Megazyme

# LACTULOSE

***(RAPID / SENSITIVE)***

(Incorporating reagents for use in the procedure described by ISO Method 11285:2004)

## ASSAY PROCEDURE

K-LACTUL 03/11

(50 Assays per Kit)

Megazyme

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## INTRODUCTION:

Thermal treatment of milk is an essential process in the dairy industry. Various heating regimens can be employed, and thus in some cases it becomes necessary to determine thermal history accurately. This can be achieved by quantitative measurement of lactulose, a disaccharide comprising D-galactose and D-fructose that is only produced from the common milk sugar lactose at elevated temperatures. In raw milk the level of lactulose is very low. However, as heat treatment increases this sugar can eventually reach ~ 2 g/L (in the case of UHT milk for example).

This kit (K-LACTUL) is suitable for the measurement of lactulose in milk-based products such as fresh milk, UHT milk, evaporated milk and powdered milk. However, this kit is unsuitable for the measurement of lactulose in samples that have been artificially sweetened, such as condensed milk, where the "free fructose" to lactulose ratio is extremely high.

Reagents included in this kit can be prepared for use in the measurement of lactulose using the procedure as described by ISO Method 11285:2004. However, the slightly modified advanced format K-LACTUL (Rapid Method) developed by Megazyme, that is both more sensitive and rapid, features an additional enzyme, 6-phosphogluconate dehydrogenase, which results in the sensitivity of the method being improved 2-fold. Additionally, optimised selection and use of highly purified enzymes results in a method that can easily be performed within one working day.

## PRINCIPLE:

Lactose and lactulose are simultaneously hydrolysed by the enzyme β-galactosidase to D-galactose, D-glucose and D-fructose (1), (2).

$$(1)\quad \text{Lactose} + H_2O \xrightarrow{(\beta\text{-galactosidase})} \text{D-glucose} + \text{D-galactose}$$

$$(2)\quad \text{Lactulose} + H_2O \xrightarrow{(\beta\text{-galactosidase})} \text{D-fructose} + \text{D-galactose}$$

Excess D-glucose is then removed from the system by conversion to D-gluconic acid by the enzymes glucose oxidase and catalase in the presence of oxygen (3), (4).

$$(3)\quad \text{D-Glucose} + O_2 + H_2O \xrightarrow{(\text{glucose oxidase})} \text{D-gluconic acid} + H_2O_2$$

$$(4)\quad 2\,H_2O_2 \xrightarrow{\text{(catalase)}} O_2 + 2\,H_2O$$

After the removal of excess D-glucose, any residual D-glucose and D-fructose are phosphorylated by the enzyme hexokinase (HK) in the presence of adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P), respectively, with the simultaneous formation of adenosine-5'-diphosphate (ADP) (5), (6).

$$(5)\quad \text{D-Glucose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{G-6-P} + \text{ADP}$$

$$(6)\quad \text{D-Fructose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{F-6-P} + \text{ADP}$$

G-6-P is oxidised by the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate (gluconate-6-P) with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (7).

$$(7)\quad \text{G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-P} + \text{NADPH} + H^+$$

The gluconate-6-P product is immediately converted to D-ribulose-5-phosphate (R-5-P), carbon dioxide ($CO_2$), and a further molecule of NADPH by the enzyme 6-phosphogluconate dehydrogenase (6-PGDH) (8).

$$(8)\quad \text{Gluconate-6-P} + NADP^+ \xrightarrow{\text{(6-PGDH)}} \text{R-5-P} + CO_2 + \text{NADPH} + H^+$$

On completion of reaction (8), F-6-P is converted to G-6-P by phosphoglucose isomerase (PGI) (9).

$$(9)\quad \text{F-6-P} \xrightarrow{\text{(PGI)}} \text{G-6-P}$$

The G-6-P formed reacts according to (7) and (8), with the amount of NADPH formed being stoichiometric to twice the amount of lactulose (as two molecules of NADPH are produced for every D-fructose molecule originating from the lactulose in the sample).

## SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for lactulose. The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 1.2 mg/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 4.8 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 1.00 mL.

