COMMISSION REGULATION (EEC) No 718/85

of 20 March 1985

amending Regulation (EEC) No 625/78 on detailed rules of application for public storage of skimmed-milk powder

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic 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 Regulation (EEC) No 1557/84 (2), and in particular Article 7 (5) thereof,

Whereas point 2 (b) of Annex I to Commission Regulation (EEC) No 625/78 (3), as last amended by Regulation (EEC) No 1128/84 (4), as regards the quality of skimmed-milk powder, lays down the control methods used for the detection of certain products;

Whereas a new method for detecting rennet whey by determination of glycomacropeptides using high-performance liquid chromatography has been developed, thanks in particular to the ring test that was developed by various research institutes and by several control laboratories in the Member States; whereas the said method has clear advantages, including in particular the rapid performance of routine testings of many samples per day; whereas provision should therefore be made for using this method by amending Regulation (EEC) No 625/78;

Whereas it seems advisable to provide for a 12-month period of transition and adaptation during which Member States may continue to use the free sialic acid method and operators may require that that method be used in cases where they do not accept the results obtained by means of the new method;

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

Regulation (EEC) No 625/78 is hereby amended as follows:

1. The second indent of point 2 (b) of Annex I is replaced by the following:

'— rennet whey: determination of glycomacropeptides by high-performance liquid chromatography (2). However, until 31 March 1986, Member States may also use determination of free sialic acid (3). Until that date, determination of free sialic acid will, at the request and expense of the party concerned, also be used where the latter does not accept the results obtained using the new method, in which case only the results obtained by means of the free sialic acid method will be considered.'

2. In Annex I, note (2) becomes note (3) and the following note is added:

'(2) The method shall be that set out in Annex V.'

3. The Annex to this Regulation is added as Annex V.

Article 2

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

(1) OJ No L 148, 28. 6. 1968, p. 13.
(2) OJ No L 150, 6. 6. 1984, p. 6.
(3) OJ No L 84, 31. 3. 1978, p. 19.
(4) OJ No L 109, 26. 4. 1984, p. 9.

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

Done at Brussels, 20 March 1985.

For the Commission

Frans ANDRIESSEN

Vice-President

ANNEX

'ANNEX V

DETECTION OF RENNET WHEY IN SKIMMED-MILK POWDER FOR PUBLIC STORAGE BY DETERMINATION OF GLYCOMACROPEPTIDES WITH THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE (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. References

IDF Standard 50A : 1980 Milk and Milk products — Guide to sampling techniques.

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. *Trichloroacetic acid solution*

Dissolve 240 g of trichloroacetic acid ($C1_3CCOOH$) 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 Regulation (EEC) No 625/78 (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 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. See IDF Standard 50 A: 1980.

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 2,000 ± 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 with 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]

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

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} [E] = 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} [5] 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 $\geqslant 1{,}000$ when the whey content is $\leqslant 5$ %.

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

Normally, the value of RRT_{III} [O] 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:*

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

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 g of rennet whey per 100 g determined in non-adulterated skimmed-milk powder of various origins. This figure was obtained experimentally;

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

S_{III} [O] — 0,9 represents the correction to be made to the relative average area 1,3 when S_{III} [O] is not equal to 0,9. Experimentally the relative average area of peak III of the control sample [O] 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 g of rennet whey per 100 g is $\leq$ 2,0 + (S_{III} [O] — 0,9).

Where :

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

(S_{III} [O] — 0,9) represents the correction to be made when the value S_{III} [O] is different to 0,9 (see 9.2).

9.4.2. If the relative area of peak III, S_{III} [E] is $\geq$ 2,0 and the relative area of peak II, S_{II} [E] is $\geq$ 160 and/or that of peak IV, S_{IV} [E] is $\geq$ 135, determine the protein content.

9.4.2.1. The protein content is < 37 g per 100 g, the sample analyzed was probably manufactured from milk of poor bacteriological quality. No conclusion can be drawn regarding the presence of whey. The resolution of peak II, III and IV must, however, be as clear as for the control samples and the relative retention time of peak III must be close to that obtained for peak S_{III} [O].

