FRAGRANCE IMPACT ON MARKETED AIR FRESHENER PRODUCTS BY BCOP ASSAY AND HISTOLOGY

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ABSTRACT

An exploratory in vitro eye irritation study of marketed solid and liquid air fresheners was conducted to investigate the impact of fragrance/fragrance type on overall eye irritation for specific product forms. The Bovine Corneal Opacity and Permeability (BCOP) assay was selected to evaluate eye irritation potential due to its robustness and ability to test both solids and liquids by direct corneal application. Fragrance concentration, formula ingredients and product delivery system influence degree of impact on eye irritation potential. Six different air fresheners containing a representative "watery-type" fragrance were initially compared to respective un-fragranced product bases. The BCOP assay-testing scheme was optimized following several trials using neat solid and liquid air fresheners at 3- and 10-minute exposures. The greatest changes were noted in the in vitro scores, after 10-minute exposures. In vitro scores ranged from 0.0 to 97.1, reflecting a wide range of epithelial and stromal damage. The "watery-type" fragrance had greatest impact on eye irritation potential of gel electrics, non-aerosol sprays and scented oils compared to un-fragranced bases. These products were selected for additional investigations on impact of fragrance type on eye irritation potential. Citrus, floral and spice-type fragrances were evaluated for each product form. In vitro scores ranged from 5.7 to 110.4. Different fragrance types appeared to have observable impacts on eye irritation potential for specific product forms. Floral, citrus and spice-type fragrances had greatest impact on gel electric, non-aerosol spray, and scented oil product forms, respectively. Histological evaluation of corneas treated with selected solid and liquid air freshener products further supports the correlation of tissue damage (e.g., epithelial effects) with in vitro scores. Based on the BCOP assay and histology results, the recommended testing protocol for a solid or liquid air freshener product is to test neat product for a 10-minute exposure and use the in vitro score as the endpoint for evaluation of eye irritation potential.

INTRODUCTION

The objective of the fragrance research was to establish in-house eye irritancy data on the air freshener product category to support the safety of accidental eye exposure by consumers. An exploratory in vitro eye irritation study of marketed solid and liquid air fresheners was conducted to investigate the impact of fragrance/fragrance type on overall eye irritation for specific product forms. The Bovine Corneal Opacity and Permeability (BCOP) assay was selected to evaluate eye irritation potential due to its robustness and ability to test both solids and liquids by direct corneal application. The BCOP assay measures two endpoints, opacity and permeability. Air fresheners as a product class can cause a wide range of epithelial and stromal damage. Consequently, the in vitro score (i.e., Mean Opacity Value + 15 x Mean OD490 Value (permeability value)) is the primary endpoint for the air freshener products. Product fragrance concentration, product formula ingredients and product delivery system influence the degree of impact on eye irritation potential. Studies by Cuellar, Lloyd et al. (2003 and 2004) and Burdick, Merrill et al. (2002) have shown that solvent components in a fragrance mixture can have a profound impact on the eye irritation potential of a product.

The research program was divided into two investigative phases. In Phase I, BCOP assays were conducted on six different marketed air freshener product forms containing a representative "watery-type" fragrance compared to respective un-fragranced product bases. Two solid air freshener products (i.e., adjustable solid and gel electric) and four liquid air freshener products (i.e., aerosol spray, non-aerosol spray, scented oil, and aerosol carpet foam) were tested.

Phase II of the research program involved testing of additional selected fragrance types in selected product forms (i.e., citrus, floral and apple & cinnamon (spice)). The selected products were tested using the optimized BCOP testing scheme established in Phase I (i.e., neat test material and a 10-minute exposure period) to establish a range of in vitro scores of eye irritation potentials that might be expected for different air freshener product forms/delivery systems and fragrance categories.

Histological evaluations were also performed on treated corneas to compare the degree of tissue damage to the in vitro scores.

MATERIALS AND METHODS

Marketed Air Freshener Products

Six marketed product categories (excluding candles) were selected as representative of solid and liquid air freshener products. They were adjustable solid (watery fragrance), gel electric (watery, citrus, floral, and spice fragrances), aerosol spray (watery fragrance), non-aerosol spray (watery, citrus and floral fragrances), scented oil (watery, citrus, floral, and spice fragrances), and aerosol carpet foam (watery fragrance). The primary ingredient composition of each air freshener product category is presented in Table 1.

Table 1. Major Ingredients in Representative Air Freshener Product Categories

Product Description	Product Form	Major Ingredients
Adjustable Solid	Solid Gel	> 96% water < 2% carrageenan gel base ~ 1% fragrance
Aerosol Spray Concentrate (no propellant)	Liquid Concentrate	> 99% water < 0.5% fragrance
Carpet Foam Aerosol Concentrate (no propellant)	Liquid Concentrate	> 97 % water 2 - 3% isopropanol ~0.5% fragrance
Scented Oil	Scented Oil	~ 80 – 90% functional ingredients (e.g., solvents) includes: • > 25% 3-Methyl-3-Methoxybutanol (MMB), Dipropylene Glycol Monomethyl Ether (DPGME) and/or Tripropylene Glycol Monomethyl Ether (TPGME) > 10% ≤ 25% Dipropylene Glycol (DPG) and/or Benzyl Acetate ~ 8 – 15% esthetic ingredients (e.g., fragrance heart)
Gel Electric	Thickened Gel	> 95% fragrance < 5.0% fumed silica (amorphous)
Non-Aerosol Spray	Pump Spray	> 89% water 5 -7% ethanol 1 - 2% surfactant ~ 1% fragrance

BCOP Assay Conditions:

The procedures used in these studies followed, in general, those described by Gautheron, Dukic et al. (1992) and Sina, Galer et al. (1995). Specific modifications of the assay have been described by Harbell and Curren (1998). The BCOP assays were conducted at the Institute for In Vitro Sciences. Inc., Gaithersburg, MD.

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals. The eyes were excised and then placed in Hanks' Balanced Salt Solution (HBSS), supplemented with Penicillin/Streptomycin, and transported to the laboratory on ice packs.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The corneas were excised such that a 2 to 3 mm rim of sclera was present around the cornea. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, supplemented with 1% fetal bovine serum (complete MEM). The corneal holders were incubated at 32 ± 1ºC for a minimum of 1 hour.

BCOP Assay Procedure

An aliquot of 750 µL of either a liquid air freshener test article (i.e., aerosol sprays, non-aerosol sprays, scented oils, and aerosol carpet foams), positive control (100% ethanol), or negative control (deionized water) was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. Viscous or solid gel air freshener test articles (adjustable solids and gel electrics) were administered directly onto the exposed corneal surface using a positive displacement pipet. The in vitro score was used as the primary endpoint for evaluation of eye irritation potential of the air freshener products.