The assay is linear over the range of 0.65 to 65 mg of lactulose per "Enzymatic Determination Reaction" (section B, page 8). This equates to a lactulose concentration range of 4.8 to 480 mg/L in an original sample processed using the Standard Assay Procedure (section A, page 7).

In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 1.00 mL, this corresponds to a lactulose concentration of approx. 1.2 to 2.4 mg/L of sample solution. If in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

## INTERFERENCE:

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments i.e. by adding lactulose to the sample in the initial extraction steps.

If the conversion of the D-fructose liberated from lactulose has been completed within the time specified in the assay, it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-fructose (approx. 5 mg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

## SAFETY:

The reagents used in the determination of lactulose are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrates contain sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

## KITS:

Kits suitable for performing 50 assays are available from Megazyme. The kits contain the full assay method plus:

**Bottle 1:** β-Galactosidase suspension (3 mL).
Stable for > 2 years at 4°C.

**Bottle 2:** Glucose oxidase and catalase, lyophilised powder.
Stable for > 2 years at -20°C.

**Bottle 3:** Buffer (6 mL, pH 7.6) plus sodium azide (0.02 % w/v) as a preservative.
Stable for > 2 years at 4°C.

**Bottle 4:** $NADP^+$ plus ATP.
Stable for > 2 years at 4°C.

**Bottle 5:** Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.2 mL.
Stable for > 2 years at 4°C.

**Bottle 6:** 6-Phosphogluconate dehydrogenase suspension (2.2 mL).
Stable for > 2 years at 4°C.

**Bottle 7:** Phosphoglucose isomerase suspension (2.2 mL).
Stable for > 2 years at 4°C.

**Bottle 8:** Lactulose (0.1 mg/mL) plus D-fructose (0.05 mg/mL) standard solution (5 mL).
Stable for > 2 years at 4°C.

## PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently store the bottle in an upright position. Swirl the bottle to mix contents before use.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 6 mL of distilled water. To avoid repetitive freeze / thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes at -20°C.
Stable for > 2 years at -20°C.

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years at 4°C.

**4.** Dissolve the contents of bottle 4 in 12 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Once dissolved, the reagent is stable for > 2 years at -20°C.

**5 & 6.** Use the contents of bottles 5 and 6 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently store the bottles in an upright position. Swirl the bottle to mix contents before use.
Stable for > 2 years at 4°C.

**7.** Use the contents of bottle 7 as supplied.
Stable for > 2 years at 4°C.

**8.** Use the contents of bottle 8 as supplied.
Stable for > 2 years at 4°C.

**NOTE:** The lactulose plus D-fructose standard solution (bottle 8) is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. To test the sample preparation process (section A, page 7), treat the standard solution as a sample and use as supplied. To test for sample interference during the Enzymatic Determination Reaction (section B, page 8) add 0.1 mL (5 mg D-fructose) of standard solution to the cuvette after the sample reaction is complete. The concentration of lactulose is determined directly from the extinction coefficient of NADPH (page 9).

## PREPARATION OF REAGENT SOLUTIONS (NOT SUPPLIED):

### 1. Sodium phosphate buffer (0.4 M, pH 7.6, 4 mM magnesium sulphate): 500 mL

Add 24 g of $Na_2HPO_4$ (Sigma cat. no. 71640), 4.3 g of $NaH_2PO_4.H_2O$ (Sigma cat. no. 71506) and 0.5 g of $MgSO_4.7H_2O$ (Sigma cat. no. M-5921) to 450 mL of distilled water. Adjust the pH to 7.6 with 1 M NaOH (40 g/L) and make the volume up to 500 mL with distilled water. Store at 4°C.

### 2. Sodium acetate buffer (2 M, pH 4.5): 50 mL

Add 13.6 g of sodium acetate trihydrate (Sigma S-8625) to 40 mL of distilled water and dissolve. Adjust the pH to 4.5 with acetic acid and make volume up to 100 mL with distilled water. Store at room temperature.

