Screening of Cosmetics Ingredients for Phototoxic Potential Using the In Vitro 3T3 Neutral Red Uptake Phototoxicity Test

[1]Ramez Labib, [1]Enrico Gilberti, [1]Stephen Gettings, [2]Hans Raabe

[1]Avon Products, Inc., Suffern, NJ, USA, [2]Institute for In Vitro Sciences, Inc., Gaithersburg, MD, USA

ABSTRACT

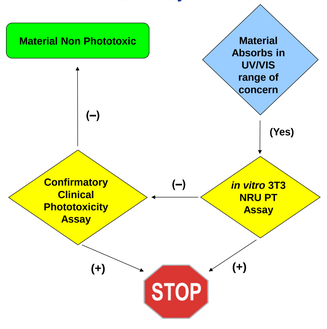
Phototoxicity is an acute toxic response after exposure to a phototoxicant and either UV radiation or visible light (UV/VIS). Phototoxicity from substances applied topically typically occurs at the site of photo-irradiation. Phototoxicity is the result of direct cellular damage caused by a non-immunological inflammatory response. Clinically, phototoxicity resembles an exaggerated sunburn (erythema, increased skin temperature, pruritis and edema). Phototoxicity reactions have been reported for both synthetic substances and those which occur naturally (e.g., botanical extracts). Although symptoms generally subside quickly, the potential for substances used in topical products to cause phototoxicity is clearly of concern for manufacturers of cosmetics, personal care and other consumer products. Historically, the potential to cause phototoxicity from substances applied topically was evaluated by utilizing various animal models. However in 1997 the 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) was validated by ECVAM's Scientific Advisory Committee as an *in vitro* method for evaluating the phototoxic potential of chemicals shown to absorb in the UV/VIS range. To illustrate the utility of the 3T3 NRU PT as a useful screening tool in the safety evaluation of potential cosmetic ingredients, the results of the evaluation of 42 botanical extracts and 25 synthetic chemicals found to absorb in the UV/VIS range are reported. Most substances evaluated were found not to be phototoxic *in vitro*; however, 9 substances were identified as potentially/probably phototoxic in the 3T3 NRU PT and were eliminated from further consideration for use as cosmetic ingredients. Several substances found to be non-phototoxic in the 3T3 NRU PT were formulated with other ingredients in a prototype cosmetic formulation and subject to clinical testing. No manifestations of phototoxicity were observed in any of the test subjects in the prototype formulation containing any of the substances identified as non-phototoxic *in vitro*.

INTRODUCTION

Phototoxicity (photoirritation) is a light-induced, non-immunological skin response to a photoreactive substance. A photoreactive substance is defined as a chemical (or mixture of chemicals) which absorb in the UVB (290 – 320 nm), UVA (320 – 400 nm) and/or visible light (>400 nm) portion of the ultraviolet/visible (US/VIS) radiation absorption spectrum (US FDA, 2003); however, the majority of substances which are known to be phototoxic absorb in the UVA portion of the spectrum (Lovell, 1993).

Photoirritation reactions resemble primary irritation reactions in that they can be elicited following a single exposure and can occur after either ingestion or dermal contact with a phototoxicant. It therefore behooves responsible manufacturers of cosmetic and personal care products to carry out an assessment of the potential for phototoxicity of novel cosmetic ingredients intended for use on areas of the body which may be exposed to sunlight. To this end, a phototoxicity screening paradigm to identify potentially phototoxic substances prior to their use in cosmetic formulations is described (*Figure 1*):

Figure 1. Testing and Evaluation Strategy of Ingredients for Phototoxicity Potential



- Preliminary assessment of potential to cause phototoxicity is conducted based on a thorough literature search for relevant safety information and an evaluation of any pre-existing toxicity (animal or clinical) data. Substances for which there is evidence of causing phototoxicity are rejected from further consideration for use as cosmetic ingredients. The UV/VIS absorbance spectrum (250-700 nm) is determined for those substances for which there was no existing data on phototoxicity potential.
- Those substances with absorbance ≥290 nm are evaluated in the *in vitro* 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT). The 3T3 NRU PT is accepted at the international level (OECD Test Guideline 432) and for regulatory purposes (both in the U.S. and elsewhere) (US FDA 2003, EC, 2000; EMEA, 2002).
- If a positive response is observed in the 3T3 NRU PT, the substance is considered unacceptable for use as a cosmetic ingredient. Assuming there are no other safety considerations precluding their use as cosmetic ingredients, substances found to be negative in the 3T3 NRU PT are evaluated in one or more prototype cosmetic formulations at the final desired use concentration in a confirmatory clinical phototoxicity test prior to their use in marketed cosmetic products.

