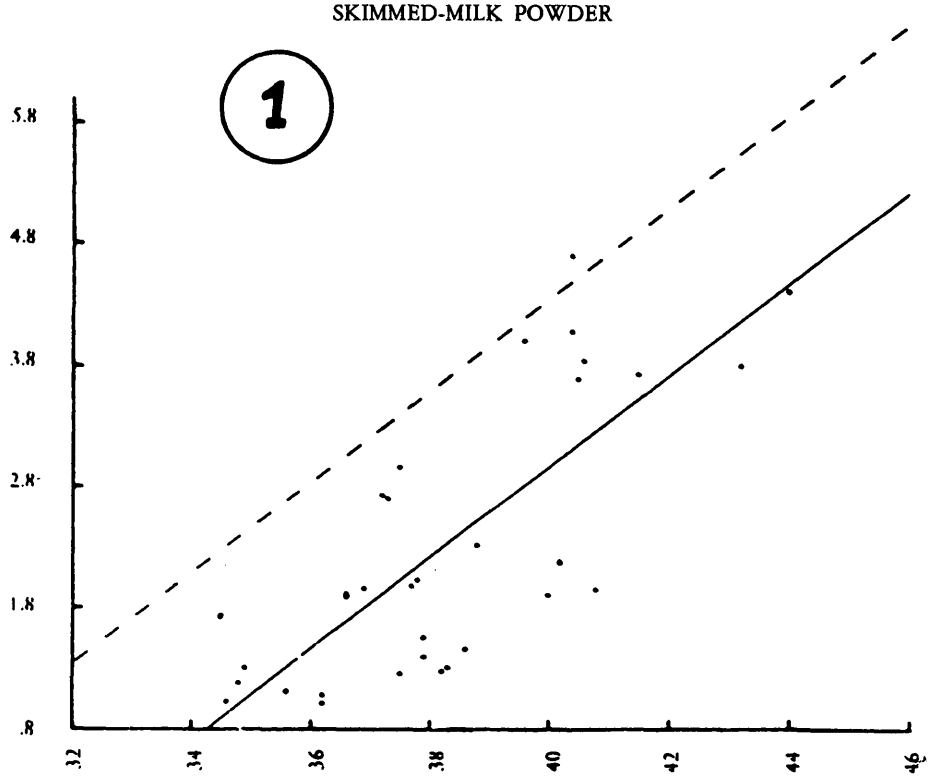
The rennet whey content is calculated according to the following formula:

$$W = T_2 + 0{,}91$$

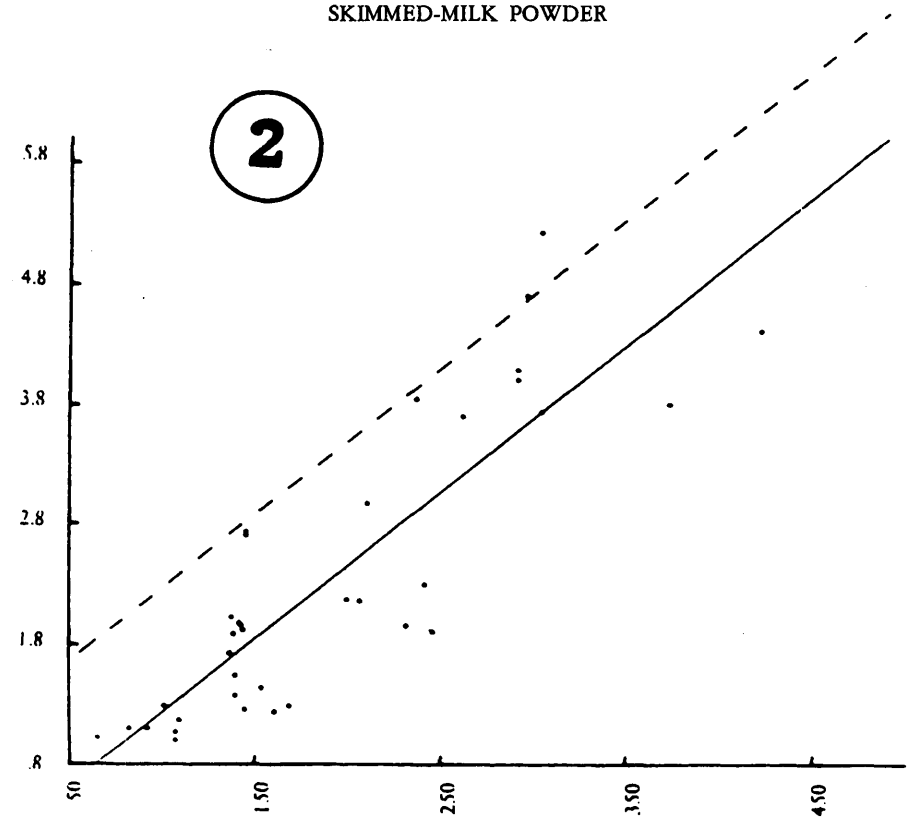
where:

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

SKIMMED-MILK POWDER



SKIMMED-MILK POWDER



ANNEX VI

SKIM-MILK POWDER. DETERMINATION OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE CONTENT

REVERSED-PHASE HPLC METHOD

1. Scope and field of application

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

2. Definition

Content of PS + PE: 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-phthaldialdehyde (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 recognized 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 shall 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-phthaldialdehyde derivatization*

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-ophthaldialdehyde (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 × 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 derivatization 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 walvelength

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 (C18), 5 µm particle size

6. Sampling

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

7. Procedure

7.1. *Preparation of the internal standard solution*

Weigh 30,0 ± 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 ± 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 min 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 × g for 10 min and collect the supernatant in a glass vial (5.10).

