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Stability Testing of Cosmetic Products

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INTRODUCTION

Products formulated by cosmetic chemists are intended to perform a variety of ''miracle'' functions, such as reshaping hair, delivering fragrance, smoothing and softening skin, imparting color to the face, and cleansing the entire body. Chemists can deliver many of these miracles by using the variety of technologies described elsewhere in this book. In using these technologies to develop products, chemists must be aware of formulation issues that might prevent the product from performing optimally. Assessing product stability is a critical part of this formulation process. This chapter discusses the basic principles of stability testing of cosmetic delivery systems. We will begin with a general definition of stability testing and move on to problems encountered by specific formula types. We will conclude this section with a discussion of stability issues that are not necessarily directly related to the formulation, such as processing and packaging.

A PRACTICAL DEFINITION OF STABILITY TESTING

Stability testing may be defined as the process of evaluating a product to ensure that key attributes stay within acceptable guidelines. In order to make this testing meaningful, it is important to accurately establish the nature of these critical product attributes, to measure how they change over time, and to define what degree of change is considered acceptable. Defining which parameters are crucial requires a combination of chemical knowledge about the formula and common sense about product usage. The chemist should be aware that cosmetic products must not only continue to function over time but must also look, feel, and smell the same each time the consumer uses them. Therefore, testing must evaluate esthetic characteristics in addition to functional properties. This is an important consideration because cosmetic products can change in a number of different ways, which may affect consumer perception. For example, fragrances become distorted, colors may fade or darken, and consistency may change, resulting in a thicker or thinner product. Chemists must determine which of these product characteristics will change over time and design appropriate testing to measure the extent of the changes. Nacht cites several technical issues to be considered, including compatibility between the delivery system and the active ingredient, compatibility with the overall formula, appropriate mechanism of release for

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the particular application, the rate of release of the active ingredient, and overall safety for the user [1]. This chapter discusses some of the key tests that the chemist can use to measure the changes in these characteristics. An important fact to remember is that no product remains unchanged forever. Depending on the intended use of the product and its anticipated shelf life, a small change over time may be inconsequential or devastatingly detrimental. In general, if a change is consumer-perceptible, the product may not be considered stable.

USEFUL INFORMATION PROVIDED BY STABILITY TESTING

Stability data are useful as an ''early warning system'' that can alert the chemist to potential formulation/package-related problems. Such advance information can be helpful in many ways.

Guiding the Chemist During Product Development

While you are formulating a product, preliminary testing of its stability can guide you in making modifications to ensure that it is stable. If you determine, for example, that an emulsion shows separation after exposure to freeze/thaw conditions, you may elect to modify the surfactant system to correct the problem and then repeat the test on the modified formula to determine whether it performs better or worse. Preliminary stability test data are an important parts of the trial-and-error development process.

Ensuring That the Product Will Continue to Be Esthetically Acceptable to the Consumer

More than other products, cosmetics are intended to be esthetically pleasing to the consumer. For this reason consumers are likely to notice subtle changes in the odor or appearance of their favorite products. Since no product remains 100% unchanged as it ages, it is critical that the chemist anticipate the changes that may occur and make sure that they stay within limits that are not consumer-perceptible. Stability testing allows you to see how the product will behave over time.

Determining That the Product Will Perform as Intended and Remain Safe to Use

Studying the performance of samples that are exposed to accelerated aging lets you assess how the product will function over time. This is particularly important for cosmetic products that use the technologies described in this book to deliver ''active'' ingredients. If the formula is not stable, the delivery of the active ingredient may be impaired. Take, for example, the case of an antiperspirant stick with an encapsulated fragrance that is released upon exposure to moisture and heat. If the delivery system is poorly designed, the fragrance may be released too soon or not at all. Properly designed stability testing can reveal such problems so that corrective action can be taken.

Forewarning the Company About Problems That Might Occur After Consumer Purchase

For example, testing can show that the product may thicken somewhat over time and may be difficult to dispense from the package. Realizing this beforehand is important to the company because it will allow the company to anticipate consumer reaction.

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Even though stability testing provides much useful information, it is not an exact science and will not guarantee a trouble-free product, but it can give an idea of the risks involved and help provide a solid scientific foundation for evaluation of future problems.

STABILITY TEST DESIGN

When faced with a situation where testing might be appropriate, ask some basic questions about the task ahead.

Why Is Testing Being Done?

Why is testing necessary? Are you concerned with product appearance or do you want to determine if specific performance characteristics change over time? The reasons for doing the tests will determine what kind of tests are required. Therefore it is critically important to approach this testing with a scientific mind set and to have a clearly defined hypothesis to be tested. Take, for example, the case of a skin lotion formula that develops an unpleasant odor. The reason for the test is to determine what is causing the odor. Your hypothesis may be that the fragrance you have selected is reacting with the formula ingredients to cause this problem. To test this hypothesis appropriately, you will need to assess the odor of the unfragranced base to determine how the fragrance affects the overall smell of the product. In this example, the unfragranced samples are the controls because the fragrance, which is the scientific variable, has been removed. Evaluation of appropriate control samples can prove or disprove the hypothesis—i.e., that the fragrance is causing the problem.

Another example illustrating the importance of conducting a properly controlled study is the case of an emulsion that separates after prolonged storage in its plastic bottle. In this case the reason for the test is to determine what is causing the separation. One hypothesis may be that the package is allowing water vapor to escape, thus leading to emulsion instability. To test this hypothesis, you will need to screen out the variable of concern: the packaging. Therefore, control samples could be packaged in glass to eliminate the possibility of moisture loss. If the control samples do not show the same instability that the packaged samples show, you have demonstrated that the packaging material is indeed having a negative effect on the product.

Finally, consider a case where the variable of interest is the viscosity of the product. If you are concerned that the product may become too thick over time and will not dispense properly, you could design a study to track product batches with varying initial viscosity. Suppose the target viscosity is 20,000 cps. You could monitor the viscosity of a series of batches with viscosities ranging from low to high. You may make batches which are initially at 5000, 10,000, 15,000, and 20,000 cps, respectively. You would then monitor the viscosity of these batches as a function of time and temperature. You may learn that viscosity does not change significantly from the initial value, which means that a very narrow specification will be required. In other words, the product must be very close to its final viscosity when it is produced. On the other hand, you may discover that as long as the initial viscosity is between 5000 and 15,000 cps, the product will build to 20,000 cps within 2 weeks and stay at that level for 2–3 years. In this case your specification can be rather broad, since—regardless of the initial value—the consumer will only be exposed to product that is 20,000 cps. In all these cases, understanding why the test needs to be done helps you establish appropriate controls, which are essential if meaningful test results are to be obtained.

What Is Being Tested?

