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Optimizations of the Reconstructed Human Epidermis-Based Phototoxicity Testing to Evaluate the Safety of Long-Lasting Cosmetic Products

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Synopsis

The safety of cosmetic products coming in contact with consumers' skin is often assessed using New Approach Methodologies, which include Reconstructed human Epidermis (RhE) tissue models that have demonstrated utility as reliable test systems. In our manuscript, we evaluated the phototoxicity potential of long-lasting cosmetic products, such as lipsticks and foundations containing UV-absorbing ingredients. Given their design to persist on application areas of skin, technical challenges were encountered in the experiments using RhE tissues as the products could not be entirely removed at the end of the exposure period. A proof-of-concept study was conducted using two prototype formulations, one long-lasting and one standard use lipstick, that were spiked with multiple concentrations of chlorpromazine, a known photoirritant. The results of the proof-of-concept study identified technical aspects that were optimized (dosing and rinsing procedures) and incorporated in subsequent studies that included a group of 10 long-lasting cosmetic products. Our manuscript reports on a successful case study that supports the adaptations and optimizations to established testing protocols, and thus allows product lines that pose technical testing challenges to be reliably assessed for safety.

INTRODUCTION

Color cosmetics represent a significant part of the market nowadays, covering different categories of make-up products designed for application to the skin, eyes, or lips.[1] Among color cosmetics, long-lasting lipsticks and foundations have continuously gained popularity due to their durability, which proved to be an appealing feature to the consumers.[1] Furthermore, long-lasting cosmetic products often offer an additional benefit through the incorporation of Sun Protection Factor (SPF) in their composition.

Product lines within this category contain compounds that significantly absorb light in the UV-visible light range (290–700 nm) and are applied on areas of skin that are exposed to

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**At the time of writing this manuscript L.H. & R.L were employees of Avon Global Research and Development.

sunlight. Therefore, they may pose phototoxicity risk to the consumers.[2–4] Phototoxicity, or photoirritation, is an adverse reaction occurring upon exposure to a photoreactive product and subsequent exposure to light.[2,4] In addition, these types of products require photosafety testing in some global markets, as is the case for countries that are part of the regional trade agreement El Mercado Común del Sur (MERCOSUR): Argentina, Brazil, Paraguay, and Uruguay.[5] Based on MERCOSUR Cosmetics Regulation, long-lasting face and lip products (foundations and lipsticks) with the added SPF benefit are subject to safety and efficacy testing for registration.[6] Therefore, it behooves the industries manufacturing these types of product lines to ensure their safety prior to launch on the market. While the regulations do not require a complete suite of human clinical phototoxicity testing *per se*, safety substantiation can be achieved by using New Approach Methodologies (NAMs), which may be used by manufacturers in conjunction with the monitoring and analysis of data collected through post-market consumer safety surveys.

NAMs based on RhE tissue models provide reliable data that can be used to address certain safety endpoints and reduce the use of animals for preclinical testing.[7–9] The RhE tissue models have been used for decades to evaluate the safety of cosmetics and personal care products.[10–11] Generally based on human keratinocytes grown at Air Liquid Interface, the tissue models replicate structurally and physiologically viable basal, spinous, and granular cell layers, ultimately differentiated to form a functional *stratum corneum*, which provides the barrier function specific to the skin.[9,10,12] The RhE models mimic the outermost layer of the skin epidermis, which makes them ideal test systems for evaluating topically applied products like cosmetics.[12]

Several regulatory Test Guidelines (TG) using the RhE models have been adopted by the Organisation for Economic Cooperation and Development (OECD) to address skin irritation (TG 439),[13] skin corrosion (TG 431),[14] and most recently, photoirritation (TG 498).[4] While the regulatory assays are designed to address hazard, a recent manuscript by Ritacco *et al.*, 2022 presented a tiered testing paradigm using an RhE model (EpiDerm™, MatTek Corporation, Ashland, MA, USA) to address risk by establishing No Observed Effect Levels for photoirritation as confirmed by no human risk clinical testing.[8] Cosmetic companies have historically been using the same RhE model to address the photosafety of their finished products using testing protocols adapted from the original prevalidation studies conducted by the Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment (BfR)[15] and the EpiDerm™ Phototoxicity DB-ALM Protocol n° 121 described in the European Union Reference Laboratory (EURL)—European Centre for the Validation of Alternative Methods (ECVAM) database.[16]

While several protocols using the RhE model have been developed and routinely used, we devised a series of assay optimizations to the standard phototoxicity protocol adapted from Liebsch *et al.*, 1999 to address specific testing challenges of long-lasting cosmetics associated with their removal from the RhE tissue models.[15] The intentional development of the long-lasting products to resist transferability, provide durability, and long wear contributed to the difficulty in their removal from the tissues. Residual product on the tissues can result in prolonging the total treatment exposure, potentially impeding the irradiation exposures, and potentially interfering with the photometric measurement of the viability endpoint. Thus, technical challenges were encountered in the experiments as the products could not be entirely removed at the end of the exposure period and led to the development of the optimized protocol we describe within the manuscript. These

optimizations were postulated after inconclusive results were obtained due to tissue detachment, tissue loss, variability between treatment replicates, and overall inconsistency in results for several long-lasting products when evaluated using the standard phototoxicity protocol as adapted from Liebsch, *et al.* 1999. A proof-of-concept study was conducted using two prototype formulations, one long-lasting and one standard use lipstick, which were spiked with multiple concentrations of chlorpromazine (CPZ), a known photoirritant. The results of the proof-of-concept experiment supported the incorporation of procedural changes. The optimized protocol was then used to evaluate 10 additional long-lasting cosmetic products. Our strategy is a successful case study exemplifying the need to adapt testing methodologies to the specifics of the products being evaluated, which can further advance the successful implementation of NAMs for cosmetic safety assessment.

