Leakage assessment of flexible pouches using dye penetration test with correlation to modeled bacterial aerosol challenge test

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Abstract Package integrity is a primary measure of a package's ability to keep the contained product inside and to keep potential contaminants out. In this study, injecting and vacuum dye penetration methods were applied for the assessment of the package integrity of retortable flexible pouches having various sizes of micro-channels. The purpose of this study is to evaluate the usefulness of dye penetration as a physical test that can be incorporated into a stability protocol and compare the results of the dye penetration test with those from the bacterial aerosol challenge test. The study found a direct correlation between the results of the vacuum dye penetration test and those of the microbial test. The critical leak size that can ensure the flexible package integrity was 15 μm . To detect defective pouches, the dye vacuum testing had a sensitivity similar to that of bioaerosol challenge test.

Keywords Leak detection · Integrity · Flexible packaging · Dye penetration · Microbial challenge

Introduction

An important aspect of product quality assurance is to demonstrate the maintenance of package integrity throughout the product shelf life. The presence of defects, such as pinholes in the material itself or micro-channels in the sealing area, can affect the stability and thus the efficacy of the product contents. Additionally, the potential for

microbiological contamination is a concern. The word *leakage* refers to the flow of a fluid through a leak without regard to the physical size or shape of the hole. When a fluid flows through a small leak, the flow rate depends on the geometry of the leak, the nature of the leaking fluid, the pressure differential, and the prevailing temperature [1, 2].

Traditionally, dye penetration and microbial challenge tests have been used to verify package integrity. Microbial challenge testing is an established technique within the food industry that simulates what can happen to a product during processing, distribution, and subsequent handling, following inoculation with one or more relevant microorganisms. Microbiological tests have been used in efforts to quantify the approximate minimum size of a critical defect [3–5]. Thus far, defect sizes between 0.2 and 80 μm in diameter for bacterial penetration have been described, with the most common size being approximately 10 μm [5]. The limitations associated with microbial testing have motivated the use of various physical tests of package integrity, such as the dye penetration test method, which food and pharmaceutical industries anticipate as a method for ensuring packaging integrity [6, 7].

The dye penetration test is generally performed to determine the integrity of the package seal and to identify the locations of any leaks in the packaging, such as pouches, trays, and blisters. According to the ASTM F1929 [8], the dye penetration test involves immersing the package in a dye solution, injecting an indicating dye into the package, or placing two or three drops of a methylene blue dye in the sealing areas. Prior to employing a physical test method (e.g., the dye penetration method) in a stability protocol, a strong correlation between the microbial ingress and the physical test methods should be established [9, 10]. Although dye penetration testing of packaging systems, has been described in the literature [6, 7, 10, 11], minimal

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information is available regarding the correlation of dye penetration with microbial ingress. The present study proposes an approach to improve defective packaging systems and determines the correlation of microbial ingress with dye penetration in flexible retortable pouches.

Standard ASTM F3039 [12] was released to expand the package integrity test offerings, not to replace or augment the F1929 [8]. This standard can be used to detect channel leaks through the seals of a flexible barrier. A channel leak can be thought of as a tunnel that runs through the two layers of material being sealed together. The presence of a channel exposes the package to a potential breach of integrity, as microbes may enter the package where the sterile product is housed. In the testing and packaging community, there is no agreement on which sizes of a channel can be considered deleterious to the product, and therefore, to potential patients or other users.

The initial goal of this study was to estimate the detection limits of the bioaerosol challenge testing technique regarding its ability to detect laboratory-generated micro-channels that simulate defects in food packages. We tested packages having micro-channels of diameters between 15 and 100 μm in the seal plane traversing the major axis of the heat seal. The objectives of this study are [1] to determine the ability of the vacuum dye penetration technique to detect and locate defects smaller than 50 μm , and [2] to determine the correlation between the results of the dye penetration test and those of the bioaerosol challenge test.

Materials and methods

Sample preparation

The ALOx retortable pouches (DNP Co. Ltd., Japan) without aluminum layers were used as the test sample; each pouch had an Aluminium oxide-Polyethylene terephthalate/Nylon/cast polypropylene (AlOx-PET/Ny/PP) structure, a total thickness of 0.154 mm, and a contents volume of 150 mL. We chose optically transparent retortable pouches for this study to enable independent channel defect verification using blue dye.

To perform the integrity vacuum dye testing and the bioaerosol challenge testing, 620 samples were prepared. The micro-channel leaks were intentionally created using a technique in accordance with ASTM F1929 [8]. To create the micro-channels, fine tungsten wires with diameters of 15, 25, 50, and 100 μm (Eynsham W558911/W557412, Oxford, England) were used. The created micro-channels were 5 mm in length and 15, 25, 50, or 100 μm in diameter. Packages without channel leaks were used as controls.