Corneal Opacity

After a minimum of 1 hour of incubation, the corneal holders were removed from the incubator. The medium was removed from both chambers and replaced with complete MEM. The opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer. Three corneas, whose opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The medium was then removed from the anterior chamber and replaced with the test article, positive control, or negative control.

Corneal Permeability

After the second opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was refilled with complete MEM, and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1ºC. The medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 µL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD490) was determined using a Molecular Devices Vmax kinetic microplate reader.

Histology

The treated corneas from Phase I and II were fixed for at least 24 hours in 10% buffered formalin. Pathology Associates International (Frederick, MD) embedded, sectioned and stained the fixed corneas. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin block so that a section of each half could be cut and placed on a single slide. Slides were stained with hematoxylin and eosin. IIVS evaluated the slides (Harbell and Curren (2005)). Photomicrographs and thickness measurements were prepared using a Spot Insight (Spot Diagnostic Instruments) digital camera and associated software. Representative photomicrographs of air freshener-treated corneas are shown in this poster.

Presentation of Data

The following formula was used to calculate the in vitro score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD490 Value)

Table 2. Optimization of BCOP Testing Scheme for Representative Air Fresheners with and without Fragrance

Product Form	Exposure Time	Opacity Value	Permeability Value (OD_{490})	*In Vitro* Score
Adjustable Solid Base – *without Fragrance*	3 minutes 10 minutes	-0.7 0.0	0.002 -0.001	-0.6 0.0
Adjustable Solid w/ Watery Fragrance	3 minutes 10 minutes	0.0 0.7	0.000 0.001	0.0 0.7
Gel Electric Base- *without Fragrance*	3 minutes 10 minutes	0.8 0.8	0.002 0.008	0.4 0.5
Gel Electric w/ Watery Fragrance	3 minutes 10 minutes	3.0 18.3	-0.001 0.200	3.0 21.3
Aerosol Liquid Base Concentrate – *without Fragrance*	3 minutes 10 minutes	0.3 0.0	0.002 0.001	0.4 0.0
Aerosol Base w/ Watery Fragrance	3 minutes 10 minutes	0.0 -0.3	-0.002 0.001	0.0 -0.3
Non-aerosol Liquid Base - *without Fragrance*	3 minutes 10 minutes	-1.0 2.3	0.007 0.037	-0.9 2.9
Non-aerosol Base w/ Watery fragrance	3 minutes 10 minutes	0.3 7.7	0.007 0.009	0.4 7.8
Aerosol Carpet Foam Liquid Base - *without Fragrance*	3 minutes 10 minutes	0.0 1.7	0.027 0.052	0.4 2.5
Carpet Foam Base w/ Watery fragrance	3 minutes 10 minutes	1.0 -0.3	0.034 0.122	1.5 1.5
Scented Oil Base - *without Fragrance*	3 minutes 10 minutes	8.7 24.0	0.128 0.481	10.6 31.2
Scented Oil w/ Watery fragrance	3 minutes 10 minutes	32.3 69.0	0.801 1.871	44.3 97.1

The composition of the air freshener base, as well as fragrance concentration and type have a major impact on eye irritation potential. In the case of scented oil, it is evident that not only the base oil has moderate eye irritation potential, but also the addition of fragrance to the oil greatly increases the eye irritation potential of the scented oil. The solvents used as functional ingredients in the scented oils are eye irritants, which appear to have a synergistic effect on eye irritation potential of the added fragrance. Similar results, although to a much lesser degree, were observed with the other product forms.

RESULTS AND DISCUSSION

The BCOP assay-testing scheme was optimized following several trials using neat test concentrations of solid and liquid air freshener products for 3- and 10-minute exposure times. The greatest overall observable differences in eye irritation potential were in the in vitro scores, which occurred using a 10-minute exposure time (Table 2). The "watery-type" fragrance had greatest impact on eye irritation potential of gel electric, non-aerosol spray and scented oil product forms compared to respective un-fragranced bases. These product forms were selected for additional investigations in Phase II on impact of fragrance types on eye irritation potential (Table 3).

Previous studies have also reported an appreciable range in irritancy potential among fragrance types Cuellar, Merrill et al. (2002). These authors also reported that the formulations with very high concentrations of fragrance oil (similar to the gel electric product type studied here) were less irritating than the similar fragrance type when ethanol was the carrier. The scented oils in this study contained 8 to 15% fragrance heart and the rest of the volume was composed of various organic solvents. Certain solvents have been shown to enhance irritation potential, perhaps by increasing penetration, while others decrease the irritation Cuellar, Lloyd et al. (2004). Given the extremely complex nature of the formulations, assessment of the final mixture (fragrance and solvents) is beneficial to assure that the product falls within the normal irritancy range.

The 10-minute exposure was selected to cover the irritancy range for these diverse product forms. It is intended to model the direct instillation of 100 µL (standard Draize test) rather than the aerosol exposure that would be used for products packaged in that form.

Table 3. BCOP Assay Results for Air Freshener Products by Fragrance Type

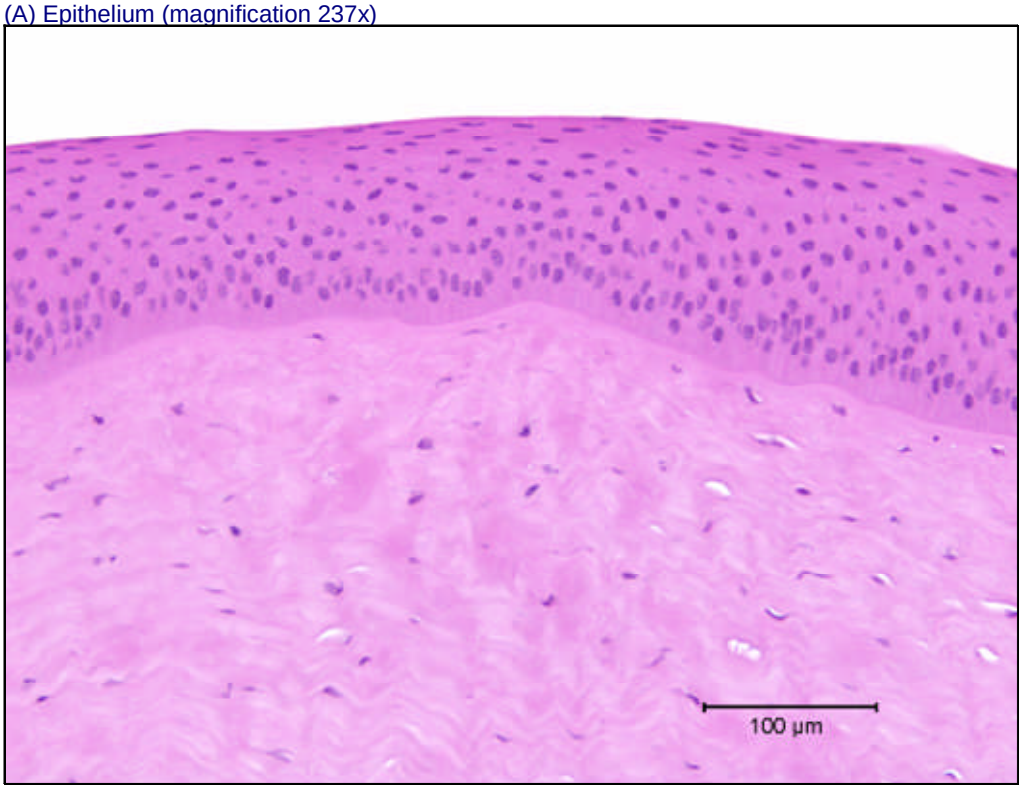
Product Form*	Fragrance Type	Opacity	Permeability Value (OD_{490})	*In Vitro* Score
Gel Electric	*Floral*	31.4	1.191	49.3
Gel Electric	*Watery*	18.3	0.200	21.3
Gel Electric	*Apple & Cinnamon - Spice*	11.4	0.075	12.5
Gel Electric	*Citrus*	5.2	0.035	5.7
Non-Aerosol Spray	*Citrus*	11.2	0.023	11.5
Non-Aerosol Spray	*Floral*	10.3	0.021	10.6
Non-Aerosol Spray	*Watery*	7.7	0.009	7.8
Scented Oil	*Apple & Cinnamon - Spice*	74.6	2.388	110.4
Scented Oil	*Floral*	73.0	1.876	101.1
Scented Oil	*Watery*	69.0	1.871	97.1
Scented Oil	*Citrus*	62.2	1.732	88.2
Scented Oil Base	No fragrance	32.4 (Mean of 2 values)	0.642 (Mean of 2 values)	42.0 (Mean of 2 values)