MATERIALS & METHODS

REAGENTS AND ASSAY CONTROLS

The RhE tissues (EPI-200) and the EpiDerm™ Assay Medium (proprietary mixture) were supplied by MatTek Corporation (Ashland, MA, USA). The assay negative control, Hanks' Balanced Salt Solution (HBSS) containing Ca^{++} & Mg^{++} and without phenol red, was provided by Fisher Scientific (Portsmouth, NH, USA). CPZ, dimethyl sulfoxide (DMSO), isopropanol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Millipore Sigma (Bedford, MA, USA). The Phosphate Buffered Saline (PBS) (pH 7.4) without Ca^{++} & Mg^{++}, Dulbecco's Phosphate Buffered Saline without Ca^{++} & Mg^{++} (CMF-DPBS), L-glutamine, Dulbecco's Modified Eagle's Medium (DMEM), and penicillin/streptomycin were supplied by Quality Biologicals (Gaithersburg, MD, USA).

Stocks of CPZ (100 mg/mL) were prepared in DMSO and then frozen at $-20^{\circ} \pm 5^{\circ}$C until use. Stocks of MTT (10 mg/mL) were prepared in PBS, filtered using a 0.45 μm Corning filter (Corning, NY, USA), and frozen at $-20^{\circ} \pm 5^{\circ}$C until use. Stocks of MTT Addition Medium were prepared by supplementing DMEM with 2 mM L-glutamine and then stored at 2° to 8°C until use.

The solvent control, HBSS containing 1% DMSO, was prepared by adding DMSO to HBSS at a 1:100 concentration. The assay positive control, 0.02% CPZ, was prepared from the 100 mg/mL stock by adding DMSO to create a solution of 2% CPZ, which was then further diluted 1:100 in HBSS to create the final 0.02% CPZ in HBSS containing 1% DMSO. The Post-Exposure Assay Medium was prepared on the day of use by adding streptomycin sulfate (100 μg/mL) and penicillin (100 IU/mL) to EpiDerm™ Assay Medium. A 1 mg/mL MTT solution was prepared on each day of use by adding 9 mL of MTT Addition Medium to 1 mL MTT stock (10 mg/mL) and used within 2 h of preparation.

Killed control (KC) tissues (*i.e.,* prepared by repeated freeze-thaw cycles from viable tissues and that have limited, if any, metabolic activity) were either received from MatTek Corporation or prepared at IIVS.

The 9 lipsticks (L#1 to L#9) and 6 foundations (F#1 to F#6) tested in our experiments were representative formulations supplied by Avon Global Research & Development in stock volumes (*e.g.,* 100 mL) in a glass vessel (*i.e.,* not in final product packaging as would be purchased by consumer).

SOLAR SIMULATOR

The tissues were irradiated (+Irr) using a Dermalight SOL3 solar simulator (UVATEC, Sherman Oaks, CA, USA) fitted with H1 filter (320–400 nm), allowing passage of UVA and visible light while attenuating UVB. The UVA intensity was checked before and after each experiment using the Model 100 broadband radiometer (G&R Labs, Santa Clara, CA, USA) equipped with UVA sensor. Tissues were exposed to an equivalent UVA of 1.7 ± 0.1 mW/cm^2, resulting in a total UVA exposure of 6 J/cm^2 after a 1-hour period. The nonirradiated tissues (dark exposure treatment group) are referred to as −Irr throughout the manuscript.

DIRECT MTT REDUCTION TEST AND KILLED CONTROL EXPERIMENTS

Prior to each experiment using RhE tissues, a preliminary test was performed for each product to determine its ability to directly reduce MTT in the absence of viable cells or interfere with the MTT measurement. An aliquot of each product (50 μL or 20 μL, depending on protocol, standard or optimized, respectively—see Figure 1) was added to a 1 mg/mL MTT solution and then incubated in the dark at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air (*i.e.,* standard culture conditions) for 1 to 3 hours. If the MTT solution turned to a purple or blue color, or if the product was dark colored and the ability to detect a color change was not possible, the product was presumed to directly reduce MTT or interfere with the measurement, respectively. All of the long-lasting cosmetic products included in our experiments were darkly colored and thus, a KC experiment was performed concurrently.

The KC tissues were treated with the products and then underwent the same procedures as viable tissues (*i.e.*, exposure time, irradiation steps, post-exposure incubation, and MMT extraction process). Additional calculations were performed as needed.

The amount of direct MTT reduction or interference was considered to have potential to impact the relative viability results if the net optical density (OD) at 570 nm (OD_{570}) values for the KC were greater than 0.150 (representing ∼10% of the OD_{570} value of the negative

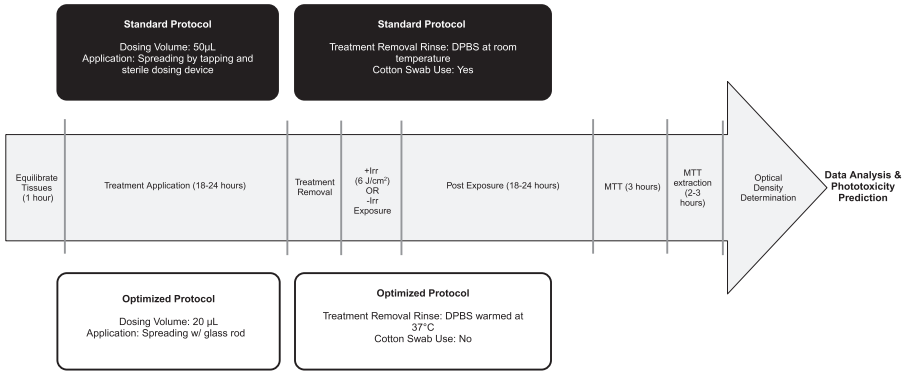


Figure 1. Flow diagram of RhE Phototoxicity assay procedures used to evaluate cosmetic products. The procedures were highly similar between protocols with exception of the treatment application and treatment removal steps as highlighted in the boxes specifying differences between the standard protocol (black boxes) and optimized protocol (white boxes). Figure created with BioRender.com.

control-treated viable tissues and determined empirically at IIVS) and were adjusted as described in the data calculation section below.