The results of using the paradigm to screen 68 candidate cosmetic ingredients with >0.1% absorbance at ≥290 nm for which there was no prior evidence of phototoxicity are reported.

MATERIALS & METHODS

All test substances were commercially available botanical extracts (or mixtures of botanical extracts) (n=42; *Table 1*) or synthetic materials (n=26; *Table 2*) under consideration for use as cosmetic ingredients. The absorption spectrum of each test substance was measured in an aqueous solution using a UV/VIS spectrophotometer with a wavelength range of 250-700 nm and cuvette path length of 1 cm. Substances were considered to exhibit significant absorption if the specific absorbance (A) for a 1% solution with a path length of 1 cm (A1% cm) was >0.1 at any wavelength between 290-700 nm. Test substances were evaluated in the 3T3 NRU PT assay if the A1% cm at any wavelength ≥290 nm was >0.1.

The *in vitro* 3T3 NRU PT protocol was a modification of the procedure described in OECD Test Guideline 432 (TG 432). In brief, Balb/c mouse derived 3T3 fibroblast cells were incubated with various dilutions of test substances in 96 well plates for 1 hour, and thereafter exposed to UV/VIS light (at wavelengths >290 nm) for 50 minutes (5 J/cm2). A second set of treated fibroblasts was exposed to the test material in the dark and evaluated in parallel. Neutral red dye uptake (NRU) was determined 24 hours later by measuring the optical density at 550 nm. Neutral red dye is only taken up and retained within the lysosomes of metabolically active, viable cells, thus providing a direct measure of cell viability (*Figure 2*).

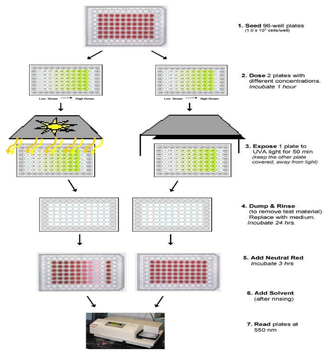


Figure 2: Overview of the 3T3 NRU PT Assay

A decrease in cell viability has been shown to correlate with phototoxicity (Spielmann *et al.*, 1994, 1998). The concentration of test material causing a 50% reduction of cell viability (IC_{50}) was calculated using an appropriate non-linear curve fitting model (*Figure 3*). To discriminate between phototoxic and non-phototoxic substances, a photo irritation factor (PIF) defined as the ratio of the IC_{50} value determined in the absence of UVA to the IC_{50} value in the presence of UVA was calculated (Spielmann *et al.*, 1994, 1996). For those substances where IC_{50} values could not be determined in either the UVA or the dark exposure groups, the mean photo effect (MPE) was determined using a comparison of the area under the curve (AUC) from the concentration response curves obtained in both the presence and absence of UV light. A test substance with a PIF < 2 or an MPE < 0.1 is predictive of "no phototoxicity"; a PIF ≥ 2 and < 5, or an MPE ≥ 0.1 and < 0.15 is predictive of "probable phototoxicity"; and a PIF ≥ 5 or an MPE ≥ 0.15 is predictive of "phototoxicity" (OECD TG 432).

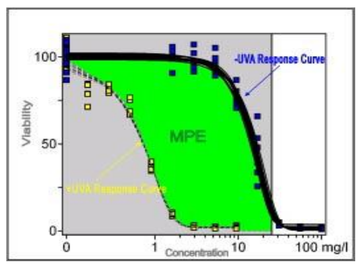


Figure 3: Comparison of the MPE Response Curves in the 3T3 cells in presence and absence of UV light