9.4.2.2. The protein content is $\geq$ 37 g per 100 g :

— if the relative areas of peaks II, III and IV of sample [E] are within the dashed lines (based on statistics of experimental data on the graphs 1-6, see Annexes), the presence of rennet whey cannot be established ;

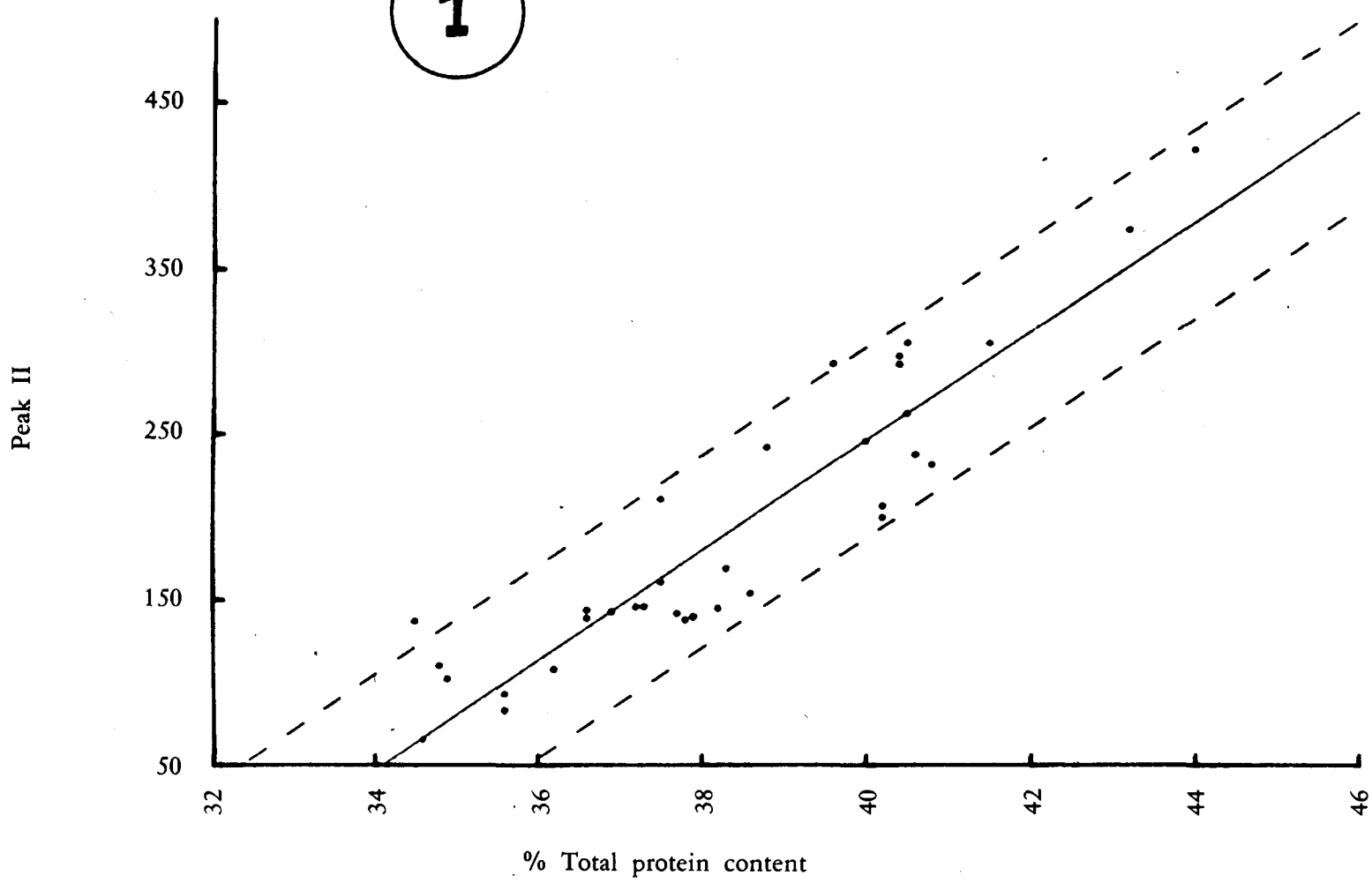
— in the case if only the relative area of peak III is above the dashed line in the graphs 2, 3 and 6 then rennet whey is present. The amount of rennet whey is calculated by subtracting from S_{III} [E], obtained as in paragraph 9.1.2, the average experimental value of peak III (solid line), which is found in graph 2 with the protein content of sample [E].