STANDARD PROTOCOL PROCEDURES

The standard protocol (Figure 1) was adapted from the method prevalidated by ZEBET[15] and with similarities to OECD TG 498[4]. Briefly, the products were applied to the apical surface of the tissues (Figure 2A) at 50 μL/tissue. To facilitate spreading over the surface of the tissue, sterile dosing devices (plastic push-pin devices about the same diameter as the tissue surface) were applied over the dose (Figure 2B). Each product was applied to the surface of six replicate viable tissues and duplicate KC tissues, where applicable. All assay control treatments were applied in quadruplicate viable tissues, or duplicate KC tissues as needed. The tissues were incubated up to 24 hours at standard culture conditions. At the completion of the exposure period, attempts were made to remove the applied products from the surface of the RhE tissues using sterile CMF-DPBS. Sterile cotton swab applicators soaked in sterile CMF-DPBS were used to facilitate removal of the products (Figure 2C). The treatments could not be completely removed from the tissues following the rinsing and swabbing process (Figure 2D). Half of the tissues from each treatment group were designated for the irradiation step (+Irr) and the remaining half for the dark exposure (−Irr). Tissues were placed into the respective wells of a 24-well plate containing 0.3 mL of HBSS per well, and plate lid applied. Tissues were then exposed to a total of 6 J/cm^2 of UVA (+Irr) or dark conditions (−Irr) for 1 hour. At the completion of the 1-hour exposure, the tissues were transferred to 6-well plates containing Post-Exposure Assay Medium and incubated at standard culture conditions for 18 to 24 hours. After the overnight incubation, tissues were incubated with 0.3 mL of 1 mg/mL MTT solution for 3 ± 0.1 hours at standard culture conditions. The reduced MTT was then extracted in 2 mL of isopropanol for 2 to 3 hours at room temperature on a plate shaker. The tissue inserts were removed from their wells and then 200 μL of each extracted sample were quantified at 550 nm or 570 nm using a Versamax plate reader (Molecular Devices, CA, USA) with shaking function selected. Duplicate samples of isopropanol (200 μL) were plated as blanks. The OD at 550 nm was initially used in alignment with previous IIVS internal protocols, but later updated to 570 nm in alignment with the OECD Test Guidelines. While both wavelengths were acceptable for the absorbance readings, OD_{570} is presented throughout the manuscript for simplification.

A preliminary screening experiment that pre-evaluated the cytotoxicity potential of the products was conducted to ensure the selection of appropriate exposure times for phototoxicity experiments. Briefly, a single viable tissue was exposed to the product at three to four exposure times (*e.g.*, 4, 8, 20 and 24 hours) in the dark conditions only (−Irr). A single tissue was also treated with HBSS (−Irr) for the longest exposure time (up to 24 hours). After the completion of the appropriate exposure time, the standard protocol as described above was followed. The cytotoxicity results were used to inform exposure times for the subsequent phototoxicity experiment, which may have been less than the standard 18 to 24 hours treatment exposure period. Products L#1, F#1, and F#2 were evaluated in the preliminary screening followed by one phototoxicity experiment.

The positive control was evaluated in each phototoxicity experiment conducted, and exposed to the tissues for 18 to 24 hours, and at reduced exposure times of 3, 8, or 20

Figure 2. Representative photographs of untreated RhE tissue (2A), sterile dosing device spreading 50 μL of product over tissue surface under the standard protocol treatment application (2B), sterile cotton swabs removing product at treatment termination (2C), tissue with residual test product after attempted treatment removal (2D), sterile glass rod spreading 20 μL of product over tissue surface under the optimized protocol treatment application (2E), tissue treated with 20 μL product covering tissue surface (2F), partial tissue loss to tissue as highlighted with arrow (2G), and a detached tissue that was separated from the semi-permeable membrane but that remained within the tissue culture insert (2H). Images captured by IIVS and figure created with BioRender.com.

hours when a shorter exposure time was prescribed from the preliminary screening results for the products being concurrently tested.

OPTIMIZED PROTOCOL

The dosing and rinsing procedures were the focus of the optimizations as they were anticipated to have the greatest potential to impact the study results. The products were

applied at 20 μL dosing volumes and then spread over the surface of the tissues using a sterile glass rod (Figure 2E) to ensure complete coverage over the surface of the tissue (Figure 2F). The sterile CMF-DPBS used to remove the treatments was warmed at 37° ± 1°C. Cotton swabs were not used to remove residual product. A summary comparison of the procedures the standard and optimized protocols is presented in Figure 1.

Products L#4 to L#6 and F#3 to F#9 were evaluated in one phototoxicity experiment. The positive control, 0.02% CPZ, was evaluated in each experiment.

PROOF OF CONCEPT STUDY

Two lipsticks were tested as part of a proof-of-concept study to investigate the impact of the optimized protocol and its capacity to predict phototoxic potential of the products when spiked with various concentrations of CPZ, a known photoirritant: L#2 was a long-lasting product, while L#3 was a standard product. The proof-of-concept study was conducted using the optimized protocol (Figure 1) and included treatment removal with and without use of cotton swabs to understand potential impact. L#2 was tested undiluted (neat) and was also spiked with two concentrations of CPZ (0.1% and 0.5%), while L#3 was tested neat and spiked with 0.5% and 1% CPZ, respectively. The CPZ-spiked lipsticks were prepared by blending the 100 mg/mL CPZ stock in DMSO with an aliquot of each designated neat lipstick loaded into a syringe and mixing between two syringes affixed with a stopcock until homogenous. L#2 and L#3 were evaluated in two phototoxicity experiments.