Confirmatory clinical phototoxicity testing (n=10) was conducted based on the method of Kaidbey and Kligman (1980) but with exposure to each test substance for 24 hours rather than 6 hours and exposure to 0.5 MED full spectrum solar-simulated radiation in addition to 10 J/cm^2 UVA (the protocol was approved by an Ethical Review Board). Measurement of the shortest exposure producing a minimally visible faint erythema 20 to 24 hours later was used to determine the Minimal Erythemal Dose (MED) for each subject on Day 1. On Day 2 approximately 40 mg of a prototype cosmetic formulation containing a test substance was applied to duplicate (2x2 cm) squares of nonwoven cotton cloth and fastened to skin with occlusive tape. Twenty four hours later, one set of patches was removed and the test sites immediately exposed to 10 J/cm^2 UVA plus 0.5 MED full spectrum solar-simulated radiation. The other set of patches served as unirradiated controls. An adjacent site was similarly treated with a vehicle (petrolatum) and exposed to the same dose of UVA plus 0.5 MED full spectrum solar-simulated radiation and served as an irradiated control. Reactions were graded immediately and at 24 and 48 hours after irradiation. A phototoxic material will produce either a wheal-and-flare response immediately after exposure or an intense erythema at either 24 or 48 hours (Kaidbey and Kligman, 1980).

RESULTS

Only materials with an A1% cm value >0.1 at any wavelength ≥290 nm were evaluated in the 3T3 NRU PT assay. Representative absorbance spectra for two test substances (a botanical extract and a synthetic material) are illustrated in Figure 4.

Figure 4: Absorbance Spectra

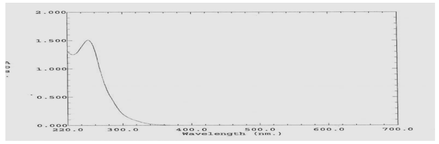


Figure 4a: Spectrum of Melicope Extract Diluted in 1% Ethanol

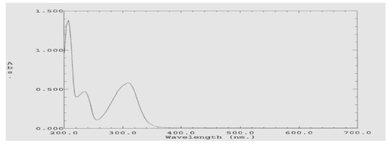


Figure 4b: Spectrum of Lauryl PEG-9 Polydimethylsiloxyethyl Dimethicone Diluted in 1% Ethanol

Most of the test substances were predicted to be non-phototoxic in the 3T3 NRU PT (ie., PIF<2 or MPE <0.1; *Tables 1 and 2*). Only 7 materials (6 botanical extracts and one synthetic material) were predicted to be phototoxic (PIF > 5 or an MPE > 0.15). Two substances, a botanical extract and a synthetic material, were classified as probable phototoxic ingredients (MPE > 0.1 and < 0.15). The remaining 59 substances (35 botanical extracts and 24 synthetic materials) were classified as non-phototoxic according to OECD TG 432.

There was no further interest in their use as cosmetic ingredients for several substances found to be non-phototoxic in the 3T3 NRU PT. The remainder were incorporated with other ingredients in one or more prototype cosmetic formulations and evaluated in a confirmatory clinical phototoxicity test.

No reactions were observed in any of the subjects participating in the confirmatory clinical phototoxicity tests (*Tables 1 and 2*).