Another important factor to understand is the status of the formula being tested. Is it a developmental prototype or the final production material? Consider a situation, as in the example provided above, where you are primarily concerned with the change in product viscosity. Furthermore, consider that the final color and fragrance of the product have not yet been firmly established, although there are several candidates under evaluation. You could prepare samples with every possible color/fragrance combination and measure their viscosity over time. This could involve thousands of samples and tens of thousands of measurements, which are both costly and time-consuming. So, bearing in mind that you are testing a prototype and not a finished product, you may instead opt to test the uncolored, unfragranced base formulation first. In this way you can expeditiously get data on the parameter of interest—in this case viscosity. By evaluating prototypes early on, you have given yourself more time to react to problems. Of course, the testing may have to be repeated once the final formula is established because the fragrance may affect viscosity. Similarly, if the final production package is not yet available, you may choose to evaluate formula stability in a packaging material that approximates the characteristics of the final container. Here too, the final formula and package combination must eventually be tested together, because the formula may interact unfavorably with the package. Asking the ''what'' question will help make your testing meaningful without forcing you to go to excessive lengths.

Where Will Test Samples Be Stored and How Many Are Necessary?

Ideally, you could gain information on formula stability by performing exhaustive tests on every variable involved in every formulation you work with, but this is not always feasible, because proper testing requires a significant commitment of time and resources. Therefore, most companies have standardized test procedures for the storage of stability samples which depend on the objective of the study. Such procedures involve evaluations of samples stored at a variety of conditions and include enough samples to be statistically significant. Usually sample storage is done at elevated temperatures, under freeze and/or freeze thaw conditions, and with exposure to various types of light. Elevated temperature storage is critical, since the rate of chemical reactions roughly doubles for every 10°C increase in temperature. Storage at higher temperatures allows you to accelerate the aging process and to see certain problems much sooner than they would appear at room temperature. Of course, the potential drawback is that, at high temperatures, you may be forcing reactions to occur that would not happen at all at lower temperatures. Cold storage evaluates conditions that may negatively affect the solubility of ingredients or stability of emulsions. Sunlight and ultraviolet (UV) light exposure can reveal problems with ingredients that are reactive to the respective wavelengths; fragrances and colors are particularly sensitive in this regard. The most common storage conditions used in this industry are 54°C or 50°C, 45°C, 37°C or 35°C, room temperature (25°C), 4°C, freeze/thaw, and exposure to fluorescent and natural light.

Since many of the tests that must be conducted to evaluate product performance will affect the sample physically (e.g., spraying an aerosol can), multiple samples are required at each storage condition to ensure there will be enough samples left for evaluation at the end of the test period. Depending on the protocol set by your organization, as

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many as one hundred or more samples may be required for a complete study. Again, you should follow your corporate guidelines to make sure that sample quantities will be enough for a thorough evaluation of all necessary conditions.

How Samples Are Evaluated and What to Look for—Identification of Instability

How samples are evaluated depends entirely on the type of product and the nature of the problems that might occur. Instability is typically identified by evaluating various product characteristics either by subjective observation of properties—such as color, odor and appearance—or by objective instrumental evaluation of pH, viscosity, particle size, and electrical conductivity. For instance, simply looking at a sample that has been stored at accelerated temperatures can often reveal significant changes such as color changes, emulsion separation, or rheological changes. Similarly, a quick olfactory evaluation can uncover major flaws in fragrance stability. More rigorous characterization of product attributes can be obtained instrumentally—for example, with a viscometer or pH meter. These instruments are highly sensitive and can distinguish small changes in products. Such changes are important to note since, as in the case of a change in pH, they may represent chemical reactions that are occurring in the formula.

Other specialized testing can be performed to quantify specific changes in formulated systems. For example, microscopic evaluation and light scattering are used to appraise changes in particle size and distribution of emulsions. A Coulter counter is also used for these determinations [2], as are conductivity measurements [3]. Nuclear magnetic resonance (NMR) and x-ray crystallography can also be used to reveal additional information regarding emulsion structure. In certain systems, specific assays are performed to measure the activity of functional ingredients. These types of tests are tailored for the compound in question. For instance, the bactericidal efficacy of preservatives or other antimicrobial compounds may be measured over the course of a stability test. In addition, chromatographic tests, spectroscopic measurements, titrametric evaluations, and other wet chemical methods can be used to detect signs of instability. Other indications of instability include incompatibility of product and package, which can lead to weight loss and package degradation (such as softening or cracking of container walls, clogging of orifices, corrosion of metal parts, etc. [4]). But perhaps the most important question to ask in assessing instability is to determine how much change is acceptable. Knowlton and Pearce have stated that a useful rule of thumb is to consider product rejection if the attributes being measured deviate by more than 20% of their original value [4]. This value is an interesting reference point; however, for some formulations, much smaller deviations may be critical. The impact of such changes must be assessed on a case-by-case basis.

SITUATIONS THAT REQUIRE STABILITY TESTING

A good chemist should have an understanding of factors that are critical to product stability, so that appropriate testing can be conducted when necessary. Situations in which stability testing is generally necessary include but are not limited to the following situations: consideration of a new formulation, qualification of new raw materials, evaluation of new manufacturing processes, and testing of different packaging components. As you will see, stability testing is not a finite, one-time task; instead, it is an ongoing, dynamic

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process that begins when the product is being developed and continues to evolve as the formula, packaging, or manufacturing processes change.

FORMULA-RELATED REASONS TO STABILITY TEST

Specific Considerations Related to Development of Particular Formula Types

The process of stability testing a product is closely tied to the process of creating the formulation. As you develop formulations, you should always screen stability samples early in the process to make sure that your efforts are headed in the direction that will lead to a stable product. Every formula will have slightly different stability testing requirements, but for the sake of this discussion, we will give primary consideration to the types of cosmetic delivery systems detailed in this text.

Emulsions

Emulsions are among the most common types of delivery systems used for cosmetic products. They enable a wide variety of ingredients to be quickly and conveniently delivered to hair and skin. While many definitions of emulsions have been proposed, we will define them as heterogeneous systems in which at least one immiscible or barely miscible liquid is dispersed in another liquid in the form of tiny droplets of various sizes [5]. Consequently, these systems are inherently unstable and eventually, given enough time or energy, will separate into separate phases.

Emulsions used for cosmetic products are typically semisolid materials composed of an oil (hydrophobic) phase and a water (hydrophilic) phase. These phases are characterized as either the internal phase or external phase, depending on the overall composition of the emulsion. The internal phase is that which is contained inside separate discrete particles surrounded by surfactants; these particles are known as micelles. The external phase is the ''solvent'' or diluent, which surrounds the micelles. Usually, the external phase is the more abundant one. Depending on the composition of each phase, simple emulsions can be either oil in water or water in oil, the type of which depends specifically on what emulsifier is used.

Although the internal-phase particles of an emulsion are polydisperse (meaning they have various sizes), their average size is often used for emulsion classification [6]. When the average diameter of internal particles is less than 100 Å, the system is called a *micellar emulsion*. A particle diameter of 2000 to 100 Å is called a *microemulsion*. Larger particles produce macroemulsions, which are the most common types found in cosmetic formulations. More complex emulsions can have multiple internal phases. These emulsions, called *multiple emulsions*, can be oil in water in oil or some combination. For cosmetic applications, they are formed by first making a water-in-oil emulsion and then mixing that emulsion with a water phase. These types are particularly useful for encapsulating materials giving prolonged release when applied to a surface such as skin [7].

