EVOLUTIONARY CHANGES IN THE CORNELL PHOSPHATASE TEST

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SUMMARY

The Cornell phosphatase test, with its single buffer for milk and all dairy products, has been adopted successfully and widely in the past 15 years. Recent changes in national and state regulations raising temperatures to high levels for acceptable pasteurization, and direct experience gained on the behavior of chemical reagents in phosphatase testing have suggested evolutionary revisions in the Cornell test, leading to greater simplicity without upsetting its demonstrated past high sensitivity and reproducibility. Changes include establishing one hour as the incubation time for the standard test, simplifying color standard preparation, substituting CQC for BQC, reducing time of color development to 5 min, emphasizing butyl alcohol extraction and selecting a lower level phenol value for defining underpasteurization.

The revised Cornell standard 1-hr phosphatase test detects 0.1% raw milk addition to pasteurized milk and a drop of between one and two degrees in HTST milk pasteurization processes. It does the above with less reagents and in a shorter time than formerly.

The Cornell phosphatase test *(3, 4)*, now 15 years old, illustrates uniquely a universal method for determining the extent of underpasteurization of milk and milk products. It applies equally well to all dairy products, whether milk or aged cheese, for only one concentration of buffer and precipitant is required. In addition, prior knowledge of the history of the samples is unnecessary.

Extensive past collaborative testing between 22 laboratories indicated the Cornell test possesses the same degree of accuracy, sensitivity, and reproducibility on milk and ripened cheese as possessed by the multibuffered AOAC standard method *(1, 4)*.

The Cornell phosphatase test has never been modified previously in any detail by the author. However, more recent studies in his laboratory on the high degree of CQC stability and on blue color development attained with sugar-containing dairy products *(2, 5)*, along with greater knowledge of phosphatase reactivation mechanisms and detection *(6, 7)*, have suggested some design changes in the Cornell method.

The changes include: *(a)* a shift from a 24-hour to a 1-hour incubation; *(b)* a simplification of color standard preparation; *(c)* the substitution of CQC for BQC and the elimination of one reagent; *(d)* the development of color at 37 C for 5 min rather than 15 min; *(e)* a preference for butyl alcohol extraction readings rather than aqueous; *(f)* an adjustment downward of the critical phenol value defining underpasteurized milk and milk products.

THE REVISED CORNELL PHOSPHATASE TEST (STANDARD 1-HR)

Principle

Milk, cheese, and other dairy products are individually incubated in a single, high strength carbonate-bicarbonate buffer substrate at about pH 9.5 for a specific period, then the mixture is acid precipitated. The resulting clear filtrate is alkalized and color developed.

Alkaline phosphatase concentration is indicated by the intensity of blue color arising from the hydrolysis of the substrate, disodium phenyl phosphate, and the reaction of the free phenol portion with the stable dye, CQC. The enzyme is inactivated at proper minimum pasteurization temperatures and thus the amount of color that is present directly relates to the degree of underpasteurization.

Materials and Reagents

Cornell standard phosphatase test materials and reagents, except for the organic phenol standards, outlined in following sections, may be obtained through the Whitman Laboratories, Inc., Norwich, New York.

Materials-Apparatus. These are as follows:

Test tubes — Pyrex, 16 x 150 mm.

Pipettes — delivery 1 ml, graduated in 0.1 ml, and 5 ml (or 10 ml), graduated in 0.1 and 1.0 ml.

Weighing balance — Torsion.

Water bath — temperature thermostatically controlled at 37 ± 1 C.

Thermometer — 0-110 C, certified against NBS.

Funnels — size suitable for 11-cm filter paper.

Filter paper — Whatman No. 42 — 11 cm.

Medicine dropper and amber bottle — 25-50 ml capacity — dropper to deliver approximately 43 drops/ml.

Color standards — Organic phenol for determining units phenol produced. Prepared inorganic standards may be used alternatively.

Comparative light plate — To effect better visual color resolution (optional equipment).

Colorimeter (optional equipment) — Bausch and Lomb Spectronic 20, or equivalent. B & L ½ in. test tubes suitable for sample containers.

Specific Reagents (All of Analytical Grade)

1. Carbonate buffer substrate — Dissolve 11.5 g anhydrous sodium carbonate, Na_2CO_3, 10.2 g anhydrous sodium bicarbonate, $NaHCO_3$, and 1.1 g pure disodium phenylphosphate in distilled water and make up to 1 liter.