TABLE 1. Phototoxicity Results for Natural Extracts

Botanical Name	*In Vitro* 3T3 NRU Phototoxicity Test			Clinical Phototoxicity
	PIF	MPE	Conclusion	Conclusion
Botanical Blend 1: Citrus Nobilis (Mandarin Orange) Peel Extract; Citrus Grandis (Grapefruit) Fruit Extract; Isopropyl Myristate; Citrus Aurantium Dulcis (Orange) Peel Extract; Mangifera Indica (Mango) Fruit Extract; Aniba Rosaeodora (Rosewood) Wood Extract; Citrus Aurantifolia (Lime) Peel Extract; Vanilla Planifolia Fruit Extract	1.9	0.09	Not Phototoxic	Non Phototoxic
Botanical Blend 2: Citrus Aurantium Dulcis (Orange) Peel Extract; Mangifera Indica (Mango) Fruit Extract; Aniba Rosaeodora (Rosewood) Wood Extract; Citrus Grandis (Grapefruit) Fruit Extract; Citrus Nobilis (Mandarin Orange) Peel Extract; Vanilla Planifolia Fruit Extract; Citrus Aurantifolia (Lime) Peel Extract; Prunus Armeniaca (Apricot) Fruit Extract	1.7	0.018	Not Phototoxic	Non Phototoxic
Botanical Blend 3: Vanilla Planifolia Fruit Extract; Prunus Armeniaca (Apricot) Fruit Extract; Vitis Vinifera (Grape) Fruit Extract; Butter Extract; Aniba Rosaeodora (Rosewood) Wood Extract; Cinnamomum Zeylanicum Bark Extract; Citrus Medica Limonum (Lemon) Peel Extract; Trigonella Foenum-Graecum Seed Extract; Theobroma Cacao (Cocoa) Extract; Eugenia Caryophyllus (Clove) Flower Extract; Lavandula Angustifolia (Lavender) Extract; Lavandula Angustifolia (Lavender) Extract	ND	0.094	Not Phototoxic	Non Phototoxic
Pouzolzia Pentandra Extract	-0.0015	ND	Not Phototoxic	Non Phototoxic
Sapindus Rarak Fruit Extract	1	-0.0185	Not Phototoxic	Non Phototoxic
Melicope Hayesii Leaf Extract	1	0.09	Not Phototoxic	Non Phototoxic
Raphia Farinifera Extract	1.096	0.02	Not Phototoxic	Non Phototoxic
Erythrina Flabelliformis Extract	ND	0.16	Phototoxic	Not Tested
Plumbago Indica Extract	1	-0.016	Not Phototoxic	Not Tested
Hymenosporum Flavum Extract	45.3	0.36	Phototoxic	Not Tested
Melaleuca Quinquernervia Extract	5.25	0.18	Phototoxic	Not Tested
Erigeron breviscapus Extract	ND	0.2	Phototoxic	Not Tested
Rhinacanthus Nasutus Extract	1	0.034	Not Phototoxic	Not Tested
Gynandropsis Gynandra Extract	ND	0.02	Not Phototoxic	Not Tested
Hedyotis Auricularia Extract	ND	0.041	Not Phototoxic	Not Tested
Thunbergia Laurifolia Extract	2.67	0.124	Probable Phototoxic	Not Tested
Plankton Extract, Arginine Ferulate	ND	0.061	Not Phototoxic	Not Tested
Simmondsia Chinensis (Jojoba) Seed Oil	ND	0.05	Not Phototoxic	Not Tested
Mentha Piperita (Peppermint) Leaf Extract, Vanilla Planifolia Fruit Extract	ND	-0.05	Not Phototoxic	Not Tested
Backhousia Citriodora Leaf Oil	1.11	0.014	Not Phototoxic	Not Tested
Citrus Grandis (Grapefruit) Seed Extract	0.92	-0.024	Not Phototoxic	Not Tested
Cinnamomum Zeylanicum Leaf Oil; Murraya Koenigii Stem Extract	1.06	0.001	Not Phototoxic	Not Tested
Salix Alba (Willow) Bark Extract	ND	-0.003	Not Phototoxic	Not Tested
Feronia Elephantum Extract	ND	0.16	Phototoxic	Not Tested
Harungana Madagascariensis Extract	1.15	0.0035	Not Phototoxic	Not Tested
Lonchocarpus Capassus Extract	ND	0.2235	Phototoxic	Not Tested
Cupressus Sempervirens Cone Extract	ND	0.027	Not Phototoxic	Not Tested
Lycium Chinense Fruit Extract	ND	-0.053	Not Phototoxic	Not Tested
Mentha Piperita (Peppermint) Extract	ND	-0.032	Not Phototoxic	Not Tested
Hippophae Rhamnoides Fruit Extract	ND	-0.46	Not Phototoxic	Not Tested
Skeletonema Costatum Extract	1.515	0.027	Not Phototoxic	Not Tested
Petasites Hybridus Leaf Extract	ND	-0.01	Not Phototoxic	Not Tested
Mentha Viridis (Spearmint) Extract	ND	-0.009	Not Phototoxic	Not Tested
Botanical Blend 4: Camellia Sinensis Leaf Ext, Aniba Rosaeodora (Rosewood) Wood Extract, Lavandula Angustifolia (Lavender) Extract, Rosmarinus Officinalis (Rosemary) Leaf Extract, Fucus Vesiculosus Extract, Prunus Persica (Peach) Fruit, Vanilla Planifolia Fruit Extract, Rose Extract, Citrus Aurantium Bergamia (Bergamot) Fruit Extract, Coriandrum Sativum (Coriander) Seed Extract, Cupressus Sempervirens Seed Extract, Jasminum Officinale (Jasmine) Flower Extract	ND	0.012	Not Phototoxic	Not Tested
Botanical Blend 5: Cucumis Sativus (Cucumber) Fruit Extract, Anthemis Nobilis Flower Extract, Rose Extract, Citrus Medica Limonum (Lemon) Peel Extract, Cucumis Melo (Melon) Fruit Extract	ND	0.026	Not Phototoxic	Not Tested
Nymphaea Coerulea Flower Extract	ND	0.042	Not Phototoxic	Not Tested
Hibiscus Sabdariffa Flower Extract	ND	-0.001	Not Phototoxic	Not Tested
Helianthus Annuus (Sunflower) Seed Oil, Chamomilla Recutita (Matricaria) Flower Extract	ND	-0.003	Not Phototoxic	Not Tested
Helianthus Annuus (Sunflower) Seed Oil, Vanilla Planifolia Fruit Extract	ND	-0.059	Not Phototoxic	Not Tested
Radish Root Ferment Filtrate	ND	-0.012	Not Phototoxic	Not Tested
Coleus Forskohlii Root Extract	ND	0.027	Not Phototoxic	Not Tested
Royal Jelly, Radish Root Ferment Filtrate	ND	-0.005	Not Phototoxic	Not Tested

