U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 27: Screening Method for Phosphatase in Cheese

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Introduction

Over the past ten years outbreaks of foodborne diseases have been caused by the consumption of dairy products made with raw or improperly pasteurized milks. Milk is pasteurized by heating at 62.8°C for 30 minutes or 71.7°C for 15 seconds. These temperatures kill all nonsporeforming pathogens and inactivate the native alkaline phosphatase (ALP) enzyme found in milk. The thermal resistance of ALP [EC 3.1.3.1] ortho-phosphoric monoester phosphohydrolase is greater than that of nonsporeforming pathogenic microorganisms; therefore, liquid milk and milk products that show a negative result when tested for phosphatases are considered properly pasteurized and safe (4). Soft cheeses produced by fermentation (e.g., blue, Swiss, Camembert) show a positive result when analyzed for ALP, because ALP is produced by the microorganisms used during fermentation of the cheeses (2,3).

The method given below is an expansion of phosphatase (residual) in milk to cheese (1). The buffer has been changed because it was found that carbonate ions are inhibitory to bovine ALPs (5). Unlike the previous AOAC (16th ed.) method (sec. 946.03), the analysis is done in a single test tube and the reagents are the same for all of the cheeses tested.

This method is a screening method. A sample which exceeds the levels in Table 1 must be reanalyzed using AOAC method 946.03 (16th ed.) which is equivalent to AOAC method 16.275-16.277 (13th ed.), cited in 21 CFR 133.5.

A. Equipment and materials

1. Pipets or digital pipetter, able to dispense volumes ranging from 100 µl to 1 ml (Eppendorf or equivalent)
2. Pipetter tips
3. Centrifuge capable of holding 10-15 ml tubes and spinning them at least at RCF 2400 × *g*
4. Ice bath
5. Test tubes, 10-15 ml
6. Two water baths. The first is a boiling water bath used for pasteurization of controls; the second is maintained at 40°C and is used to incubate the test mixture.
7. Spectrophotometer capable of measuring at 650 nm.

B. Reagents

1. **AMP buffer.** Dissolve 10.0 g 2-amino-2-methyl-1-propanol in water. Adjust pH to 10.1 with 6 M HCl, add 10 ml Tergitol type 4, and dilute to 1 L with distilled water.

2. **Buffer substrate.** Dissolve 0.5 g phenol-free crystalline disodium phenyl phosphate in AMP buffer solution and dilute to 500 ml with AMP buffer. Prepare fresh daily.

3. **n-Butyl alcohol**, n-BuOH, b.p. 116-118°C

4. **Catalyst.** Dissolve 200 mg $CuSO_4 \cdot 5H_2O$ in distilled water and dilute to 100 ml.

5. **CQC solution.** Dissolve 40 mg crystalline 2,6-dichloro-quinonechloroimide in 10 ml MeOH and transfer to dark bottle. Or prepare solution by dissolving 1 Indo-Phax tablet (containing catalyst) (available from Applied Research Institute, 141 Lewis St., Perth Amboy, NJ 08861) in 5 mL MeOH. Store in refrigerator. Discard after 1 week or when brown.

6. **CQC-catalyst solution.** Mix equal volumes of CQC solution and catalyst together. **Prepare fresh daily.**

7. **6 M HCL solution.** To 50 ml water add 50 ml concentrated hydrochloric acid. **ADD ACID TO WATER.**

8. **Phenol standard solutions.**

    a. **Stock solution**--Accurately weigh 1.000 g pure phenol, transfer to 1 L volumetric flask, dilute to volume with 0.1 N HCl, and mix (1 ml = 1 mg phenol). Solution is stable several months in the refrigerator.

    b. **Working solution**--Dilute 100 µl solution (a) to 100 ml with AMP buffer, and mix (1 ml = 1 µg phenol). **Prepare fresh daily.**

    c. **Color standard solutions**--Dilute 0.0, 0.25, 0.5, 1.0, 2.5, and 5.0 ml of working solution (b) to 5.0 ml with AMP buffer in a series of test tubes. Add 0.5 ml water to each tube.

9. **Tergitol type 4 (also called Niaproof type 4).** This is an anionic surfactant, 7-ethyl-2 methyl-4 undecanol hydrogen sulfate, sodium salt (Sigma No. 4). **No substitutions**.

10. **Water**. Distilled or deionized.