The RRT_{III} [E] value should correspond to the expected one on the basis of the amount of rennet whey found ;

— In any other case no conclusions can be drawn regarding the presence of rennet whey.

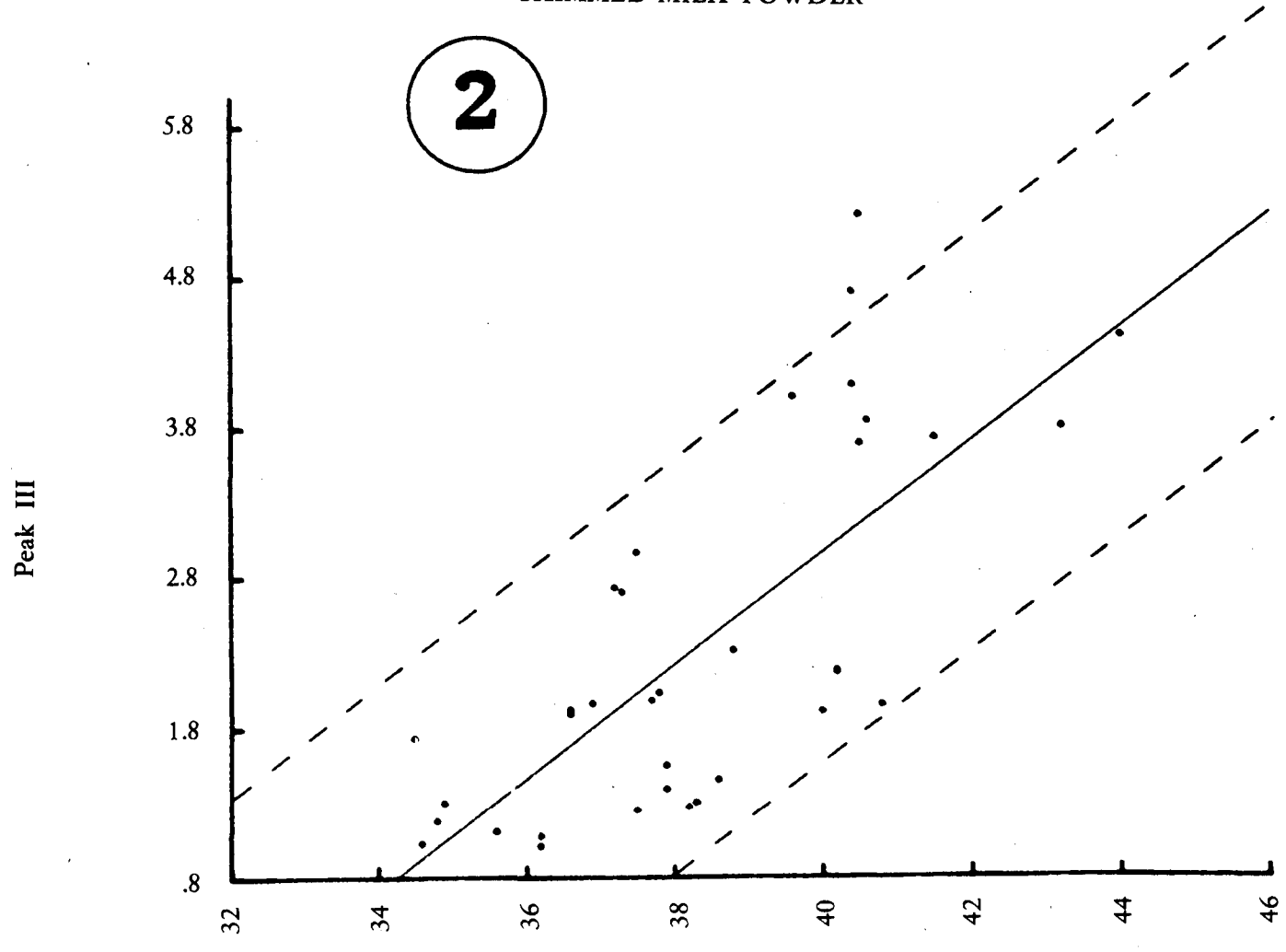
SKIMMED-MILK POWDER

1



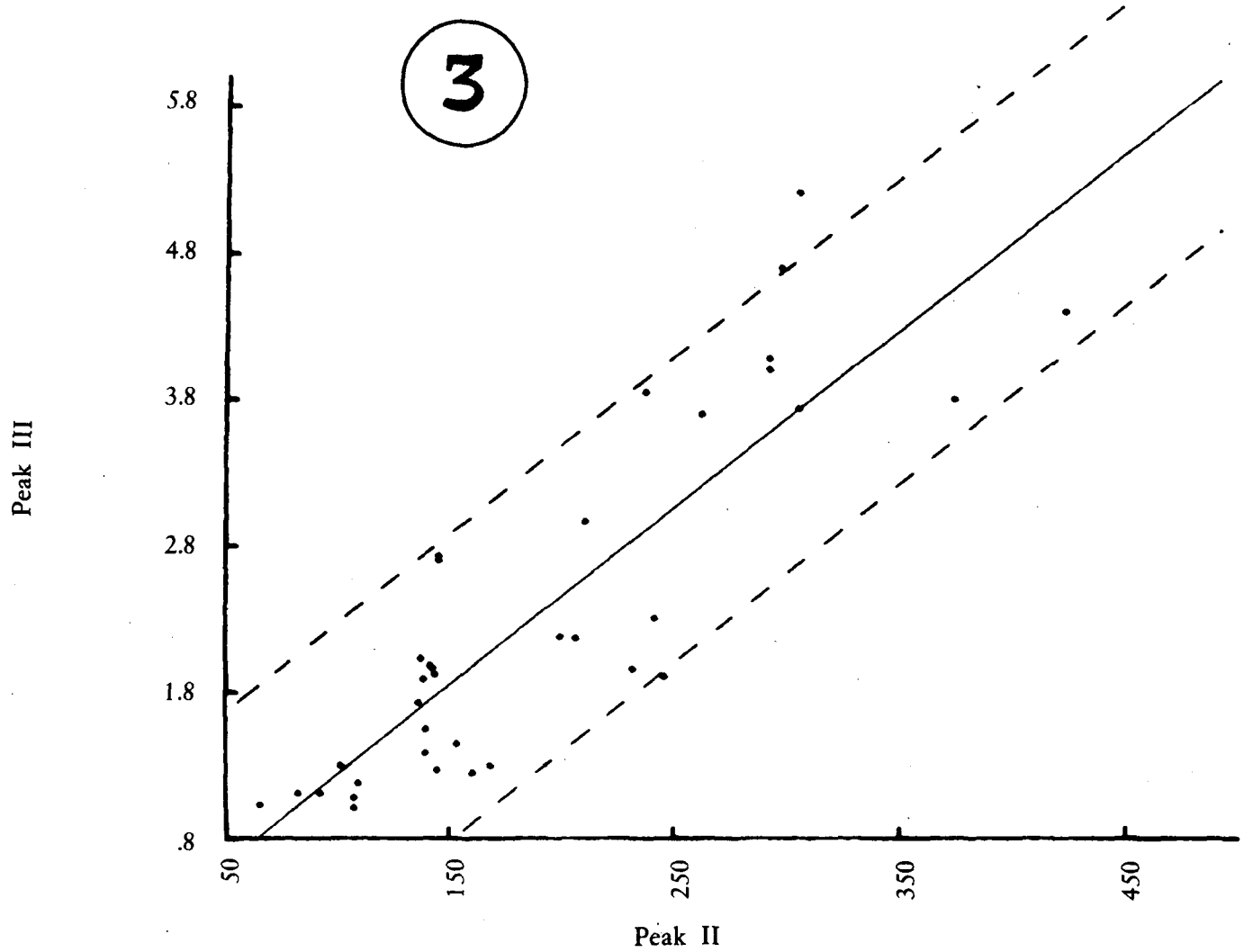
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2