DATA AND CALCULATIONS

Data were analyzed using Excel. A corrected OD_{570} for each tissue was calculated using the following formula:

$$Corrected\ OD_{570} = Product\ or\ Control\ Raw\ OD_{570} - Blank\ Mean\ OD_{570}$$

When KC tissues were used, the amount of direct MTT reduction or interference by the product was determined using the following calculation:

$$Net\ OD_{570}\ of\ the\ Product = Raw\ OD_{570}\ Product\ KC - Raw\ OD_{570}\ Negative\ Control\ KC$$

The % relative tissue viability was determined based on the following calculation:

$$\%Viability = \frac{Corrected\ OD_{570}\ of\ Product\ or\ Positive\ Control}{Corrected\ OD_{570}\ of\ Negative\ Control} \times 100$$

The individual tissue % relative viability values were averaged to calculate a mean % of control for tissues in the (+Irr) and (−Irr) treatment groups. One standard deviation of the % of control of the tissue replicates was calculated and presented using error bars in the data (see the Results section).

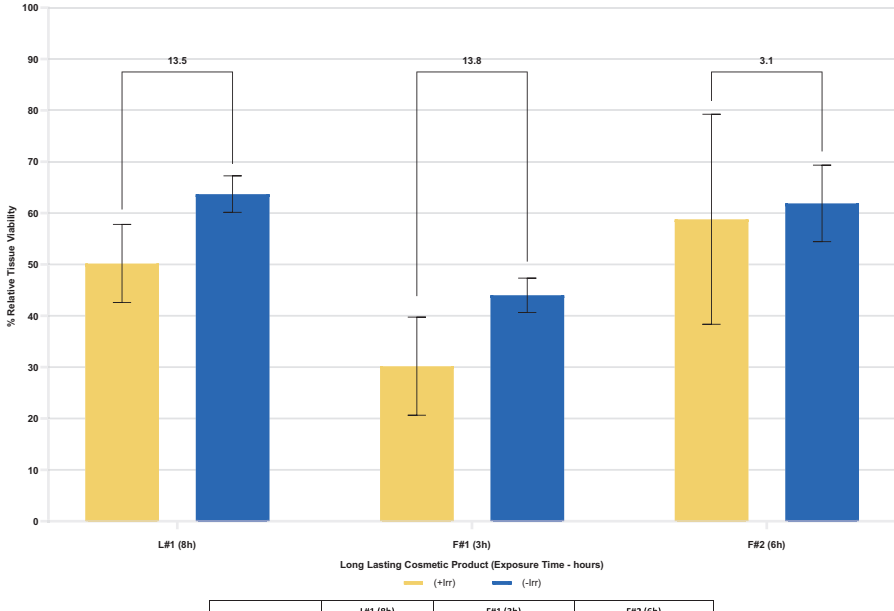
EVALUATION OF CYTOTOXICITY AND PHOTOTOXICITY POTENTIAL

In alignment with the prediction model presented in the protocol by ZEBET[14] and OECD TG 498[4], a product was considered to have exhibited phototoxicity potential if the difference in viability between the tissues in the (+Irr) and (−Irr) treatment groups was ≥30%. This evaluation was applicable in all of our phototoxicity experiments. In addition, the tissues exposed in the absence of irradiation (−Irr) were expected to have sufficient viability (*e.g.*, >35% in alignment with OECD TG 498, 2023)[4] to appropriately evaluate for phototoxicity potential. If the viability was less than 35% (−Irr), the results were considered to indicate cytotoxicity.

RESULTS

INCONCLUSIVE PHOTOTOXICITY RESULTS OBTAINED FOR LONG-LASTING COSMETIC PRODUCTS WHEN EVALUATED USING THE STANDARD PROTOCOL

Figure 3 presents the summary of the results obtained when the standard protocol was used to evaluate three long-lasting cosmetic products (one lipstick and two foundations).



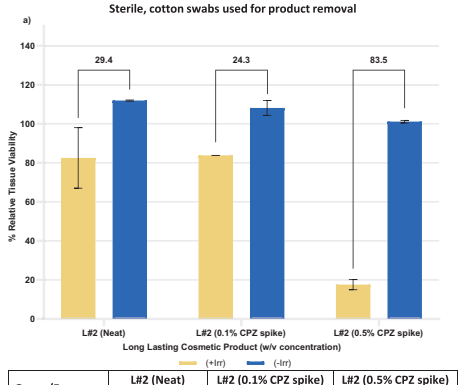
Group/Exposure	L#1 (8h)		F#1 (3h)		F#2 (6h)	
	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)
Mean % Viability	50.2	63.7	30.2	44.0	58.8	61.9
S.D. of Viability	15.2	7.1	19.1	6.7	40.9	14.9

Figure 3. Example of inconclusive results obtained when long-lasting cosmetic products were tested using the standard RhE-based phototoxicity protocol. The mean % relative viability values (calculated from three tissues per treatment and +Irr or −Irr exposure) pertaining to the tissues exposed in the presence of irradiation (+Irr) are presented in the yellow bars, while the blue bars represent the % mean relative viability values of the tissues exposed in the absence of irradiation (−Irr). Standard deviation (1) of the % viability of three tissue replicates was calculated and presented using error bars. The difference between tissues in the (+Irr) and (−Irr) treatment groups was calculated and presented above bars in respective treatment group graphs. Individual mean % viability and standard deviation of % viability for (+Irr) and (−Irr) are presented in the table below the graphics. F: foundation; Irr: irradiation; L: lipstick.

