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An impedimetric method for rapid screening of cosmetic preservatives

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An efficient impedance method was developed for rapid evaluation of cosmetic preservatives. The method used decimal reduction time or D-value to assess preservative efficacies. The D-value, which was calculated from the plot of Log CFU ml^{-1} versus time by linear regression analysis, could be obtained within 48 h. Thus, the time required for the challenge test was reduced from 4–8 weeks with the standard procedures (eg US Pharmacopeia), to 2 days with the current method. A calibration curve ($r = -0.95$) was established by plotting the Log CFU ml^{-1} versus capacitance detection time (DT) of 108 samples. With the calibration, CFU can be estimated directly from the impedance test without plating. Two commercial biocides and several other chemicals were evaluated in a shampoo by the impedance procedure against *Pseudomonas aeruginosa*. The D-values obtained from the impedance test were not significantly different from those produced by the conventional plate count method. The technique was found to be particularly useful when screening a large number of compounds to find novel preservatives and synergistic preservative combinations.

Keywords: impedance; D-value; cosmetic preservative; *P. aeruginosa*; screening method

Introduction

Preservative efficacy testing is conducted to determine whether a cosmetic formulation is adequately preserved during manufacture, distribution, storage and use. It is usually performed by exposing the preserved material to challenge organisms and determining the number of surviving organisms during several weeks of incubation. The current methods for preservative efficacy testing are the procedures of the Cosmetic, Toiletry & Fragrance Association [1], the United States Pharmacopeia [2] and the British Pharmacopeia [3]. These standard procedures are very similar in many aspects, and suffer from the major disadvantage of being time consuming. The test usually lasts for four weeks, and if rechallenge is required it may take 2 months. It is impractical to test a large number of samples for screening new preservatives by these procedures. More rapid and automated methods are needed to accelerate the screening process.

The linear regression method [11–14] was developed as a rapid procedure for determining preservative efficacy. The method provides a rapid, quantitative expression of cell inactivation rate designated by a D-value in a preserved product. The D-value, which is the time required for achieving 90% reduction, can be obtained from the plot of Log CFU ml^{-1} versus time within 48 h. However, the method is still costly in labor and test materials because of the necessity for plating. It is desirable to use an alternative technique to substitute for conventional plate counting in the linear regression method.

The impedance method is such an alternative that can be used for counting purposes if a proper calibration between CFU and impedance detection time (DT) can be established. Impedance devices use the impedance microbiology principle to measure the electrochemical changes in a microbiological culture [4, 7–9]. Impedance changes occur in a culture medium as the chemical composition of the medium changes due to growth and metabolic activity of the organisms. The population density of the organism is correlated to the impedance DT. The DT is referred to the time required to produce a detectable acceleration in the impedance (or capitance, or conductance) curve [8]. Connolly *et al* [5] compared three rapid methods for preservative efficacy testing: the direct epifluorescence technique, the ATP bioluminescence method and the impedance method; the impedance method offered the most satisfactory results. The same authors [6] described an impedance procedure for preservative efficacy testing of pharmaceuticals and cosmetics. Data obtained by Noble *et al* [10] indicated that the impedance procedure provided a suitable alternative to conventional plate counting methods for estimating the bacterial loads in surface water samples. This paper describes a rapid impedance procedure for evaluating the efficacy of cosmetic preservatives. The objective of the study was to assess the feasibility of using the impedimetric technique to replace the traditional plate count method for rapid screening of compounds for finding novel cosmetic preservatives.

Materials and methods

Microorganism, media and culture conditions

Pseudomonas aeruginosa isolated from a naturally contaminated paint was used for the study. The organism was identified by using the Biolog, Microstation™ system (Manual of Biolog Microstation™ version 3.5, pp D1–D20, Biolog, Inc, Hayward, CA, USA, 1993). It was chosen as the challenge organism because it was already available in

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our laboratory and because it was easier to acclimate this organism in the shampoo (described below) than typical ATCC organisms. *P. aeruginosa*, especially naturally occurring strains, are generally considered among the most common contaminating bacteria in the cosmetic industry. The organism was acclimated in the shampoo at room temperature for more than 1 month to obtain adequate growth before testing. The contaminated shampoo containing the organism was used as the actual inoculum for all challenge tests. The plate count medium was Letheen Agar (BBL®, Becton Dickinson and Company, Cockeysville, MD, USA) containing (g L^{-1}): pancreatic digest of casein, 5.0; dextrose, 1.0; beef extract, 3.0; lecithin, 1.0; polysorbate 80, 7.0; agar, 15.0; deionized water, 1.0 L, final pH 7.0. The dilution medium for plate counts was Letheen Broth (BBL®) containing (g L^{-1}): peptic digest of animal tissue, 10.0; beef extract, 5.0; lecithin, 0.7; polysorbate 80, 5.0; sodium chloride, 5.0; deionized water, 1.0 L, final pH 7.0. The impedimetric test medium, (Bactometer medium) was Letheen Broth (BBL®) supplemented with 0.4% glucose and 0.4% tryptone; this medium produced the best signal response. All media were sterilized in an autoclave at 121° C for 20 min.