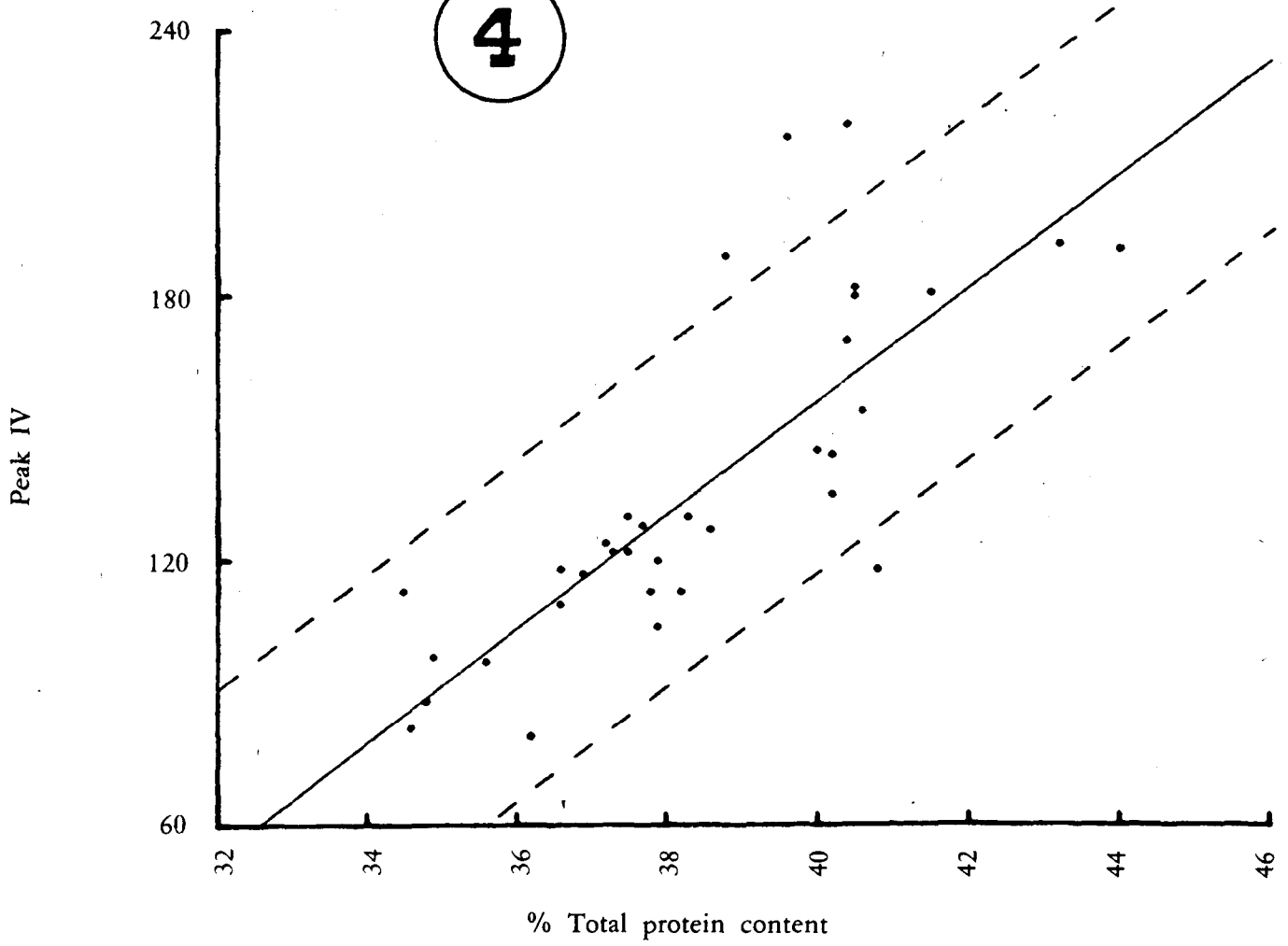
SKIMMED-MILK POWDER

3



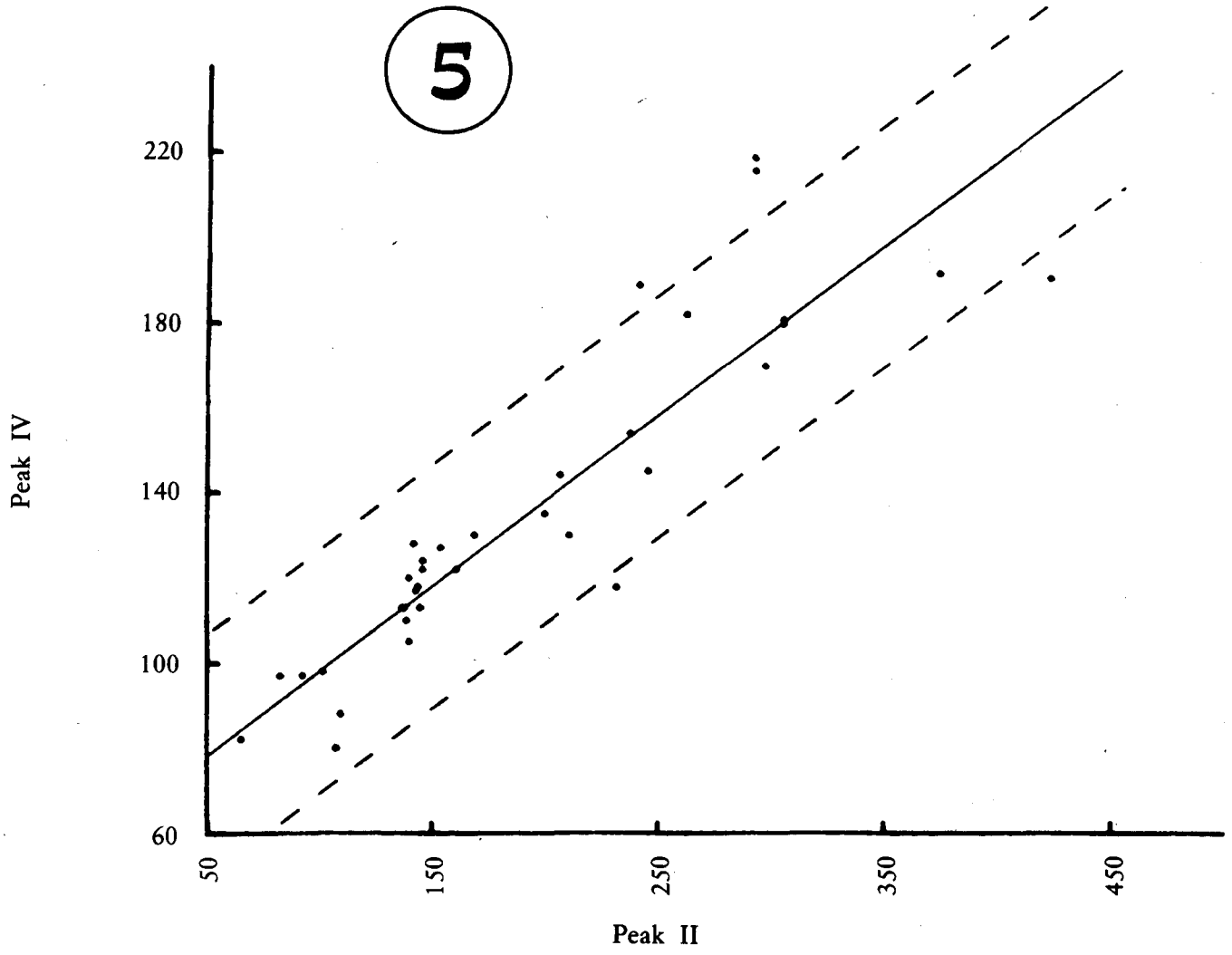
SKIMMED-MILK POWDER

4



SKIMMED-MILK POWDER

5



SKIMMED-MILK POWDER

6

