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**Identification of Reconstituted
Milk in Pasteurized and UHT Milk**

巴氏杀菌乳和 UHT 灭菌乳中复原乳的鉴定

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Identification of Reconstituted Milk in Pasteurized and UHT Milk

1 Scope

This Standard specifies the identification method of reconstituted milk in pasteurized and UHT milk.

This Standard is applicable to the pasteurized and UHT milk.

2 Normative References

The following documents are essential to the application of this document. For the dated documents, only the versions with the dates indicated are applicable to this document; for the undated documents, only the latest version (including all the amendments) are applicable to this document.

GB 5009.5 Determination of Protein in Foods

GB/T 6682 Water for Laboratory Use – Specifications

GB/T 10111 Generation of Random Numbers and Procedures Applied to Sampling Inspection for Product Quality

3 Terms and Definitions

For the purposes of this document, the following terms and definitions apply.

3.1 Raw milk

The normal milk extruded from the breasts of the healthy dairy animals, it meets the relevant national requirements and has no ingredient changes.

3.2 Reconstituted milk

The milk obtained by mixing a dried or concentrated dairy product with water in proportion.

3.3 Heat treatment

4.1.1 Principle

Hydrolyze the specimen by hydrochloric acid, then determine its protein content; after diluting, the hydrolysate shall be analyzed by high performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UPLC) under ultraviolet (wavelength 280nm) detector and quantify by external standard method.

4.1.2 Reagents and materials

Unless otherwise specified, all reagents used in this method shall be analytical reagents; and the water shall be Class-I water in the laboratory specified in GB/T 6682.

4.1.2.1 Methanol (CH_3OH): chromatographically pure.

4.1.2.2 Concentrated hydrochloric acid (HCl , density 1.19g/mL).

4.1.2.3 Trifluoroacetic acid: chromatographically pure.

4.1.2.4 Ammonium acetate.

4.1.2.5 Furosine: $\text{C}_{12}\text{H}_{17}\text{N}_2\text{O}_4 \cdot x\text{HCl}$.

4.1.2.6 Hydrochloric acid solution (3mol/L): add 2.5 mL of concentrated hydrochloric acid to 7.5mL of water; mix evenly.

4.1.2.7 Hydrochloric acid solution (10.6mol/L): add 88mL of concentrated hydrochloric acid to 12mL of water, mix evenly.

4.1.2.8 Ammonium acetate solution (6g/L): accurately take 6g of ammonium acetate to dissolve into water; make constant volume to 1L; pass through the 0.22 μm aqueous phase membrane; ultrasonically degas for 10min.

4.1.2.9 Ammonium acetate (6g/L) containing 0.1% trifluoroacetic acid solution: accurately take 6g of ammonium acetate; dissolve into partial water; add 1mL of trifluoroacetic acid; make constant volume to 1; pass through 0.22 μm aqueous phase membrane; ultrasonically degas for 10min.

4.1.2.10 Furosine standard stock solution (500.0mg/L): convert the furosine standard substance as per the Net Peptide Content provided by the standard substance certificate; then use 3mol/L hydrochloric acid solution to formulate into a standard stock solution. It can be stored for 24 months at -20°C .

Example:

If the Net Peptide Content marked on the furosine standard substance certificate is 69.1%, then take 7.24mg of furosine standard substance; use 3mol/L hydrochloric acid solution to dissolve and make constant volume to 10mL; the concentration of standard stock solution is 500.0mg/L.

equivalent.

Column temperature: 35°C.

Mobile phase: 6g/L ammonium acetate containing 0.1% trifluoroacetic acid aqueous solution is mobile phase A; methanol is mobile phase B; while pure water is mobile phase C.

Elution conditions: mobile phase A. Isocratic elution, 0.4mL/min.

2) Determination

The mobile phase pure water and methanol should be used to wash the chromatographic system; before the instrument is used, use mobile phase pure water to transit; use mobile phase A to equilibrate the chromatographic column at the flow rate of 0.4mL/min. Inject 0.5μL of 3mol/L hydrochloric acid solution to check the purity of the solvent. Inject 0.5μL to-be-tested solution to determine the furosine content. See Appendix A for chromatograms.

