LACTOSE ANHYDROUS
Stage 4, Revision 4 (CP: EP)

BRIEFING NOTE

1. The revision is now refered as "Revision 4" as an expedited revision (Rev.3) was signed in June 2008.

2. **Appearance of solution**: it has been specified to allow the solution to cool before examination. Reference solutions have been introduced for comparison for a more objective evaluation of clarity and color.

3. **Acidity or alkalinity**: the changes included in the expedited revision (Rev.3) signed in June 2008 have been taken into account.

4. **Test on alpha and beta lactose**: based on the results from an experimental study (in attachment) the derivatisation procedure has been optimised and the use of a classical injection port has been introduced as an alternative to the cold on-column injection system. It has been shown in the experimental study that more reproducible results are obtained with the cold on-column injection system. However precision is within a range which is acceptable for the purpose of this test and the classical injection system is also acceptable. In addition, the size of the injection vials is not given anymore following a comment from JP.

EP would like to seek agreement from PDG that this test could be included in the non-mandatory section FRC in the EP monograph.

5. **Microbial contamination**:
Acceptance criterion for TYMC: JP has proposed a limit of 50 CFU/g. During the elaboration of harmonised chapters on microbial contamination of non-sterile products, microbiologists have agreed to express the limits as 10^x. In this context, it is suggested to specify TYMC with a limit of 10^1. However, EP experts have considered this to be very strict and unnecessary for lactose. We suggest reconsidering the need for this criterion at a later stage depending on public enquiry comments.

Test for absence of Salmonella: JP has requested the inclusion of a test for absence of Salmonella. We acknowledge the potential presence of Salmonella in dairy products, but suggest collecting data during the public enquiry to evaluate whether the production processes used eliminate this risk sufficiently.
Therefore the test has been included into the Stage 4 proposal with a view to re-evaluating it after the comment period.

Stage 4 draft for Rev. 4

ANHYDROUS LACTOSE

Anhydrous Lactose is *O*-β-D-galactopyranosyl-(1→4)-β- D-glucopyranose (β-lactose) or a mixture of *O*-β-D-galactopyranosyl-(1→4)-β- D-glucopyranose and *O*-β-D-galactopyranosyl-(1→4)-α- D-glucopyranose (α-lactose).

Clarity and color of solution—Dissolve 1 g in 10 mL of boiling water. Allow to cool. The solution is clear and nearly colorless: : its clarity is the same as that of water or its opalescence is not more pronounced than that of reference suspension I, and it is not more coloured than reference solution BY_7.
Determine the absorbance of this solution at a wavelength of 400 nm. The absorbance divided by the path length in centimeters is not more than 0.04.

Specific rotation— Dissolve 10 g by heating in 80 mL of water to 50 degrees. Allow to cool, and add 0.2 mL of 6 N ammonium hydroxide. Allow to stand for 30 minutes, and dilute with water to 100 mL: the specific rotation, calculated on the anhydrous basis, determined at 20 degrees, is between +54.4 degrees and +55.9 degrees.

Acidity or alkalinity—Dissolve 6 g by heating in 25 mL of carbon dioxide-free water, cool, and add 0.3 mL of a solution of phenolphthalein (1 g in 100 ml of alcohol): the solution is colorless, and not more than 0.4 mL of 0.1 N sodium hydroxide is required to produce a pink or red color.

Loss on drying—Dry it at 80° for 2 hours; it loses not more than 0.5% of its weight.

Residue on ignition—not more than 0.1%. Ignition temperature is 600 ± 50°.

Water, Karl Fischer — not more than 1.0%, determined on a preparation containing anhydrous lactose in a mixture of methanol and formamide (2:1).

Protein and light-absorbing impurities -- Measure the light absorption of a 1% (w/v) solution in the range of 210 to 300 nm. The absorbance divided by the path length in centimeters is not

more than 0.25 in the range of 210 to 220 nm and is not more than 0.07 in the range of 270 to 300 nm.

Content of alpha and beta anomers—

Gas chromatography.

Test solution. Introduce 10 mg of the substance to be examined in a vial with a screw cap. Add 4 ml of a mixture of 19.5 per cent of dimethyl sulfoxide, 22.0 per cent of trimethylsilylimidazole and 58.5 per cent of pyridine. Sonicate for 20 min at room temperature. Transfer 400 µl to a injection vial. Add 1 ml of pyridine. Close the vial and mix well.

Reference solution. Prepare a mixture of alpha-lactose monohydrate and beta-lactose having an anomeric ratio of about 1:1 based on the labeled anomeric contents of the alpha-lactose monohydrate and the beta-lactose. Introduce 10 mg of this mixture in a vial with a screw cap. Add 4 ml of a mixture of 19.5 per cent of dimethyl sulfoxide, 22.0 per cent of trimethylsilylimidazole and 58.5 per cent of pyridine. Sonicate for 20 min at room temperature. Transfer 400 µl to a injection vial. Add 1 ml of pyridine. Close the vial and mix well.

Pre-column:

— *material*: intermediate polarity deactivated fused-silica [(1)] ,

— *size*: *l* = 2 m, Ø = 0.53 mm,

([(1)] Restek Guard column is suitable)

Column:

— *material*: fused-silica,

— *size*: *l* = 15 m, Ø = 0.25 mm,

— *stationary phase*: *poly(dimethyl)(95)(diphenyl)(5)siloxane (film thickness = 0.25 µm)* [(2)] .

([(2)] Varian CP Sil 8 CB is suitable)

Carrier gas: helium for chromatography.

Column pressure: 70 kPa.

Temperature:

— *temperature programme* as follows:

	Time (min)	Temperature (°C)
Column	0-1	80
	1-3	80 ->150
	3-15.5	150 ->300
	15.5-17.5	300
Injection port		275 or use cold-on column injection
Detector		325

Detection: flame-ionisation.