2. Trichloroacetic-hydrochloric acid precipitant — Dissolve 25 g trichloroacetic acid crystals in 50 ml water, add 50 ml concentrated HCl (approximately 36 per cent), and mix well but carefully.

3. Sodium carbonate solution (8 per cent) — Dissolve 80 g anhydrous sodium carbonate, Na_2CO_3, in distilled water and make up to 1 liter.

4. Copper sulfate-Calgon solution — Dissolve 0.5g copper sulfate ($CuSO_45H_2O$) and 50 g sodium hexametaphosphate crystals, or Medi-Calgon, in distilled water and make up to 1 liter. Calgon solution sequesters calcium which presents turbidity.

5. CQC solution — Dissolve 0.2 g dichloroquinonechloromide, crystalline, in 25 ml absolute ethyl, or absolute methyl, alcohol.

6. n-Butyl alcohol — B.P. 117.5 C. It is not necessary to neutralize the n-butyl

7. Preparation of Phenol Color Standards

a. Stock Phenol Solution
Dissolve 1 g phenol crystals in water and make up to 1 liter. Preferably made up fresh or store at 5 C.

b. Buffer Solution
Dissolve 11.5 g anhydrous sodium carbonate, Na_2CO_3, and 10.2 g anhydrous sodium bicarbonate, $NaHCO_3$, and 0.1 g copper sulfate, $CuSO_4 \cdot 5H_2O$, in distilled water and make up to 1 liter.

c. Diluted Phenol Solution
To 2 ml of stock phenol solution, 7a, add enough buffer solution, 7b, to make up to 500 ml. This solution contains 4 μg phenol per ml.

d. Proportioning Solutions for Standards and Final Development
Pipette into test tubes (16 x 150 mm) portions in the ratios listed in first two colums, Table 1. With one exception, later adjusted, all mixtures are 10 ml volumes before color development.

Add to each of above 10 ml volumes in table 1 exactly 2 drops CQC with quick stirring and make 2 full inversions of test tube. Incubate at 37 C for 5 min. Add 5 ml n-butyl alcohol to each test tube after color development. Invert test tube 5 times to extract color. No Calgon is required in preparing standards. Seal tops with proper stoppers, wax, or with parchment paper and store in refrigerator.

A standard curve may be produced using a colorimeter or spectrophotometer at 650 mμ. Remove approximately 3 ml alcohol from extracted standards and pipette into small size cuvettes or ½ in. dia. test tube. Read directly against alcohol extract from 0.0 μg phenol solution with transmission set at 100%. Plot, on regular coordinate paper, L = 2 — log G, where G = % transmission, against phenol concentrates. Standard curve will result.

8. Controls and Reagent Blanks

Use reageant blanks and negative controls. To obtain a negative control, heat 1.0 ml milk or 0.5 g cheese, or other product under test, in a test tube to 85 C for at least 1 min. Cool. Then conduct procedure as prescribed. Subtract value of control and/or reagent blank from actual test value.

TABLE 1. PROPORTIONING OF SOLUTIONS

Buffer solution (7b) (ml)	plus	Diluted phenol solution (7c) (ml)	After color development 10 ml portions of mixtures give respective phenol concentration (μg/test tube)
10.0		0	0
15.6		0.4	1
(Remove 6 ml mixture and discard. Develop color on remaining 10 ml)			
9.5		0.5	2
9		1	4
8		2	8
7		3	12
6		4	16
5		5	20
0		10	40

PROCEDURE FOR THE CORNELL PHOSPHATASE TEST

Sampling.

For milk, cream, chocolate milk, buttermilk, whey, ice cream mix, and condensed milk. Pipette 1 ml of milk or fluid product into test tube (16 x 150 mm), add 10 ml of warm 37 C carbonate buffer substrate and mix.

For ripened cheese, cottage and cream cheese, butter, and milk powder. Transfer 0.5 g cheese, or solid product, to a test tube (16 x 150 mm). Mascerate the solid thoroughly with a glass rod. Add 1 ml warm (37 C) carbonate buffer substrate and stir the solid into a paste. Then add an additional 9 ml carbonate buffer substrate and stir.

Incubation.