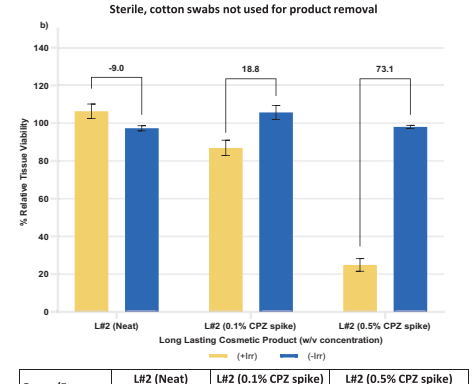
As highlighted in Figure 3, high variability between tissue replicates resulted from tissue loss (F#1, −Irr) (Figure 2G) and tissue detachment (F#2, +Irr & −Irr) (Figure 2H). The tissues treated with L#1 had a higher-than-expected cytotoxicity based on previous results (data not shown). Furthermore, in most cases the data indicated higher than expected cytotoxicity potential of the products not exposed to irradiation (−Irr). Therefore, based on the data obtained in these experiments, an inconclusive prediction for phototoxicity potential was drawn for L#1, F#1, and F#2.

PROOF-OF-CONCEPT STUDY

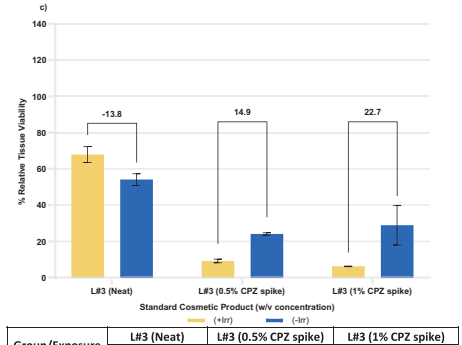
Figure 4 summarizes the results of the experiments using the optimized protocol (Figure 1). L#2 and L#3 (undiluted) were evaluated in two independent experiments, and the data



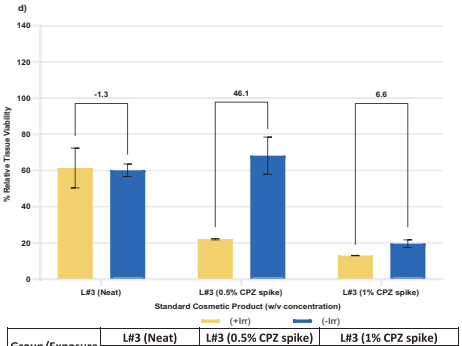
Group/Exposure	L#2 (Neat)		L#2 (0.1% CPZ spike)		L#2 (0.5% CPZ spike)	
	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)
Mean % Viability	82.5	111.9	83.8	108.1	17.6	101.1
S.D. of Viability	15.5	0.6	0.1	7.6	5.2	1.3



Group/Exposure	L#2 (Neat)		L#2 (0.1% CPZ spike)		L#2 (0.5% CPZ spike)	
	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)
Mean % Viability	106.3	97.3	86.9	105.7	24.9	98.0
S.D. of Viability	7.6	2.7	8.1	7.4	6.7	1.7



Group/Exposure	L#3 (Neat)		L#3 (0.5% CPZ spike)		L#3 (1% CPZ spike)	
	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)
Mean % Viability	67.9	54.1	9.2	24.1	6.2	28.9
S.D. of Viability	8.8	6.4	2.0	1.3	0.4	21.9



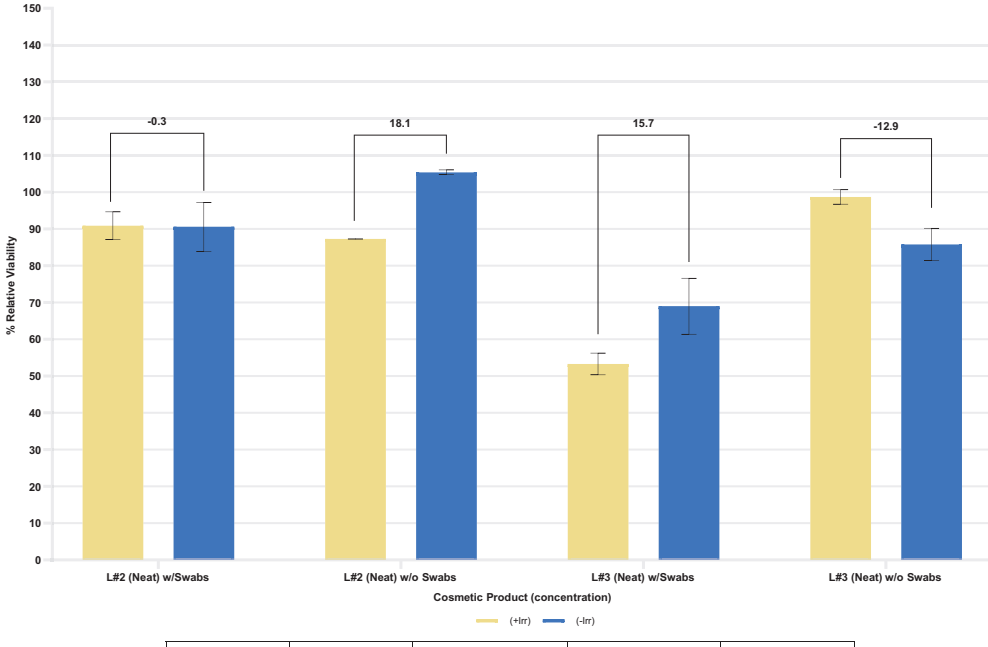
Group/Exposure	L#3 (Neat)		L#3 (0.5% CPZ spike)		L#3 (1% CPZ spike)	
	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)
Mean % Viability	61.4	60.1	22.1	68.2	13.0	19.6
S.D. of Viability	22.0	6.9	0.8	20.5	0.3	4.2

Figure 4. Proof of concept experimental results outlining the impact of swabs used to remove the lipsticks spiked with various concentrations of a known phototoxic ingredient (CPZ) on RhE tissue viability. The mean % relative viability values (calculated from two tissues per treatment and +Irr or −Irr exposure) pertaining to the tissues exposed in the presence of irradiation (+Irr) are presented in the yellow bars, while the blue bars represent the % mean relative viability values of the tissues exposed in the absence of light (−Irr). Standard deviation (1) of the % viability of tissue replicates was calculated and presented using error bars. The difference between tissues in the (+Irr) and (−Irr) treatment groups was calculated and presented above bars in respective treatment group graphs. Individual mean % viability and standard deviation of % viability for (+Irr) and (−Irr) are presented in the table below the corresponding graph. L: lipstick; CPZ: chlorpromazine; Irr: irradiation.

presented in Figure 2 pertain to the first experiment. The data generated in the second experiment were similar (Figure 5).