Stability Considerations

Since emulsions represent a mixture of two or more materials that are not miscible in each other, they are, according to the second law of thermodynamics, inherently unstable. This means that eventually the two phases will separate. The degree and speed of instabil-

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ity are quite variable. For example, a mixture of mineral oil and water when shaken will form a macroemulsion, which immediately separates upon standing. Other emulsions can remain stable for years, but eventually all emulsions will separate. While the second law of thermodynamics suggests that emulsions will separate over time, it does not provide a mechanism of this destabilization. Investigation into how emulsions destabilize has revealed three primary processes leading to instability: flocculation, creaming, and coalescence [8].

Flocculation

This process is characterized by a weak, reversible association between droplets of the emulsion's internal phase. Each individual droplet maintains its own identity; thus there is no change in the basic droplet size [8]. Flocculation represents a less serious sign of instability, which can be reversed by shaking the system [9].

Creaming

When particles of an emulsion aggregate, there is a tendency for upward sedimentation. This causes a partial separation of the emulsion into two emulsions, one of which is richer in the internal phase and the other richer in the external phase [9]. As in the case of flocculation, this stability problem can be reversed by agitation.

Coalescence

An aggregation between two particles can, if the two particles combine, lead to the formation of one larger particle. This process, known as coalescence, represents a more serious stability problem. A related phenomenon is that of Ostwald ripening, in which the particles all tend to become the same size. Both of these processes are irreversible and can eventually lead to complete separation of the internal and external phases of the emulsion [10]. An alternative consequence of these forms of instability is phase inversion, in which the internal phase becomes the external phase and vice versa [9]. For stability considerations, this change is typically undesirable, since it will change the physical properties of the product.

All emulsions are potentially subject to all of these destabilizing processes simultaneously, and the resulting effects on any given emulsion will vary. For example, microemulsions and micellar emulsions are initially transparent. Over time, the size of their internal-phase particles may increase, and they will develop translucent appearance. Since macroemulsions are opaque, a similar change in appearance will not be notable; however, there may be changes in viscosity and measurable separation. Multiple emulsions are typically less stable than monoemulsions. Over a short period of time, the number of multiple emulsion particles tend to be reduced. This results in the ''leaking out'' of some of the encapsulated material and reduces the duration of prolonged release.

In addition to the inherent processes that destabilize emulsions, other factors may be involved. Storage temperature has been shown to affect emulsion product stability. Generally, elevated temperatures result in destabilization, while reduced temperatures improve emulsion stability. Aqueous-phase evaporation may also contribute to instability over the life of a product. Microbial contamination can also cause a breakdown of emulsion stability. Finally, chemical reactions within the emulsion can lead to a change in the stability of the emulsion. While these types of reactions can be initiated by temperature increases, they can also be prompted by UV light or other types of electromagnetic radiation.

VESICULAR SYSTEMS—LIPOSOMES AND NIOSOMES

Definition/Description

Vesicular systems encompass a number of delivery technologies, including liposomes and niosomes. Both of these systems employ a ''vessel'' to contain active ingredients within a formula and to provide controlled delivery of these ingredients. Nacht defines controlled delivery as a ''system that would result in a predictable rate of delivery of its active ingredients to the skin'' [1]. Liposomes are a classic example of this technology, in which phospholipids are used to create lipid ''capsules'' that can be loaded with various ingredients. Although liposomes are enjoying tremendous popularity in cosmetics today, they have their roots back in the early 1960s. At that time Professor Bangham, at the Institute for Animal Physiology in Cambridge, U.K., was one of the first to speculate that lipids such as phosphatidyl choline could be used to create sealed vesicles with bilayer membranes similar to cell membranes [1]. Niosomes are another delivery technology related to liposomes; the difference is that, unlike liposomes, niosomes are based on nonionic surfactants. L'Oréal pioneered the development of nonionic liposomes using nonionic surfactants such as polyoxyethylene alkyl ethers combined with fatty alcohols or fatty acids [1].

Stability Considerations

Liposome and niosome stability may be referred to in terms of leakage of contents, presence of oxidation products, or changing particle size due to aggregation formation and fusion. They are rather fragile capsules, and certain precautions must be taken to make sure that they remain intact and are able to deliver their contents. Leakage can be caused by mechanical forces like high-shear processing, which should be avoided. Similarly, excessive heat, which may destabilize the lipid bilayers, should be avoided. Perhaps most notably, liposomes may be solubilized by surfactants that may be present, and therefore they are not suitable for use in detergent systems. This is particularly true of systems such as shampoos and body washes, which contain strong anionic surfactants that can dissolve the lipid walls. In fact, even though liposomes are often used in creams and lotions, the emulsifiers used in these formulas may also be enough to disrupt the fragile walls. For these reasons, many formulators believe that gels are the ideal vehicle for liposomes because they lack the high HLB (hydrophilic lipophilic balance) surfactants present in many conventional emulsions, which might disrupt the lipid bilayers [10]. There is hope for using liposomes in emulsion. K. Uji et al. report that stable liposome suspensions can be prepared by using a cross-linked acrylic acid/alkyl acrylate copolymer at very low concentrations, because it can effectively stabilize lecithin liposomes in o/w emulsions [11]. Furthermore, there is some evidence in the patent literature that the addition of collagen, albumin, or gamma globulin to the liposomes can decrease the harmful effects of detergents [10].

In addition to leakage, vesicle systems may fuse together and no longer be available as discrete units for the delivery of active agents. According to Weiner, such fusion can occur for several reasons, including preparation below their transition temperature, the presence of contaminants such as fatty acids and divalent cations, changes in pH, or the addition of nonelectrolyte hydrophobic molecules [12]. Furthermore, phase separation of bilayer components can occur upon extended storage. In an excellent review on the subject, Fox refers to an article by Crommelin et al., that reports on preserving the long-term

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stability of liposomes. Crommelin discusses the chemical pathways by which phospholipids can degrade: by hydrolysis of the ester groups or oxidation of the unsaturated acyl chains. This research points to an optimal pH for liposome stability. For phosphotidylcholine liposomes, the pH for the lowest hydrolysis rate was found to be 6.5. The stability of liposomes was further enhanced by using phospholipids with fully saturated acyl chains (like those made from hydrogenated soybeans, so the opportunity for oxidation is reduced) [10]. Similarly, liposomes may be stabilized by sugar esters, for example, maltopentose monopalmitate have been used to improve stability of cosmetic systems [13].

For a more detailed discussion of the morphology of liposomal bilayers, we refer the reader to Liposomes: From Biophysics to Therapeutics [12]. The author provides an excellent discussion of the elastic properties and tensile strength of liposomes as well as the effect of solvents and osmotic effects on liposomal structures.