TABLE 2. Phototoxicity Results for Synthetics

Chemical Name	*In Vitro* 3T3 NRU Phototoxicity Test			Clinical Phototoxicity
	PIF	MPE	Conclusion	Conclusion
Mesyloxybenzyl Isobutylbenzenesulfonamide	1.003	-0.004	Non Phototoxic	Not Tested
S-Phenyl-L-Cysteine	ND	0.09	Non Phototoxic	Not Tested
3-Acetoxyacetophenone	ND	0.019	Non Phototoxic	Not Tested
Chloro Benzyl Substituted Oxobutanoic Acid -2-{(4-Chlorobenzyl)Sulfanyl]-4-(4-Chlorophenyl)-4-Oxobutanoic Acid	1.19	0.094	Non Phototoxic	Non Phototoxic
Chloro Phenyl Substituted Oxobutanoic Acid -2-{(4-Chlorophenyl)Sulfanyl]-4-(4-Ethoxyphenyl)-4-Oxobutanoic Acid	1.84	0.152	Probable Phototoxic	Not Tested
Benzophenone-1	1.11	-0.012	Non Phototoxic	Not Tested
Trisodium Ascorbyl Palmitate Phosphate	1.04	-0.022	Non Phototoxic	Not Tested
Sodium Phytate/Aq/Alc	ND	-0.05	Non Phototoxic	Not Tested
Batyl Alcohol/Chimyl Alcohol	ND	-0.11	Non Phototoxic	Not Tested
2-(4-Benzylpiperidin-1-Yl)-5-Cinnamamido-N-(3-Ethoxypropyl)Benzamide	ND	0.013	Non Phototoxic	Not Tested
2-{2-(3-Methylbenzyl)-1h-Benzo[D]Imidazol-1-Yl)-1-Morpholinoethanone	9.25	0.54	Phototoxic	Not Tested
N-Butyl-3-Oxo-4-M-Tolyl-1-Thia-4,8-Diazaspiro[4.5]Decane-8-Carboxamide	ND	0.012	Non Phototoxic	Not Tested
3-Hydroxy-4,5-Dimethyl-2(5h)-Furanone	ND	0.036	Non Phototoxic	Not Tested
Benzophenone-3 (Oxybenzone)	ND	0.018	Non Phototoxic	Not Tested
Benzophenone-9	ND	0.029	Non Phototoxic	Not Tested
Ethylhexyl Salicylate (Octisalate as active ingredient)	ND	0.0015	Non Phototoxic	Not Tested
Polyurethane-34	ND	0.027	Non Phototoxic	Not Tested
Dimethicone/Divinyldimethicone/Silsesquioxane Crosspolymer	0.963	-0.017	Non Phototoxic	Not Tested
Polyglyceryl-6 Polyricinoleate, Polyglyceryl-2 Isostearate, Disteardimonium Hectorite	ND	-0.022	Non Phototoxic	Not Tested
Hydroxyphenyl Propamidobenzoic Acid	ND	0.017	Non Phototoxic	Not Tested
Lauryl Peg-9 Polydimethylsiloxyethyl Dimethicone	0.83	-0.035	Non Phototoxic	Not Tested
Silicone Quaternium-18/Trideceth-6/Trideceth-12, Silicone Quaternium-18/Trideceth-6/Trideceth-12	1.00	-0.0345	Non Phototoxic	Not Tested
Sodium Trideceth Sulfate	0.98	-0.013	Non Phototoxic	Not Tested
Cetearyl Dimethicone Crosspolymer, Caprylyl Methicone, PEG/PPG-20/23 Dimethicone	ND	0.07	Non Phototoxic	Not Tested
Octocrylene	ND	0.019	Non Phototoxic	Not Tested
Polyester-8	ND	-0.092	Non Phototoxic	Not Tested

