A Reflectance Colorimeter Instrument for Measurement of Microbial and Enzymatic Activities in Milk and Dairy Products

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ABSTRACT

A reflectance color meter has been combined with a Zymate II robot and incubator to measure microbial and enzymatic activity in dairy and food products. Microwells are automatically filled with samples, dyes, and media, and the plates are intermittently removed during incubation to measure color changes of the dye(s). Traditional pH, metabolic, or O/R dyes can be used. The instrument can be programmed and media/dye selected for more rapid estimation of antibiotics, microbial numbers, abnormal milks, coliform counts, product shelf life stabilities, yeast counts, staphylococcal counts, enzymes and culture activity tests, etc. Antibiotic test data are similar to that obtained with impedance instrumentation. Where fewer samples per day are processed, models requiring manual sample preparation are described.

Measurements of microbial activity or parameters of unwholesomeness (19) are potentially more useful in food quality control laboratories than microbial numbers obtained with the standard plate count (SPC) (16). The usefulness of the latter technique is improved with preincubation. The SPC requires 48 h for colony forming units (CFU) to contain about log 8 organisms and become visible. Modern instrumentation, sensitive to the conversion of energy sources to acids or alcohols, can quantify microbial activity in a few minutes to 30 h depending upon the load (1,6). The SPC only estimates the original number of bacteria in a sample while impedance microbiology, for example, estimates rate of microbial growth, the contribution of each microbial cell to unwholesomeness of food, the inhibition or stimulation phenomena in addition to providing counts (1). This instrumentation can detect numbers below 1 CFU/ml when combined with preincubation (9). It measures bacteriophage activity (21), abnormal milk (8), yeasts in yogurts and other foods (22,23), and lactic culture activity (12). Impedance microbiology methods described by Okigbo and Richardson (14) are more sensitive to penicillin and streptomycin (both <0.001 IU/ml) than present reference methods and can be used to quantify the antibiotics.

Reflectance colorimetry is described in this paper (17). It has the same applications as impedance microbiology but measures changes in dye pigmentation rather than electrical signals and is therefore not as sensitive to temperature effects (5), and stray voltages. A more economical, disposable sample tray can also be used. Dyes that produce color changes with pH, oxidation/reduction potential, or free amino groups, can again be useful in dairy laboratories; i.e. litmus, resazurin, and methylene blue. This paper describes configurations of the instrument and discusses typical examples of applications that have been evaluated.

MATERIALS AND METHODS

Sample plates

Sterile micro test plates containing 96 wells and flat-bottoms were used [i.e. plate, MicroTest III®, sterile, disposable, polystyrene, Falcon®No. 3070. Lids were added where the Zymark sampler robot (Z-II) was not used]. The volume of each well is 0.3 ml. Thus if 0.2 ml sample and 0.1 ml reagent/dye were blended, the lower limit of detection would be 5 CFU/ml (mean 1 CFU/0.2 ml), compared to the lower estimate of <1,000 CFU/ml when 0.001 ml is used in the plate loop count (16). Lower limits can be reduced through preincubation or by using micro test plates with larger wells. Plates containing 6 and 24 wells hold 7.5 and 2.0 ml and allow the detection of 0.13 and 0.5 CFU/ml respectively. Changes in computer software would make it possible to change from one plate configuration to another. Per sample costs for the disposable tray with 96 wells would be approximately 1¢, about 2% that of impedance well costs.

Reagents

Reagents for media and dyes were those traditionally used in culture media. Concentrations of DIFCO reagents (DIFCO

1Contribution number 3562 of the Utah Agricultural Experiment Station. Approved by the Director.
2Station Expérimentale Laitière, Place du Champ de Foire, Poligny (Jura), France.
Labs, PO Box 1058A, Detroit, MI 48232) were changed where appropriate to conform with media used in approved methods such as with impedance instrumentation (7) and to produce final concentrations equal to reference methods, except where indicated.