Shampoo ingredients

The shampoo used in the study contained: 45% triethanolamine lauryl sulfate, 7% cocamidopropyl amino betaine, 3% lauramine oxide, and 45% deionized water, final pH 7.0.

Impedance device

The impedance equipment used was the Bactometer® model-128 microbial monitoring system (BioMerieux Vitex, Inc, Hazelwood, MO, USA). It has eight plastic modules in one unit. Each module contains sixteen molded wells. Each well, containing two stainless steel electrodes, can hold up to 2 ml of culture medium. A total of 128 samples can be monitored simultaneously in one unit. The instrument uses three detection modes: impedance, capacitance and conductance. Instrumental signals were measured by the processing unit every 6 min. At the end of each 6-min sampling cycle, the data were transferred to a Nerve Center II computer (Vitek) where the data were collected, stored, and processed.

Chemicals

The chemicals evaluated in the study as potential antimicrobial preservatives were: dimethylhydroxymethyl pyrazole (DHMP); trihexametaphosphate pyrimidine (THPP); 1-methyl-3,5,7-triaza-1-azoniatricyclo-[3.3.1.$1^{3,7}$] decane chloride (MTADC); dodecyl morpholine; and dodecyl imidazole. The concentrations of the chemicals are given in Table 1. Stock solutions (10% w/v) of the chemicals were prepared either in deionized water or in acetone. All the chemicals used in the study were obtained from Buckman Laboratories International Inc, Memphis, TN, USA. A control test with 1% acetone alone (the maximum concentration of acetone incorporated into the test system was 1% v/v) demonstrated that 1% acetone did not affect growth of *P. aeruginosa*.

Test procedure

Chemical stock solutions were added to glass bottles containing 10 ml of unpreserved shampoo to give the desired biocide concentrations. The treated shampoo was inoculated immediately by adding 0.5 ml of contaminated shampoo to provide a final bacterial concentration of about 10^8 organisms ml^{-1}. The inoculated shampoo was placed at room temperature for 24 h. At 0, 4, 8, and 24 h, 1 ml of sample was withdrawn from each bottle and diluted in 9 ml of Bactometer medium. This dilution ratio was found to produce the best instrumental response. One milliliter of this diluted sample was then added to the Bactometer wells, and the instrument was run at 37° C under capacitance mode for 24–48 h. A control sample without preservative was included for comparison. The samples were also plated on Letheen agar to obtain the actual CFU ml^{-1} using Letheen Broth as the dilution blanks. All plates were incubated at 37° C for 48 h. The D-value for each preservative was calculated from the plot of Log CFU ml^{-1} versus treatment time as described previously [11].

Calibration

A calibration was conducted to correlate plate count (CFU ml^{-1}) with capacitance DT. An inoculated shampoo (containing about 10^9 *P. aeruginosa* ml^{-1}) was diluted in the Bactometer medium to give a bacterial concentration range from 10^2 to 10^8 CFU ml^{-1}. One milliliter of sample was withdrawn from each dilution for determining the DT using the impedance procedure, and another 1.0 ml of sample was taken simultaneously from the same dilution for obtaining CFU using the conventional plate count method. All samples were run in duplicate in the Bactometer and in plate counting. This procedure was repeated until 108 active data points were accumulated. The calibration curve was constructed by plotting Log CFU ml^{-1} versus DT of 108 active samples. An active sample was considered a sample that produced no more than 10% variation between the duplicate DTs and no more than 30% variation between the duplicate plate counts (Bactometer Calibration Manual, pp 3–5, BioMerieux Vitek, Inc, 1991).

All experiments were performed in triplicate and the results presented are the average values of three parallel samples, except that the experiment for calibration was done in duplicate.

Results

Detection of bacterial growth by capacitance in unpreserved shampoo

In an initial test, an unpreserved shampoo inoculated with 3.5×10^8 *P. aeruginosa* ml^{-1} was monitored using the three detection modes. Change in electrochemical signal was observed only using the capacitance mode. The capacitance DT for the inoculated culture was 2.65 h, whereas no DT was obtained for the sterile sample (Figure 1). Instrumental response was not observed with the impedance and conductance modes (data not shown). The growth detected by capacitance was confirmed by subculturing on Letheen agar. Capacitance was selected as the detection mode for all the subsequent experiments.

