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Determination of shampoo preservative stability and apparent activation energies by the linear regression method of preservative efficacy testing

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Synopsis

Preservative efficacy tests were performed on stability samples of a shampoo during storage for 18 mo. at 3°, 20°, 38°, and 49°C. The shampoo preservative system deteriorated with increasing storage time and temperature, as measured by the "linear regression method." Thus, the decimal reduction time (D-value), which was used as the measure of the rate of death for *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus* sp., increased from about 4 hr to over 30 hr after accelerated aging at 49°C for 3 mo. The D-values for *Staphylococcus aureus* increased more slowly.

Apparent activation energies (Ea′) for the change in shampoo preservative potency were calculated from the D-values at different temperatures. The Ea′ values decreased from −7, −6, and −4 Kcal/mole at 1 mo. to −16, −10, and −9 Kcal/mole at 12 mo. for shampoo challenged with *E. coli, P. aeruginosa,* and *Bacillus* sp., respectively. This loss of preservative system potency appeared to follow first-order or pseudo first-order reaction kinetics, while the magnitude of the Ea′ varied with the length of storage and the test organisms used.

This work illustrates the need for using different microorganisms when conducting preservative efficacy tests of cosmetic products and shows how the quantitative values obtained by the linear regression method are well-suited to monitoring stability tests. The use of this method for determining the apparent molar concentration of a preservative from D-values and for predicting the stability of cosmetic preservative systems from Ea′ values is discussed.

INTRODUCTION

Stability testing is the final step in the development of cosmetic products. The objective of this testing is to demonstrate that a product does not change significantly during its expected shelf life. Product stability is necessary because several months or years may elapse between the time a cosmetic is manufactured and used up by the consumer.

Recognizing this, current Good Manufacturing Practices (cGMP) for human OTC drug products stipulate that products must be stable for at least 3 yr. and that this must be supported by appropriate stability data (1). In many instances, cGMP are used as a basis for supporting documentation for cosmetic products. Thus, stability tests may be per-

formed by storage of products at various temperatures for up to 5 yr. to demonstrate satisfactory shelf life.

Microbial spoilage may occur in aqueous products and in anhydrous products that are exposed to water; consequently, cosmetic preservatives are included in formulations to inhibit the growth of bacteria, yeasts, and molds while products are in trade channels and in the hands of the consumer. The principles of preservative efficacy testing have been reviewed (2,3), including the need for demonstrating that products have adequate stability (2).

Although several methods of preservative testing are available (4–6), our laboratory uses the "linear regression method" because it provides a rapid, quantitative expression of the rate of death of specific test organisms in a product when using defined test conditions (4). The rate of death determined by the linear regression method is expressed as the decimal reduction time (D-value), which is the time required for inactivation of 90% of the population of test organisms.

The rationale for using D-values is that every organism has a characteristic rate of death when subjected to a specific lethal treatment (4). This enables a laboratory to provide quantitative results on the rate of inactivation of specific test organisms in a product. Thus, the linear regression method can be used to determine the effect of formulation changes and component interaction on the stability of the preservative system.

Since the linear regression method was adopted, we have observed changes in preservative efficacy of some formulations during the course of stability studies. This report illustrates the value of performing preservative efficacy tests on stability samples by showing how a shampoo preservative system deteriorates with age.

EXPERIMENTAL

TEST ORGANISMS

The test organisms used in these studies were taken from the Jergens culture collection and consisted of *Staphylococcus aureus* (FDA 209 strain), *Pseudomonas aeruginosa* (PRD 10 strain), *Bacillus* sp. (isolated from a contaminated cosmetic product), and *Escherichia coli* (ATCC 8739). These organisms were cultured and used for challenging the test samples, as described in a previous report (4).

TEST SAMPLES

The test samples consisted of a proprietary formulation of a shampoo in high-density polyethylene containers. The shampoo contained ammonium lauryl sulfate, cocamidopropyl betaine, propylene glycol, polysorbate 20, hydrolyzed animal collagen, tetra sodium EDTA (and other ingredients), and was preserved with methylparaben [MP], chloromethylisothiazolinone [CMIT], and methylisothiazolinone [MIT].

STABILITY TEST

One bottle of freshly prepared shampoo was used for the initial determinations (i.e., 0-mo.). Several bottles of the test samples were stored at refrigerator temperature, room

temperature, 100°F, and 120°F (i.e., 3°, 20°, 38°, and 49°C, respectively) for the duration of the stability study. One bottle of product stored at each temperature was removed at specified times and subjected to preservative efficacy testing. After sampling, the bottles were placed in the sample storage archives.