**Reflectance Colorimeter Instruments**

The instrument obtains color measurements from the bottom of microtest wells at frequent intervals; these measurements are then related to biochemical changes caused by the organisms or enzymes. When these changes occur, they indicate the concentration of the biological units of interest. A computer calculates and prints out the desired estimates.

The Omnispec 192 (OS192) (two plates x 96 wells = 192 sample capacity) is comprised of an x,y-recorder table surface with a mechanism that can move one or two microtest plates horizontally over the detector that is located below in the center of the table. The table surface is temperature controlled at 32°C. Samples and reagents are added manually to wells. A ribbon cable connects the instrument with a computer.

The Omnispec 960 Auto (OS960) combines a Zymate™ II Laboratory Automation System (Z-II) (Zymark Corp., Zymark Center, Hopkinton, MA 01748) and the OS192. The Z-II can be programmed to conduct a sequence of tasks. With the two devices complete automation is possible, from removing milk or food homogenate from test tubes to printing out the desired data. The testing rate is about 50 samples/h for one analysis and 24 samples/h for four different simultaneous tests. The OS192 in the automatic system monitors color changes while the Z-II places a prepared plate upon the OS192 table and commands initiation of the reading sequence. The OS192 signals the Z-II when analyses are complete or plate change is indicated. A Zymate™ incubator shelf module allows the system to store and analyze up to 960 samples. The shelves provide an evaporation seal above plates, thus eliminating the need for lids. Alternatively, a Parafilm™ seal was placed over wells when long incubation periods were involved.

**Detector**

Color parameters from the bottom of sample wells were obtained with a Minolta Chroma Meter CR-121 and processed with a Minolta Data Processor DP-100 into the L’a’b’ color notation system (Minolta Corp., 10 Williams Drive, Ramsey, NJ 07446). While reflectance color readings are primarily sought because transmittance or turbidity readings cannot be obtained with milk and many other foods, this detector can be used for clear and turbid solutions, as demonstrated by the mean values obtained with milk and water containing brom cresol purple (BCP), automatically pipeted into eight wells, and pasteurized milk sample, were mixed with brom cresol purple indicator. The Omnispec 192 positioning system was equal to or insignificantly better than when using the Z-II positioning system.

**Computer**

A TeleCAT 286 (Televideo Systems, Inc., Sunnyvale, CA 94086) with 20 MB hard disc controlled the OS192, communicated with the Z-II, through a power and event control station (Zymark Inc.) and stored the data from the Minolta data processor.

**Software**

Easylab™ (Zymark Corp.) computer language was used to drive the Z-II. Software was written to operate the Z-II and process the data. Estimated endpoints were based on the time taken for the S-shaped curve to pass through the point half way between the initial baseline and the extreme value obtained when the color had changed. Earlier detection was possible by estimating the early rate changes, similar to the method used to establish impedance detection times (5), but the former method should be more accurate. Other endpoints were modified depending upon the method. For example, with antibiotic assays, the concentrations were best estimated at a particular time, such as in 2 h (Fig. 9).

**Microbial plate count**

Microbial counts were run using standard procedures on a spiral plate counter (16). Only those plates containing between 20 and 150 visible colonies in appropriate areas were used. Coliform counts were prepared using conventional plating with an overlay as specified (16).

**RESULTS AND DISCUSSION**

**Precision statistics**

The data reported below include any errors associated with the ability of the Z-II/OS192 to prepare the samples, repeatedly locate the sample well above the CR, and to obtain accurate readings. Eight milk samples from one pasteurized milk sample, were mixed with brom cresol purple (BCP), automatically pipeted into eight wells, and measured by the system every 15 min for 7 h. No significant differences were found between wells [Fd<Fr<0.05 (df = 7 & 216)]. The means and standard deviations for the three color parameters were \( L^* = 5.02 \pm 0.133, a^* = 18.735 \pm 0.465 \), and \( b^* = -10.254 \pm 0.225 \), with coefficients of variability of 2.6, 2.5, and 2.2%, respectively. The Zymark system can repeat any position within \( \pm 1 \) mm of the cylindrical operating space (64 cm diameter x 376 circular x 34 cm high). The precision data associated with the OS192 positioning system was equal to or insignificantly better than when using the Z-II positioning system alone.