Table 1 D-values from capacitance test and plate count method against *P. aeruginosa*

Chemical name	Concentration (ppm)	D-Value (h, mean ± SD) (from capacitance test)	D-Value (h, mean ± SD) (from plating)
Control	–	143.4 ± 16.7	166.0 ± 23.4
DHMP	100	27.9 ± 0.2	34.2 ± 3.1
	500	7.7 ± 0.3	7.6 ± 0.2
	1000	3.3 ± 0.1	2.9 ± 0.1
THPP	1000	2.4 ± 0.1	2.6 ± 0.1
MTADC	750	31.4 ± 1.5	31.0 ± 2.5
Dodecyl morpholine	1000	75.5 ± 8.6	73.8 ± 6.7
Dodecyl imidazole	1000	122.5 ± 11.4	55.2 ± 6.3

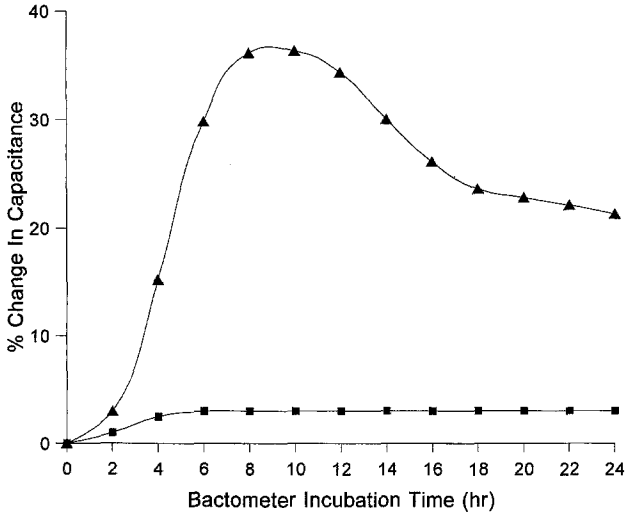

Figure 1 Capacitance response caused by *P. aeruginosa* growing in an unpreserved shampoo. Symbols: –▲–, inoculated sample; –■–, sterile sample

Calibration

In order to estimate CFU by using impedance technique, it is desirable to construct a calibration curve to correlate plate counts with DTs. Figure 2 shows the calibration curve based on 108 active data points in an unpreserved shampoo with *P. aeruginosa*. A linear regression line was the best fitting curve for the data. The line equation was: Log (CFU ml^{-1}) = $-0.29 \times DT + 9.48$, with a correlation coefficient of -0.95 ($P < 0.01$, Student's *t*-test). The maximum DT was 25.6 h and the minimum DT was 1.4 h. The minimum and the maximum DTs were referred to the earliest and the latest DTs of the 108 active data points used to generate the calibration. The bacterial concentration range used to establish the calibration was between 2.3×10^1 to 8.4×10^8 CFU ml^{-1}.

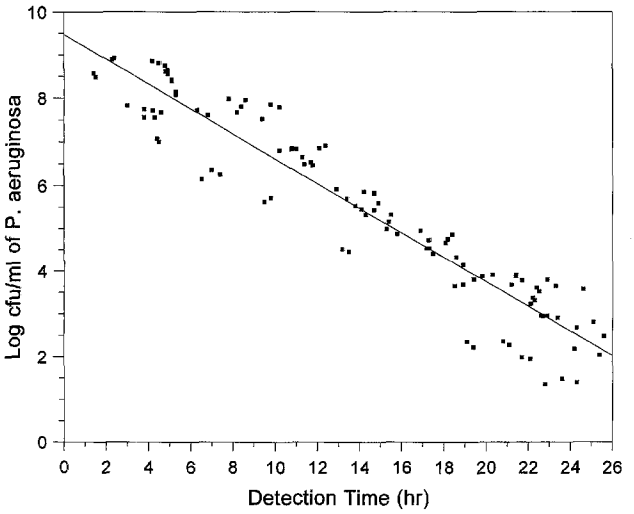

Figure 2 Calibration curve of Log CFU ml^{-1} versus capacitance DT in an unpreserved shampoo with *P. aeruginosa*. $r = -0.95$ ($P < 0.01$, Student's *t*-test)

Evaluation of preservative-treated shampoo by capacitance and plate count

Figure 3 illustrates the capacitance response of a shampoo treated with DHMP against *P. aeruginosa*. At 0 h (before treatment), an acceleration in capacitance occurred as early as 1.6 h. When the shampoo was treated with the preservative for 4 or 8 h, capacitance was detected at 10.2 and 18.5 h, respectively. The treatments resulted in approximately a 9- and a 17-h delay in DT.