TEST PROCEDURE

A 0.1-mL aliquot of test organism suspension, containing about 10^8 organisms/mL, was added to ca. 50-mL portions of each test sample in 100-mL screw-capped bottles. Aerobic plate counts were determined, and D-values were calculated as described in an earlier report (4).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Test samples of shampoo were diluted to 1% (wt/vol) in mobile phase. After filtration, the samples were assayed by injecting 250-μL aliquots onto a 250 × 4.6 mm i.d. LiChrosorb® RP-18 (5 μm) column with a 40 × 4.6 mm i.d. guard column containing Perisorb® RP-18, 30–40 μm. Chromatographic conditions were as follows: mobile phase = water:methanol (45:55) with 0.2% acetic acid, flow rate 0.5 mL/min, temperature 25°C, and detector range 0.05 AUFS. The column effluent was monitored at a UV wavelength of 275 nm.

CALCULATION OF APPARENT ACTIVATION ENERGY

The apparent activation energy (Ea′) for the shampoo preservative system was determined for each test organism at each time period of the stability study (i.e., 1, 3, 6, or 12 mo.). A plot of Log 2.303/D-value *vs* 1/T (where T is the absolute temperature in °K) was calculated for each test organism, and the slope of each line was determined by linear regression. The Ea′ has a value of $-2.303R$ (slope), which is the same calculation as for Ea (17). Preservative efficacy tests were not performed on samples stored beyond 6 mo. at 49°C or 12 mo. at 38°C. The D-values obtained on samples stored for 18 mo. at 3° and 20°C were not used in calculation of Ea′ values because D-values at the higher temperatures were not available.

RESULTS

Figure 1 shows the changes in preservative efficacy that occurred during storage of the shampoo at 3°, 20°, 38°, and 49°C for 18 mo. when using *E. coli* as the challenge organism. It is apparent that the preservative system was not stable and that the D-values increased with increasing storage time and temperature. Thus, the D-values for *E. coli* changed from ≤4 hr at the outset of the study to 35 hr after storage for 1 mo. at 49°C. The preservative efficacy decreased more slowly in samples stored at 38°C than at 49°C, and samples stored at 3° and 20°C were affected much less than those stored at 38°C, as indicated by smaller changes in D-values during the test.

The preservative efficacy test results obtained when *P. aeruginosa* was inoculated into shampoo samples that had been stored up to 18 mo. at different temperatures are presented in Figure 2. The findings obtained with *P. aeruginosa* were similar to those

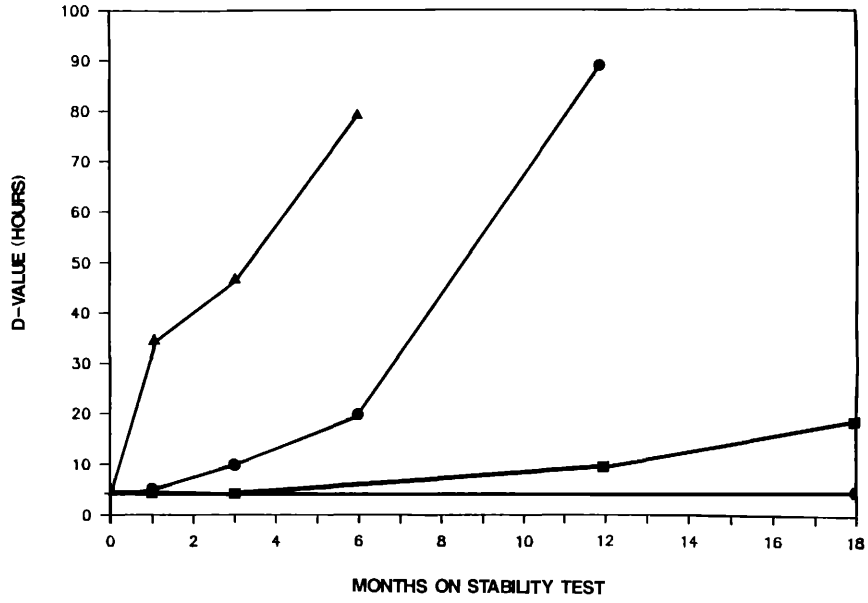

Figure 1. D-values for *E. coli* in shampoo after storage at 3°, 20°, 38°, and 49°C for 1, 3, 6, 12, and 18 mo. Explanation of symbols: ▲—▲, shampoo stored at 49°C; ●—●, shampoo stored at 38°C; ■—■, ■—■, shampoo stored at 20°C; and ◗—◖, shampoo stored at 3°C.

obtained with *E. coli;* however, comparison of Figures 1 and 2 reveals that the test strain of *E. coli* was slightly more resistant to the preservative system than the test strain of *P. aeruginosa* used in these studies.