The range of values indicated that the instrument could...
measure subtle color changes; even those of coliform medium (CM) as acids are produced by coliforms in chocolate ice cream.

**Microbial numbers**

Resazurin (RES) and methylene blue O/R dyes currently are seldom used to measure milk quality where low initial microbial numbers are present (16). Samples are usually tested in 1 to 3 h but, with the OS192, samples can be incubated longer until the numbers of microbes increase sufficiently to reduce and change the color of the dyes. Total microbial numbers were estimated using litmus, RES, BCP, or triphenol tetrazolium chloride (TTC). These dyes are sensitive to O/R, pH, or metabolic breakdown. These and many other dyes can be used with the OS192 system because the instrument measures changes in the total visual spectrum. Classification of groups may be possible through dye selection. For example, some psychrotrophs reduce dyes poorly so performance in two substrates with different dyes may help identification.

One example of estimating total numbers is shown in Fig. 2. A culture of *Streptococcus cremoris* UC 320+ containing log 9 CFU/ml, 0.2 ml, was placed in a well, and 0.1 ml sterile reconstituted (10%) nonfat dry milk (RNDM) containing 3 x SPC nutrients (16) was added to produce the same final concentration of nutrients as in the normal SPC. BCP was also included in the reagent to produce a final concentration of 0.015%. Decimal dilutions of the culture were made into neighboring wells, each of which contained RNDM with SPC nutrients and BCP. Figure 2 summarizes the changes in BCP dye as the organisms changed the pH indicator from blue to yellow. It graphically shows the ease of detecting endpoints. The procedures involved in impedance microbiology (5) could be used to predict color detection points. Alternatively, we programmed the computer to select the endpoint at the midpoint (b' = 8 on Fig. 2) between the baseline and extreme color value of the S-shaped curve. We considered this subject to fewer errors.

Similar results were obtained with RES with temperature abused raw milk with an initial microflora content of log 9 CFU/ml that was decimally diluted in the microtest plates. RES made it possible to measure the change of b' (from blue to “less blue”), a' (from pink to less pink) or the change in L' (from colored to colorless).

Raw milk samples were obtained from bulk tanks and divided into four lots. One lot was tested without modification, the second was incubated 18 h at 13°C (preliminary incubation technique (16)), the third was incubated 18 h at 21°C to obtain very high total numbers, and the fourth was diluted 1/100 to obtain very low total numbers. The abused, diluted, and unmodified samples were mixed with SPC nutrients, BCP or TTC and tested on the instrument. Spiral plate results between 20 and 150 limited the total numbers from over 500 to 140. The results were compared to those obtained on a Spiral plater and plotted in Fig. 3 and 4. The R = 0.97 and 0.96 respectively for estimates from log 0.4 to 9.5.

![Figure 2](image-url)

**Figure 2.** Time required for diluted lactic culture sample to increase the b' values using BCP indicator dye. The sample was diluted from log -1 through log -6.

![Figure 3](image-url)

**Figure 3.** Regression line and correlation coefficient of data between spiral plate and OS192 methods for estimating total viable microorganisms in 140 raw milk samples. High numbers were obtained by abusing samples at 13 or 21°C for 18 h before sampling. Low numbers were obtained by diluting raw milk samples log -2 and log -3. The dye was TTC and L' was measured.

**Coliforms**

Presumptive coliform counts were estimated using either BCP, RES, or TTC dyes, some of which gave better results than others. Samples, 0.2 ml, were added to 0.1 ml medium containing 3 x tryptone bile broth (17). *Escherichia coli* ATCC #29522 was used to evaluate the instrument (Fig. 5).