*All products were tested neat and for 10-minute exposure times.

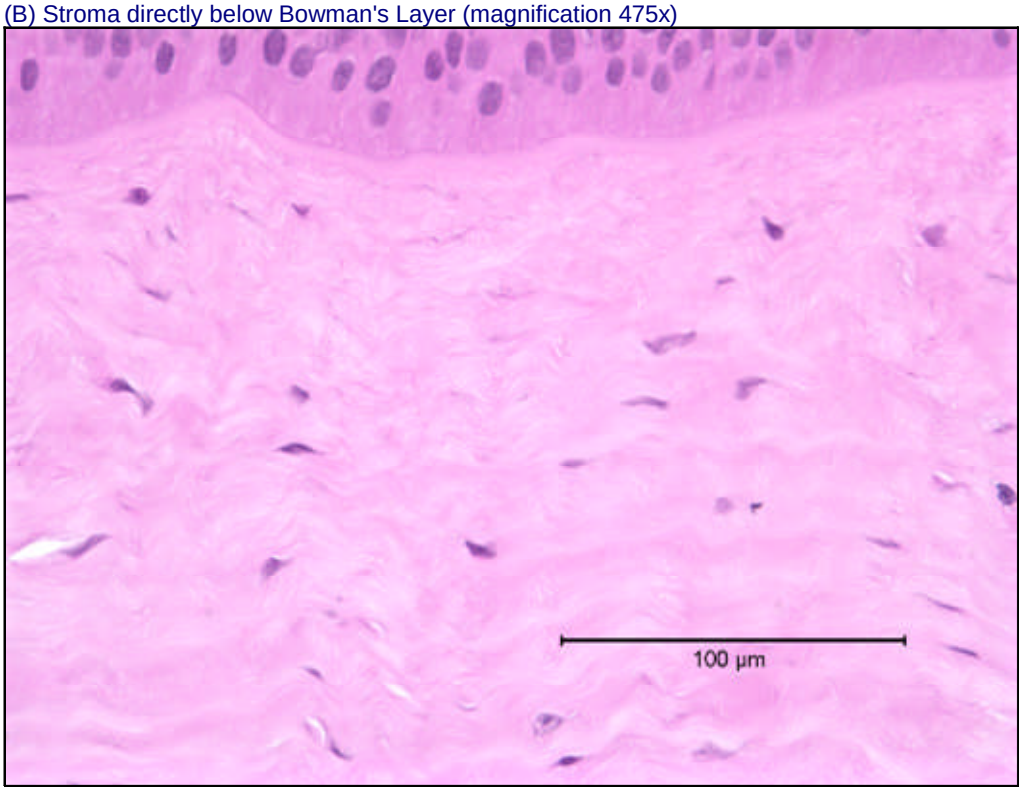
The fragrance type (i.e., watery, citrus, floral, and spice) appears to have an impact on eye irritation potential depending on the product form (i.e., gel electric, non-aerosol spray, and scented oil). As noted by in vitro scores in Table 3, the greatest impact on eye irritation potential of the gel electric, non-aerosol spray and scented oil product forms, was with the floral, citrus, and spice fragrance types, respectively.

Figure 1. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure

(A) Epithelium (magnification 237x)



(B) Stroma directly below Bowman's Layer (magnification 475x)



(C) Full thickness (magnification 48x)

