Spectrophotometric Determination of Hydrogen Peroxide in Milk [1]

V. M. AMIN and N. F. OLSON
Department of Food Science and Industries, University of Wisconsin, Madison

Abstract

A method was developed using titanium salts for quantitative determination of hydrogen peroxide in milk. Samples of sterilized reconstituted (11%) nonfat dry milk, sterilized homogenized whole milk, and raw clarified whole milk were prepared for analysis by precipitating the proteins with 70% (w/v) trichloroacetic acid. The yellow-colored complex of hydrogen peroxide and titanium salts was measured at the absorption maxima of 400 and 415 mμ for titanium sulfate and titanium tetrachloride, respectively, and levels of hydrogen peroxide determined from standard curves. Analyses of known quantities of hydrogen peroxide in milk indicated that both reagents reacted stoichiometrically to give accurate determinations of the concentrations of hydrogen peroxide. The titanium tetrachloride method was more accurate, with coefficients of variability ranging between 0.79 to 3.83% for replicate determinations of several different samples of each of the three types of milk and between 0.49 to 1.71% for replicate determinations of single samples of each of the three types of milk.

Qualitative tests using reagents such as benzidine (3), iodine (12, 13), perchromic acid (15), and vanadium pentoxide (11) are available for detecting the presence of hydrogen peroxide (H_2O_2) in milk. Ceric sulfate (8), iodine (12), and potassium permanganate (7, 13) have been used for quantitative determination of H_2O_2 in inorganic and simple organic systems. However, these reagents have limited application for quantitative determination of H_2O_2 in milk. Ceric sulfate (9) and potassium permanganate (10, 13) may combine with milk proteins. Use of iodine is limited to solutions which do not contain compounds that oxidize the iodide ion (12, 13). Vanadium pentoxide has been used in a method for spectrophotometric determination of the amount of H_2O_2 in milk, but the method apparently lacks sensitivity (11).

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Various workers have used titanium to determine the concentration of H_2O_2 in simple and complex systems. Richardson (14) in 1893 used titanic acid to estimate the levels of H_2O_2 in urine by the Nessler tube method. Allsopp (1) determined H_2O_2 concentrations photoelectrically at 470 mμ with titanium sulfate. Berry and coworkers (5) used titanium sulfate for determination of H_2O_2 in a semisynthetic microbiological medium. Titanium tetrachloride has been used to determine the concentration of H_2O_2 spectrophotometrically at 415 mμ in diethyl ether (17).

Titanium salts would seem to have several advantages in quantitative determination of H_2O_2 in milk. Hydrogen peroxide and titanium combine in equimolar proportions to form a stable yellow-colored complex (4). Acidic titanium is not affected by other oxidizing agents or air. Sutterfield and Bonnell (16) reported that inorganic peroxides produced a yellow-colored complex, but only if converted to H_2O_2. The only other peroxide with which titanium will form a yellow-colored complex is hydroxyl akyl peroxide [RCH(OH)OOH]. This weak addition compound of an aldehyde and H_2O_2 is not likely to occur naturally in milk.

The present paper describes a method for quantitative determination of hydrogen peroxide in milk using titanium salts.

Methods

Preparation of titanium reagents. Two titanium salts were used in this study—titanium sulfate and titanium tetrachloride. Titanium sulfate was prepared by dissolving titanium dioxide in concentrated sulfuric acid by boiling. The solution was then cooled to room temperature and diluted with an equal volume of distilled water, cooled again to room temperature, and the excess titanium dioxide centrifuged out at 3,000 × *g* for 30 min.

The acidic titanium tetrachloride reagent was prepared according to the method of Wolfe (17) from a clear solution of titanium tetrachloride (Cat. no. T-308, Fisher Scientific Co., Chicago, Illinois). Preparation of the titanium tetrachloride reagent was simpler and did not require boiling of the concentrated acid or centrifuging of 6 N sulfuric acid solution as required in preparation of titanium sulfate.

Preparation of standard curves. Standard

curves were prepared for water, sterilized reconstituted (11% solids) nonfat dry milk (NDM), sterilized homogenized whole milk, and clarified raw whole milk by first adjusting the concentration of H_2O_2 to 500 μg/milliliter in each sample at 4 C. Each sample was mixed, and 1-, 2-, and 3-ml aliquots pipetted into 100-ml volumetric flasks to give 5, 10, and 15 μg/milliliter, respectively, in the final diluted solutions used to make the standard curves. Immediately after pipetting, 70% (w/v) trichloroacetic acid was added to the flasks to precipitate milk proteins and arrest any catalase or peroxidase activity. Optimum amounts of trichloroacetic acid for 1-, 2-, and 3-ml samples of milk were 1, 1.5, and 2 ml, respectively. After 1 to 2 min the mixtures were diluted to 100 ml with distilled water and filtered through a double thickness of filter papers (Whatman no. 42 or 50). Preliminary experiments indicated that 3 ml of milk diluted to 100 ml was the maximum level which could be assayed by the present method.