MOLECULAR CARRIERS

Definition/Description

Molecular carriers represent a delivery system in which one compound is used to bind another compound to a substrate, thereby changing the former's characteristics. This allows the bound material to be delivered to a surface and released when conditions are appropriate. One example of this type of technology is cyclodextrin chemistry. Cyclodextrins are created from starch-derived glucopyranose units and are classified as cyclic oligosaccharides. When formed, they contain a hydrophobic cavity capable of entrapping molecules of different sizes, shapes, and polarities. Molecules entrapped as such are found to be more resistant to environmental stresses and therefore more stable [14]. They can be used to entrap various types of compounds such as fragrances, vitamins, pigments, and dyes. Cyclodextrins have been used in cosmetic products for a variety of reasons, such as to reduce odor in mercaptan-containing systems [15], improve the stability of hair dyes [16], and as an active ingredient to treat acne [17].

Stability Issues

The complex of the cyclodextrin with a guest molecule is typically quite stable under ambient temperatures and dry conditions. However, in the presence of certain materials the guest molecule can be prematurely displaced thereby reducing the effectiveness of the delivery system [18]. This factor is of major concern when developing and particularly when assessing the stability of a formula.

PARTICULATE SYSTEMS—MICROCAPSULES, BEADS, AND MICROSPHERES

Definition/Description

Microcapsules are one of the oldest controlled release technologies. They were developed to produce carbonless carbon paper and are composed of a core with the active ingredient surrounded by a shell, analogous to an egg. Microcapsules may have a multilayer construction with multiple cores containing the active. The active ingredients are released either by rupture of the capsule walls or by diffusion/permeation of the contents [1]. Fairhurst and Mitchnick list a range of materials that are typically used in this regard including

adhesives, drugs, colors, fragrances, flavors, agricultural chemicals, solvents and oils. Classic shell materials include gelatin or gum arabic, cellulosic polymers, or synthetic polymers [19]. Starch based capsules are often used to deliver fragrance and cosmetic ingredients.

Beads and microspheres are small solid particles onto which other ingredients can be adsorbed for later delivery. Nylon particles, for example, are useful for delivery of certain active ingredients. Antiperspirant salts are said to be more efficacious when delivered via nylon spheres, and the esthetics of the product are said to be improved. Coloring agents may be delivered in this manner as well; Schlossman discloses a patented method (U.S. patent 5,314,683) of coupling cosmetic pigments to microspheres to provide uniform reflectivity, improved dispersion, and superior viscosity characteristics [10]. Tokubo et al. describe a process for preparing spherical hectorite particles, with a diameter of about 100 Å, which can be used to deliver glycerin and solid pigments such as titanium dioxide, zinc oxide, and ferric oxide.

Stability Considerations

Microcapsules are somewhat fragile physically and care must be taken to avoid premature rupture and release of the contents. Excessive temperature should be avoided by adding microencapsulated ingredients late in the manufacturing process. Likewise, refrain from formulating with materials that may act as solvents on the capsules walls. Finally, avoid high-shear processing, such as milling and homogenizing, which can physically disrupt the capsules. Additional techniques for enhancing the stability of microcapsules can be found in the technical literature. Fox refers to an interesting Shiseido patent for improving the stability of gelatin microcapsules by coating the surface of the capsule with a basic amino acid or its polymer [10]. In general, microcapsules are a stable, efficacious method of delivering chemicals in cosmetics. In fact, when properly formulated, microcapsules can actually enhance stability of systems by protecting the ingredients they carry from external forces. For instance, in an example provided by the Mono-Cosmetic Company, ascorbic acid particles are coated with silicone or a polymer—e.g., ethyl cellulose, to protect the ascorbic acid against oxidation [10]. Similarly, in delivering cosmetic materials via beads and microspheres, care must be taken not to disturb the matrices physically. As with microcapsules, excessive shear can be a problem, for if the capsules are broken, their ability to retain the ingredient to be delivered will be impaired.

GENERAL CONSIDERATIONS RELATED TO FORMULA MODIFICATION

Regardless of which delivery technology you choose to utilize in a formulation, there are certain fundamental stability considerations that you must deal with. For each of the technologies discussed above, factors such as raw material sources, manufacturing process, and packaging composition all play a role in product stability.

Raw Material Substitution

Often it becomes necessary to substitute one raw material for another similar material. This frequently occurs because a supplier discontinues one of the raw materials used in your formula. In exchange, a different, yet supposedly ''identical,'' material may be offered. Depending on the chemistry of the materials involved, there is no way to anticipate if such a change will affect formula stability. Therefore, in such situations you must con-

duct testing to ensure your formula will remain stable. Similarly, you may wish to substitute another material that is cheaper but is not anticipated to function differently. For example, in a shampoo formula, you may substitute sodium lauryl sulfate for ammonium lauryl sulfate. Given the functional similarities between the two, you would not anticipate significant problems; nonetheless, some degree of stability testing would be prudent.

Alternate Vendor Qualification

You may also elect to qualify alternate raw material suppliers for ingredients in the formula. It is desirable to have secondary sources for most raw materials to ensure a steady supply and competitive pricing. Unfortunately, even though raw materials from different suppliers may have the same CTFA (Cosmetics, Toiletries, and Fragrance Association) designation, they may not be chemically identical, because chemical feedstocks and processing conditions vary between suppliers. Therefore, a raw material from one supplier cannot always be automatically inserted into a formula developed with a different supplier's raw material. The impact of even seemingly inconsequential change in raw materials must be established by stability testing.

NON–FORMULA-RELATED REASONS

Processing Issues

In addition to the formulation and raw material issues described above, there are processing issues that can affect product stability. For example, stability testing is typically required the first time a new formulation is made on a large scale. This is because the way in which the product is made on a large scale can have a dramatic effect on its stability. This is particularly true of emulsions, because the energy used in processing determines particle size and distribution, which helps determine product stability. The only way to fully assess the impact of the chosen manufacturing method on product stability is to evaluate samples made under actual production conditions. This may require that a trial production batch be made prior to commercialization of the formula. At the very least, stability testing should be done on the first production batch of any new product, so that the impact of actual production processing conditions may be evaluated.

Once a manufacturing process has been shown to be successful, any changes to that process may require additional testing. Alterations in the order of raw material addition may be necessary to reduce processing time; changes in heating and cooling rates may occur due to differences in heat transfer in large batches; and different mixing conditions will all affect the amount of shear the product experiences. Any one of these changes will cause stability problems.

Packaging Issues

Even with the formulation and manufacturing processes held constant, variations in packaging material can cause problems that require stability testing. Not all packages are created equal: glass and plastic behave differently, and different kinds of plastic vary in properties such as oxygen permeability, color fastness, and thermal resistance. Certainly a new combination of formula and package should be tested, and even a change in an existing packaging material or the supplier of that material merits evaluation. The stability of aerosol systems, for example, is extremely package-dependent, since the package com-

position will help to determine how resistant the final product is to corrosion. The overall objective is to be alert for changes to the formulation/manufacturing/packaging system that may necessitate additional testing, so that you can be confident that your product will remain stable. Of course, your observations should not be limited to the formula itself. Changes that result from formulation and packaging interaction may be critical to total product integrity. To this end, weight loss, changes in plastic color and odor, and other package-related observations are important. The objective is to gain as much knowledge as possible regarding the behavior of the product over time.