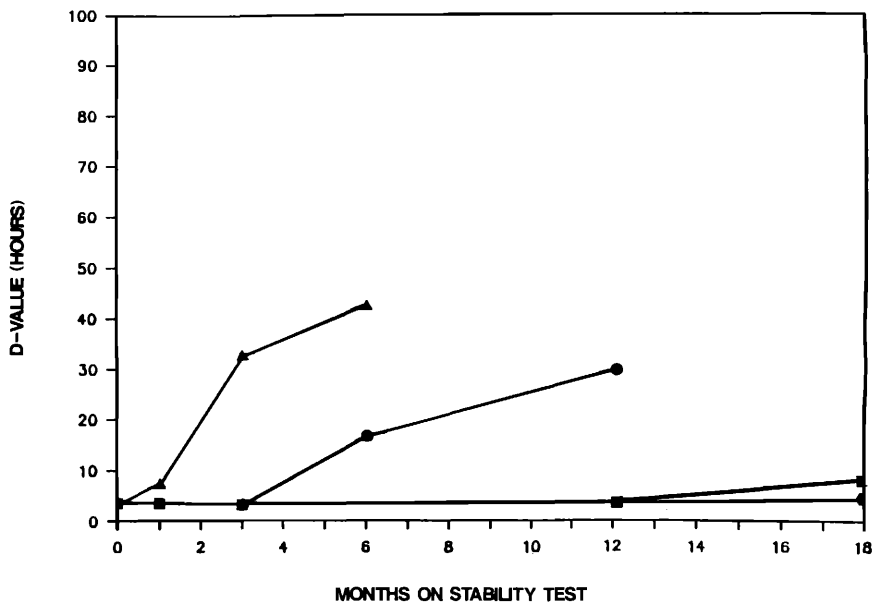

Figure 2. D-values for *P. aeruginosa* in shampoo after storage at 3°, 20°, 38°, and 49°C for 1, 3, 6, 12, and 18 mo. Explanation of symbols: ▲—▲, shampoo stored at 49°C; ●—●, shampoo stored at 38°C; ■—■, shampoo stored at 20°C; and ◗—◖, shampoo stored at 3°C.

Figure 3 illustrates the stability profile of the shampoo when challenged with *Bacillus* sp. The D-values obtained with this gram-positive spore former at several points in time were similar to those obtained with *P. aeruginosa*. Although *S. aureus* was much less resistant to the preservative system than the other test organisms, storage of the shampoo decreased the potency of the preservative system for this organism. Thus, the D-values changed from around 4 hr at the outset of the stability study to 7.6 hr after storage for 6 mo. at 49°C and to 9.8 hr after storage for 12 mo. at 38°C (Figure 4).

The preliminary HPLC analyses for MP, CMIT, and MIT revealed that the concentration of MP was unchanged and that the concentration of isothiazolinones appeared to have decreased in all shampoo test samples that had been used in the stability study. A shampoo spiked with MP, CMIT, and MIT and assayed by HPLC gave the chromatogram shown in Figure 5. Here, the CMIT and MIT peaks are clearly evident. This may be contrasted with the chromatogram obtained from a shampoo aged for 18 mo. at 38°C (Figure 6), in which no MIT peak is evident. Similar findings were obtained with shampoo samples stored at the higher temperatures.

The Ea′ values for the change in the shampoo preservative system were calculated by substituting 2.303/D-values for the reaction rate constants (k) in the Arrhenius equation (7). The results presented in Table I show that the Ea′ values for the shampoo preservative system increased negatively from −7, −6, and −4 Kcal/mole at 1 mo. to −16, −10, and −9 Kcal/mole at 12 mo. when performing preservative efficacy tests with *E. coli, P. aeruginosa* and *Bacillus* sp., respectively. Insufficient data were available for calculating a change in Ea′ values during the first 6 mo. of the study when testing with *S. aureus* because this organism was inactivated so quickly in the shampoo. The