When Coliform Medium (CM) (7) was used to test for the presumptive coliforms in 80 samples of raw milk, correlations of R = 0.94 were obtained with the coliform
plate count (Fig. 6) (16). Some samples were abused by preincubation for 18 h at 30°C to obtain the high numbers. Subtle color changes in VRB medium made it possible to detect coliforms even in chocolate ice cream (Fig. 7).

Antibiotics/culture activity

Sterile RNDM, 0.1 ml, was fortified with the same nutrients used in either A-1 or A-4 antibiotic media (16), either TTC, RES, or BCP dyes, and 10% lactic culture. This mixture was added to the 0.2 ml sample in each well.

The lactic culture that gave best results was a proteinase positive strain of *Streptococcus cremoris*, UC 310+. Any of the media blends or dyes produced satisfactory results. To test the activity of a culture, 0.2 ml milk for cheesemaking was added to each well and color changes during incubation were compared with changes in wells containing antibiotic-free sterile RNDM. This method could be used to screen agglutinating cultures because color would change more rapidly at the bottom of the well if the culture agglutinated (unpublished data).
Figure 8 shows the results when 0.000 to 10 IU streptomycin/ml were quantitated using TTC. The delay in color development made it possible to detect amounts as low as 0.005 IU/ml. The L* value could also be used for this purpose. Kanamycin was also detected to 0.005 IU/ml. Penicillin was detectable to 0.0005 IU/ml (Fig. 9). Results are available within 2 h (Fig. 4 & 5) if the lactic culture is active (14).

Figure 8. Effects of different concentrations of streptomycin on the time for TTC color (a') changes due to metabolism by lactic culture UC 310+.

Figure 9. Effects of different concentrations of penicillin on the time for BCP color (b') changes due to acid production by lactic culture UC 310+.

Figure 10. Effects of diluting raw milk sample log 0 to log -5 on the time for BCP color (b') changes due to acid production by lactic culture UC 310+. Sample contained unknown concentration of antibiotic.

The ability of the instrument to quantitate sulfamethazine in milk is summarized in Fig. 11. Penicillin Medium (16) broth constituents were added to milk containing the sulfa drug. It could be estimated at between 3 and 30 ppb. This was lower than found when the Delvotest Multi™ was read on the color meter (Fig. 12).

Abnormal milk
Abnormal milk with high salt content (12) can be detected using a modified Mohr test (3). Diluted raw milk (1/100), 1 ml, was added to a well (in 48-well plates) containing 10 µl of 1% NaOH, 5% K2Cr2O7 and 15 µl 0.1M AgNO3. There was no need for incubation and the initial color reading was adequate (Fig. 13). Somatic cell counts on 70 samples were obtained on a model 215.
Figure 12. Effects of different concentrations of sulfamethazine on the time for BCP color (b*) changes due to acid production by Bacillus sterothermophilus var. calidolactis in Delvotest-Multi™

\[ y = -11.472 + 2.956X \quad R = 0.90 \]

y = 6.926 + 2.329X \quad R = 0.89

Figure 13. Regression line and correlation coefficient of data from 70 raw milk samples tested for NaCl content (L*) and somatic cell count.

Fossomatic (16) with R = 0.89. Increasing amounts of NaCl were added to RNDM to note the effects upon the modified Mohr test (12). A linear pattern was obtained.

NAGase (10) activity provided a better estimate of abnormal milk. Into a 48-well plate, 0.2 ml sample and 0.3 ml 3.3 mM substrate solution (10) were mixed, and heated 15 min at 50°C. One milliliter of 1M glycine containing 1% DOC sodium deoxycholate adjusted to pH 10 with 1 M NaOH was added to stop the reaction. Readings on the solutions produced a wider range of data (Fig. 14) than with the NaCl method though the reagent costs were higher. The curve became nonlinear at higher concentrations. Note that L*, a*, or b* data are used in different figures (Examples: Fig. 13, 14 vs. 5), depending upon which gave the widest range.

\[ y = -11.472 + 2.956X \quad R = 0.90 \]

Figure 14. Regression line and correlation coefficient of data from 70 raw milk samples tested for NAGase activity (b*) and somatic cell count.