CONCLUSION

This chapter is intended to provide insight into the issues associated with the stability testing of cosmetic products. For the beginning chemist, we stress the importance of careful, methodical observation to ensure that as many stability problems as possible are identified. For the veteran formulator, we urge periodic review of the latest technical literature so that it will be possible to keep pace with new developments in stabilizing the specific delivery systems discussed in this book. Hopefully the references we have provided will be helpful in this regard.

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Stability Control: Microbiological Tests

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MICROBIOLOGICAL CONTROL OF RAW MATERIALS

Microbial Health Hazards by Contaminated Products

The microbial spoilage of cosmetics has been reported in the literature for many years [1–3]. One of the first reported incidents [4] is the death by tetanus of four babies in New Zealand in 1946, the vector being a contaminated talcum powder. The same vector was the source of two other cases of tetanus in an English hospital [5]. Since the 1960s, cases of cosmetic-induced infections were described in parallel with the awareness of the problem for topical drugs [6–12]. The isolated organisms were Gram-negative bacteria from the genus *Klebsiella*, *Enterobacter*, *Serratia*, and *Pseudomonas* [13,14]. The organism *Pseudomonas aeruginosa*, a particularly virulent hospital pathogen transmitted by eye cosmetics, led to cases of infections and even blindness [15–20], or folliculitis from sponges [21]. Studies were then conducted to evaluate the importance of the problem [22–29] and to investigate the primary contaminating sources such as raw materials, personnel, water, and packaging, as well as secondary sources, such as the consumer [30].

Sources of Contamination

These can be divided into three groups [11,28,31,32]:

1. The microbiological quality of raw materials, including water;
2. The manufacturing process; and
3. The galenical form (which is made with vegetable and/or animal extracts) of the product.

Microbiological Quality of Raw Materials, Including Water

Their quality depends upon their origin. Raw materials from animal or vegetable origin can be heavily contaminated with 10^6 or even more organisms per gram or milliliter [33–35]. Fecal bacteria are regularly identified. In contrast, synthetic raw materials are rela-

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tively free from contamination, with the exception made for some that have steps in their manufacture, such as kaolin, some sugars and vitamins, some synthetic surfactants (e.g., sodium lauryl ether sulfate [SLES]), or partially hydrated salts. A recent study in our laboratory (Boussard et al., unpublished data) showed that out of 188 different synthetic tested raw materials, only 48, or 25.5%, gave results higher than 100 organisms per gram or milliliter. The recovered organisms were bacilli or Gram-positive cocci. A microbiological testing program of the raw materials must be set up.

Water remains one of the most important contamination factors of a product. Species like *Pseudomonas*, *Achromobacter*, *Aeromonas*, or *Flavobacterium* are recovered from natural waters [36]. Softening or deionization treatments frequently alter the microbiological water quality. These systems must be well maintained and the water microbiologically treated, using, e.g., ultraviolet (UV) lamps or/and bacterial filtration to ensure optimal quality. Microbiological control of production water should be made at least each working day, and a validation program of the water quality set up.

Manufacturing Process

During the manufacturing process, contamination can occur through contact by the operators, the manufacturing equipment, and the air. The micro-organisms capable of contaminating a cosmetic from human sources are part of the rhinopharyngal, buccal skin, hair, hand skin, and, in some circumstances, intestinal floras. Among these, fecal streptococci, staphylococci, enterobacteria, and *Pseudomonas* have sufficient vitality to survive and even to multiply in a product.

The manufacturing equipment is also an important source of contamination, coming from maintenance materials (oils, greases), from poor cleaning and/or disinfection on a regular basis, and from product changeover. The design of the equipment is also participating in this process: a piece of equipment that cannot be totally emptied is critical; the equipment storage conditions must also be optimized to avoid product residues stagnant in the system. The design of cleaning in place (CIP) systems must be carefully evaluated: a CIP that leaves a small quantity of stagnant water together with diluted product will have a negative effect instead of a beneficial one.

Attention must be paid to the air quality of the manufacturing rooms. The number of workers and the importance of their movements contributes to 80% of aerial contamination [37]. Air conditioning contributes to 15% of this contamination, and the room structure (materials used) to 5%. It is thus necessary to fix acceptable levels for the biocontamination of the air and to control the air quality. According to the European Good Manufacturing Practices (GMPs) [38], the limits of the class D rooms should be used (200 organisms/m^3).

Galenical Form of the Product

A parameter of crucial importance in the microbiological stability of a formulation is its water availability, or a_w. This aspect will be discussed at the end of this chapter. Some processes, such as manufacturing at high temperature (e.g., lipsticks) can help to reduce or avoid bacterial contamination. Thus high-risk products are aqueous-based products containing raw materials from biological origin such as lotions, suspensions, creams, gels, and emulsions, especially if they are manufactured at room temperature.

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Establishment of Microbial Limits

For many years there have been discussions on whether total count would be sufficient to guarantee the microbiological quality of a cosmetic, or if the exclusion of specified microorganisms, pathogens, or potential pathogens would also be required. The current trend is to require quantitative and qualitative microbial limits. Acceptance criteria for cosmetics and control methods will be issued in the Seventh Amendment of the European Cosmetic Directive. Nevertheless, the acceptance criteria will be minimal criteria that fulfill the public health expectations, such as:

1. *Microbial limits for finished products*. Maximum 1000 organisms/g or mL, and absence of *Staphylococcus aureus*, *Candida albicans*, enterobacteria, and *Pseudomonas aeruginosa* in one gram or milliliter of the product. Exceptions are baby-care products, eye products, and products for intimate hygiene—maximum 100 organisms/g or mL, and absence of *Staphylococus aureus*, *Candida albicans*, enterobacteria, and *Pseudomonas aeruginosa* in one gram or milliliter of the product.
2. *Microbial limits for raw materials*. Maximum 100 organisms/g or mL, and absence of *Staphylococcus aureus*, *Candida albicans*, enterobacteria, and *Pseudomonas aeruginosa* in one gram or milliliter. Limits for water as raw material could be fixed at maximum 100 organisms/mL and absence of coliforms and *Pseudomonas aeruginosa* in 100 mL.

However, what must be the attitude of a manufacturer if one of the following germs is identified in a product: Gram-negative bacilli other than enterobacteria and *Pseudomonas aeruginosa*, staphylococci different from *Staphylococcus aureus*, or fecal streptococci? What is the significance of this regarding manufacturing hygiene? Are these organisms harmless? Furthermore, in addition to the human safety, it must be emphasized that contamination of products with nonharmful organisms can partially or totally destroy the product aesthetic (e.g., perfume, color) and can alter the product performance. The rise of these questions emphasizes the need of internal quantitative and qualitative microbiological safety margins and of a quality-assurance system.