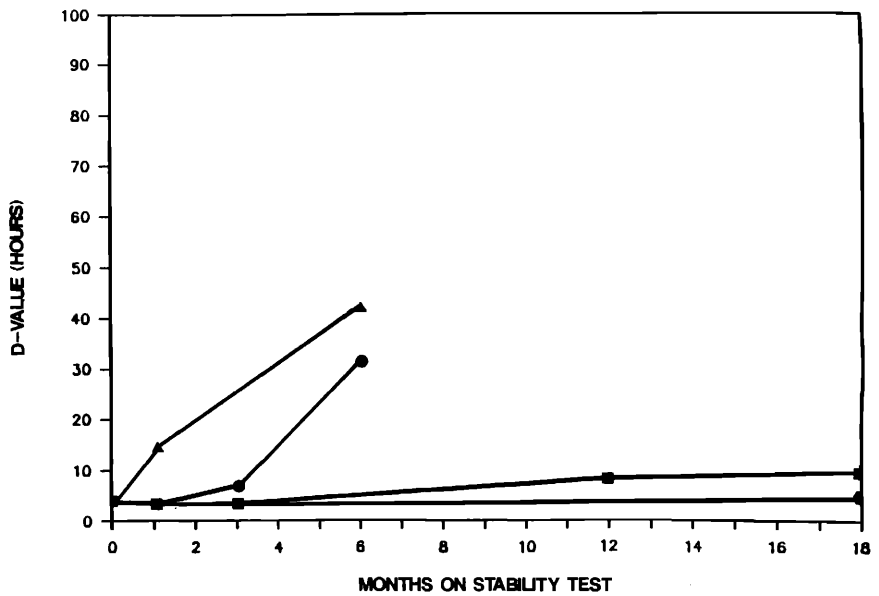

Figure 3. D-values for *Bacillus* sp. in shampoo after storage at 3°, 20°, 38°, and 49°C for 1, 3, 6, 12, and 18 mo. Explanation of symbols: ▲—▲, shampoo stored at 49°C; ●—●, shampoo stored at 38°C; ■—■, shampoo stored at 20°C; and ◗—◖, shampoo stored at 3°C.

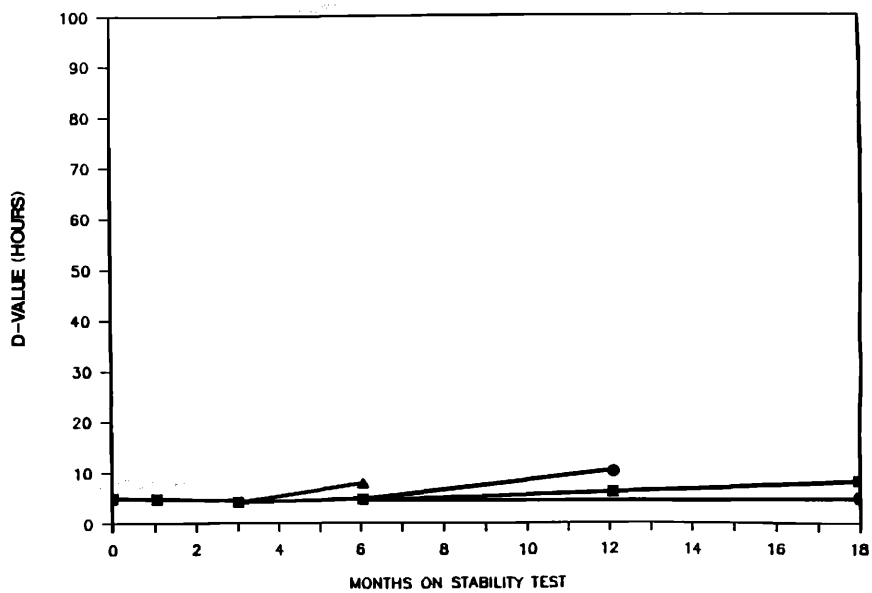

Figure 4. D-values for *S. aureus* in shampoo after storage at 3°, 20°, 38°, and 49°C for 1, 3, 6, 12, and 18 mo. Explanation of symbols: ▲—▲, shampoo stored at 49°C; ●—●, shampoo stored at 38°C; ■—■, shampoo stored at 20°C; and ◗—◖, shampoo stored at 3°C.

Ea′ values obtained with *S. aureus* were calculated to be −2 and −4 Kcal/mole from D-values obtained after the shampoo was stored for 6 and 12 mo. respectively.

Regression lines for the plot of Ea′ *vs* months of storage were calculated for the test organisms (Figure 7). The slopes of these regressions showed a similar progression for all of the test organisms, exhibiting values of −0.8, −0.4, −0.6, and −0.3 Kcal/mole/month and the corresponding correlation coefficients (r) of −1.00, −0.93, −0.75, and −1.00 for *E. coli, P. aeruginosa, Bacillus* sp., and *S. aureus,* respectively. These data suggest that the loss of preservative system potency in the shampoo followed first-order or pseudo first-order reaction kinetics.