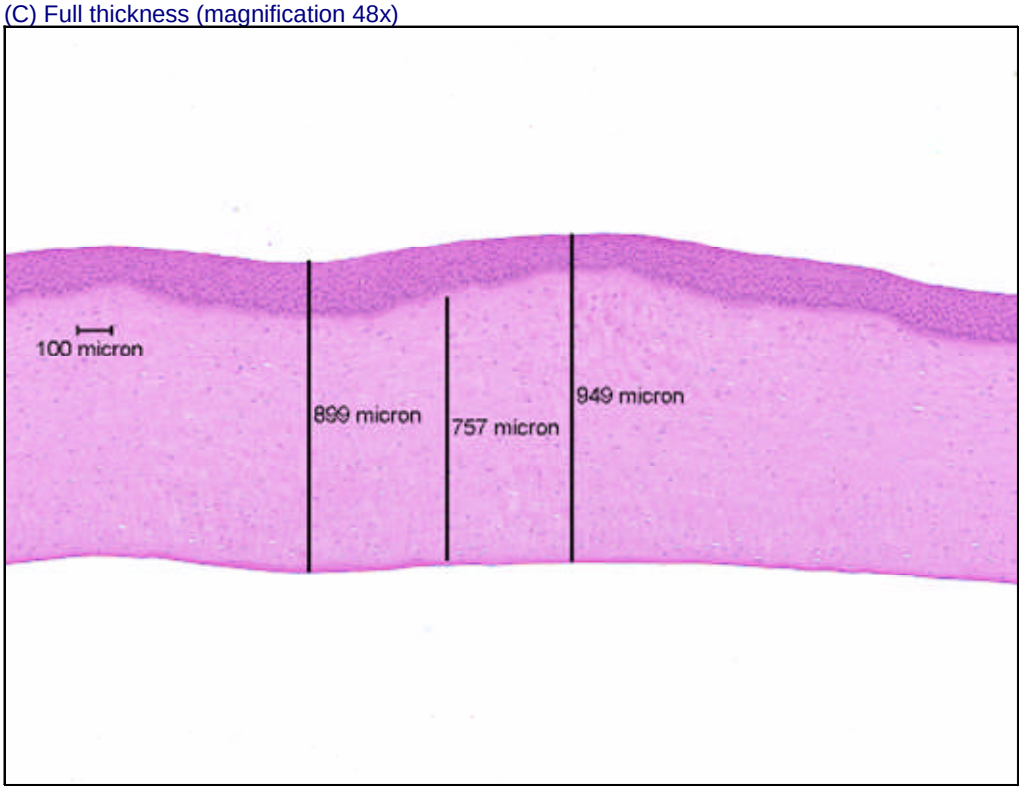
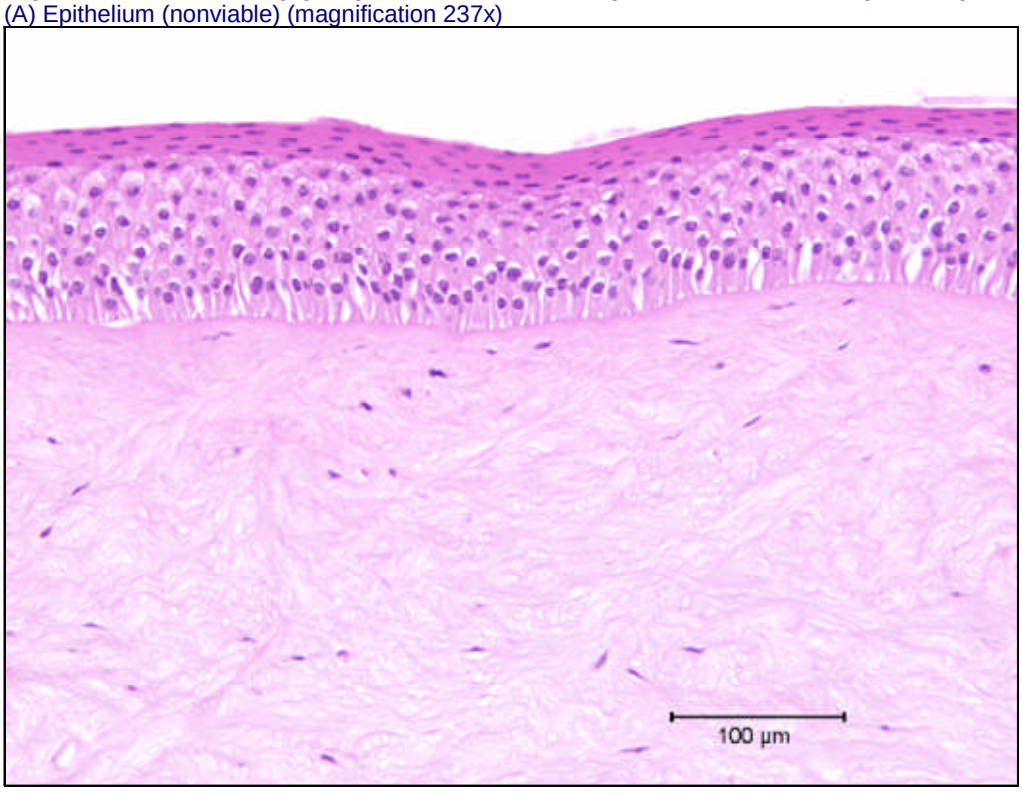
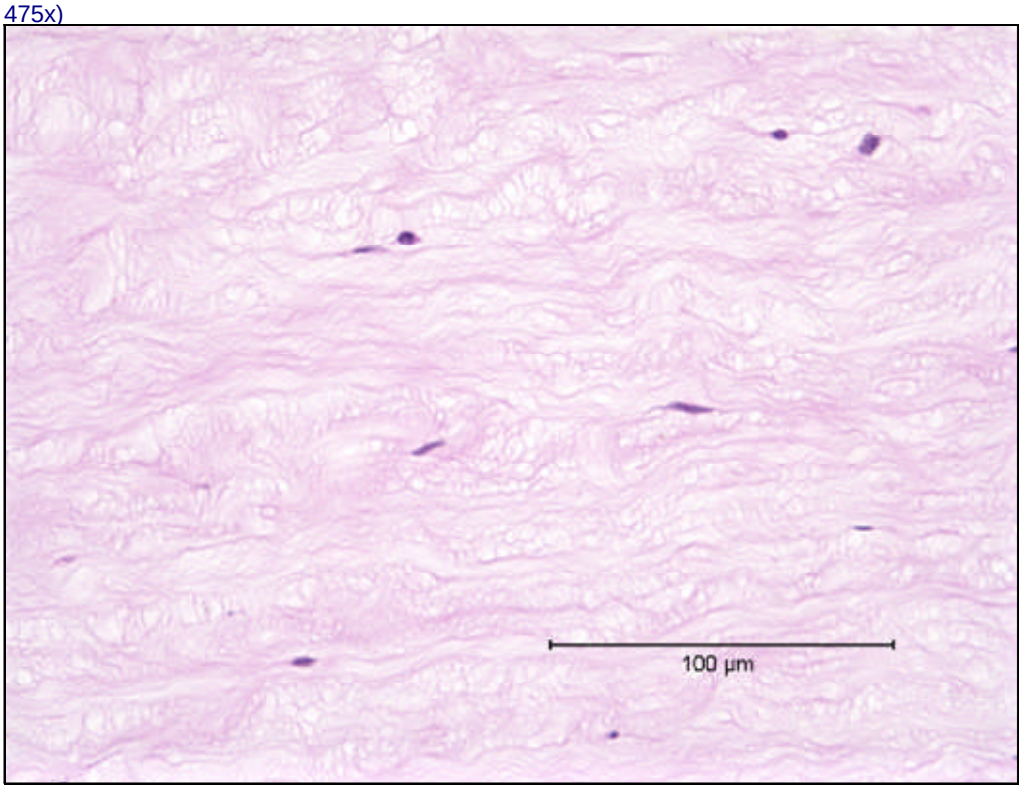


Figure 2. Scented Oil (Spice), neat, 10-minute exposure, 120-minute post-exposure

(A) Epithelium (nonviable) (magnification 237x)