### 3. TEA buffer (1 M, pH 7.6 plus 10 mM magnesium sulphate): 100 mL

Add 14.9 g of TEA buffer (Acros cat. no. 421630025) and 0.25 g of $MgSO_4.7H_2O$ (Sigma cat. no. M-5921) to 80 mL of distilled water. Adjust the pH to 7.6 and make volume up to 100 mL with distilled water. Store at 4°C.

### 4. Concentrated Carrez I solution: 200 mL

Dissolve 30 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6.3H_2O$} (Sigma cat. no. P-9387) in 200 mL of distilled water. Store at room temperature.

### 5. Concentrated Carrez II solution: 200 mL

Dissolve 60 g of zinc sulphate {$ZnSO_4.7H_2O$} (Sigma cat. no. Z-4750) in 200 mL of distilled water. Store at room temperature.

### 6. Sodium hydroxide (0.33 M): 200 mL

Add 2.64 g of sodium hydroxide pellets (Merck cat. no. 1.06482.5000) to 180 mL of distilled water and dissolve. Make to volume (200 mL) with distilled water. Store at room temperature.

### 7. Octanol (~ 99 % v/v; Sigma cat. no. O-4500)

Store at room temperature.

### 8. Hydrogen peroxide (~ 30 % v/v; Sigma cat. no. H-1009)

Store at room temperature.

## EQUIPMENT (RECOMMENDED):

1. Glass beaker (100 mL).
2. Volumetric flask (50 mL).
3. Disposable 1.5 mL polypropylene microfuge tubes e.g. Sarstedt cat. no. 72.690 (www.sarstedt.com).
4. Disposable 1.5 mL polypropylene screw cap microfuge tubes e.g. Sarstedt cat. no. 72.692 (www.sarstedt.com).
5. Disposable 13 mL polypropylene tubes e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
6. Disposable plastic micro-cuvettes (1 cm light path, 1.5 mL) e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 7591 15 (www.brand.de).
7. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
8. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.05 mL aliquots of Buffer 3 and $NADP^+$/ATP (solution 2)].

10. Analytical balance.
11. Spectrophotometer set at 340 nm.
12. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
13. Heated water bath (set at 40°C).
14. Microfuge (required speed 13,000 rpm).

## STANDARD ASSAY PROCEDURE (Rapid Method):

### A. SAMPLE PREPARATION (Liquid Milk):

1. To a 1.5 mL polypropylene microfuge tube, add the following with mixing after each addition:

| Pipette into microtube | Volume |
|---|---|
| * sodium phosphate buffer (0.4 M) | 0.20 mL |
| distilled water | 0.70 mL |
| milk sample | 0.50 mL |
| * concentrated Carrez II solution | 0.05 mL |
| * concentrated Carrez I solution | 0.05 mL |
| Mix by vortex and then centrifuge at 13,000 rpm for 10 min. Carefully pipette the clear supernatant for use in step 2. | |

2. To a 1.5 mL polypropylene screw cap microfuge tube, add:

| | Sample | Blank |
|---|---|---|
| supernatant (from step 1) | 0.50 mL | 0.50 mL |
| * sodium acetate buffer (2 M) | 0.10 mL | 0.10 mL |
| distilled water | - | 0.05 mL |
| suspension 1 (β-galactosidase) | 0.05 mL | - |
| Mix by vortex and incubate in a water bath set at 40°C for 1 hour. Then add the following with mixing by vortex after each addition: | | |
| * TEA buffer (1 M) / $MgSO_4$ (10 mM) | 0.50 mL | 0.50 mL |
| * sodium hydroxide (0.33 M) | 0.05 mL | 0.05 mL |
| * octanol (~ 99 % v/v) | 0.01 mL | 0.01 mL |
| suspension 2 (GOX/catalase mixture) | 0.05 mL | 0.05 mL |
| * hydrogen peroxide (30 % v/v) | 0.02 mL | 0.02 mL |
| Immediately cap the tubes and incubate in a water bath set at **40°C** for **15 min**. After 15 min, centrifuge at 13,000 rpm for 10 min. **Slowly release the cap and carefully pipette 1.0 mL of the clear supernatant for use as the "sample solution" in the "Enzymatic Determination Reaction" (see section B, page 8).** | | |

* These components should be prepared as described on pages 5 and 6.

**NOTE:**
1. Care must be taken when releasing the screw cap from the microfuge tube at the end of step 2. The cap should be released slowly to release pressure inside the tube generated by the glucose oxidase / catalase reaction.

2. For the preparation of milk powder or evaporated milk samples refer to "Modified Sample Extraction" on page 10.

## B. ENZYMATIC DETERMINATION REACTION:

**Wavelength:** 340 nm
**Cuvette:** 1 cm light path (glass or plastic; 1.5 mL semi-micro)
**Final volume:** 1.16 mL
**Sample solution:** 0.65-65 mg of lactulose per cuvette (in 0.1-1.0 mL sample volume)

**Read against air** (without cuvette in the light path) or against water

| Pipette into cuvettes | Sample | Blank |
|---|---|---|
| sample solution (see section A)<br>solution 3 (buffer)<br>solution 4 (NADP+/ATP) | 1.00 mL<br>0.05 mL<br>0.05 mL | 1.00 mL<br>0.05 mL<br>0.05 mL |
| Mix*, read absorbance of the solutions ($A_1$) after approx. 3 min and start the reactions by addition of: | | |
| suspension 5 (HK/G-6-PDH)<br>suspension 6 (6-PGDH) | 0.02 mL<br>0.02 mL | 0.02 mL<br>0.02 mL |
| Mix*, read absorbance of the solutions ($A_2$) at the end of the reaction (approx. 10 min). Then add: | | |
| suspension 7 (PGI) | 0.02 mL | 0.02 mL |
| Mix*, read absorbance of the solutions ($A_3$) at the end of the reaction (approx. 15 min). | | |

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

**NOTE:**
Where a sample has generated absorbance differences ($A_2$-$A_1$) > 0.300 or ($A_3$-$A_2$) > 1.500 refer to "Troubleshooting" on page 10.

## CALCULATION:

Determine the absorbance difference ($A_3$-$A_2$) for both blank and sample. Subtract the absorbance difference ($A_3$-$A_2$) of the blank from the absorbance difference ($A_3$-$A_2$) of the sample, thereby obtaining $\Delta A_{lactulose}$.

The concentration of lactulose can be calculated as follows:

$$c = \frac{V \times MW \times F}{\varepsilon \times d \times v \times 2} \times \Delta A_{lactulose} \quad [g/L]$$

**where:**

V = final volume [mL]
MW = molecular weight of lactulose [g/mol]
F = dilution factor
= 7.68 [standard sample preparation for "Liquid Milk"]
$\varepsilon$ = extinction coefficient of NADPH at 340 nm
= 6300 [1 x $mol^{-1}$ x $cm^{-1}$]
d = light path [cm]
v = sample volume [mL]
2 = 2 moles of NADPH produced for each mole of lactulose

***It follows for lactulose:***

$$c = \frac{1.16 \times 342.3 \times 7.68}{6300 \times 1 \times 1.0 \times 2} \times \Delta A_{lactulose} \quad [g/L]$$

$$= 0.2420 \times \Delta A_{lactulose} \quad [g/L]$$

If the sample has been diluted in addition to the dilution during the standard sample preparation for "Liquid Milk", the result must also be multiplied by the additional dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

***Content of lactulose:***

$$= \frac{c_{lactulose}\ [g/L\ sample\ solution] \times 100}{weight_{sample}\ [g/L\ sample\ solution]} \quad [g/100\ g]$$

**NOTE:** These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

## CONSIDERATIONS: (Rapid Method)

## A. MODIFIED SAMPLE PREPARATION:

### Milk Powder:

Accurately weigh 5 g of milk powder into a 100 mL beaker. Add 40 mL of distilled water and stir on a magnetic stirrer until fully dissolved. Transfer to a 50 mL volumetric flask, make to volume (50 mL) with distilled water and mix thoroughly. Continue from step 1 of Sample Preparation (Liquid Milk) (section A, page 7).

### Evaporated Milk:

Pipette 2 mL of evaporated milk into a 50 mL volumetric flask, make to volume (50 mL) with distilled water and mix thoroughly. Continue from step 1 of Sample Preparation (Liquid Milk) (section A, page 7). In this instance an additional dilution factor (F') of 25 should be used for the calculation of lactulose content (see Calculation, page 9).

***Hence, it follows for lactulose:***

$$= \quad 0.2420 \times \Delta A_{lactulose} \times 25 \qquad [g/L]$$

## B. TROUBLESHOOTING:

### Samples with ($A_2$-$A_1$) above 0.300 or ($A_3$-$A_2$) above 1.500:

Recommendations for re-processing samples that have generated absorbance differences ($A_2$-$A_1$) above 0.300 or ($A_3$-$A_2$) above 1.500 when processed using the Standard Assay Procedure (section A and B, pages 7 and 8) are given in the table below. The suggested recommendations are dependent upon the combination of the two absorbance values generated:

| ($A_2$-$A_1$) | ($A_3$-$A_2$) | Recommendation |
|---|---|---|
| > 0.300 | < 1.500 | Repeat the Sample Preparation (section A, page 7) and increase the second incubation of step 2 (glucose oxidase / catalase) to 30 min. |
| > 0.300 | < 1.500 | Appropriately dilute the original sample with distilled water and repeat the Sample Preparation (section A, page 7). |
| < 0.300 | > 1.500 | Appropriately dilute the prepared sample with distilled water prior to the Enzymatic Determination Reaction (section B, page 8) to obtain an absorbance difference (A3-A2) between ~ 0.100 to ~ 1.500. |

## ISO METHOD 11285:2004: REAGENT PREPARATION

This section describes the preparation of reagents (supplied and not supplied) for use in the measurement of lactulose using the procedure as described by ISO Method 11285:2004.

**NOTE:**

1. This kit does not supply any literature describing the procedure of ISO Method 11285:2004.
2. Once the supplied reagents have been prepared for use in the procedure described by ISO Method 11285:2004 these reagents cannot subsequently be used for the procedure of K-LACTUL (Rapid Method).
3. The procedure described by ISO Method 11285:2004 does not require the use of the supplied reagent bottles 3 and 6.
4. Further products are required for the preparation of the supplied reagents; these are listed under "Preparation of Reagents (Not Supplied)".
5. Since NADP and ATP are added to the assay as a 0.1 mL mixture, an additional 0.1 mL of distilled water should be added to account for this.

## REAGENTS (SUPPLIED):

**Bottle 1:** β-Galactosidase suspension (3 mL).
Stable for > 2 years at 4°C.

**Bottle 2:** Glucose oxidase and catalase, lyophilised powder.
Stable for > 2 years at -20°C.

**Bottle 3:** $NADP^+$ plus ATP.
Stable for > 2 years at 4°C.

**Bottle 4:** Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.2 mL.
Stable for > 2 years at 4°C.

**Bottle 5:** Phosphoglucose isomerase suspension (2.2 mL).
Stable for > 2 years at 4°C.

**Bottle 6:** Lactulose (0.1 mg/mL) plus D-fructose (0.05 mg/mL) standard solution (5 mL).
Stable for > 2 years at 4°C.

## PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

**1.** Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently store the bottle in an upright position. **Prior to use, swirl the bottle to mix contents** then remove

an aliquot of the enzyme suspension and dilute 26-fold in 3.2 M ammonium sulphate (e.g. add 0.1 mL enzyme suspension to 2.5 mL 3.2 M ammonium sulphate) to give a final concentration of **150 U/mL**. Stable for > 2 years at 4°C.

2. Add 2.77 mL catalase (Sigma cat no. 60630) and 0.33 mL of distilled water to bottle 2 and mix thoroughly. This will give the following final concentrations: **glucose oxidase (4,000 U/mL) and catalase (1.3 MU/mL).** Store at 4°C.

3. Dissolve the contents of bottle 4 in 24 mL of distilled water then add 750 mg adenosine 5'-triphosphate (e.g. Megazyme cat. no. R-ATP20) and 1.25 g sodium bicarbonate (e.g. Sigma cat. no. 71628) and dissolve. This will give the following final concentrations: **NADP (10 mg/mL), ATP (50 mg/mL) and sodium bicarbonate (50 mg/mL).** Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Once dissolved, the reagent is stable for > 2 years at -20°C.

4. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper then add 1.1 mL of 3.2 M ammonium sulphate to give the following final concentrations: **hexokinase (280 U/mL) and glucose 6-phosphate dehydrogenase (140 U/mL).** Subsequently store the bottle in an upright position. Swirl the bottle to mix contents before use. Stable for > 2 years at 4°C.

5. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper then add 0.94 mL of 3.2 M ammonium sulphate to give a final concentration of **700 U/mL.** Subsequently store the bottle in an upright position. **Swirl the bottle to mix contents before use.** Stable for > 2 years at 4°C.

## PREPARATION OF REAGENT SOLUTIONS (NOT SUPPLIED):

### 1. Catalase (Sigma cat. no. 60630) ~ 65,000 U/mg; ~ 20 mg/mL

Use to supplement bottle 2 of the supplied reagents.

### 2. Sodium bicarbonate (Sigma cat. no 71628)

Use to supplement bottle 4 of the supplied reagents.

### 3. Adenosine 5'-triphosphate (Megazyme cat. no. R-ATP20)

Use to supplement bottle 4 of the supplied reagents.

**4. Concentrated Carrez I solution: 200 mL**

Dissolve 30 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6.3H_2O$} (Sigma cat. no. P-9387) in 200 mL of distilled water. Store at room temperature.

**5. Concentrated Carrez II solution: 200 mL**

Dissolve 60 g of zinc sulphate {$ZnSO_4.7H_2O$} (Sigma cat. no. Z-4750) in 200 mL of distilled water. Store at room temperature.

**6. Sodium phosphate buffer (0.4 M, pH 7.6, 4 mM magnesium sulphate): 500 mL**

Add 24 g of $Na_2HPO_4$ (Sigma cat. no. 71640), 4.3 g of $NaH_2PO_4.H_2O$ (Sigma cat. no. 71506), and 0.5 g of $MgSO_4.7H_2O$ (Sigma cat. no. M-5921) to 450 mL of distilled water. Adjust the pH to 7.6 with 1 M NaOH (40 g/L) and make the volume up to 500 mL with distilled water. Store at room temperature.

**7. TEA buffer (0.4 M, pH 7.6 plus 4 mM magnesium sulphate): 400 mL**

Add 24 g of TEA buffer (Acros cat. no. 421630025) and 0.4 g of $MgSO_4.7H_2O$ (Sigma cat. no. M-5921) to 300 mL of distilled water. Adjust the pH to 7.6 and make volume up to 400 mL with distilled water.

**8. Octanol (~ 99 % v/v; Sigma cat. no. O-4500)**

**9. Sodium hydroxide (0.33 M): 200 mL**

Add 2.64 g of sodium hydroxide pellets (Merck cat. no. 1.06482.5000) to 180 mL of distilled water and dissolve. Make to volume (200 mL) with distilled water. Store at room temperature.

**10. Hydrogen peroxide (~ 30 % v/v; Sigma cat. no. H-1009)**

**11. TEA buffer (1 M, pH 7.6 plus 10 mM magnesium sulphate): 100 mL**

Add 14.9 g of TEA buffer (Acros cat. no. 421630025) and 0.25 g of $MgSO_4.7H_2O$ (Sigma cat. no. M-5921) to 80 mL of distilled water. Adjust the pH to 7.6 and make volume up to 100 mL with distilled water.

## REFERENCE:

1. Mendoza, M. H., Olano, A. & Villamiel, M. (2005). Chemical Indicators of Heat Treatment in Fortified and Special Milks. *J. Agric. Food Chem.*, **53 (8)**, 2995 -2999.

## NOTES:

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9. Stop clock.