Use of Validated Methods to Control Products and Water

Microbiological Control of Finished Products and Raw Materials

The method described here is based on the method for microbiological analysis of nonsterile pharmaceuticals in the 3rd edition of the *European Pharmacopeia* [39,40] and from a publication of a working party of the ''Fédération Internationale Pharmaceutique'' [41].

Sample Preparation. A 10% homogeneous solution or suspension of the product is prepared with a sterile neutralizing solution or a sterile buffered peptone saline solution at pH 7. The neutralizing solution is used in case of the presence of known or suspected antimicrobial substances in the product. The pH 7 solution is used in case of preservative-free raw materials. For nonsoluble products, 0.1% of tween 80 or heating at a temperature not higher than 40°C for half an hour maximum can help in the homogenization. The neutralizing solution is basically letheen broth (Difco) supplemented with various inhibitors of the preservatives or disinfectants. The 10% homogenate is then used to perform the bacterial and fungal counts and to investigate the presence of specified microorganisms. If, for technical reasons, the use of 10 g sample is not possible, 5, 2.5, or even 1 g can be mixed for a total suspension of 100 mL.

Validation of the Preservative's Inactivation. The efficacy of the neutralizing solution must be validated in order to avoid false-negative results. For this purpose, 1 mL of the preserved sample or 1 mL sterile normal saline is added to 9 mL neutralizing solution. The two tubes are mixed well and let to rest for 10 minutes. 0.1 mL of a mixed suspension of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027 at 10^4 bacteria/mL are then added to the tubes, which are mixed again. The colony-forming units in each tube are estimated. The difference in the results must be lower than $1/2$ log between the tubes.

Bacterial and Fungal Counts. From the 10% homogenate, an appropriate number of successive tenfold dilutions in the sterile buffered peptone saline at pH 7 are carried out. A plate count is then made by transferring duplicates of 1 mL of the dilutions in sterile Petri dishes, followed by the addition of 15 mL melted agar. Tryptic Soy agar is used for the bacteria and Sabouraud Chloramphenicol agar for yeast and moulds. For the bacterial counts the dishes are incubated at 30 to 35°C for 5 days, and for the yeast and moulds, 20 to 25°C for 5 to 7 days. The Petri dishes used for the fungal counts are also used to check the presence of *Candida albicans*.

Investigations for the Presence of Specific Microorganisms

1. Enterobacteria and other gram-negative organisms. One milliliter or 1 g of the 10% homogenate is mixed with 100 mL enterobacteria enrichment broth (EEB) and incubated at 35 to 37°C for 24 to 48 hours. Subcultures are then carried out on violet red bile dextrose VRBG agar dishes and incubated at 35 to 37°C for 18 to 24 hours. The colonies of presumptive Gram-negative organisms are then identified.
2. *Escherichia coli.* One milliliter or 1 g of the 10% homogenate is mixed with 100 mL Mac Conkey broth and incubated at 43 to 45°C for 18 to 24 hours. Subcultures are carried out on Mac Conkey agar dishes incubated at 43 to 45°C for 18 to 24 hours. The colonies of lactose-fermenting gram-negative organisms are then identified.
3. *Pseudomonas aeruginosa* and other gram-negative organisms growing on Cetrimide agar. One milliliter or 1 g of the 10% homogenate is mixed with 100 mL Tryptic Soy broth (TSB) and incubated at 35 to 37°C for 24 to 48 hours. Subcultures are carried out on Cetrimide agar dishes incubated at 35 to 37°C for 18 to 24 hours. The colonies are then identified.
4. *Staphylococcus aureus.* One milliliter or 1 g of the 10% homogenate is mixed with 100 mL TSB and incubated at 35 to 37°C for 24 to 48 hours. Subcultures are carried out on Baird Parker agar dishes incubated at 35 to 37°C for 18 to 24 hours. The black colonies are then identified.

Validation of the Sterility of the Media. Sterility of all the media must be checked. For example, sterile saline is used instead of the sample and the bacterial counts and the appropriate investigations for specific organisms are performed. No microbial growth must be recorded in this assay.

Validation of the Growth-Promoting Properties of the Selective Media. The following reference strains are incubated separately in TSB at 30 to 35°C for 18 to 24 hours: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and, *Escherichia coli* ATCC 8739. Each bacterial suspension is diluted to obtain around 1000 organisms per milliliter. The three suspensions are equally mixed together and 0.3 mL of the mixture (containing about 100 organisms of each strain) are used as the inoculum to

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perform the investigations for the specific micro-organisms. The organisms must be detected in the media used for this assay.

Microbiological Control of Water

The microbiological quality of water is of particular importance and can be checked quantitatively and qualitatively. For the quantitative determinations of a potential water contamination, 100 mL or 10 mL of water are filtered through bacteriological filters (porosity of 0.45 μm). After filtration, the filters are deposited on the surface of Tryptic Soy agar Petri dishes. Amounts of 1 mL and 0.1 mL of water are also incorporated in two melted tryptic soy agar for a plate count in duplicate. All the dishes are incubated at 30 to 35°C for 3 to 5 days. For the qualitative determinations, 100 mL of water are filtered through 0.45 μm sterile filters. The filters are laid down on sterile Mac Conkey Petri dishes for the coliform bacteria and on Cetrimide agar Petri dishes for *Pseudomonas*. These are incubated at 30 to 35°C for 3 to 5 days. Questionable colonies are identified.

CHALLENGE TEST FOR THE EFFICACY OF PRESERVATION

Aim of Preservation

It is generally accepted that adequate preservation of a finished product, with preservatives or based on active preservation of a formulation, implies that the product remains stable and safe during storage (shelf-life) and consumer use [1,42–46]. From a public-health point of view, preservation must avoid infection of the consumer, and for product-quality reasons it must prevent a deterioration of the preparation. It is especially important to point out that the use of preservatives must not mask a lack of hygiene during manufacture. It is thus imperious to manufacture any cosmetic product according to Good Manufacturing Practices (GMPs) [34] such as imposed by the 6th Amendment of the European Directive 76/768/CEE [47], and to reach at the end of the manufacture the microbiological quality level discussed earlier in this chapter. Furthermore, the challenge test to evaluate the efficacy of preservation must not be simply performed on a lot-per-lot basis. The test has to be essentially connected with each development phase of the preparation [48]. It must be as simplified as possible for routine use, easy to standardize, and reproducible. Moreover, the test method must be able to show the potential intrinsic antibacterial efficacy of a formulation and should thus be performed on each finished product in its intact original container as well. Indeed, changes in the composition of the preparation have a tremendous influence on preservation [49,50]. Even minor changes in perfumes or dyes can affect the global behavior of the product [2,51,52]. Moreover, the material of the container and its type of closure influences the efficacy of the preservation and the protection of the product during use [45,53–55]. Rubber closures are, for example, known to absorb some amount of preservative from a solution [56,57]. Shave foams are often presented in containers under pressure with a propeller gaze such as butane. These storage conditions can widely influence the survival of some aerobic contaminants. Moreover, refrigeration can alter the preservative efficacy [58]. The preservatives may be inactivated by the components of the product [59].