The results obtained for the long-lasting cosmetic product L#2 (Figure 4A, 4B) showed that regardless of the use of swabs for the removal of the lipstick from the tissues, the test system was capable to identify the product spiked with 0.5% CPZ as phototoxic. The standard deviation of mean tissue viability values were overall tighter when swabs were not used in the experiments (Figure 4B).

The results obtained for the standard cosmetic product L#3 (Figure 4C, 4D) showed that the viability values of the tissues treated with the neat and spiked product were lower than those obtained for L#2. L#3 spiked with 1% CPZ was cytotoxic, and the phototoxicity potential could not be evaluated (*i.e.,* viability of tissues in the −Irr treatment group was <35%) (Figure 4C, 4D). Furthermore, the use of swabs likely contributed to the lower viability values obtained for the product spiked with 0.5% CPZ (Figure 4C). Phototoxicity potential was identified only when swabs were not used (Figure 4D).



Group/Exposure	L#2 (Neat) w/Swabs		L#2 (Neat) w/o Swabs		L#3 (Neat) w/Swabs		L#3 (Neat) w/o Swabs	
	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)	(+Irr)	(-Irr)
Mean % Viability	90.9	90.6	87.3	105.4	53.3	69.0	98.7	85.8
S.D. of Viability	7.5	13.3	0.3	1.3	5.9	15.2	3.9	8.8

Figure 5. Proof-of-concept results from experiment #2 outlining the impact of swabs used to remove the lipsticks (undiluted) on RhE tissue viability. The mean % relative viability values (calculated from three tissues per treatment and +Irr or −Irr exposure) pertaining to the tissues exposed in the presence of irradiation (+Irr) are presented in the yellow bars, while the blue bars represent the % mean relative viability values of the tissues exposed in the absence of light (−Irr). Standard deviation (1) of the replicates was calculated and presented using error bars. The difference between tissues in the (+Irr) and (−Irr) treatment groups was calculated and presented in graphs. Individual mean % viability and standard deviation of viability for (+Irr) and (−Irr) are presented in the table below the graph. L: lipstick; Irr: irradiation.

PHOTOTOXICITY RESULTS OBTAINED FOR LONG-LASTING COSMETIC PRODUCTS WHEN EVALUATED USING THE OPTIMIZED ASSAY PROTOCOL

The phototoxicity potential of 10 long-lasting cosmetic products, six lipsticks (L#4 to #9) and four foundations (F#3 to #6), were evaluated using the optimized protocol and are presented in Table I. The long-lasting products were well tolerated by the tissues, as supported by the lack of cytotoxicity in the nonirradiated treatment groups (−Irr) (*i.e.,* viability >35%). None of the long-lasting products induced at least 30% difference in viability of tissues in the +Irr treatment group as compared to tissues in the −Irr treatment group, and therefore were not predicted to have phototoxicity potential.

DISCUSSIONS

The use of cosmetics has been described for centuries dating back to 10,000 B.C.[9,17] and their makeup and functionality have evolved tremendously ever since to include specific types of product lines, like the long-lasting cosmetics evaluated in our experiments. Long-lasting cosmetics are typically formulated with higher levels of film-formers, due to their ability to adhere well to the skin.[1] Film foamers also aid in application, wear, and the nontransfer properties of a product, making them ideal for lip and face cosmetics.[1] The lips are subject to constant abrasion from eating, drinking, the licking of the lips as well as stretching movements associated with speaking.[1] As a result, the long-lasting lip products in this manuscript were formulated with film foamers to withstand the high abrasion native to the lips, provide durability, and adhesion to the lips for long-lasting wear. The long-lasting

Table I
Prediction of the Phototoxicity Potential of Long-Lasting Cosmetic Products (Lipsticks and Foundations) Evaluated Using the Optimized Protocol

Product tested	(+Irr) treatment group		(−Irr) treatment group		% Difference[a]	Phototoxicity potential[b]
	Tissue viability (%)	SD (%)	Tissue viability (%)	SD (%)		
L#4	98.4	2.0	94.3	3.3	−4.1	No
L#5	97.3	6.7	89.5	4.6	−7.8	No
L#6	99.4	4.1	101.7	4.8	2.3	No
L#7	109.7	3.4	105.6	3.0	−4.1	No
L#8	95.7	2.2	85.5	5.9	−10.2	No
L#9	100.5	7.7	90.8	4.3	−9.7	No
F#3	97.8	1.4	94.2	5.6	−3.6	No
F#4	107	1.3	99.4	2.1	−7.6	No
F#5	105.9	1.5	106.7	3.9	0.8	No
F#6	112.8	19.9	87.2	3.7	−25.6	No

F: foundation; +Irr: irradiated; −Irr: nonirradiated; L: lipstick.

(a) Calculation of difference in % mean relative viability of tissues exposed in the presence (+Irr) or absence (–Irr) of UVA/visible light.

(b) According to the prediction models presented in ZEBET[14], OECD Test Guideline 498[4], and study protocol used in our experiments, a product is predicted to have phototoxicity potential if the difference between the viability of the tissues exposed in the presence of irradiation (+Irr) as compared to the absence of irradiation (–Irr) is ≥30%.

foundations were formulated similarly, targeting long wear, adhesion to the skin, and durability that could withstand normal facial movements and physical stressors.[1] This was achieved with the inclusion of varying levels of silicone film-formers which interact with solvents, pigments, and fillers within the foundation to provide long-lasting color, water and sebum resistance, and superior adhesion to the skin.