C. Sampling of cheeses

Hard cheese. Take sample from interior with **clean** spatula or knife.

Soft and semisoft ripened cheese. Harden cheese by chilling in freezer. Avoid contaminating sample with microbial phosphatases from contamination that may be present on the surface. Sample by either of the following methods:

- Cut portion from end of loaf or side of cheese, extending in 5 cm (2 inches) if possible, to a point somewhat beyond center in case of small cheese. Cut slit 6-12 mm (1/4-1/2 inch) deep at least half way around portion and midway between top and bottom. Break portion into 2 parts, pulling apart so that break occurs on line with slit and taking care not to contaminate freshly exposed broken surface. Remove sample from freshly exposed surface at or near center of cheese.

- Remove surface of area to be sampled (e.g., end and adjacent sides) with clean knife or spatula to depth of 6 mm (1/4 inch). Clean instruments and hands with hot water and phenol-free soap, and wipe dry. Remove freshly exposed surface from same or greater depth, and repeat cleaning. Take sample from center of freshly exposed area, preferably at or near center of cheese if cheese is small.

Processed cheese and cheese spreads. Take sample from beneath surface with clean knife or spatula.

D. Screening test for phosphatase (residual) in cheese

Collect and weigh two 0.5 g samples into test tubes using the directions in section C. Add 0.5 ml water (one tube is the test portion; the second tube is for the boiled control or blank). Mash with a glass rod. Heat controls in a boiling water bath for 2 min, and rapidly cool in ice bath.

To test portion and boiled controls add 5 ml buffer substrate and mix by vortex or inversion. Immediately incubate test portions, boiled controls, and color standard solutions in water bath 15 min at 40 ± 1°C (allow 1 min warm-up time for total of 16 min). Mix samples once during incubation.

Remove from water bath and add 0.2 ml of CQC catalyst solution or 0.1 ml of Indo-Phax solution. Mix, and immediately place back in 40°C water bath for 5 min. Remove from bath and cool in ice-water bath 5 min.

Add 3 ml butanol and extract by inverting the parafilm covered tubes 6 full turns. Chill in ice water bath 5 min. Centrifuge 5 min at 2400 × *g*.

Remove butanol (upper) layer into cuvettes with Pasteur pipet. Read absorbance of butanol extracts with spectrophotometer at 650 nm. Standard curve of µg phenol against absorbance should be a straight line. Subtract the µg phenol/g cheese of the boiled blank from the

corresponding sample to get the µg phenol/g cheese for the sample. If less than zero, record as zero.

E. Interpretation of results

Table 1 lists different types of cheese, maximum amounts of phenol equivalents/g of cheese, and appropriate CFR reference. Any sample exceeding the levels listed in Table 1 is violative. If a particular cheese is not listed in Table 1, then it shall be considered violative if it exceeds 12 µg phenol equivalents/g cheese.

Table 1. List of cheeses[(a)] from CFR 21

Cheese	µg phenol/g cheese	CFR Reference
Brick	20	133.108(a)(2)
Cheddar	12	133.113(a)(2)
Colby	12	133.118(c)(2)
Cook cheese, Koch Kaese	12	133.127(a)(2)
Washed curd and soaked curd	12	133.136(a)(2)
Edam	12	133.138(a)(2)
Gouda	12	133.144(a)(2)
Gruyere	12	133.149(a)(2)
Hard	12	133.150(c)(2)
Limburger	16	133.152(a)(2)
Monterey & Monterey Jack	12	133.153(a)(2)
Mozzarella & Scamorza	12	133.155(a)(2)
Low moisture Mozzarella & Scamorza	12	133.156(a)(2)
Muenster	12	133.16 (a)(2)
Pasteurized process	12	133.169(a)(2)
Process cheese food	12	133.173(a)(2)

Cheese	µg phenol/g cheese	CFR Reference
Pasteurized Neufchatel, cheese spread with other foods	12	133.178(a)(2)
Provolone	12	133.181(a)(2)
Samsoe	12	133.185(a)(2)
Semisoft	20	133.187(c)(2)
Semisoft part-skim	20	133.188(c)(2)
Spiced	12	133.190(a)(2)
Swiss & Emmentaler	12	133.193(a)(2)

[a] NOTE: Different cheeses have different levels. The value per gram of cheese is obtained by multiplying the value listed in the CFR by 4.

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