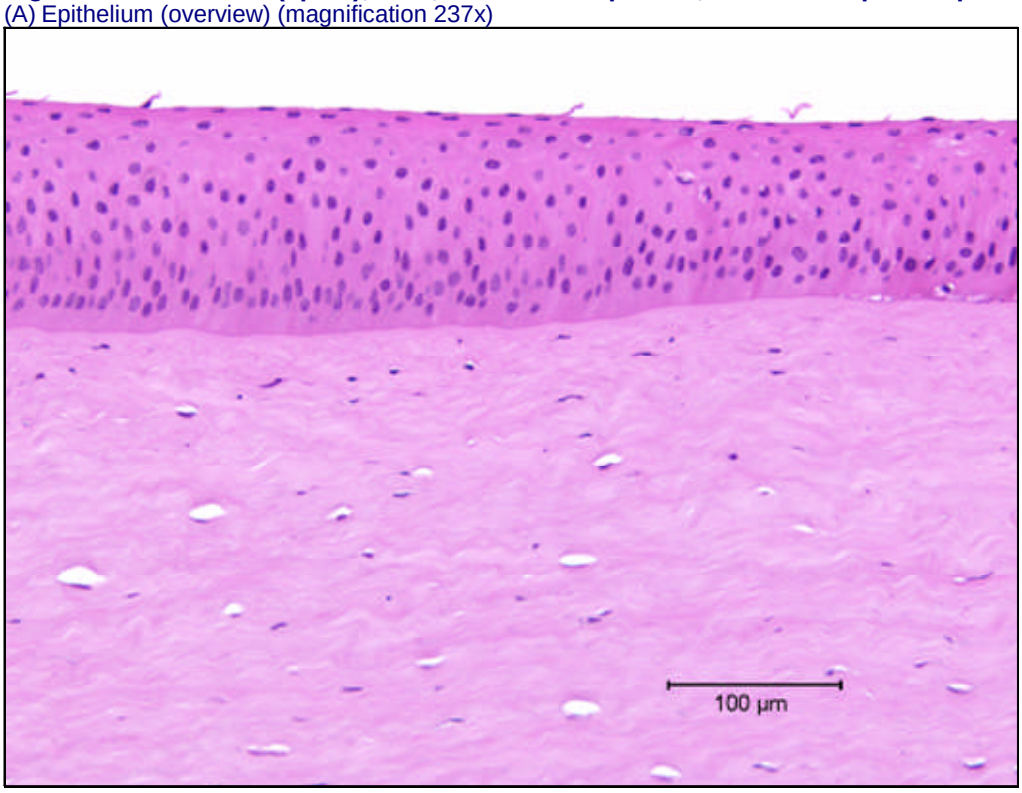


(B) Stroma at 20% depth showing extreme collagen matrix vacuolization and the marked increase in keratocytes with abnormal chromatin condensation (probably not viable at the time of fixation) (magnification 475x)

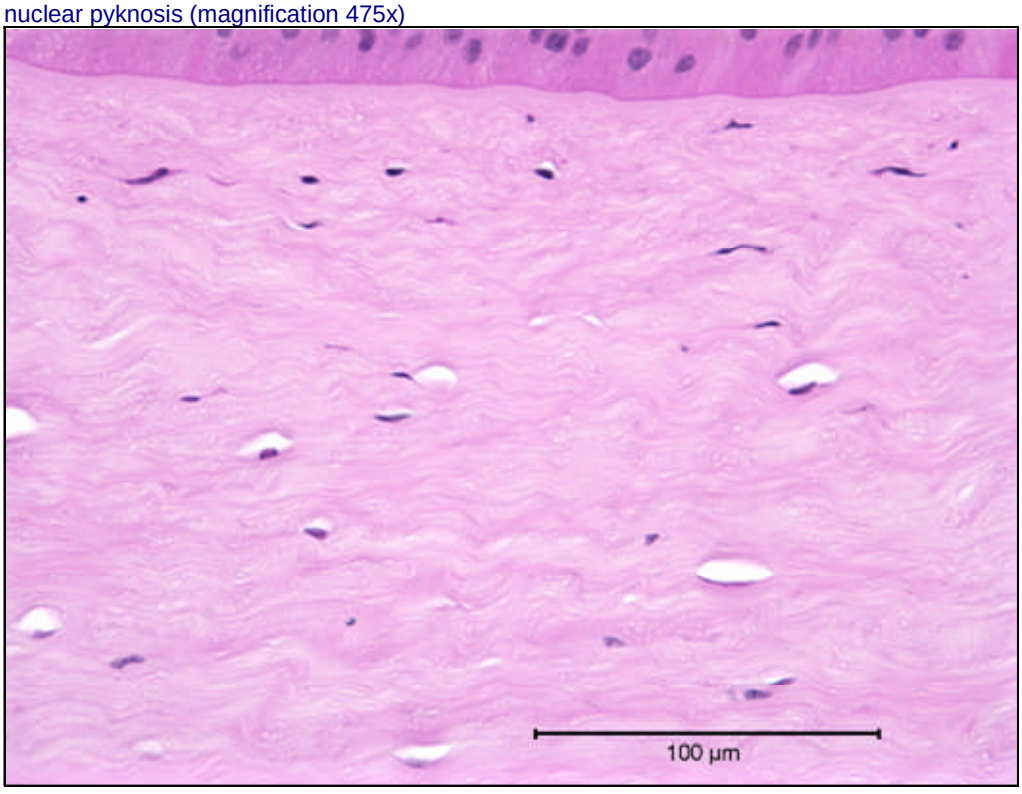


(C) Full thickness (magnification 48x)

Figure 3. Gel Electric (spice), neat, 10-minute exposure, 120-minute post-exposure
(A) Epithelium (overview) (magnification 237x)



(B) Stroma directly below Bowman's Layer showing slight collagen matrix vacuolization and keratocyte nuclear pyknosis (magnification 475x)



(C) Full thickness (magnification 48x)