Microstructure of channels

The micro-channels were verified to be open and clear prior to the tests. We used optical microscopy with a calibration grid (600 $\times$) (Xi-CAM, Bestec Vision Co., Korea) to check the channel shapes and sizes. Moreover, we verified the diameter and shape of each micro-channel using a scanning electron microscope (SEM; Quanta FEG250, FEI Co. Ltd., Oregon, USA). The deformations of the channel cross sections due to the surface shear force imparted as the wires were withdrawn appear to be minimal, according to a scanning electron microscopy assessment of the cross sections.

Injecting dye penetration test

The dye solution used in all the dye penetration testing consisted of 1% methylene blue (Alfa Aesar, A Johnson Methy Co., USA) and 0.25% sodium dodecyl sulfate (SDS; DaeJung, Korea) in deionized water. The SDS was added to decrease the surface tension of the dye solution. Then, the dye solution was filtered through a 0.2 mm cellulose acetate filter (DISMIC-25cs, ADVANTEC, Japan) in order to preserve the consistency, prior to use in all tests.

This method involved injecting dye into each 200 package using a syringe. The ASTM F3039 standard calls for dye penetrant to be injected into the package in a volume relative to the longest edge of the pouch or tray [12]. The dye was allowed to contact the sealed edge for 5 s. Then, the package was rotated in order to expose the dye to contact with the other seals of the package. The total visual inspection time was approximately 20 s [8]. After allowing the dye to run along and penetrate all the seals, a visual inspection of the seals was performed. The seals of the transparent packages were visually examined for entrance of dye penetrating into the seal areas.

Vacuum dye penetration test

The vacuum condition was achieved according to ISO 8362-2 [13]. The samples were filled with deionized water to a headspace depth of approximately 15 mm for the dye penetration tests. Numbered defected pouches were inverted and placed into a pan filled with methylene blue dye solution, along with three non-defective samples as negative controls. Then, the chamber was evacuated for 30 min to 75 kPa (563 mm Hg) using a JSR Vacuum drying oven (JSVO-60T, Korea), and then, the vacuum was quickly released to allow the pressure inside the pouches to equilibrate with the outside pressure. The pouches remained inverted in the pan an additional 30 min to take up the dye. The requirement that the vacuum be quickly released was not included in the ISO procedure; however, previous

experiments and studies by Artz et al. [7] indicated that the vacuum dye method is more effective if the vacuum is sharply released. We performed this dye penetration test by running it for 210 pouches: 50 pouches of each micro-channel diameter and 10 pouches as controls.

Visual inspection

Laboratory personnel were each given one pouch labeled “blank” (negative control) that contained only purified water. The personnel compared the pouches tested by the vacuum dye penetration method with the blank pouches, visually inspecting them for signs of dye ingress if the dye was present. Five people were selected to determine the limit of detection for visual dye analysis, identifying each sample as either “containing dye” or “not containing dye.”

Spectrophotometric analysis

The existence of dye in the flexible pouches was confirmed by a spectrophotometric technique. The absorbance of the dye solutions was measured at 668 nm using a spectrometer (V-650 UV/VIS Spectrophotometer, Jasco, Japan). The dye penetration (injecting and vacuum) studies involved the evaluation of pouches having the four different micro-channel sizes. The absorbance of water in the pouches used as blanks was assumed as 0.0001 at 668 nm. We prepared diluted dye solutions with concentrations ranging from 0.002 to 200 ppm of dye to generate a standard curve for dye concentration comparison.

Microbial ingress procedure

For the microbial challenge test, 135 mL of tryptic soy broth (TSB; Becto, USA) was piped into each pouch before completely sealing it. A total of 210 pouches were tested. *DH 5α Escherichia coli* (*E. coli*) and *KCCM 11335 Staphylococcus aureus* (*S. aureus*) (obtained from the KCCM, Korean culture center of microorganisms) have been used in bioaerosol microbial package integrity testing, with a concentration of microorganisms in a solution of about 3.0×10^8 CFU/mL [14]. Equal volumes of *E. coli* and *S. aureus* cell suspensions were combined to give approximately equal populations of each culture.

Defective pouches as described above were placed in a chamber (constructed of acrylic with approximate dimensions of 0.13 m^3), which connected to the nebulizer (Pari LC SPIRINT 0123, Germany). Then, the microorganisms inoculum were nebulized into the chamber for 60 min, followed by waiting for 3 h to obtain a static condition. A 14-day incubation period was conducted at 37°C , throughout which the pouches were visually inspected and periodically checked for the presence of microbial growth,

which would indicate microbial ingress. A pouch with no defect was injected with 0.2 mL of a 10^3 CFU/mL solution of the organisms to serve as a positive control. We applied this method to another type of packaging in our previous work [15].