Nowadays, cosmetic and personal care products are regularly assessed for their potential to induce toxicity to the areas of the human body they come in contact with.[9] An additional layer of complexity is brought by products intended to be applied onto the skin for extended periods of time (long lasting) and that may come in contact with UV and visible light. If any chemical or combination of ingredients that are part of the composition of these products is excited by UV absorption or visible light, it has the potential to become phototoxic.[9,18,19] Subsequently, photosensitized molecules have the capacity to induce oxidative damage which can further lead to directly or indirectly induced damage to the keratinocytes within the epidermal layers.[9,18,20] With sufficient irradiation exposure, a phototoxic compound can induce adverse reactions in most individuals, occurring within minutes to hours upon exposure.[21] Clinical manifestations of phototoxicity can include erythema, edema, burning, and inflammation.[9,18,21] As a result, product lines like long-lasting cosmetics (lipsticks, foundations, etc.) that contain UV or visible light-absorbing ingredients require safety assessment for potential phototoxicity.

Depending on the market, the safety assessment requirements may be specific regarding effective test methods to investigate the phototoxicity potential. The products tested in our experiments were intended for launch in the MERCOSUR countries (Argentina, Brazil, Paraguay, and Uruguay). The National Agency for Sanitary Surveillance (ANVISA) in Brazil defines personal care products, cosmetics and perfumes as "preparations consisting of natural or synthetic substances for external use on various parts of the body, skin, capillary system, nails, lips, etc., with the sole or main purpose of cleaning them, perfuming them, altering their appearances, correcting body odors, and or protecting or maintaining them in good condition."[22] Based on the risk involved, the products are separated into two classes (I and II). From that perspective, lipsticks and eye and facial make-up preparations (without sunscreen) are considered Class I and do not require detailed information on their labeling regarding their mode of use and their restrictions of use.[22] To this end, a reliable and reproducible test system, like the RhE model, can be used to substantiate the safety of cosmetic products intended for this market.[9]

The RhE-based phototoxicity test was previously reported for assessment of sunscreens,[23] sunscreen additives like titanium dioxide (TiO_2),[24,25] or of personal care product ingredients.[26,27] Previous reports on lipsticks' safety focused primarily on their photostability[18,28] and/or photoprotective properties,[29] or on the presence of metals in their composition and the risk posing to the consumers.[30,31] Unlike the test system in OECD TG 432 based on a monolayer of Balb/c 3T3 mouse fibroblast cells,[3] the RhE model is a multi-layer reconstructed tissue model comprised of human cells that can overcome the challenges of solubility and is tolerant to UVB. Further, the RhE tissue is a more relevant test model given the intended use (*i.e.,* application to the skin) of the cosmetic products.[9] There are limited studies published on the phototoxic assessment of lipsticks and foundations, and our experiments report for the first time on the use of the RhE phototoxicity assay for safety substantiation of color cosmetics and specifically for long-lasting products.

Our manuscript brings forward the concept of adapting an existing established protocol to the specifics of a product line posing technical challenges during the experimental

investigations. In the development of the optimized protocol, we considered intended product use, as well as technical aspects for study execution. As such, a balance was needed between application, exposure, and removal of the products from the tissue surface. These products are applied to the skin as a thin layer, and therefore, the application volume was lowered (20 μL) and applied over the surface of the tissues using a sterile glass rod (Figure 2E), providing full coverage across the surface of the tissue (Figure 2F). In our experiments, all treatments were attempted to be removed prior to irradiation to ensure adequate UV exposure since the tissues are irradiated (+Irr) from a top-down approach (*e.g.,* at the apical surface). As described in the ZEBET protocol[15] and OECD TG 498,[4] treatments are removed after irradiation (+Irr), but additional guidance in OECD TG 498[4] discusses that opaque or dark colored materials that may impede the light exposure should be removed prior to UV exposure (+Irr).[4] The removal also aligned with the clinical confirmatory testing approaches where excess product not absorbed into the skin during the initial treatment period was removed prior to irradiation.[32] For phototoxicity testing, products are applied on the *stratum corneum* of the tissue model (preclinical) (OECD TG 498)[4] and human skin (clinical)[30] for a specific time period, allowing penetration into the tissue prior to the irradiation exposures. The initial treatment periods (up to 24 hours) were expected to be sufficient exposure times to allow penetration into the tissues (OECD TG 498).[4] Previous reports communicated that toxicity and phototoxicity induced by ingredients or finished products to human skin depend on their penetration rates through the *stratum corneum*. In that regard, multiple studies showed that the penetration rates through *in vitro* skin models were greater than that of human native skin,[33–37] thus providing additional support to our optimized protocol. The removal of treatments prior to irradiation was supported in practice by the results of the tissues treated with the positive control (0.02% CPZ) for 3 to 24 hours exhibiting phototoxicity potential (Table II). In our initial experiments using the standard protocol (Figure 1), cotton swabs soaked in buffer solution were utilized to facilitate treatment removal, as previously described in OECD guidelines procedures when standard rinsing processes are not sufficient.[4,13] However, when cotton swabs were used, the results were inconclusive (Figure 3), and their use was more likely to cause mechanical

Table II
Summary of Positive Control (0.02% CPZ) Experimental Results

Exposure time[a]	+Irr		−Irr		% Difference[b]	Phototoxicity potential?[c]
	Tissue viability (%)	SD (%)	Tissue viability (%)	SD (%)		
8 h	7.8	0.8	89.7	12.7	81.9	yes
3 h	12.2	2.1	90.6	2.0	78.4	yes
18–24 h	28.1	3.0	99.9	2.4	71.8	yes
18–24 h	15.5	2.5	94.5	1.5	79.0	yes
18–24 h	31.3	2.0	98.9	5.2	67.6	yes
18–24 h	61.9	1.4	105.1	2.1	43.2	yes
18–24 h	31.7	1.2	102.0	1.1	70.3	yes

+Irr: irradiated; −Irr: nonirradiated.