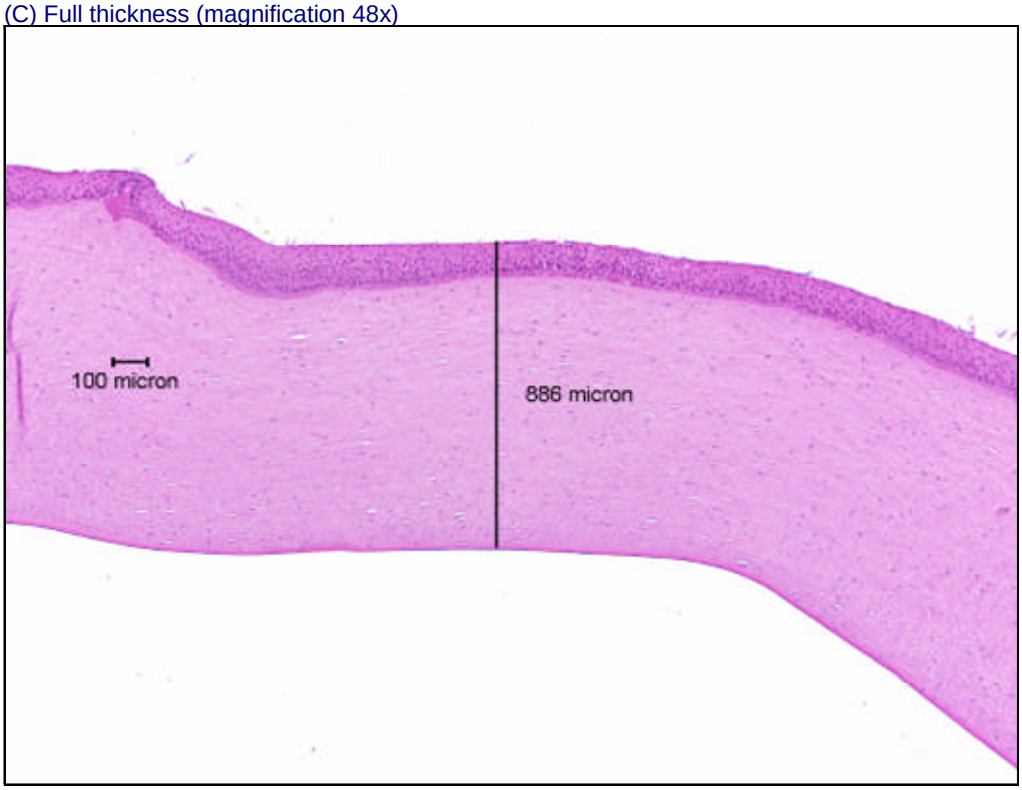
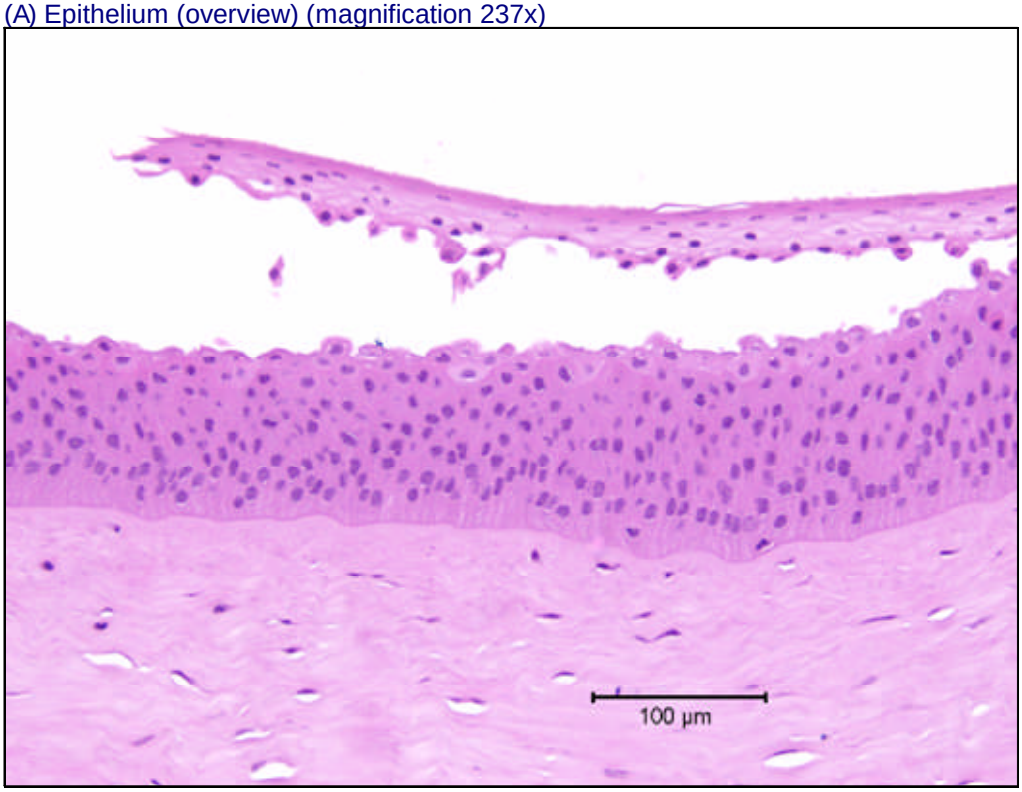
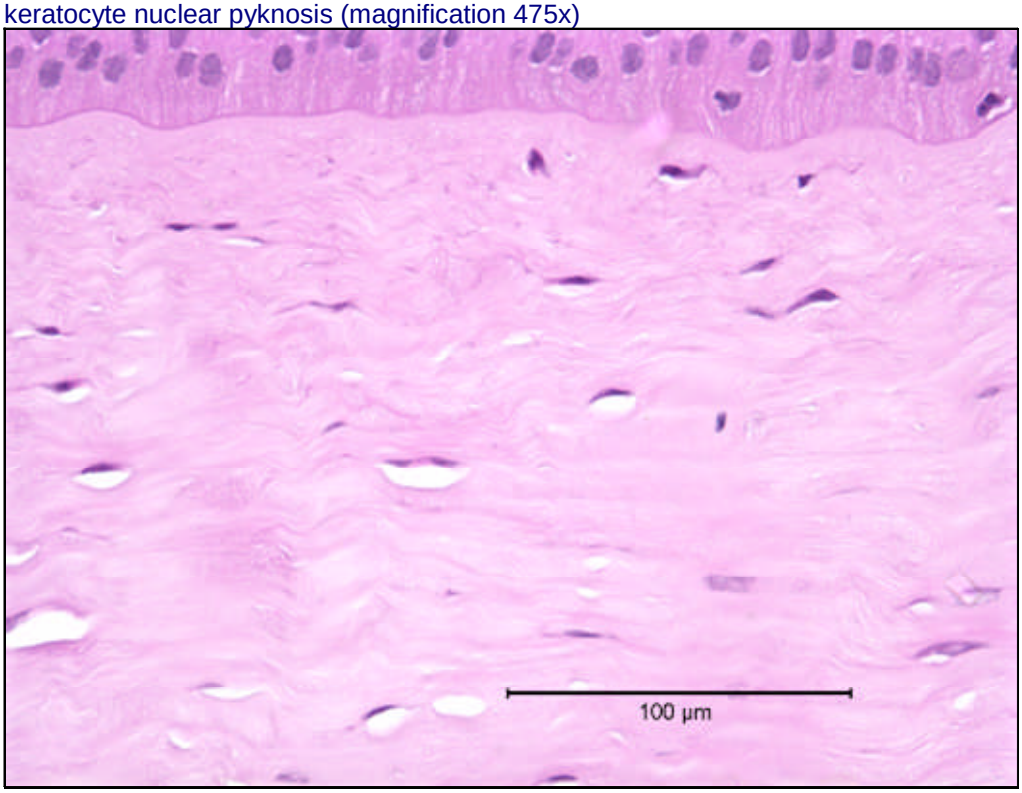


Figure 4. Non-Aerosol Spray (citrus), neat, 10-minute exposure, 120-minute post-exposure
(A) Epithelium (overview) (magnification 237x)



(B) Stroma directly below Bowman's Layer showing slight collagen matrix vacuolization and keratocyte nuclear pyknosis (magnification 475x)