4.1.6 Result calculation

4.1.6.1 Furosine content in the specimen

The furosine is calculated by mass fraction F ; the value of which is expressed in mg/100g protein; and calculated as per Formula (1):

$$F = \frac{A_t \times C_{std} \times D \times 100}{A_{std} \times m} \dots\dots\dots (1)$$

Where:

A_t – value of furosine peak area in the tested sample;

A_{std} – value of furosine peak area in the furosine standard solution;

C_{std} – concentration of furosine standard solution, in mg/L;

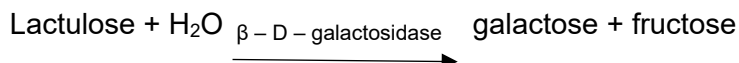
D – when determining, the dilution factor ($D=6$);

m – protein concentration in the sample hydrolysate, in g/L,

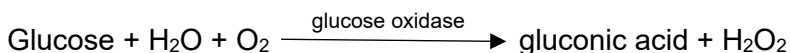
The calculation result shall be retained to one digit after the decimal point.

4.1.6.2 Furosine content at the end of sterilization of pasteurized milk

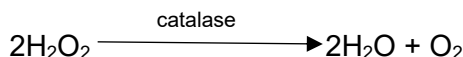
At the end of sterilization of pasteurized milk, the furosine content is calculated by FT ; the value of which is expressed by mg/100g protein; and calculated as per Formula (2):



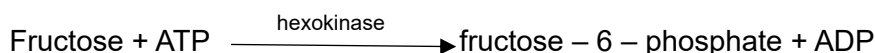
Then add glucose oxidase (GOD); oxidize most of the glucose into gluconic acid:



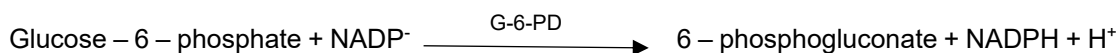
The above reaction generates the hydrogen peroxide, which can be removed by the catalase:



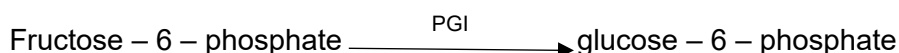
A small amount of unoxidized glucose and lactulose are hydrolyzed to generate the fructose; under the catalysis of hexokinase (HK), react with Adenosine Triphosphate (ATP); separately generate glucose – 6 – phosphate and fructose – 6 – phosphate:



The generated glucose – 6 – phosphate, under the catalysis of glucose – 6 – phosphate dehydrogenase (G – 6 – PD), reacts with oxidized coenzyme II, namely, nicotinamide adenine dinucleotide phosphate (NADP^+), and generates reduced coenzyme II, namely, reduced nicotinamide adenine dinucleotide phosphate (NADPH):



The generated NADPH can be determined at the wavelength 340nm. However, fructose – 6 – phosphate shall use phosphoglucose isomerase (PGI) to transfer into glucose – 6 – phosphate:



The generated glucose – 6 – phosphate reacts with NADP^+ ; and measure the absorbance at the wavelength of 340nm. Calculate the lactulose content by the difference of the above two measurement results. The original fructose in the sample can be measured and deducted by the blank sample. The determination of the blank sample is the same as the determination of the sample; only add no β – D – galactosidase.

4.2.2 Reagents and materials

Unless otherwise specified, all reagents used in this method are analytical reagents; while the water shall be Class-I water in the laboratory specified in GB/T 6682.

4.2.2.1 Sterilized water.

4.2.2.2 Hydrogen peroxide (H_2O_2 , mass fraction of 30%).

4.2.2.24 Sodium hydroxide solution (1mol/L): dissolve 4g of sodium hydroxide into 100mL of water.

4.2.2.25 Ammonium sulfate solution (3.2 mol/L): dissolve 42.24g of ammonium sulfate into 100mL of water.

4.2.2.26 Buffer Solution A (pH 7.5): take 4.8g of disodium hydrogen phosphate, 0.86g of sodium dihydrogen phosphate and 0.1g of magnesium sulfate; then dissolve into 80mL of water; use 1mol/L sodium hydroxide solution to adjust the pH to be 7.5 ± 0.1 (20°C); make constant volume of 100mL.

