# Commission Implementing Regulation (EU) 2016/1240 of 18 May 2016 laying down rules for the application of Regulation (EU) No 1308/2013 of the European Parliament and of the Council with regard to public intervention and aid for private storage (Text with EEA relevance)

ANNEX V

## SKIMMED MILK POWDER

[F1 Appendix I

## 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 OF THE METHOD

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 shall be of recognised analytical grade. Water shall be distilled or water 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 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-phthaldialdehyde (OPA)*

4.4. **HPLC elution solvents**

4.4.1. *Elution solvents shall be prepared using HPLC-grade reagents.*

4.4.2. *HPLC-grade water*

4.4.3. *Methanol of tested fluorimetric purity*

4.4.4. *Tetrahydrofuran*

4.4.5. *Sodium dihydrogen phosphate*

4.4.6. *Sodium acetate*

4.4.7. *Acetic acid.*

5. APPARATUS

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

5.2. **Beakers, 25 and 100 ml capacity**

5.3. **Pipettes, capable of delivering 1 and 10 ml**

5.4. **Magnetic stirrer**

5.5. **Graduated pipettes, capable of 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, capable of operating at 27 000 × g**

5.10. **Glass vials, about 5 ml capacity**

5.11. **Graduated cylinder, 25 ml capacity**

5.12. **pH-meter, accurate to 0,1 pH units**

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, capable of maintaining the column at 30 °C ± 1 °C*

5.13.4. *Fluorescence detector, capable of operating 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 LiChrospher® — 100 column (250 × 4,6 mm) or an equivalent column packed with octadecylsilane (C 18), 5 µm particle size.*

6. SAMPLING

Sampling shall be carried out in accordance with ISO Standard 707.

7. PROCEDURE

7.1. **Preparation of the internal standard solution**

7.1.1. *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)*

7.1.2. *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**

7.2.1. *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 ± 1 °C by a pipette (5.3) and stir with a magnetic stirrer (5.4) for 30 minutes in order to dissolve any lumps*

7.2.2. *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*

7.2.3. *Centrifuge (5.9) at 27 000 g × 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**

7.3.1. *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 ± 1 °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*

7.3.2. *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 ± 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 brown glass 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 ± 0,1 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. *Determine 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 calculate the response factor*

*Note:* The column shall 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:

$C = 55{,}36 \times ((A_2)/(A_1)) \times ((T_1)/(T_2))$

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. ACCURACY OF THE METHOD

*Note:* The values for repeatability were calculated according to the IDF International Standard (*).

9.1. **Repeatability**

The relative standard deviation 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.

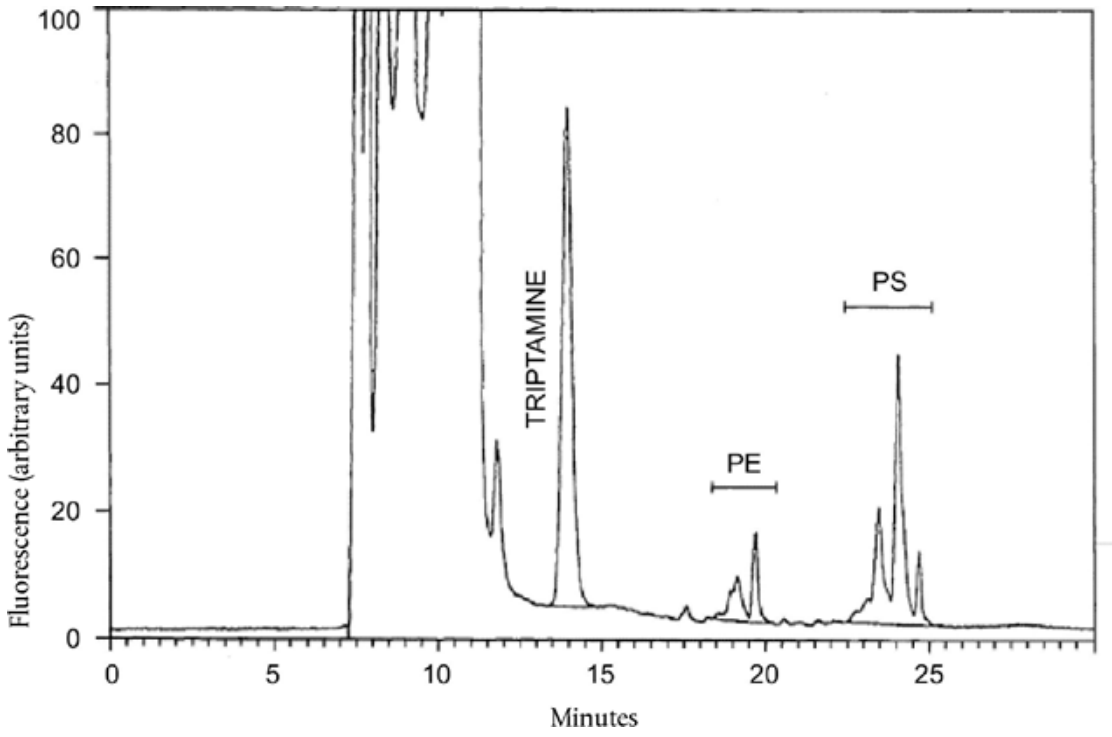
9.2. **Reproducibility**

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).*

*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]**



**Status:**
Point in time view as at 07/02/2018.

**Changes to legislation:**
There are currently no known outstanding effects for the Commission Implementing Regulation (EU) 2016/1240, Appendix I.