(C) Full thickness (magnification 48x)

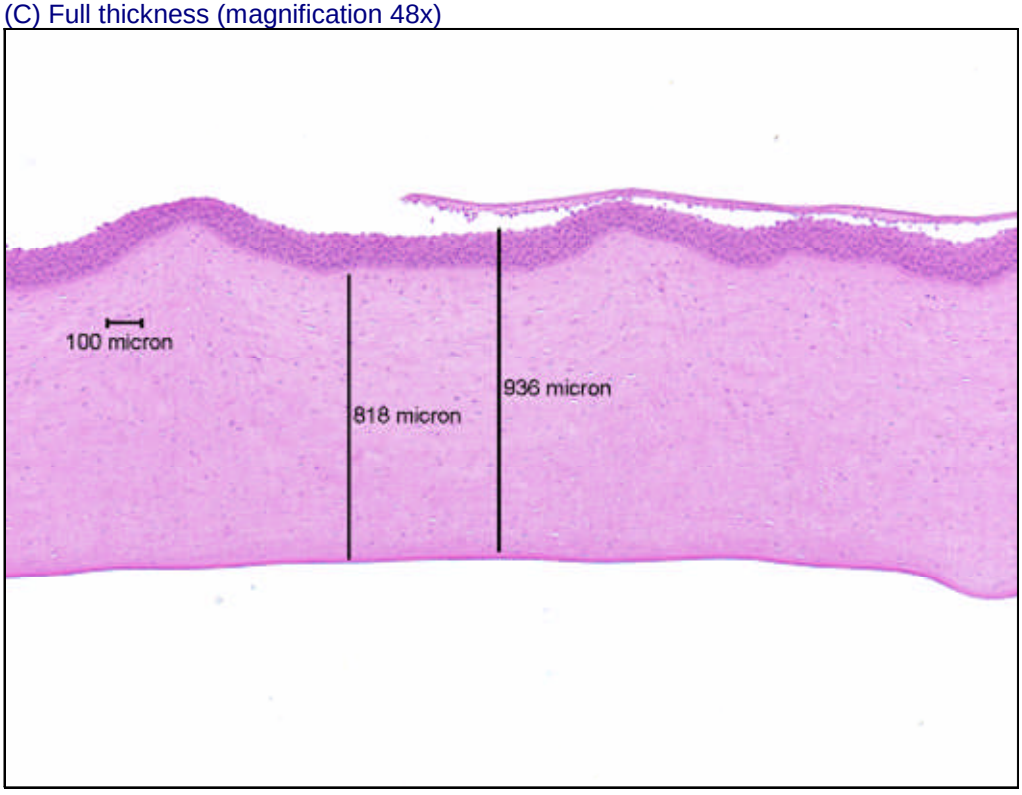
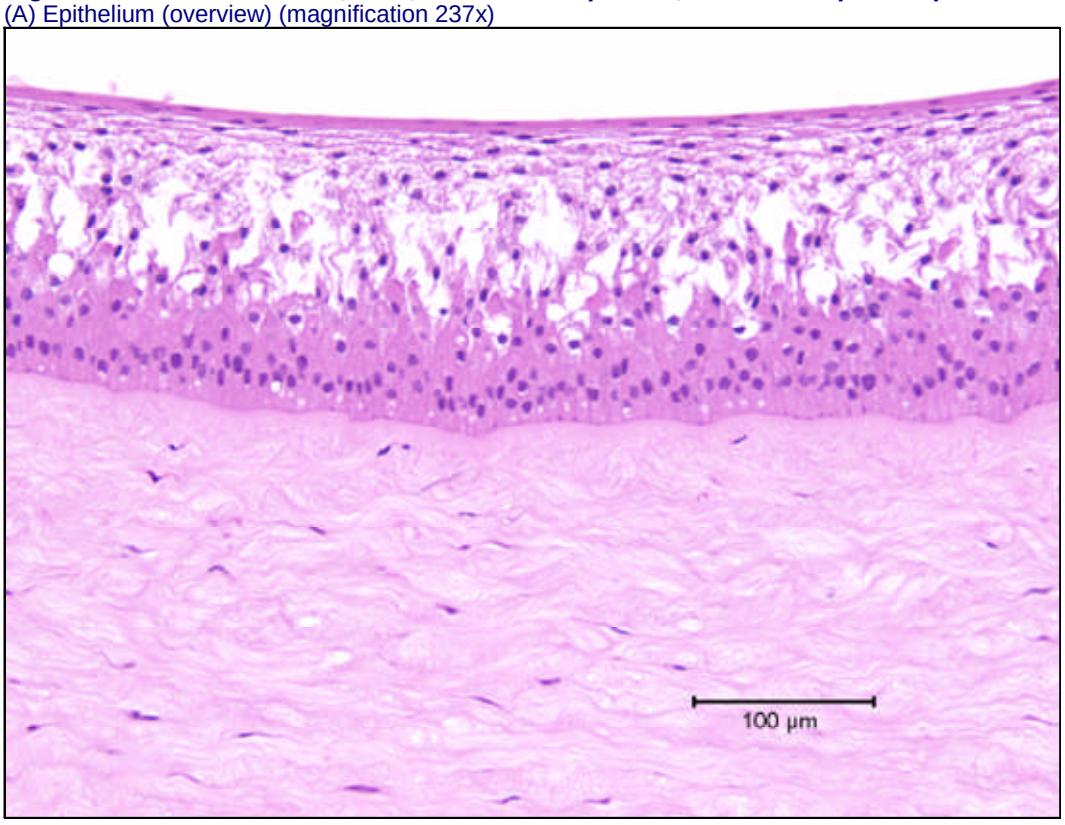
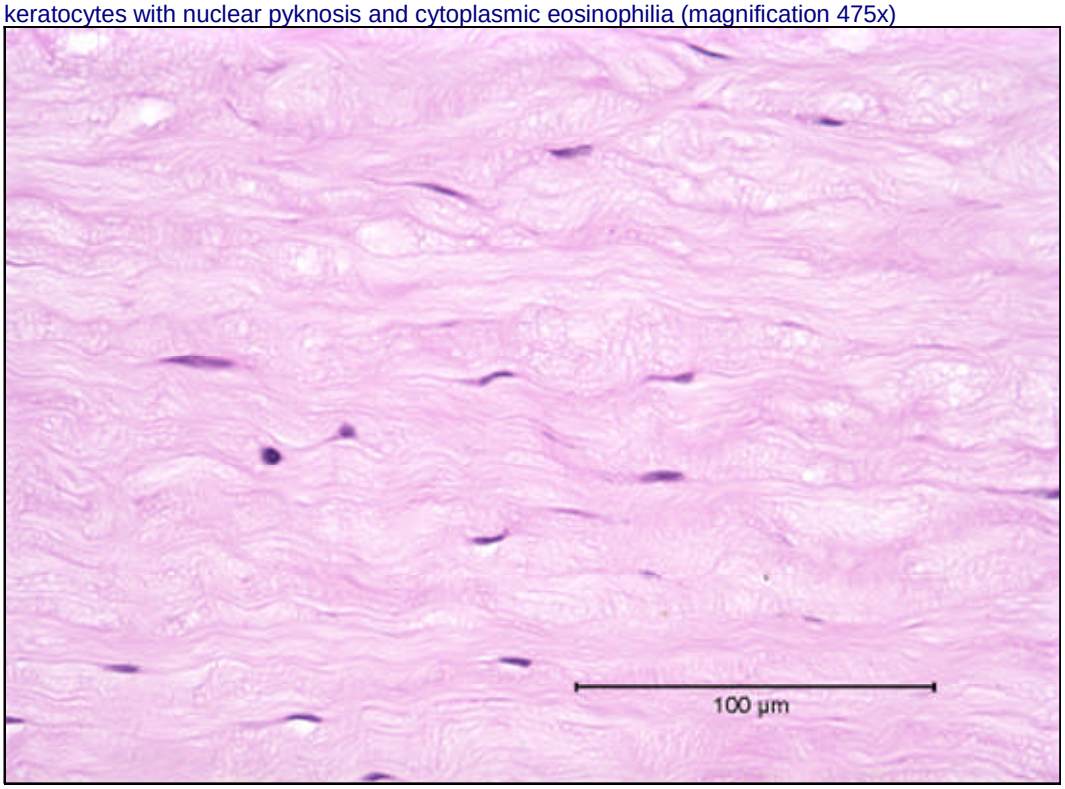