Activity Spectrum of a Preservative

The use of the word ''antimicrobial'' preservative raises the need to define exactly what kind of activity is needed for a preservative. What are the organisms of concern: bacteria,

fungi, viruses, or even spores? The scale of the activity spectrum is based on almost three parameters: (1) the survival, or even multiplication, of particular organisms in a wide range of products; (2) the pathogenicity of these organisms by the route of administration; and (3) the possibility to find effective chemicals at nontoxic concentrations.

Sporicidal action must not be considered because sporicidal chemicals are very rare (e.g., aldehydes are too toxic to be used in a cosmetic product at effective concentrations). Moreover, infectious problems induced by spore formers are very seldom, as previously discussed for the talcum powder in this chapter. Even if aerobic spore formers are often found in raw materials and finished products, according to Davis [13] they should not be a hazard to human health.

Virucidal action is not considered for cosmetics. These facts restrict the spectrum of a cosmetic preservative to bacteria and fungi. According to the most widespread opinion, a bactericidal and a fungicidal effect is needed so that the contaminating organisms accidentally introduced in the preparation will be killed. A bacteriostatic or fungistatic action could eventually be accepted to stabilize a preparation during the shelf-life of a unidose, nonsterile product. For the fungicidal and bactericidal actions, the concentration of the preservative must be toxicologicaly acceptable.

Test Organisms

As previously discussed, the range of organisms must contain bacteria and fungi. Within these we must find Gram-positive and Gram-negative bacteria because the structure of the bacterial wall influences the penetration and thus the efficacy of the preservating agent. For the fungi, representatives of the two fungal forms must be used, namely the vegetative yeast cell and the mould spore. The choice of species is directed by their skin and mucosal pathogenicity for cosmetics. Product degradation capabilities are also taken into account to choose the species. So among the Gram-positive species, *Staphylococcus aureus* is an important skin pathogen, as is *Pseudomonas aeruginosa* for the Gram-negative bacteria. This latter organism is also able to use many compounds, such as preservatives or even disinfectants, as a carbon source and is very adaptative in adverse environmental conditions even in pure water [60,61]. For the yeast, *Candida albicans* is a skin pathogen and *Aspergillus niger* is a representative of the degradation flora. The choice of strains for a standardized assay must be guided by the need to compare results obtained in different laboratories, and in this way culture-collection strains are chosen coming from the American Type Culture Collection (ATCC). The strains normally used are as follows: (1) *Staphylococcus aureus* ATCC 6538, (2) *Pseudomonas aeruginosa* ATCC 9027, (3) *Candida albicans* ATCC 10231, and (4) *Aspergillus niger* ATCC 16404. These strains are to some extent resistant to the antimicrobials, and some are also used for testing disinfectants or antibiotics. For a representative preservation-efficacy test, it is also recommended to add strains isolated from the environment, water, or contaminated products. These strains live in the vicinity of or even inside the product, are well adapted to adverse conditions, and are often resistant to preservatives or even disinfectants [62–64]. Nevertheless, after a few passages in culture media, this particular resistance can disappear. Precautions must be taken to avoid this, such as immediate storage in appropriate medium by deep freezing or in liquid nitrogen.

Test Conditions and Validations

The challenge test consists in an artificial contamination of the tested sample and counting of the survivors during a period of 4 weeks maximum. Even if several preservative efficacy

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tests exist as described in the USP23 [65], the Japanese Pharmacopeia [66], or the CTFA test [67–74], the general conditions of the test described here are those of the European Pharmacopeia [43], adapted from a Federation Internationale Pharmaceutique (FIP) working party publication [44]. Several points, such as validations and strain maintenance, are described here in more detail.

Maintenance of Microbial Strains

The cultures can be maintained as described in the CEN 216 PrEN 12353 document [75]. Stock cultures are maintained at a temperature below −18°C. To prepare the working culture, subcultures are originated from the culture stock by streaking onto adequate agar medium slopes. The second and/or third subcultures can be used as the working cultures.

Preparation of the Inoculum

The subcultures to be used in the test are plated on Petri dishes of suitable media, e.g., Tryptic Soy Agar (TSA) for the bacteria and Sabouraud Dextrose Agar (SDA) for the fungi. After adequate incubation—18 to 24 hours for the bacteria, 48 hours for the yeast, and 3 to 5 days for the mould—the cultures are collected with sterile, normal saline. The suspensions are then calibrated against a Mac Farland scale or by using any suitable calibration system. This calibrated suspension homogenized at a maximum ratio of 1:100 (0.2 mL in 20 µg or mL, for example) of the tested sample must give between 5.10^5 and 5.10^6 organisms per millilitre or gram. Such a high inoculum density is imposed not only by the counting technique of the survivors, or the ''plate count,'' but also by the importance of the logarithmic reduction asked for the products.

Test Conditions

The first day of the challenge test, the product and two controls—one comprising the tested product without preservatives and one of normal saline with 1% peptone—are inoculated with each microbial strain. A microbial count is immediately performed after homogeneization on this group of three vials. Counts are performed after dilution of 1 g or mL of the sample, with 9 mL of neutralizer. The neutralizing solution used is the same as in the first part of this chapter. Further dilutions are made in normal saline in order to perform a plate-count technique according to the estimate contamination. Sampling is performed in the same way for the preserved samples, after 2, 7, 14, and 28 days of storage of the inoculated product kept at room temperature in the dark or in its normal storage conditions.

To estimate the starting value 100%, the product effect must be evaluated on the inoculum. So, the inoculum level is estimated in a nonpreserved test product, if available, and compared with the level measured into normal saline containing 1% of peptone. If the following occur: (1) data obtained in the nonpreserved product are equivalent to those obtained in saline, and this value is chosen as the starting level (100%); (2) the data obtained in the product is $<$ or $=$ 1 log from the saline data, the value obtained in the saline control is chosen as the starting value; and (3) if the product data are $>$ 1 log from the saline control, this is an indication of product contamination and the test is invalid. The results of the test are expressed as logarithmic reduction versus time of the value taken as 100%.

Validation of the Contamination of the Sample

The contamination of the sample consists of a homogeneous incorporation into the sample of a single strain at a maximum ratio of 1% of calibrated suspension. Most of the time,

the inoculum is aqueous and dispersed in an aqueous phase; for some products, addition of tween 80 ou isopropylic myristate could be useful to homogenize the inoculum. In some cases, a dried inoculum suspended in isododecane is used to contaminate fatty products. It is indispensable to ascertain that the inoculum can homogeneously be dispersed through the product. This is nearly immediate for liquids but much more difficult for oily products such as creams or mascaras. A validation is thus performed using a nonpreserved product that is inoculated with the calibrated suspension and homogenized. At least three different samples are taken from the product and the results of the counts obtained for these samples are compared. The difference between samples must be less than 1 log.