Color development. Two milliliters of titanium reagent were mixed with 5 ml of the milk filtrate and allowed to stand for 5 min. The absorbancy of the yellow color which developed immediately was measured at the absorption maxima of 400 mμ for titanium sulfate complex and at 415 mμ for titanium tetrachloride complex against water-reagent blanks, using a Bausch & Lomb Spectronic 20 colorimeter.

Results and Discussion

Standard curves of hydrogen peroxide concentrations in water and milk samples are plotted in Figures 1 and 2. Line A in Figure 1 and Line C in Figure 2 represent the standard curves for water, whereas Lines B and D in Figures 1 and 2 represent the standard curves for all three types of milk samples. A single line was drawn for the three milk samples, since individual lines would not be distinguishable in the figures.

The functional relationships $y = a + bx$ for the different test solutions are given in Table 1. The lower absorbance levels and negative Y intercepts for the milk samples as compared to water may have been caused by decomposition of H_2O_2 by milk constituents. It is known that H_2O_2 is decomposed by many metallic ions such as Ca^{++}, Mg^{++}, Cu^{++}, Fe^{++}, and Fe^{+++} (6). In addition, H_2O_2 combines with proteins and sugars (2).

In spite of these effects, the method is accurate and sensitive, as indicated by data in Table 2. Means, standard deviations, and coefficients of variability are given for different levels of added H_2O_2 in the three types of milk and water. The coefficients of variability would indicate that greater uniformity was attained between replicates when titanium tetrachloride was used as the reagent rather than titanium sulfate.

Some of the variability in Table 2 was undoubtedly caused by variations in standardizing H_2O_2 concentrations between replicate determinations. To test the reproducibility of the

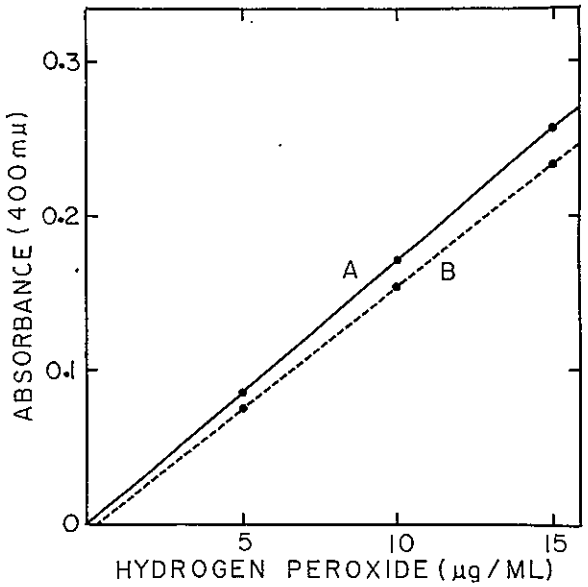

FIG. 1. Standard curves for hydrogen peroxide concentrations determined by the titanium sulfate method in water (A) and sterilized reconstituted NDM, sterilized whole milk, and raw clarified whole milk (B).

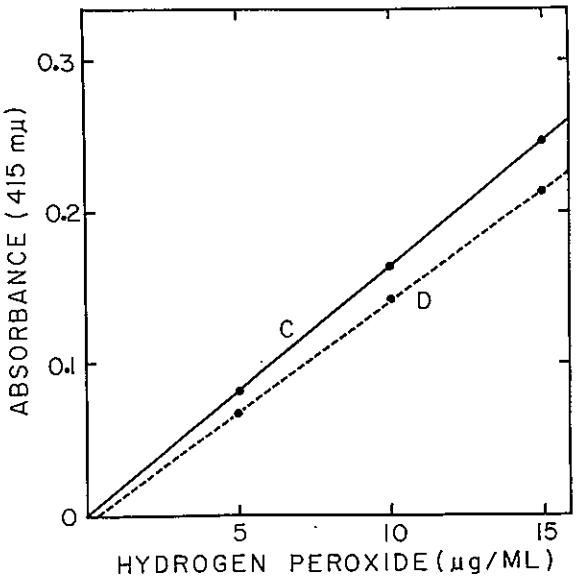

FIG. 2. Standard curves for hydrogen peroxide concentrations determined by the titanium tetrachloride method in water (C) and sterilized reconstituted NDM, sterilized whole milk, and raw clarified milk (D).