DISCUSSION

Accelerated aging, or storage at elevated temperatures, has been used to study the shelf life of cosmetic products and/or to determine the effect of physical and chemical parameters of the formula on preservative stability (8,9). Several reports indicate that cosmetic preservative systems may interact with components of the formula or packaging materials, with concomitant loss of preservative potency (9–14).

In the present study, the preservative system of the shampoo was found to be unstable, as indicated by the change in D-values with time and temperature. Higher storage temperatures produced fairly rapid deterioration in shampoo preservation, as determined with *E. coli, P. aeruginosa,* and *Bacillus* sp. This inactivation of the preservative system was not as obvious when using *S. aureus* as the challenge organism (Figure 4) because the net antibacterial effect of the shampoo (discussed below) inactivated *S.*

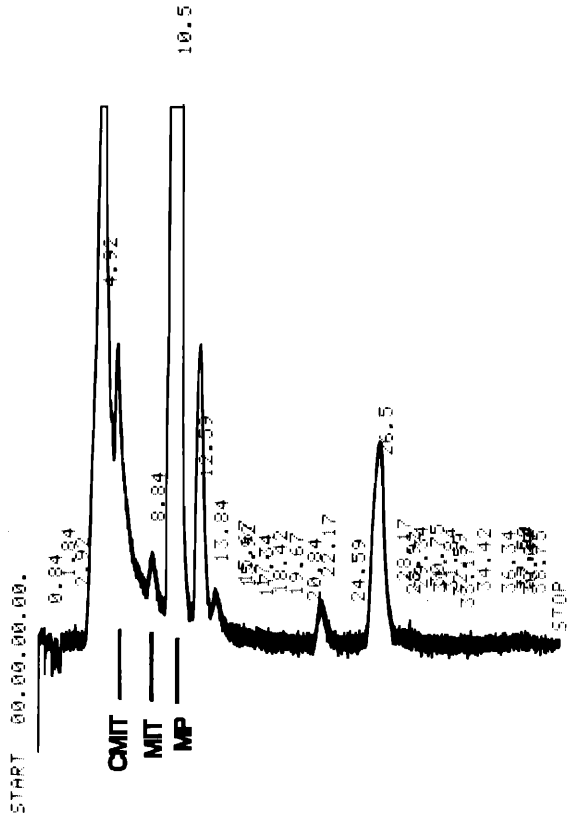

Figure 5. HPLC chromatogram of shampoo spiked with methylparaben (MP), chloromethylisothiazolinone (CMIT), and methylisothiazolinone (MIT).

aureus rapidly. The differences in the rates of inactivation of the preservative system occurring at 3° to 49°C were not due to differences in the shampoo samples, because all samples used at each time/temperature period came from the same bottle.

The shampoo stored at 49°C for over one month was the least resistant to contamination by *E. coli,* as evidenced by the larger D-values obtained in comparison with those observed when using the other test organisms. This may be due, in part, to limited activity of MP against gram-negative organisms (2) and, in part, to the ability of coliforms to grow in the presence of anionic surfactants. For example, 0.01% sodium lauryl sulfate is used to increase the selectivity for coliforms in enrichment media such as Lauryl Tryptose Broth (16).

These findings show that different microorganisms do not respond monotonically to the preservative system in the shampoo. This is why it is essential that preservative efficacy testing be performed using test organisms with all of the physiological characteristics expected to be a potential problem in the formula. As a minimum, gram-negative organisms with diverse metabolic capabilities, such as *P. aeruginosa,* a representative of the coliform group, such as *E. coli,* a gram-positive coccus, such as *S. aureus,* and a gram-positive, spore-forming rod (*i.e., Bacillus* sp.) should be included in preservative testing. These bacteria, in addition to selected yeasts and/or molds not discussed in the