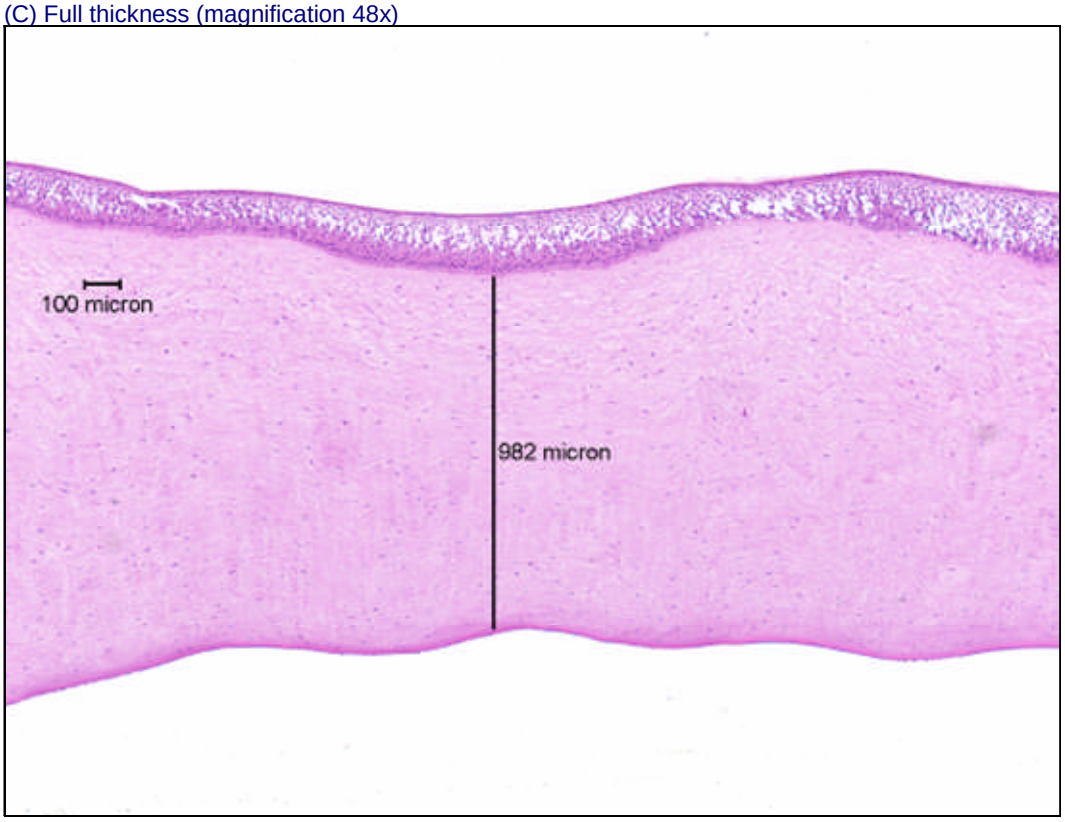
Figure 5. Scented Oil Base, neat, 10-minute exposure, 120-minute post-exposure
(A) Epithelium (overview) (magnification 237x)



(B) Stroma at 20% depth showing moderate collagen matrix vacuolization and the increased frequency of keratocytes with nuclear pyknosis and cytoplasmic eosinophilia (magnification 475x)



(C) Full thickness (magnification 48x)



CONCLUSIONS

The BCOP test results provide guidance on acceptable in vitro score ranges for air freshener product forms/delivery systems, different fragrance categories and correlation with acceptable eye irritation potential for currently marketed air freshener products. The following conclusions are made based on the results of the BCOP assays and histological evaluations.

The recommended testing protocol for a solid or liquid air freshener product is to test the neat product for a 10-minute exposure and use the in vitro score as the endpoint for evaluation of eye irritation potential.
In vitro scores for air freshener products (with and without fragrance) ranged from 0.0 to 110.4, reflecting a wide range in epithelial and stromal damage. Both opacity and permeability effects are accounted for by the in vitro scores. It is not recommended that either the opacity or the permeability (OD490) value be used as the sole BCOP assay endpoint for evaluating the air freshener product forms, due to the wide range of eye damage observed with the air freshener products and fragrance variants.
Scented oils produced the highest in vitro scores, followed in order of decreasing in vitro scores by gel electrics, non-aerosol sprays, carpet foam aerosol liquid concentrate (no propellant), air freshener aerosol liquid concentrate (no propellant), and adjustable solid. The composition of an air freshener (e.g., solvents) has a major impact on eye irritation potential.
Different fragrance types (i.e., watery, citrus, floral, or spice) appear to have observable impacts on eye irritation potential compared to respective un-fragranced air freshener product bases. In addition, certain fragrance categories appear to have a greater effect on specific product forms. Floral, citrus and spice-type fragrances had the greatest impact on gel electric, non-aerosol spray, and scented oil product forms, respectively.
Histological evaluation of corneas treated with selected solid and liquid air freshener products further supports the correlation of tissue damage (e.g., epithelial and stromal effects) and in vitro scores. Stromal keratocyte damage has been shown to be associated with increased irritation potential (both an increased degree and persistence of the lesions) Jester, Li et al. (1998) and Maurer, Parker et al. (2002).

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