Note:

Test sample solution shall be stored at 4 °C till 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) 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:

Standard sample solution shall be stored at 4 °C till the HPLC analysis is performed.

7.4. *Preparation of the derivatizing reagent*

Weigh 25,0 ± 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 derivatizing reagent shall be stored at 4 °C in a dark vial and is stable for one week.

7.5. *HPLC determination*

7.5.1. Elution solvents (4.4)

Solvent A:

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 derivatizing 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 min, then set at A:B = 40:60 and equilibrate at 1 ml/min for 15 min.

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 min.

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

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 min 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 peak groups

Under the described conditions (Figure 1), PS elutes as 2 main partially unresolved peaks preceded by a minor peak. PE elutes as 3 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:

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

where:

C = PS or PE content (mg/100 g powder) in the test sample

A_1 = PEDP peak area of the standard sample solution (7.3)

A_2 = PS or PE peak area of the test sample solution (7.2)

T_1 = tryptamine peak area of the standard sample solution (7.3)

T_2 = 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 'Guidelines for the interpretation of analytical results and the application of sensory evaluation in relation to milk and milk products under the common market organization' (Commission document VI/2721/95).

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. **Bibliography**

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

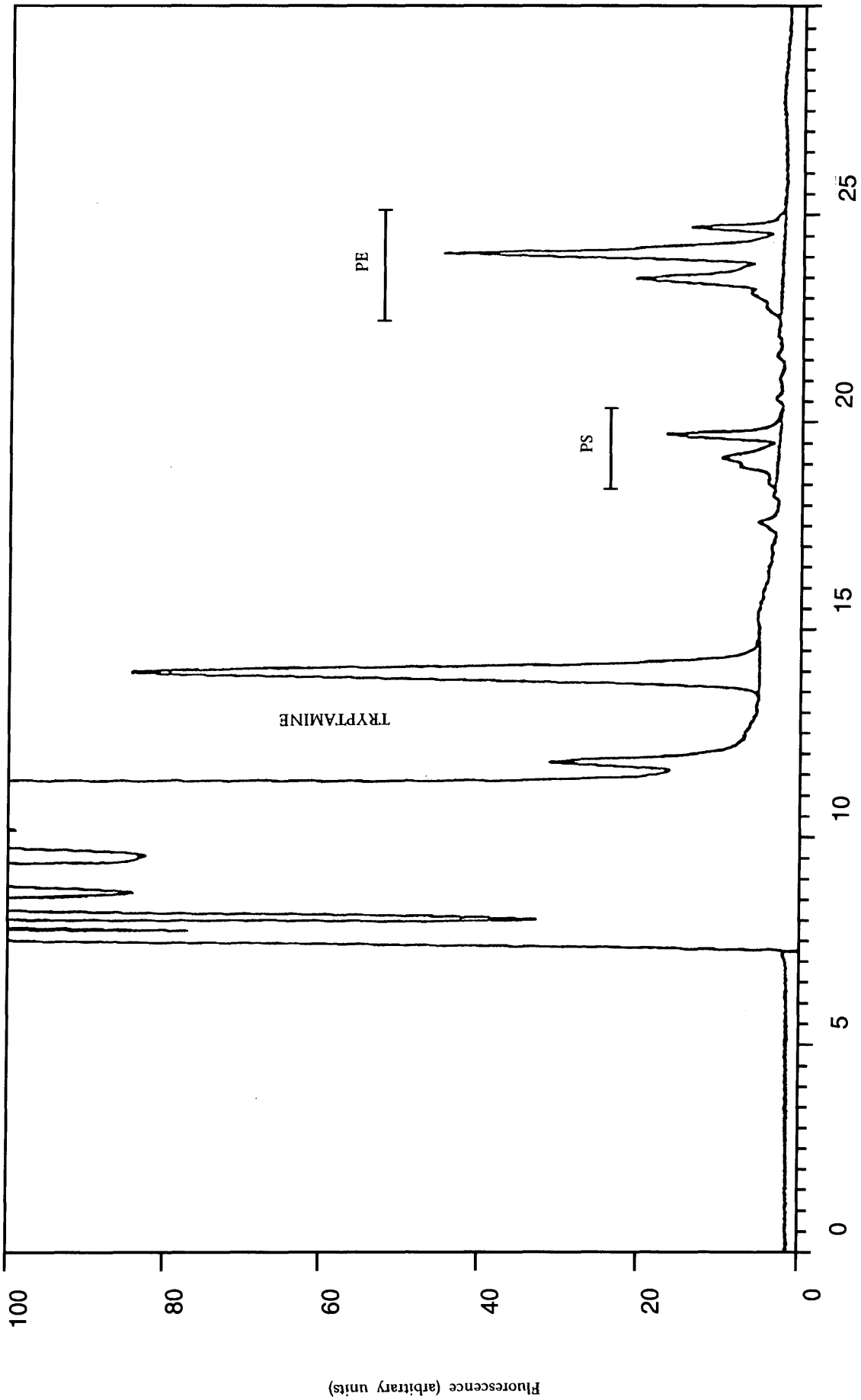


Figure 1: HPLC pattern of OPA-derivatives of phosphatidylserine (PS) and phosphatidylethanolamine (PE) in methanol extract of reconstituted skim-milk powder. Integration mode for the peaks of PS, PE and tryptamine (internal standard) is reported.

ANNEX VII

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 test kits are available and can be used if they have the required sensitivity for Benzylpencillin 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 ß-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 pencillinase + p-amino benzoic acid to the test system:

   Positive result: Inhibiting substance cannot be identified by this procedure.

   Negative result: Inhibiting substances are a ß-lactam antibiotic and a sulfonamide/dapson.

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