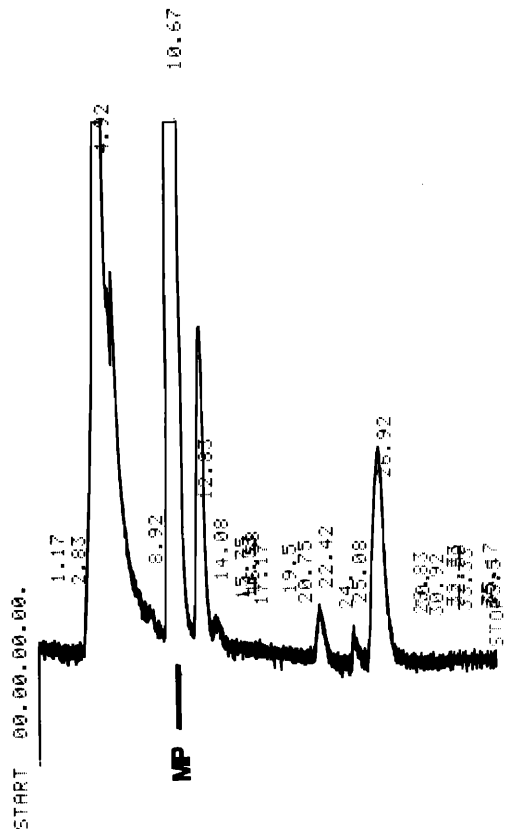

Figure 6. HPLC chromatogram of shampoo after storage at 3°C for 18 months.

current work, provide sufficiently diverse morphological and physiological characteristics to give reasonable assurance that the test results offer a good indication of the resistance of the product to contaminants that could come into contact with the product during production or while in the hands of the consumer. Other considerations in the selection of test organisms have been discussed (2).

It is known that some cosmetic raw materials affect preservative efficacy. In addition, it should be recognized that the preservative system of a cosmetic product may involve

Table I
Change in Apparent Activation Energies (Ea′) of Shampoo During Stability Testing

	Ea′ (Kcal/mole)*				
Test Organism	0 mo	1 mo	3 mo	6 mo	12 mo
E. coli	–**	−7	−9	−11	−16
P. aeruginosa	–	−6	−6	−9	−10
Bacillus sp.	–	−4	−3	−10	−9
S. aureus	–	–	–	−2	−4

* Calculated by substitution of 2.303/D-value for k in Arrhenius equation (see equation {3}).
** Calculation not performed due to lack of sufficient data.

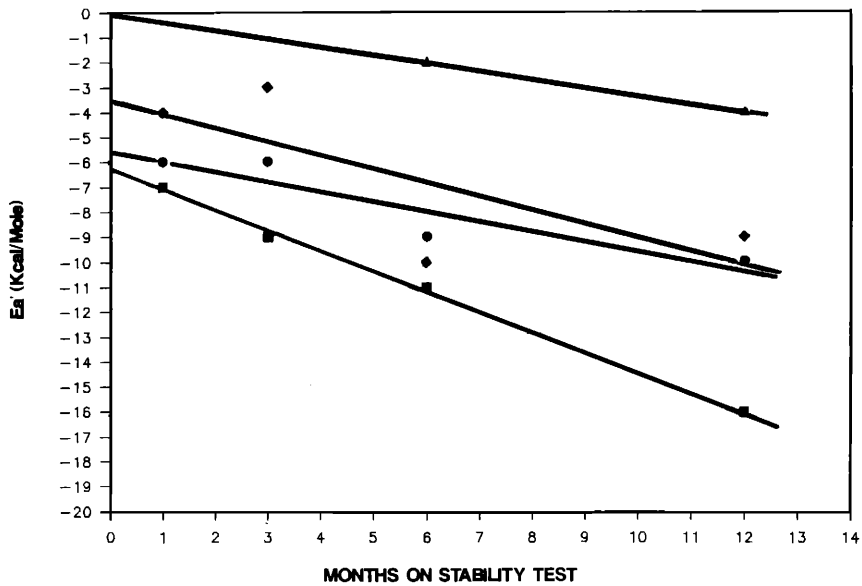

Figure 7. Change in apparent activation energies (Ea′) for the shampoo preservative system during the stability study, determined using test organisms. Explanation of symbols: ■—■, *E. coli;* ●—●, *P. aeruginosa;* ◆—◆, *Bacillus* sp.; and ▲—▲, *S. aureus.*

more than chemicals with known antimicrobial activity. Factors such as pH, water activity, nutrient availability, surfactant concentration, sequestering agents, and other interferences will determine the extent to which preservative action is manifested in any given formulation. Thus, the preservative system of a product involves both specific preservative chemicals and the physicochemical constitution of the entire formulation.

The inhibition of bacterial growth in a shampoo may conceivably be due to more than one mechanism (i.e., surfactant destabilization of cell membranes, preservative action on cellular metabolism, sequestration of divalent metal ions by tetrasodium EDTA, unavailability of nutrients, etc.). One would not expect organisms with different metabolic capabilities to be inactivated at the same rate in any cosmetic product. In the current study, it was found that the four test organisms responded differently to the net antibacterial effect of the shampoo.