4.2.2.27 Buffer Solution B (pH 7.6): take 14.00g of triethanolamine hydrochloride and 0.25g of magnesium sulfate; dissolve into 80mL of water; use 1mol/L sodium hydroxide solution to adjust pH to be 7.6 ± 0.1 (20°C); make constant volume of 100mL.

4.2.2.28 Buffer Solution C: pipette 40.0mL of Buffer Solution B; use water to make constant volume of 100mL; mix evenly.

4.2.2.29 β - D - galactosidase suspension (150mg/mL): use 3.2mol/L ammonium sulfate solution to prepare the β - D - galactosidase with activity of 12.6 IU/mg into suspension with concentration of 150mg/mL. It shall be prepared for current use; and not oscillate when preparing.

4.2.2.30 Glucose oxidase (GOD) suspension (20mg/mL): use sterilized water to prepare the glucose oxidase with activity of 200 IU/mg into suspension with concentration of 20mg/mL. It shall be prepared for current use.

4.2.2.31 Catalase suspension (20mg/mL): use sterilized water to prepare the catalase with activity of 65000 IU/mg into suspension with concentration of 20mg/mL. It shall be stored at 4°C ; shake it before use to make it uniform.

4.2.2.32 Hexokinase (HK) / glucose - 6 - phosphate dehydrogenase (G - 6 -PD) suspension: in 1mL of 3.2mol/L ammonium sulfate solution, add 2mg of hexokinase with activity of 140 IU/mg, and 1mg of glucose - 6 - phosphate dehydrogenase with activity of 140 IU/mg; gently shake to form suspension. It shall be stored at -20°C .

4.2.2.33 Phosphoglucose isomerase (PGI) suspension (2mg/mL): use 3.2 mol/L ammonium sulfate solution to prepare the phosphoglucose isomerase with activity of 350 IU/mg into suspension with concentration of 2mg/mL; it shall be stored at 4°C .

4.2.2.34 5'-adenosine triphosphate (ATP) solution: dissolve 50mg of adenosine 5'-triphosphate disodium salt ($5' - \text{ATP} - \text{Na}_2$) and 50mg of sodium bicarbonate into 1mL of water; it shall be stored at -20°C .

4.2.2.35 Nicotinamide adenine dinucleotide phosphate (NADP) [Translator Note: here it should be (NADP⁻)] solution: dissolve 10mg of triphosphopyridine nucleotide

Where:

ΔA_L – net absorbance difference of the sample;

M_L – molar mass of lactulose (342.3 g/mol);

ε – molar absorbance of NADPH at 340nm ($6.3L \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$);

V_1 – total volume of liquid in cuvette (3.240 mL);

V_2 – volume of filtrate in the cuvette, in mL;

d – cuvette light path length (1.00cm);

8 – dilution factor.

The calculation result shall be retained one digit after the decimal point.

4.2.7 Precision

The absolute difference between two independent test results obtained under the repeatability conditions is no more than 10% of the arithmetic mean.

The absolute difference between two independent test results obtained under the reproductivity conditions is no more than 20% of the arithmetic mean.

4.2.8 Detection limit

The detection limit is 4.2mg/L.

4.3 Calculation of ratio between lactulose and furosine

$$R = \frac{L}{FT} \dots\dots\dots (8)$$

The calculation result shall be retained two digits after the decimal point.

5 Identification of Reconstituted Milk

5.1 Pasteurized milk

When $L < 100.0\text{mg/L}$, it shall be judged as follows:

- a) When $12.0\text{mg}/100\text{g protein} < FT \leq 25.0\text{mg}/100\text{g protein}$, if $R < 0.50$, then it shall be judged as containing reconstituted milk.
- b) When $FT > 25.0\text{mg}/100\text{g protein}$, if $R < 1.00$, then it shall be judged as containing

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