Results and discussion

Dye penetration

A total of 100 pouches per each diameter size were evaluated in the dye penetration (injecting and vacuum) study. No dye penetration was detected in the negative controls, and the $15 \mu\text{m}$ test units were negative for dye penetration as well. As can be seen in Fig. 1, the blue color of the dye cannot detect on the micro-channel with $15 \mu\text{m}$ size. However, the test units with $25 \mu\text{m}$ or larger micro-channels were penetrated by the dye solution. Visual inspection indicated that the dye penetration varied from 4 to 30% for the 25 and $50 \mu\text{m}$ micro-channels with the injection method, and the vacuum dye penetration method showed 64% for the $25 \mu\text{m}$, and 96% for the $50 \mu\text{m}$ micro-channels. ASTM F3039 is to be applied for the detection of edge seal channel leaks through a nonporous package that are $50 \mu\text{m}$ or greater in diameter [12]. The traditional ASTM F1929 dye penetration standard is used for porous packaging material, and ASTM F3039 is intended for nonporous packages—both for transparent (e.g., nylon–nylon) pouches and for opaque (e.g., foil–foil) packages [8, 12]. The dye penetration method depends on several experimentally controlled physical parameters, including dye concentration, temperature, pressure differential, viscosity of the dye solution, and surface tension [16].

A standard calibration curve showing the relationship between the absorbance and the dye concentration is presented in Fig. 2. The absorbance of known amounts of dye solutions (from serial dilution) varied from 0.0001 to 0.0810. For instance, a solution with a concentration of $0.002 \mu\text{g/mL}$ of dye in water had an absorbance of approximately 0.0045 at 668 nm. Therefore, the pouches with absorbances greater than 0.004 were considered positive for dye penetration. However, the dye solution visual detection results were reported as binary data (exist/not exist).

Table 1 gives the theoretical amount of liquid that should enter each pouch by the end of the dye penetration experiment. The results of the dye penetration test were compared with the theoretically calculated results by using Poiseuille’s Law for viscous flow through a micro-channel (Table 1). Poiseuille’s equation [17] is defined as

$$Q = (\pi r^4 / 8nL)(P_1 - P_2), \quad (1)$$

Fig. 1 Optical digital microscope images from throughout the micro-channels after dye penetration test. ($\times 600$), (A) 100 μm , (B) 50 μm , (C) 25 μm , and (D) 15 μm (the blue color is 0.2% methylene blue solution). (Color figure online)

Fig. 2 Standard curve showing the relationship between dye concentration and absorbance

where Q (mL/s) is the volumetric flow rate, r (μm) is the radius of the channel, L (μm) is the length of the channel, n (cps) is the viscosity of the liquid, P_1 is the upstream pressure, and P_2 is the downstream pressure in pascals. P_1 and P_2 are defined as the atmospheric pressure and 75 kPa, respectively. Poiseuille's equation predicts a breached detection limit between 15 and 25 μm , which is similar to the experimental detection limit of 15 μm (Fig. 3). Poiseuille's Law is a useful guide for estimating the limit of detection of the vacuum dye testing method, even when not all conditions of Poiseuille's Law are met.

Kirsch et al. [10] described an additional factor that should be considered: the presence of "airlocks" resulting from trapped air within the micro-channel's lumen, which may form during the preparation of defective pouches or upon applying a vacuum. Depending on the solution viscosity, contact angle, and diameter of the micro-channel, the presence of airlocks can prevent liquid ingress. One

Table 1 Comparison of actual dye ingress and theoretical flow rate based on the Poiseuille's equation

Micro-channel inner diameter (μm) ^a	Theoretical flow rate ($\mu\text{L/s}$)	Absorbance at 668 nm	Calculated final dye concentration in pouches ($\mu\text{g/mL}$)
15	5.44×10^3	0.0002	4.82×10^{-4}
25	4.20×10^4	0.0253	6.11×10^{-2}
50	6.72×10^5	0.0589	1.43×10^{-1}
100	1.07×10^7	0.0610	1.47×10^{-1}

^a The viscosity of the dye solution was 1.2 cps and the micro-channel length was 5 mm

Fig. 3 Percentage of pouches detected by the microbial challenge, vacuum, and injecting dye penetration methods

method for removing airlocks from micro-channels is to apply a pressure differential. LaPlace's equation is a useful tool for estimating the amount of pressure needed to remove airlocks, and it also emphasizes the importance of the solution surface tension in the liquid tracer (dye testing) packaging integrity test [18]. LaPlace's equation can be written as

$$P = 4\gamma \cos \Theta / D \quad (2)$$

where P is the driving gage pressure at the micro-channel outlet, γ is the surface tension, Θ is the contact angle between the surface of the liquid and the micro-channel wall, and D is the diameter of the micro-channel. The smaller the micro-channel diameter is, the larger the gage pressure that is required to break the surface tension and cause leakage. We assumed the cosine of the contact angle between the surface of the liquid and the micro-channel wall to be unity.