(a) Initial treatment exposure time of positive control to the RhE tissue prior to +Irr or –Irr.

(b) Calculation of difference in % mean relative viability of tissues exposed in the presence (+Irr) or absence (–Irr) of UVA/visible light.

(c) According to the prediction models presented in ZEBET[14], OECD Test Guideline 498[4], and study protocol used in our experiments, phototoxicity potential was predicted if the difference between the viability of the tissues exposed in the presence of irradiation (+Irr) as compared to the absence of irradiation (–Irr) is ≥30%.

damage to the tissues, including tissue loss and detachment from the insert membranes (Figures 2G, 2H), resulting in variability between replicates, as highlighted in the proof of concept experiments (Figure 4). This was not unexpected for long-lasting cosmetic products since they are designed to be durable to sustain exposure extending several hours (*e.g.,* often up to a full day of wear). These products may be so durable that their removal would be facilitated through a cosmetic make-up remover (according to products' labels). Therefore, the swabs were not used during the treatment removal process in the subsequent optimized protocol (Figure 1). Collectively, these optimizations, as initially investigated in the proof-of-concept study, supported that the optimized protocol allowed the identification of the phototoxicity potential of the CPZ-spiked lipsticks (Figure 4). Therefore, these changes were incorporated into the optimized protocol (Figure 1) for subsequent evaluation of phototoxicity potential of 10 additional long-lasting products.

As expected with the optimized protocol, residual cosmetic product was observed on all tissues treated with the long-lasting cosmetic products (Figures 2D, 2F). Residual product can affect assay results by prolonging the total treatment exposure (*e.g.*, the initial exposures up to 24 hours and then throughout the post-exposure incubation period of 18 to 24 hours), impeding the irradiation exposures, and interfering with the measurement of the MTT endpoint. The long-lasting products were well tolerated on the tissues for up to 48 total hours, as supported by the viability of the neat lipsticks (Figure 4) and products evaluated with the optimized protocol (Table I). The proof-of-concept experiment demonstrated that the exposure conditions did not impede the light exposure, as evidenced by the dose-dependent responses of the spiked lipsticks (Figure 4) where phototoxicity potential was determined with the 0.5% CPZ spike (L#2 and L#3). In addition to the residual cosmetic product's impact on the technical aspects of the assay, compounds that absorb light in the optimal range as MTT (OD_{570} nm $\pm$ 20 nm) and are extracted with the MTT may result in artificially increased absorbance values.[4] As described in OECD TG 498,[4] the KC net OD_{570} values were accounted for in calculations to provide a more accurate measure of tissue viability for the viable tissues. As expected, the nonswabbed KC tissues resulted in higher net OD_{570} values than the swabbed tissues, and the KC tissues treated with the long-lasting cosmetic product (L#2) had higher net OD_{570} values than the standard cosmetic product (L#3) (Table III). Although having potential to impact results, the residual test product was able to be addressed, technically, and when using the optimized protocol, it did not impede the ability to detect phototoxicity potential in this test system.

CONCLUSION

The NAMs-based testing strategy used in our manuscript is a successful case study of adapting an established methodology to meet the specific testing needs implemented for the safety assessment of a particular product line. The optimizations brought to the established RhE phototoxicity protocol took into account and aimed to mimic the specifics of the long-lasting cosmetic products of interest and their application and removal by an end-user. Our results obtained using the optimized RhE phototoxicity protocol demonstrate that the adaptations to the standard protocol were justified and allowed for a reliable, reproducible, and standardized evaluation of cosmetic products that may be difficult to remove upon application. All 10 long-lasting cosmetic products evaluated using the optimized protocol did not have phototoxicity potential, which was supported in the

Table III
Summary of the Killed Control Experiments (Proof-of-Concept Study) Assessing the Use of Swabs for the Removal of Cosmetic Products From the Tissues

Product tested, conditions, and experiment ID	KC net OD_{570} value[a]			
	(−Irr) treatment group		(+Irr) treatment group	
	w/ swabs	w/o swabs	w/ swabs	w/o swabs
L#2 (neat) (experiment #1)	0.163	0.339	0.209	0.436
L#2 (neat) (experiment #2)	0.079	0.401	0.145	0.343
L#2 (0.1% CPZ)	0.272	0.428	0.057	0.455
L#2 (0.5% CPZ)	0.121	0.354	0.138	0.378
L#3 (neat) (experiment #1)	0.004	0.012	0.003	0.016

HBSS: Hanks' Balanced Salt Solution; +Irr: irradiated; −Irr: nonirradiated; KC: killed control; L: lipstick; OD: optical density.

(a) Net KC OD_{570} value determined by subtracting the raw OD_{570} value of the cosmetic product KC tissue from the raw OD_{570} value of the negative control (HBSS) KC tissue.

Note: Since the net KC OD_{570} values for L#3 were minimal (*i.e.*, <0.150) in experiment #1, additional KC testing was not conducted for experiment #2.

Note: L#2 was the long-lasting lipstick and L#3 was the standard lipstick.

clinical confirmatory patch testing of the products in which patches were applied to the test subjects back under occlusion. While patch testing does not mimic consumer use, it is considered a gold standard in the evaluation of the safety of topical cosmetics and the results accurately reflect the severity of skin irritation that would be observed from actual use of the product. In addition, post-market surveys demonstrating actual consumer use of the product showed no adverse reactions in end-users. Our strategy was used to meet the specific testing requirements within the context of safety substantiation for launches on MERCOSUR markets. This work paves the way to the utilization of reliable and relevant testing platform incorporating tailored optimizations that can benefit industry for safety evaluation of these specific product lines.

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