Temper test tube and its contents for 5 min and then incubate at 37 C for 1 hr in same water bath.

Precipitation.

Following incubation remove test tube and carefully add down its side 1 ml trichloroacetic-hydrochloric precipitant. Filter after a few seconds into a clean test tube (16 x 150 mm), preferably of a type calibrated and marked at 5 ml.

Color Development and Measurement.

To 5 ml clear filtrate add 1 ml copper sulfate-Calgon solution. Then pipette into tube 5 ml of 8 per cent sodium carbonate solution and mix. Add exactly 2 drops CQC solution and without delay invert tube twice. Permit color to develop at 37 C for exactly 5 min. Add 5 ml n-butyl alcohol to each tube after color development. Invert tube 5 times to extract color. Read against alcohol standards.

Optionally, in place of visual reading, remove with clean pipette approximately 3 ml alcohol solution from each test tube after extraction step into a small cuvette, or ½ in. dia. test tube. Measure directly light transmission in colorimeter or spectrophotometer at 650 mμ against an alcohol extract from a suitable negative control set at 100% transmission. Determine phenol concentration by reference to standard curve prepared under section 7, Reagents.

Interpretation.

Express values directly without factor multiplication of any type. A value over 1.0 μg phenol per 0.5 ml milk and other fluid milk product, or per 0.25 g cheese or other solid milk product indicates underpasteurization and/or recontamination with raw milk. Use of filtrates at half volume in actual test determined 0.5 ml and 0.25 g units of expression.

RESULTS AND DISCUSSION

Experiments designed to check the performance of the revised Cornell standard phosphatase test were carried out on milks and cheeses of known history.

Fresh raw whole milk from the mixed University herd supply was carefully heat treated in a Cherry-Burrell HTST Unit (5000 lb capacity/hr) at the following temperatures: 161 F, 160 F, 159 F, and 158 F, each for 16.5 sec. Portions of these milks then were removed to small cheese vats and made into Cheddar cheese following standard procedures. For additional comparisons, to other lots of milk properly pasteurized at 161 F – 16.5 sec were apportioned 0.1, 0.2, and 0.5% fresh raw milk. Cheddar cheese also was made from these treated lots. All cheese were ripened four weeks at 50 F prior to testing. Results are presented in Table 2.

It is readily apparent that sensitivity of underpasteurization detection and raw milk contamination is high with the revised Cornell standard (1 hr) phosphatase test (Table 2). A drop of between 1 and 2 C in the HTST pasteurizer or an addition of only 0.1% raw milk is detected in milk or in ripened Cheddar cheese made from such milk. Milk and cheese properly pasteurized essentially produced no color after one hr incubation following subtraction of a negative control value, indicating that the new critical dividing value of 1.0 μg per 0.5 ml milk or 0.25 g cheese is sufficiently high to assure the proper classification of legally minimum pasteurized milk or cheese. These low phenol values correspond to those obtained from earlier comparative laboratory studies on the Cornell phosphatase (1 hr) test *(1, 4)*. Among 22 cooperating laboratories the vast majority reported less than 1.0 μg on properly pasteurized milks or Cheddar cheese made from properly pasteurized milk. The early data and the results from Table 2 show that with properly pasteurized products most phenol values obtained fall between 0.0 and 0.5 μg per 0.5 ml milk or 0.25 g cheese.

TABLE 2. PERFORMANCE OF THE REVISED CORNELL STANDARD PHOSPHATASE (1 HOUR) TEST AGAINST MILK AND CHEDDAR CHEESES OF KNOWN HISTORY

History of milks	Phenol concentration in treated milk	Phenol concentration in Cheddar cheese made from treated milks of Col. 1
	μg/0.5 ml	μg/0.25
161 F-16.5 sec	0.0	0.0
160 F-16.5 sec	0.0	0.0
159 F-16.5 sec	0.2	0.2
158 F-16.5 sec	5.0	3.0
Past. milk–161 F-16.5 sec	0.0	0.0
Past. milk + 0.1% raw	1.3	1.3
Past. milk + 0.2% raw	2.0	3.0
Past. milk + 0.5% raw	3.0	5.0

A value for the revised Cornell standard test, read directly, of over 1.0 μg per the 0.5 ml milk or 0.25 g cheese indicates underpasteurization or raw milk contamination. Values above obtained by visual butyl alcohol comparisons.

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