For a surface tension of 72.75 Nm/m² (surface tension of the water inside the defective pouches), a pressure differential of 20 kPa was sufficient to remove the airlocks from a micro-channel with an inner diameter of 15 μm or more (Table 2). The real contact angle is actually less than one, which may explain why dye was detected in a micro-channel with an inner diameter of 25 μm. Theoretical

Table 2 The theoretical calculated pressure for removing airlocks from the micro-channel based on Laplace's equation

Micro-channel inner diameter (μm)	Pressure (kPa)
15	19.40
25	11.64
50	5.82
100	2.91

calculations are based on the worst case scenario, in which the contact angle is assumed to be one.

Comparison of bioaerosol challenge test and dye penetration test

Package integrity evaluation methods that employ bioaerosols and simulate the conditions that the package is expected to tolerate during storage and distribution are relevant and gaining prominence. None of the negative controls (pouches without defects) were positive for microbial ingress. The positive controls exhibited growth after 1 day, thus validating the test conditions for detecting microbial growth. The number of microbial failures and the corresponding micro-channel size are also shown in Fig. 3. The cutoff for the microbial ingress study was 15 μm, which was identical to the cutoff for the dye penetration study for this particular type of leak. The samples with defect sizes of 50 and 25 μm took similar amounts of time to show microbial contamination inside a package, which means that a clear micro-channel for microbial ingress was 25 μm [15]. However, the pouches with 15 μm micro-channels did not show microbial growth. The logical explanation for this observation lies in the threshold pressure inside the test bag [16]. The dye test is considered a qualitative test, although it has been reported to have a sensitivity of 10⁻⁷ Pa·m³/s [19]. This may be too sensitive of a test if it is being used to detect packages that may lose their hermetic seal. Theoretically, microbial penetration should not occur if the leakage rate is below 10⁻⁵ Pa·m³/s [20].

The differences in the critical defect sizes found for microbial ingress among different studies could be due to differences in the test methods employed, the contact materials that affect the surface tension of the liquid, rigidity of the package at the test, package geometry, and pressure differential across the sterile barrier. Based on these results, it is clear that defects larger than 15 μm cause sterility breaches in flexible pouches. Therefore, integrity testing for on-line package testing must detect 15 μm defects to ensure product sterility. The vacuum dye penetration test was sensitive to detection across the same range of micro-channel sizes as the bioaerosol challenge method, and both methods also displayed comparable detection likelihood. Furthermore, the dye penetration test was performed to identify the location of any leaks because the microbial challenge test only indicates the presence/absence of leaks. A comparison of the dye penetration test and the bioaerosol challenge test is presented in Table 3. Compared to assessment via the microbial ingress method, the dye penetration method demonstrates significant advantages in terms of implementation, such as cost and time savings. One advantage of the microbial challenge test over the dye penetration test is that it simulates the real

Table 3 Characteristics of the test methods used in this research

Integrity test	Dye penetration (Injecting)	Dye penetration (vacuum)	Microbial challenge test (bioaerosol)
Advantages	Widely used for decades Industry and regulatory familiarity Identifies the locations of leaks Inexpensive Easy Widely accepted leak testing	Can detect defects as small as 20 μm Identifies the location of leaks No need for needle/syringe Less messy Faster with multiple samples Sensitive leak detection method	Simulates real conditions Can detect the critical defect size to ensure sterility of the product Destructive Not on-line Very labor intensive ^a Not suitable for routine checking ^a Probabilistic nature of the test ^b Requiring a relatively large sample size for adequate statistics ^a Detect viable microorganisms capable of growth only in the specific product/medium
Disadvantages	Less sensitive Cannot detect defects smaller than 50 μm Destructive Not on-line Labor intensive Messy Relatively slow Not quantitative	Destructive Not on-line	

^a Morrical et al. [21]

^b A problem with the microbial challenge test is the probabilistic nature of the test in that even if a leak is large enough for microbes to enter there still has to be a microbe that is in the vicinity of the leak in order for ingress to occur. This means that a leak can pass one microbial challenge test but fail under a different set of conditions, thus requiring a relatively large sample size for adequate statistics

conditions that the package will encounter during its shelf life. Thus, the bioaerosol microbial challenge test is a useful method for assessing package integrity and determining the critical leak size for package sterility. The results of this study indicate that the vacuum dye penetration test is also an excellent method for evaluating package integrity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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