Figure 15. Time required for dilutions of Citrobacter freundii culture to increase energy transmittance (L*) in RNDM substrate.

Shelf life predictions

Impedance microbiology is the best available laboratory method to predict shelf life of pasteurized milk (1) and cottage cheese (2). With the instruments, data concerning shelf life were available in <32 h instead of 7 to 10 d. Market milk has to be withdrawn before printed pull dates due to spoilage caused by very low levels of microorganisms that survive pasteurization. (Floyd W. Bodyfelt, personal communication). The citrus beverage industry has
successfully used the method for tests to predict shelf life stability. Results are available in 8 h (Ruth Eden, personal communication). The OS192 would perform similarly after changing the method to detect the endpoint and optimizing the media/dye combinations. If CFU/ml are very low, accuracy would be enhanced by preincubation. For example, Fahad et al. (9) were able to detect <1 coliform/ml in 14 h.

**Psychrotrophs/Proteolytic activity**

A culture of *Citrobacter freundii*, isolated from cottage cheese, was inoculated into SPC nutrient-fortified RNDM with RES indicator. Incubation at 32°C improved the energy transmittance (Fig. 15) more rapidly as CFU/ml increased. Lower temperatures may be required to count some psychrotrophic organisms, however, heat-resistant sporeformers (Ibid.) could be tested more rapidly at the higher temperatures.

The OS192 probably could be used for several proteinase activity tests. Even though the instrument measures reflectance from turbid or opaque systems, it can also be used when solutions become less turbid and more transparent. Clear solutions have measurable color values (Fig. 1) even though readings into a nonreflecting environment, such as into space, produce unreadable signals in the colorimeter.

When brilliant blue R (BBR) dye was added to sterile RNDM and inoculated with different concentrations of a 0.2% trypsin solution (Fig. 16), it was possible to determine levels of enzyme by measuring the reduction of blue color. In another trial the BBR was left out and the reduction in turbidity ($L^*$) was measurable (Fig. 17).

The instrument's ability to measure low levels of proteinase activity may mean it can be used to determine residual levels of enzyme coagulant in foods (11) or to profile the peptidases of lactic cultures (4). The ability to measure plasmin activity in milk (18) was confirmed by increasing substrate (H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide) concentrations in raw milk (Fig. 18).

**Lipase Activity**

Reduction in turbidity ($L^*$) of tributyrin or change in color ($b^*$ or $a^*$) of pH indicator dyes can be used to determine lipase activity (15). When BCP and 0.017% phenol (to inhibit microbial activity) were added to homogenized raw milk, pH changes due to the natural milk lipase activity were detected in 16 h.

Pasteurized, homogenized whole milk substrate containing BCP indicator was used to measure pancreatic lipase...
activity (Fig. 19). A 1% solution of pancreatic lipase (Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178) was added at from 5 to 25 ul.

**Yeast** other potential applications

The instrument should estimate the numbers of yeast contaminants in yogurt and similar products using the techniques described by Zindulus (22,23). The pH change causes the major adjustment in impedance instrumentation and therefore permits pH dye utilization in the OS 192.

The numbers of dyes and media usable in specialty tests for biological activity suggest that there are many more applications for the OS 192 concept. While this paper has concerned methods of immediate value to the dairy industry, the instrument could also be used in food laboratories. If samples are incubated before analysis, the instrument could be used to detect levels of *Staphylococcus* (9), *Salmonella*, *Listeria spp.*, and other pathogens. The instrument should also have applications in environmental and medical laboratories.

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