The treated shampoo was simultaneously subjected to the conventional plate count method. The CFUs obtained from actual plating at different treatment times were compared

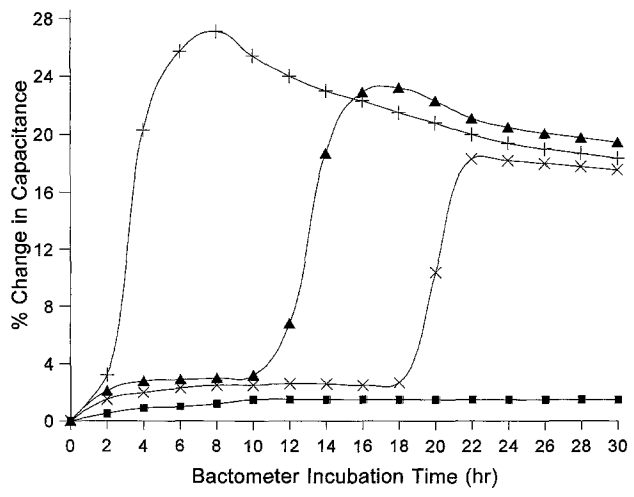

Figure 3 Capacitance curves obtained from a shampoo treated with 1500 ppm of DHMP. The shampoo was inoculated with 7.2×10^8 *P. aeruginosa* ml^{-1} before treatment. Symbols: –+–, treated for 0 h (before treatment); –▲–, 4 h; –x–, 8 h; –■–, 24 h

Table 2 Correlation of DTs with plate counts in a shampoo treated with 1500 ppm of DHMP against *P. aeruginosa*

Treatment time (h)	Mean CFU ml^{-1} ± SD (by plating)	Mean CFU ml^{-1} ± SD (estimated from calibration equation)	Mean DT (h) ± SD
0	$7.2 \pm 1.31 \times 10^8$	$8.4 \pm 1.70 \times 10^8$	1.6 ± 0.25
4	$6.4 \pm 1.26 \times 10^6$	$3.3 \pm 0.62 \times 10^6$	10.2 ± 0.80
8	$4.2 \pm 0.87 \times 10^4$	$1.3 \pm 0.47 \times 10^4$	18.5 ± 1.23
24	<10	<10	No detection

with those calculated from the calibration equation by using the corresponding DTs (Table 2). No significant differences were found between the CFUs determined by plating and those estimated from the equation ($P > 0.05$). In addition, capacitance DTs were linearly correlated ($r = -0.99$, $P < 0.01$, Student's t-test) with the CFUs for preservative-treated cells (Table 2). The longer the treatment time, the lower the plate count and the greater the DT. When the shampoo was treated for 24 h, the plate count was <10 CFU ml^{-1}, and there was 'no detection' recorded for the DT (>30 h, data not shown).

Efficacy testing by capacitance using the calibration curve

After satisfactory calibration was established, the calibration curve was used to determine CFU from the corresponding DTs in the capacitance test. Figure 4 shows the results obtained for five potential preservative chemicals against *P. aeruginosa* using the capacitance test. Antibacterial activity of each chemical was demonstrated by the rate of bacterial inactivation within 24 h or by the D-value under the treatment. The efficacies of the chemicals were simultaneously evaluated by the plate count method. The D-values obtained from the capacitance test were not significantly different from those determined by the plate count ($P > 0.05$, Student's t-test), except in the case of dodecyl imidazole ($P < 0.01$, Student's t-test) (Table 1).

A good dose-response relationship was observed for DHMP as indicated by the increased activity with increased concentrations of DHMP (Figure 4 and Table 1). Dodecyl morpholine and dodecyl imidazole had large D-values, indicating that these two compounds were not effective under the test conditions. THPP was the most effective compound tested with the lowest D-value by both the capacitance test and plate count method.