Although the goal of this investigation was not to determine the cause of change in preservative efficacy, the HPLC results revealed the presence of all three preservatives initially, but only MP was unchanged after one week of storage at room temperature (Figures 5 and 6). This suggests that CMIT and/or MIT reacted with some component in the formula—possibly hydrolyzed animal collagen, because these isothiazolinones are known to react with amines (15).

It is known that the rate constants for chemical reactions are influenced by temperature, as represented by the Arrhenius equation (17) {1}:

$$k = Ae^{-Ea/RT} \qquad \{1\}$$

where k is the reaction rate constant, A is the pre-exponential factor, Ea is the activation energy, R is the gas constant, and T is the absolute temperature. Differentiating

equation {1} with respect to temperature and integrating between limits yields equation {2} (17):

$$\log \frac{k_2}{k_1} = \frac{Ea\ (T_2 - T_1)}{2.303R\ (T_1T_2)} \qquad \{2\}$$

Stumbo (7) reported that the D-value = 2.303/k; consequently, k = 2.303/D-value. Substituting 2.303/D-values for k enables one to use an expression that relates D-values, Ea and T, as in equation {3}:

$$\log \frac{\text{D-value}_1}{\text{D-value}_2} = \frac{Ea\ (T_2 - T_1)}{2.303R\ (T_1T_2)} \qquad \{3\}$$

The rates of reactions in biological systems are affected similarly by temperature, so that the rate of inactivation of a given organism in the presence of preservative chemicals (or other physicochemical conditions that are bacteriocidal) increases with temperature. Since D-values become smaller as the rates of microbial inactivation increase, it would be expected that the D-value for a given organism in a test sample would decrease with an increase in preservative efficacy test temperature—as long as the preservative system was not altered by the assay conditions.

The situation is different in the current study since the shampoo preservative potency was evaluated at constant conditions (i.e., by performing all preservative efficacy tests at room temperature) after test samples had been stored for specified times at different temperatures. The preservative system was found to be unstable when tested by the linear regression method. This decrease in preservative potency with time and temperature of storage resulted in decreases in the slopes of the survivor curves (4) and corresponding increases in D-values for each test organism.

Although the slope of the Arrhenius activation energy plot is negative when k increases with temperature, the decrease in rates of bacterial inactivation with temperature observed in this study gives a positive slope in the Arrhenius activation energy plot, as is illustrated for the data obtained with the test samples stored for 12 mo. and challenged with *E. coli* (Figure 8). The Ea calculated from these results are negative; consequently, they are designated Ea′.

The Ea′ values calculated for shampoo preservative potency during the first 12 mo. of the stability study appear in Table I. The progressive decrease in Ea′ for all test organisms reflects the decrease in preservative system potency. Here, the rate of change of preservative system potency was greatest for *E. coli* (i.e., the organism most resistant to the shampoo preservative system) and smallest for *S. aureus* (the test organism least resistant to the preservative system).

Although the negative Ea′ values appear to be contradictory to conventional systems in which Ea is determined, one should note that the parameter being measured—preservative system potency—decreased with increasing temperature, as determined by the kinetics of inactivation of the test organisms.

In general, the rate of a chemical reaction, as expressed by k, is a function of the concentration of the reactants. If the concentration of a reactant (i.e., preservative) is changed as a result of storage at elevated temperatures for different times, determining D-values and using 2.303/D-value enables one to determine k at different temperatures. These k values may be compared with k values obtained in systems of known