Injection: 0.5 µl splitless or by cold on-column injection.

Relative retention with reference to beta-lactose (retention time = about 12 min): alpha-lactose = about 0.9.

System suitability: reference solution:

— *resolution*: minimum 3.0 between the peaks due to alpha-lactose and beta-lactose.

Calculate the percentage content of alpha-lactose from the following expression:
$100\ S_a / (S_a + S_b)$

Calculate the percentage content of beta-lactose from the following expression:
$100\ S_b / (S_a + S_b)$

S_a = area of the peak due to alpha-lactose

S_b = area of the peak due to beta-lactose

Microbial contamination (internationally harmonized methods) - TAMC: acceptance criterion 10^2 CFU/g. TYMC: acceptance criterion 10^1 CFU/g. Absence of Escherichia coli and of Salmonella.

REAGENTS

Hydrazine sulphate solution. Dissolve 1.0 g of hydrazine sulphate in water and dilute to 100.0 ml with the same solvent. Allow to stand for 4-6 h.

Hexamethylenetetramine solution. In a 100 ml ground-glass-stoppered flask, dissolve 2.5 g of hexamethylenetetramine in 25.0 ml of water.

Primary opalescent suspension (formazin suspension). To the hexamethylenetetramine solution in the flask add 25.0 ml of the hydrazine sulphate solution. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Standard of opalescence. Dilute 15.0 ml of the primary opalescent suspension to 1000.0 ml with water. This suspension is freshly prepared and may be stored for up to 24 h.

Reference suspension I. To 5.0 ml of standard of opalescence add 95.0 ml of water. Mix and shake before use.

Yellow primary solution. Dissolve 46 g of ferric chloride ($FeCl_3,6H_2O$) in about 900 ml of a mixture of 25 ml of concentrated hydrochloric acid (35.0 to 39.0 per cent *m/m* of HCl) and 975 ml of water and dilute to 1000.0 ml with the same mixture. Titrate and adjust the solution to contain 45.0 mg of $FeCl_3,6H_2O$ per millilitre by adding the same acidic mixture. Protect the solution from light.
Titration. Place in a 250 ml conical flask fitted with a ground-glass stopper, 10.0 ml of the solution, 15 ml of water, 5 ml of concentrated hydrochloric acid (35.0 to 39.0 per cent *m/m* of HCl) and 4 g of potassium iodide, close the flask, allow to stand in the dark for 15 min and add 100 ml of water. Titrate the liberated iodine with 0.1 M sodium thiosulphate, using 0.5 ml of starch solution, added towards the end of the titration, as indicator.
1 ml of 0.1 M sodium thiosulphate is equivalent to 27.03 mg of $FeCl_3,6H_2O$.

Red primary solution. Dissolve 60 g of cobalt chloride ($CoCl_2,6H_2O$) in about 900 ml of a mixture of 25 ml of concentrated hydrochloric acid (35.0 to 39.0 per cent *m/m* of HCl) and 975 ml of water and dilute to 1000.0 ml with the same mixture. Titrate and adjust the solution to contain 59.5 mg of $CoCl_2,6H_2O$ per millilitre by adding the same acidic mixture.
Titration. Place in a 250 ml conical flask fitted with a ground-glass stopper, 5.0 ml of the solution, 5 ml of hydrogen peroxide solution (3 per cent) and 10 ml of a 300 g/l solution of sodium hydroxide. Boil gently for 10 min, allow to cool and add 60 ml of dilute sulphuric acid (98 g/l of H_2SO_4) and 2 g of potassium iodide. Close the flask and dissolve the precipitate by shaking gently. Titrate the liberated iodine with 0.1 M sodium thiosulphate, using 0.5 ml of starch solution, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink.
1 ml of 0.1 M sodium thiosulphate is equivalent to 23.79 mg of $CoCl_2,6H_2O$.

Blue primary solution. Dissolve 63 g of copper sulphate ($CuSO_4,5H_2O$) in about 900 ml of a mixture of 25 ml of concentrated hydrochloric acid (35.0 to 39.0 per cent *m/m* of HCl) and 975

ml of water and dilute to 1000.0 ml with the same mixture. Titrate and adjust the solution to contain 62.4 mg of $CuSO_4,5H_2O$ per millilitre by adding the same acidic mixture.
Titration. Place in a 250 ml conical flask fitted with a ground-glass stopper, 10.0 ml of the solution, 50 ml of water, 12 ml of dilute acetic acid (115 g/l to 125 g/l of $C_2H_4O_2$) and 3 g of potassium iodide. Titrate the liberated iodine with 0.1 M sodium thiosulphate, using 0.5 ml of starch solution, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slight pale brown colour.
1 ml of 0.1 M sodium thiosulphate is equivalent to 24.97 mg of $CuSO_4,5H_2O$.

Starch solution.
Triturate 1.0 g of soluble starch with 5 ml of water and whilst stirring pour the mixture into 100 ml of boiling water containing 10 mg of mercuric iodide.
Carry out the test for sensitivity each time the reagent is used.
Test for sensitivity. To a mixture of 1 ml of the starch solution and 20 ml of water, add about 50 mg of potassium iodide and 0.05 ml of iodine solution. The solution is blue.

BY (brownish-yellow) standard solution. Mix 2.4 ml of yellow primary solution, 1.0 ml of red primary solution, 0.4 ml of blue primary solution and 6.2 ml of hydrochloric acid (10 g/l HCl).

BY_7 reference solution. Mix 2.5 ml of BY standard solution and 97.5 ml of hydrochloric acid (10 g/l HCl).