DISCUSSION & CONCLUSIONS

The purpose of this communication is to describe a phototoxicity screening paradigm based on review of existing published and unpublished phototoxicity data, UV absorption, *in vitro* assessment of phototoxicity using the 3T3 NRU PT and confirmatory clinical testing. To illustrate how the paradigm is used in practice we report the results from screening 68 candidate cosmetic ingredients for which there was no prior evidence of phototoxicity but which exhibited significant absorption in the range 290-700 nm. In contrast to OECD TG 432 (in which a test substance is not considered phototoreactive if its molar extinction/absorption coefficient is <10 L mol^{-1} cm^{-1} at 290 nm [equivalent to A1% cm >1.0]), we considered a test substance as exhibiting significant absorption if its A1% cm was >0.1 at any wavelength ≥290 nm.

Of the 68 test substances we evaluated, all had an A1% cm >0.1 at one or more wavelengths ≥290 nm and most had significant peaks at the upper end of the UV/VIS spectrum (>300 nm). The only synthetic chemicals we identified as either phototoxic or probably phototoxic in the 3T3 NRU PT were chloro phenyl substituted oxobutanoic acid and benzo imidazol derivatives. In contrast, several of the botanical extracts we evaluated were phototoxic or probably phototoxic in the 3T3 NRU PT and a number of these have traditional uses:

•The roots of *Lonchocarpus capassus* (*Figure 5a*), a plant native to Madagascar, are used to treat stomach disorders, hookworms, and coughs (Leistner, 2005).

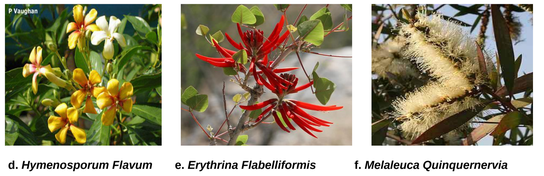
•The pulp of the fruit of *Feronia elephantum* (*Figure 5b*) a plant native to the Indian sub continent has been reported in traditional medicine as a curative for various ailments such as diarrhea, pruritis, impotence, jaundice, dysentery, heart disease, vomiting, and anorexia (Sharma *et al.*, 2012);

•Breviscapine, a flavonoid isolated from the traditional Chinese medicinal herb *Erigeron breviscapus* (*Figure 5c*) has been shown to be effective in protecting the brain against ischaemic damage, but the mechanisms remain unknown (Yiming *et al.*, 2008).

Of the other botanicals we identified as phototoxic or probably phototoxic, *Hymenosporum flavum* (*Figure 5d*) is a garden plant found in Australia (Holliday, 1998) and *Erythrina flabelliformis* (*Figure 5e*) and *Melaleuca quinquernervia* (*Figure 5f*) are field plants found in the US (Martin, 2009). None of these plants or extracts have known medicinal uses.

Figure 5: Plants from which Botanical Extracts are Predicted Phototoxic in 3T3 NRU PT

a. *Lonchocarpus Capassus* **b. *Feronia Elephantum*** **c. *Erigeron Breviscapus***



d. *Hymenosporum Flavum* **e. *Erythrina Flabelliformis*** **f. *Melaleuca Quinquernervia***

It is generally agreed that materials which test negative in the 3T3 NRU PT are not expected to have phototoxic reactions when tested in a clinical setting (Ceridono *et al.*, 2012). In our hands, none of the test substances described here which were non-phototoxic in the 3T3 NRU PT were phototoxic in the clinical test. Nonetheless, if there is an interest in the future in using any of these test substances at a higher concentration it will be necessary to repeat clinical testing at the new concentration in a prototype cosmetic formulation. Although we declined to give further consideration to those substances which were phototoxic or probably phototoxic in the 3T3 NRU PT as cosmetic ingredients, additional photo safety assessment may be necessary before a conclusive determination of the phototoxic potential can be made (Ceridono *et al.*, 2012).

In conclusion, we have found the tiered approach described here an effective tool in ensuring that marketed cosmetic products do not contain ingredients with phototoxic potential.

REFERENCES

A reprint of this poster and the complete list of references can be obtained at www.iivs.org or by scanning the QR code: