	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	

Title: Measurement of Total Antioxidant Capacity in Milk			
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Measurement of Total Antioxidant Capacity in Milk

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to provide instructions for measurement of the total antioxidant capacity in milk using the Ferric Reducing Antioxidant Power (FRAP) Assay. We believe that the total antioxidant capacity of a milk sample may be related to the milk's vulnerability to formation of oxidized sensory defects.

1.2 Scope

This SOP applies to the Milk Quality Improvement Program laboratory.

1.3 Definitions

Total Antioxidant Capacity (TAC): A unit of measure of the nonenzymatic antioxidant activity level of a substance as determined by the FRAP assay.

Ferric Reducing Antioxidant Potential (FRAP) Assay: A colorimetric assay that measures the total combined activity of the electron donating reductants (redox active antioxidants) in a test sample. The assay uses the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) as the signal, or indicator, reaction, and this is tied to a color change in the indicator compound TPTZ. This color change is quantified by absorbance at 593 nm using a spectrophotometer. Absorbance changes are linear over a wide concentration range.

1.4 Safety

Glacial acetic acid and concentrated hydrochloric acid are extremely hazardous. Lab coats, safety glasses, and nitrile gloves must be worn while working with these acids, and they must only be handled inside the fume hood. The stock solutions prepared from these substances do not require any special precautions.

Normal precautions should be taken with the remaining reagents. Do not inhale or ingest them, and rinse skin thoroughly if contact is made.

SECTION 2 MATERIALS

Reagents

- Sodium acetate trihydrate (Fisher Catalog # S609-500)
- Glacial acetic acid (Fisher Catalog # BP2401-212)
- 2,4,6-tripyridyl-s-triazine (TPTZ) (Fisher Catalog # AC16807-0050)
- Concentrated HCl (Fisher Catalog # A481-212)
- $FeCl_3$ * $6H_2O$ (Fisher Catalog # I88-100)
- $FeSO_4$ * $7H_2O$ (Fisher Catalog # I146-500)

Supplies

- Beakers, 250 and 500 mL
- Graduated cylinder, 100 mL
- Volumetric flask, 1,000 mL
- Volumetric flask, 100 or 200 mL
- Serological pipettes, 25 mL, 10 mL, 5 mL, and 2 mL
- Pipette tips, 1,000 µL
- Magnetic stir bars
- Parafilm
- 1 L glass bottles
- 250 mL glass bottle
- 4 oz dairy vial
- Disposable syringe, 5 ml
- Disposable syringe filters, 0.45 µM
- 18 x 150 mm disposable glass culture tubes
- 16 x 125 mm disposable glass culture tubes
- 16 x 125 mm screw-cap tubes
- Transfer pipettes
- Disposable spectrophotometer cuvettes

Equipment

- Analytical balance
- Pipette controller
- Micropipette, 100-1,000 µL
- Magnetic stir plate
- Water bath (37°C)
- Timer
- Test tube racks
- Vortex mixer
- Spectrophotometer (593 nm)

SECTION 3 PROCEDURES

3.1. Prepare Stock Solutions

1. **Acetate Buffer (300mM, pH 3.6)**
    a. Add a magnetic stir bar to a 500 mL beaker. Add approximately 250 mL of Milli-Q water.
    b. Weigh 3.1 g of sodium acetate trihydrate and add to the beaker. Place the beaker on a magnetic stir plate inside the fume hood and turn on the stirring.
    c. Working inside the fume hood, use a 25 mL serological pipette to measure and add 16 mL of glacial acetic acid to the beaker. After it has stirred for a few seconds, turn off the stir plate.
    d. Pick up the beaker and hold a second stir bar to the bottom of the beaker to secure the stir bar inside of the beaker. Carefully pour the entire contents of the beaker into the 1 L volumetric flask. Do not spill anything.
    e. Add additional Milli-Q water to the beaker, rinsing the stir bar and down the sides of the beaker. Pour this water into the volumetric flask. Repeat rinsing 2 more times. Do not add water beyond the line etched on the neck of the volumetric flask.
    f. Add additional Milli-Q water to the volumetric flask until the bottom of the meniscus reaches the line etched on the neck of the volumetric flask. Use a transfer pipette to slowly and carefully add the final volume of water to the flask to avoid overfilling.
    g. Seal the mouth of the volumetric flask with Parafilm. Invert the flask several times to ensure the contents are uniformly mixed.
    h. Remove the Parafilm and pour the contents of the flask into a labelled 1 L glass bottle. Store in the dark at room temperature.
2. **10mM TPTZ (2,4,6-tripyridyl-s-triazine - M.W. 312.34) in 40mM HCl (M.W. 36.46)**
    a. Add a magnetic stir bar to a 250 mL beaker. Add approximately 100 mL of Milli-Q water.

b. Weigh 0.625 g of TPTZ and add to the beaker. Place the beaker on a magnetic stir plate inside the fume hood and turn on the stirring. It is normal for the TPTZ to not dissolve at this point.
c. Working inside the fume hood, use a micropipette to measure and add 670 µL of concentrated HCl to the beaker. Allow it to stir until all of the TPTZ has dissolved, and then turn off the stir plate.
d. Pick up the beaker and hold a second stir bar to the bottom of the beaker to secure the stir bar inside of the beaker. Carefully pour the entire contents of the beaker into the 200 mL volumetric flask. Do not spill anything.
e. Add additional Milli-Q water to the beaker, rinsing the stir bar and down the sides of the beaker. Pour this water into the volumetric flask. Repeat rinsing 2 more times. Do not add water beyond the line etched on the neck of the volumetric flask.
f. Add additional Milli-Q water to the volumetric flask until the bottom of the meniscus reaches the line etched on the neck of the volumetric flask. Use a transfer pipette to slowly and carefully add the final volume of water to the flask to avoid overfilling.
g. Seal the mouth of the volumetric flask with Parafilm. Invert the flask several times to ensure the contents are uniformly mixed.
h. Remove the Parafilm and pour the contents of the flask into a labelled 250 mL glass bottle. Store at 4°C.

3. **20mM $FeCl_3$ * $6H_2O$ (M.W. 270.30)**
   a. Add a magnetic stir bar to a 250 mL beaker. Add approximately 100 mL of Milli-Q water.
   b. Weigh 1.081 g of $FeCl_3$ * $6H_2O$ and add to the beaker. Place the beaker on a magnetic stir plate and turn on the stirring. Allow to stir until all of the $FeCl_3$ has dissolved.

c. Pick up the beaker and hold a second stir bar to the bottom of the beaker to secure the stir bar inside of the beaker. Carefully pour the entire contents of the beaker into the 200 mL volumetric flask. Do not spill anything.
d. Add additional Milli-Q water to the beaker, rinsing the stir bar and down the sides of the beaker. Pour this water into the volumetric flask. Repeat rinsing 2 more times. Do not add water beyond the line etched on the neck of the volumetric flask.
e. Add additional Milli-Q water to the volumetric flask until the bottom of the meniscus reaches the line etched on the neck of the volumetric flask. Use a transfer pipette to slowly and carefully add the final volume of water to the flask to avoid overfilling.
f. Seal the mouth of the volumetric flask with Parafilm. Invert the flask several times to ensure the contents are uniformly mixed.
g. Remove the Parafilm and pour the contents of the flask into a labelled 4 oz dairy vial. Seal with Parafilm and store at 4°C.

Note: 100 mL volumes of TPTZ and $FeCl_3$ solutions can be prepared using the amounts below and a 100 mL volumetric flask.

- **TPTZ**: 0.312 g TPTZ and 335 µL of HCl
- **$FeCl_3$**: 0.541 g $FeCl_3$ * $6H_2O$

Stock solution storage:

- Acetate buffer can be stored at room temperature in the dark for up to 1 month. It should appear clear and colorless.
- TPTZ solution can be stored at 4°C for 2 weeks. It should appear clear and colorless.
- $FeCl_3$ solution can be stored at 4°C for 2 weeks provided no precipitate or obvious darkening is observed.

Stock solution disposal: Unused stock solutions can be flushed down the drain with large quantities of water.

3.2. Prepare Standard Solution

1. **1,000 µM $FeSO_4$ * $7H_2O$**

    a. Add a magnetic stir bar to a 500 mL beaker. Add approximately 250 mL of Milli-Q water.

    b. Weigh 0.278 g of $FeSO_4$ * $7H_2O$ and add to the beaker. Place the beaker on a magnetic stir plate and turn on the stirring. Allow to stir until all of the $FeSO_4$ has dissolved.

    c. Pick up the beaker and hold a second stir bar to the bottom of the beaker to secure the stir bar inside of the beaker. Carefully pour the entire contents of the beaker into the 1 L volumetric flask. Do not spill anything.

    d. Add additional Milli-Q water to the beaker, rinsing the stir bar and down the sides of the beaker. Pour this water into the volumetric flask. Repeat rinsing 2 more times. Do not add water beyond the line etched on the neck of the volumetric flask.

    e. Add additional Milli-Q water to the volumetric flask until the bottom of the meniscus reaches the line etched on the neck of the volumetric flask. Use a transfer pipette to slowly and carefully add the final volume of water to the flask to avoid overfilling.

    f. Seal the mouth of the volumetric flask with Parafilm. Invert the flask several times to ensure the contents are uniformly mixed.

    g. Remove the Parafilm and pour the contents of the flask into a labelled 1 L bottle. Store at 4°C.

    This standard solution should be prepared as close to the analysis as possible. While it can be stored refrigerated for a few days, in less than a week it forms an iron oxide precipitate that renders it unusable.

Unused $FeSO_4$ standard solution can be flushed down the drain with large quantities of water.

3.3. Prepare FRAP working solution

The FRAP working solution is prepared by mixing the acetate buffer, TPTZ, and $FeCl_3$ stock solutions in a ratio of 10:1:1. An example is given below for preparing 120 mL of working reagent.

1. Using a graduated cylinder, measure 100 mL of acetate buffer and add to a 250 mL bottle.
2. Using a serological pipette, measure 10 mL of TPTZ solution and add to the bottle. The TPTZ solution adheres to the inside of the pipette, so it should be dispensed slowly to avoid leaving droplets on the pipette walls that would affect the volume delivered.
3. Using a serological pipette, measure 10 mL of $FeCl_3$ solution and add to the bottle.
4. Close the bottle and mix by gently swirling or shaking.

The FRAP working solution should be prepared in the volume needed just before use, and it should be used within 3 hours of preparation.

Unused FRAP working solution can be flushed down the drain with large quantities of water.

3.4. Prepare Standards and Generate Standard Curve

A standard curve should be generated each time a batch of FRAP working solution is mixed. The standard curve should be generated before analyzing test samples to ensure that the assay is functioning correctly.

1. Label 7 18 x 150 mm glass culture tubes with the numbers 1 through 7.

2. Using 2 and 5 mL serological pipettes, add the volumes of $FeSO_4$ standard solution from the table below to the tubes. Pipette carefully and hold the pipette at eye level to ensure accurate measurement.
3. Using a 10 mL serological pipette, add the volumes of Milli-Q water from the table below to the tubes. Pipette carefully and hold the pipette at eye level to ensure accurate measurement.
4. Cap the tubes and vortex to mix.

Standard No.	**$FeSO_4$ * $7H_2O$ Concentration (µM)**	**$FeSO_4$ Standard Solution Volume (mL)**	**Milli-Q Water Volume (mL)**
1	50	0.5	9.5
2	100	1.0	9.0
3	200	2.0	8.0
4	300	3.0	7.0
5	400	4.0	6.0
6	500	5.0	5.0
7	600	6.0	4.0

5. Label 7 16 x 125 mm glass culture tubes with the numbers 1 through 7.
6. Using a micropipette, pipette 300 µL of each standard solution from the 18 x 150 mm tube into the corresponding 16 x 125 mm tube.
7. Using a 5 mL serological pipette, add 4.5 mL of FRAP working solution to each 16 x 125 mm tube. The FRAP working solution adheres to the interior walls of the pipette, so dispense slowly to avoid leaving droplets on the pipette walls that would affect the volume delivered.
8. Cap the tubes and vortex to mix.
9. Place the tubes in a 37°C water bath and remove after exactly 4 minutes.

10. While the tubes are incubating, turn on the spectrophotometer and set the wavelength to 593 nm. Use a transfer pipette to fill a cuvette with FRAP working solution, and use this to zero the spectrophotometer.
11. The standards should be measured as soon as they are removed from the water bath. To measure each standard, briefly vortex the tube to mix and then use a transfer pipette to fill a cuvette with the tube contents. Place the cuvette in the spectrophotometer, close the lid, and record the measurement in the appropriate cell of the standard curve spreadsheet.
12. The R^2 value for the standard curve should be > 0.99, and higher is better. If the R^2 value is lower than 0.99, then new standards should be prepared and the entire process repeated.

3.5. Test Milk Samples

1. Label 2 16 x 125 mm screw-cap tubes for each test sample.
2. Gently invert the milk sample 25 times to mix.
3. Avoiding foam, pipette 300 µL of the milk sample into each of the 2 tubes.
4. Using a 5 mL serological pipette, add 4.5 mL of FRAP working solution to each 16 x 125 mm tube. The FRAP working solution adheres to the interior walls of the pipette, so dispense slowly to avoid leaving droplets on the pipette walls that would affect the volume delivered.
5. Cap the tubes and vortex to mix.
6. Place the tubes in a 37°C water bath and remove after exactly 4 minutes.
7. For each tube, briefly vortex to mix, uncap, and insert the end of a 5 mL syringe into the tube, pressing the bottom of the syringe barrel against the lip of the tube to form a seal.
8. Maintaining this configuration, quickly invert the tube and pull up on the syringe plunger to aspirate approximately 4 mL of the solution into the syringe.
9. Attach a 0.45 µM syringe filter to the end of the syringe. Depress the syringe plunger, collecting the filtrate in a spectrophotometer cuvette.
10. Immediately measure the absorbance of the filtered sample, entering the result in the TAC calculation spreadsheet.

SECTION 4 TROUBLESHOOTING

Problems with the standard curve or problems with high differences between sample replicates are likely caused by errors made when measuring volumes of standards, reagents, or samples. When using serological pipettes, be sure to hold the pipette at eye level while drawing up the sample to obtain the most accurate volume possible. When dispensing the TPTZ stock solution and the FRAP working solution from the serological pipette, be sure to do so slowly to avoid leaving droplets on the inside of the pipette.

Issues could also be caused by reagents that are too old or that were made improperly. The SOP specifies the acceptable storage time and expected appearance of the reagents. If something appears to be wrong with one of the reagents, it should be discarded and remade.

SECTION 5 REFERENCES

Dr. Jayendra K. Amamcharla, personal communication.

Benzie, I. F. F. and M. Devaki. 2018. The ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: concepts, procedures, limitations and applications. Pages 77-106 in Measurement of Antioxidant Activity & Capacity: Recent Trends and Applications. R. Apak, E. Capanoglu, and F. Shahidi, ed. John Wiley & Sons. https://doi.org/10.1002/9781119135388.ch5.

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1		Sam Reichler	Original SOP