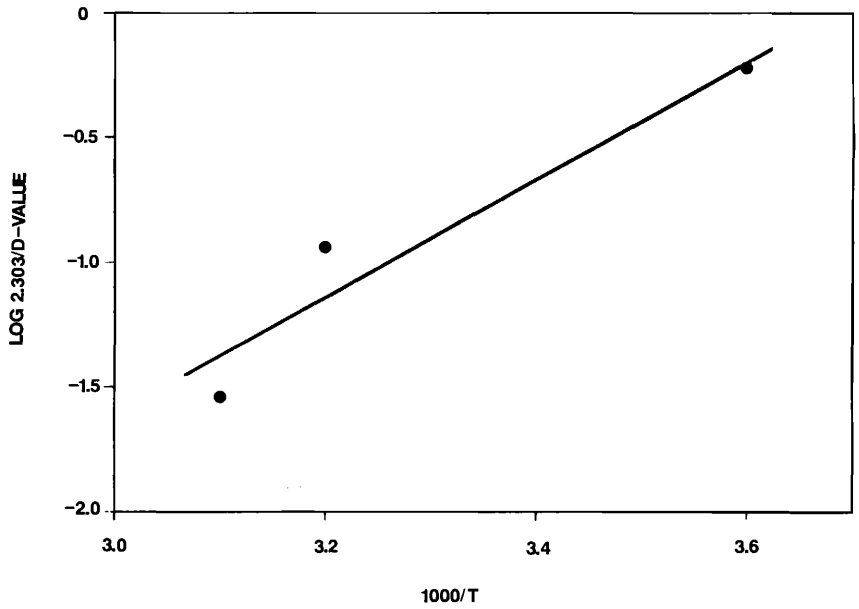

Figure 8. Arrhenius activation energy plot of shampoo preservative system potency determined using *E. coli* as the challenge organism in preservative efficacy tests on samples stored at 3°, 38°, and 49°C for 12 months.

preservative molar concentration, so that one may determine the molar concentration (or the apparent molar concentration) of active preservative in the formula after any given time and temperature of storage. Calculations of this type may be useful in systems containing only one preservative chemical. Obviously, calculations will be quite complex in products containing multiple surfactants and preservative chemicals because the loss of preservative potency may follow higher-order reaction kinetics. It is believed that determining D-values and Ea′ values may be useful in studying the kinetics of bacterial death, in determining the apparent molar concentration of a preservative, and in monitoring the performance of cosmetic preservative systems during stability studies.

In discussing accelerated stability testing, Pope used Ea values of 10–20 Kcal/mole for predicting good probability of formulation stability, which he defined as one that degraded no more than 10.5% in 3 mo. at 45°C (18). He noted that formulations that degrade through solvolysis have Ea values of 10–30 Kcal/mole and that systems with Ea values of this magnitude show marked increases in reaction rates at elevated temperatures. The findings in this study revealed that the unstable shampoo preservative system had Ea′ values of −2 to −16 Kcal/mole, depending on the time period of the determination and the test organism used. It is believed that the difference between the Ea values proposed by Pope and the Ea′ values observed in this study (including both absolute magnitude and algebraic sign) may be due to 1) the difference in k for the solvolysis reactions cited by Pope and complexation reactions, such as those involved in the interaction of isothiazolinones and amines (15); and 2) the way in which Ea and Ea′ were defined and derived.

The shampoo preservative system was satisfactory when examined at the outset of these studies (i.e., at 0 mo.), but it deteriorated during the aging study. Hence, the Ea′

values for the shampoo decreased with time. By way of contrast, determination of D-values and Ea′ values for the test organisms in a proprietary lotion preserved with MP and Quaternium-15 revealed no changes in these values for test samples stored at 3° to 49°C for the duration of the stability study. This indicated that the preservative system of the lotion was not changing detectably, as evaluated by the linear regression method. These examples illustrate a useful caveat: the Ea′ values should deviate little from the initial, acceptable values if a product is to have satisfactory shelf life.

These studies demonstrate that the linear regression method is useful for quantitating the rates of inactivation of bacteria inoculated into stability test samples and illustrate the value of this method for monitoring preservative efficacy of stability test samples. To our knowledge, this is the first report on the use of a quantitative method, which provides via the kinetics of bacterial death, a method to monitor preservative potency at various times during stability studies of cosmetic products.

In most instances, preservative efficacy testing demonstrates that the cosmetic preservative system inactivates test organisms rapidly and that the D-values are positive. The preservative system is judged to be inadequate when D-values are greater than acceptance criteria (4) or when they are negative. For example, the preservative system fails when test organisms grow in the sample. The slope of the survivor curve is positive when growth occurs, which means that the D-value is negative. Negative D-values are rarely, if ever, reported in published literature because they indicate preservative system failure and the need for reformulation.

Although negative Ea′ values appear to be somewhat anomalous, one must keep in mind that they represent a decrease in potency of the preservative system that occurs on storage and that the rate of preservative deterioration is accelerated by increases in temperature. As noted above, negative D-values are obtained when test organisms grow in the test samples. Negative D-values cannot be used in calculating Ea′ values because one cannot take the log of a negative number.

This report shows that the linear regression method may be used for predicting the stability and the apparent molar concentration of a preservative system in addition to its already-documented utility in determining the cosmetic preservative efficacy. Thus, examination of the preservative efficacy of a formula after storage at 49°C for 1 to 3 mo. may indicate the system is unstable and that reformulation is necessary. It is recommended that formulation chemists quantitate preservative system potency during accelerated aging studies to determine the likelihood of preservative system failure.

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