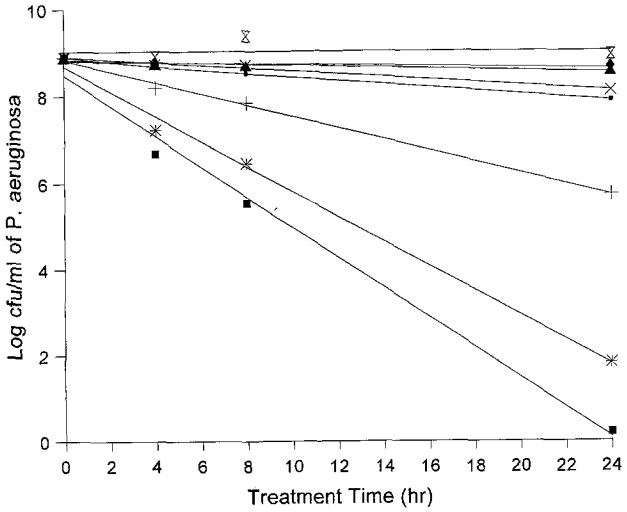

Figure 4 Survivor curves showing the effects of different chemicals or different chemical concentrations on bacterial inactivation rate in the shampoo inoculated with 7.9×10^8 ml^{-1} of *P aeruginosa*. The test was conducted using the impedance procedure without plating. The Log CFU ml^{-1} was calculated by inserting the capacitance DT into the calibration equation. Symbols: ⧖, control (without preservative); –◆–, dodecyl imidazole (1000 ppm); –▲–, dodecyl morpholine (1000 ppm); –x–, MTADC (750 ppm); –■–, DHMP (100 ppm); –+–, DHMP (500 ppm); –*–, DHMP (1000 ppm); –■–, THPP (1000 ppm)

Discussion

Data in Tables 1 and 2 suggest that the impedance technique can be used as an alternative to the conventional plate count methods for rapid evaluation of preservative efficacy. The current method is designed to offer a rapid procedure to screen preservatives for cosmetic formulations. With the method, judgement can be made in 2 days on candidate compounds. The method is particularly useful in screening a large number of chemicals in a short period of time. With the D-values generated by the impedance procedure, one can make a quick comparison among many candidates and select the most effective preservatives.

In addition to the quick results, the impedance method offers major advantages in savings in labor, laboratory space and testing materials. With the calibration curve, CFU ml^{-1} can be estimated directly from the impedance test without actual plating. Orth *et al* [14] used a D-value method to demonstrate synergism between methyl paraben and acrylic acid polymers against several *Pseudomonas* species in a nonionic lotion. However, in their procedure, a heavy workload and large amounts of testing materials were involved in determining aerobic plate counts. This costly process could be avoided by incorporating the impedance technique to the D-value procedure. The impedance method can provide a more cost-effective alternative to the traditional plate count methods for testing synergistic combinations. The impedance method is also suitable for product stability testing where a large number of identical samples are assayed to determine whether the preservative efficacy is maintained during the shelf life and consumer use under various environmental conditions.

Connolly and coworkers [6] concluded that good corre-

lations were obtained between the impedance DT and CFU in the untreated cultures of several organisms, including *P. aeruginosa*, and in cultures treated with chlorhexidine, methyl paraben and phenoxyethanol. Our results also demonstrated good correlations in the untreated culture of *P. aeruginosa* (Figure 2), and in the culture treated with DHMP (Table 2).

One potential problem with the impedance method is that individual calibration curves are required for different microorganisms. A calibration established for one microorganism may not be suitable for another. Different correlations of impedance DT with CFU were found among four different organisms: *P. aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* [6]. The construction of calibration curves can be time consuming, because at least 100 data points evenly distributed over a 5-log range in CFU are required for each calibration (Bactometer Calibration Manual, pp 4–2, BioMerieux Vitek, Inc, 1991). However, most evaluating companies use the same standard organisms for all challenge tests. This indicates that only a few calibration curves are needed for evaluating or screening cosmetic preservatives. Once a calibration is established, the impedance method can be considered a more economic alternative to the conventional plate count methods.

The cause for the wide difference in the D-values of dodecyl imidazole between the impedance and plating methods has not been determined yet. It could be possible that the chemical interferes with the impedance detection in the culture solution. The impedance method might have some disadvantages to evaluate certain compounds like dodecyl imidazole. Further investigation is required to find the exact reason why the D-values of dodecyl imidazole were different when determined by the two different methods.

The high ionic concentration in the growth medium (Letheen Broth) could contribute to the problem of no instrumental response with the conductance mode, because conductance is insensitive to the metabolic changes in high-conductivity media [10]. Driving frequency, metal composition of the electrodes, pH changes, and bacterial species are all influential factors that may affect signal detection. Stainless steel electrodes and lower driving frequencies are favored conditions for the detection of capacitance signals [8]. However, the exact reasons for lack of instrumental response with the impedance and conductance modes in this study are unknown.

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