Validation of the Neutralizing Solution

Because a neutralizing solution is used as first dilutant when counting the survivors, the efficacy of the neutralizing solution must be validated in order to avoid false-negative results. For this purpose, 1 mL of the preserved sample or 1 mL sterile normal saline are added to 9 mL neutralizing solution. The two tubes are well mixed and let at rest for 10 minutes. 0.1 mL of a 10^{-3} dilution of the calibrated suspension are then added and mixed to both tubes. The colony-forming units in each tube are estimated, and the difference in results between the tubes must be less than 1 log.

Interpretation of the Results

The criteria taken by the European Pharmacopeia for the topically applied product are a good base of discussion [43]. For bacteria, the recommended criteria (level A) are a 2 log reduction after 2 days, 3 log after 7 days, and no increase in the recovered bacteria after 28 days. For fungi, a 2 log reduction is requested after 14 days with no increase of the counts after 28 days. This requirement of no increase of the counts at the end of the test period is of particular importance. Indeed, even if the logarithmic reduction attained by a product is greater than the requirement, a regrowth of the organisms during the examination period is unacceptable. This would indicate that the micro-organisms are able to adapt their metabolic capacities to use the product, and its preservative in particular, as carbon source. In the European Pharmacopeia, it is also stated that, in justified cases, e.g., when adverse reactions could occur, level B criteria can be used to interpret the results. These are: for bacteria, a 3 log reduction after 14 days and no increase of the counts after 28 days; and for fungi, 1 log reduction after 14 days and no increase of the counts after 28 days.

DETERMINATION OF WATER AVAILABILITY OR a_w

Water availability (a_w) is defined as the water available for bacterial metabolism and is evaluated by measurement of the water vapor pressure at the surface of a product or a raw material. It can be defined as the following ratio:

$$A_w = \frac{\text{water vapor pressure over substance at t°}}{\text{water vapor pressure over pure water at t°}}$$

It depends on temperature and on formulations. It is not correlated with the total water content of a formula but depends of the quantity of water ''trapped'' into the formula chemicals. Ingredients such as humectants, gums, or others, use the water to swell and so this water is no longer available for bacterial growth. As water is a critical growth factor for micro-organisms, one of the means to preserve a formula is to decrease the level of water availability, optimizing a formula by the inclusion of ingredients that fix

the water. Most *Pseudomonas* cannot grow if the a_w is less than 90%, and under 70% the probability of microorganism growth in the product is lowered [76].

The a_w of a product is evaluated through the use of a moisture-sensing device that measures the head space relative humidity on the top of the product surface contained in a closed jar or dish after equilibration. This device must first be calibrated, using calibration standards. The standards are selected to represent low, medium, and high value operation or to bracket the area of interest. In general, the standards are saturated salt solutions such as NaCl ($a_w = 0.75$), $BaCl_2$ ($a_w = 0.90$), and LiCl ($a_w = 0.11$). As the a_w measurement is temperature dependent, it is recommended to perform the calibrations and measures at controlled room temperature. Table of commonly used standards and their temperature variations can be found in Ref. 77.

CULTURE MEDIA, NEUTRALIZING SOLUTION, AND BUFFERS

TABLE 1 Sterile Neutralizing Solution

Lecithin	4.0 g
Polysorbate 80	30.0 g
Peptamin	10.0 g
Beef extract	5.0 g
Histidine	1.0 g
Sodium laurylsulfate	4.0 g
Sodium chloride	5.0 g
Distilled water	1000 mL

TABLE 2 Sterile Buffered Peptone Saline at pH 7

Monopotassium phosphate	3.56 g
Dihydrated disodium phosphate	7.23 g (equivalent to 0.067 M)
Sodium chloride	4.30 g
Meat or casein peptone	1.0 g
Purified water	1000 mL

Note: 1 g/L or 10 g/L of polysorbate 20 or 80 can be added to the solution. Sterilize in the autoclave at 121°C for 15 minutes.

TABLE 3 Tryptic Soy Agar

Tryptone	15.0 g
Soya peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 mL

Note: Sterilize in the autoclave at 121°C for 15 minutes. pH must be 7.3 ± 0.2.

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TABLE 4 Sabouraud Chloramphenicol Agar

Meat and casein peptone	10 g
Dextrose	40 g
Chloramphenicol	0.05 g
Agar	15 g
Purified water	1000 mL

Note: Sterilize in the autoclave at 121°C for 15 minutes.

TABLE 5 Enterobacteria Enrichment Broth (EEB Mossel)

Tryptose	10.0 g
Dextrose	5.0 g
Disodium phosphate	8.0 g
Monopotassium phosphate	2.0 g
Oxgall	20.0 g
Brilliant green	0.0135 g
Purified water	1000 mL

Note: Heat to 100°C for 30 minutes, cool immediately. pH 7.2 ± 0.2.

TABLE 6 Agar with Crystal Violet, Neutral Red, Bile Salts, and VRBG Agar with Glucose

Yeast extract	3.0 g
Peptone	7.0 g
Bile salts	1.5 g
Lactose	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Neutral red	0.03 g
Crystal violet	0.002 g
Dextrose	10.0 g
Purified water	1000 mL

Note: Heat to boil, do not autoclave. pH 7.4 ± 0.2.

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TABLE 7 Mac Conkey Broth

Peptone	20.0 g
Lactose	10.0 g
Oxgall	5.0 g
Brom cresol purple	0.01 g
Purified water	1000 mL

Note: Sterilize by autoclave at 121°C for 15 minutes. pH 7.3 ± 0.2.

TABLE 8 Mac Conkey Agar

Casein peptone	17.0 g
Meat peptone	3.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Purified water	1000 mL

Note: Sterilize by autoclave at 121°C for 15 minutes. pH 7.1 ± 0.2.

TABLE 9 Tryptic Soy Broth

Casein peptone	17.0 g
Soja peptone	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Dextrose	2.5 g
Purified water	1000 mL

Note: Sterilize by autoclave at 121°C for 15 minutes. pH 7.3 ± 0.2.

TABLE 10 Cetrimide Agar

Peptone	20.0 g
Magnesium chloride	1.4 g
Dipotassium sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Glycerol	10.0 mL
Purified water	1000 mL

Note: Sterilize by autoclave at 121°C for 15 minutes. pH 7.2 ± 0.2.

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TABLE 11 Baird Parker Agar

Peptone	20.0 g
Beef meat extract	5.0 g
Yeast extract	1.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g

Note: Sterilize by autoclave at 121°C for 15 minutes. Cool to 45–50°C and add 10 mL of a sterile potassium tellurite solution at 10 g/L and 50 mL of an egg yolk emulsion.

TABLE 12 Sabouraud Dextrose Agar

Peptone	10.0 g
Dextrose	40.0 g
Agar	15.0 g

Note: Sterilize by autoclave at 121°C for 15 minutes.

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