TABLE 1

Functional relationships of standard Curves A and B from Figure 1, and Curves C and D from Figure 2

Curve	Media	Functional relationship
A	Water	$Y = 0.0172\ X$[a]
B	Sterilized reconstituted NDM (11% Solids)	$Y = -0.0057 + 0.0151\ X$
	Sterilized homogenized whole milk	$Y = -0.009 + 0.0171\ X$
	Clarified raw whole milk	$Y = -0.0054 + 0.0155\ X$
C	Water	$Y = 0.01639\ X$
D	Sterilized reconstituted NDM (11% solids)	$Y = -0.0027 + 0.01445\ X$
	Sterilized homogenized whole milk	$Y = -0.0047 + 0.01449\ X$
	Clarified raw whole milk	$Y = -0.0069 + 0.01471\ X$

[a] X = Concentration of hydrogen peroxide in μg/ml; Y = absorbance.

method, without the effects of standardizing the level of H_2O_2, the H_2O_2 concentration was adjusted to 500 μg/milliliter in single samples of each of the three types of milk. Five aliquots from each milk were prepared for assay and diluted to give an H_2O_2 concentration of 10 μg/milliliter. Concentrations of H_2O_2 were determined, using titanium tetrachloride. Standard deviations and coefficients of variability of the absorbances for all types of milk were extremely low (Table 3). Coefficients of variability were 50% less for measurements on

TABLE 2

Measurements of accuracy and variability of the two test methods for determining hydrogen peroxide in water and different types of milk

Media used for determination	Levels of added H_2O_2 (μg/ml)	No. of replicates	Mean absorbances	Standard deviations of absorbances	Coefficients of variability of absorbances (%)
			Titanium tetrachloride reagent		
Water	5.0	10	0.0819	0.00100	1.22
	10.0		0.1626	0.00186	1.14
	15.0		0.2467	0.00194	0.79
Sterilized reconstituted NDM	5.0	5	0.0687	0.00257	3.83
	10.0		0.1435	0.00255	1.78
	15.0		0.2133	0.00464	2.17
Sterilized homogenized whole milk	5.0	8	0.0689	0.00203	2.95
	10.0		0.1406	0.00255	1.81
	15.0		0.2137	0.00410	1.92
Clarified raw whole milk	5.0	9	0.0653	0.00091	1.39
	10.0		0.1429	0.00309	2.16
	15.0		0.2124	0.00497	2.34
			Titanium sulfate reagent		
Water	5.0	10	0.0850	0.00176	2.08
	10.0		0.1711	0.00325	1.90
	15.0		0.2573	0.00441	1.72
Sterilized reconstituted NDM	5.0	8	0.0705	0.00355	5.03
	10.0		0.1444	0.00631	4.37
	15.0		0.2218	0.00784	3.54
Sterilized homogenized whole milk	5.0	6	0.0781	0.00459	5.88
	10.0		0.1593	0.00634	3.98
	15.0		0.2494	0.00799	3.20
Clarified raw whole milk	5.0	6	0.0742	0.00318	4.29
	10.0		0.1454	0.00571	3.93
	15.0		0.2291	0.00357	1.56

TABLE 3
Variability of titanium tetrachloride method for determining levels of H_2O_2 in single samples of milk[a]

Type of milk	Mean absorbances	Standard deviations of absorbances	Coefficients of variability of absorbances
			(%)
Sterilized reconstituted NDM	0.1477	0.0012	0.81
Sterilized homogenized whole milk	0.1423	0.0007	0.49
Clarified raw milk	0.1344	0.0023	1.71

[a] Five replicate tests of a single sample of each type of milk.

sterilized milks in Table 3, as compared to measurements on the same types of milk in Table 2. This would indicate that deviations in standardizing H_2O_2 levels contributed to the coefficients of variability in Table 2. The greater variation of the test for raw milk samples was undoubtedly caused by varying levels of decomposition of the H_2O_2 by catalase or peroxidase in the subsamples of the milk prior to addition of trichloroacetic acid.

The titanium tetrachloride method can be used as follows to determine H_2O_2 levels in milk: Three milliliters of milk are pipetted into 100-ml volumetric flasks and 2 ml of 70% (w/v) trichloroacetic acid added. Contents of the flask are made to 100 ml and filtered. Two milliliters of titanium tetrachloride are added to 5 ml of the filtrate and the color measured at 415 mμ. Levels of H_2O_2 are determined from a standard curve developed for the type of milk assayed. The volume of milk used must be adjusted to give 1 to 15 μg of H_2O_2/milliliter of the final test solution (filtrate). The suggested 3 ml of milk will give H_2O_2 levels within this range, if the concentration is 33 to 500 μg/milliliter in the original milk. Smaller volumes of milk can be used if H_2O_2 levels exceed this range.

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