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Assessment of cosmetic ingredients in the *in vitro* reconstructed human epidermis test method EpiSkin™ using HPLC/UPLC-spectrophotometry in the MTT-reduction assay

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ABSTRACT

Cosmetics Europe recently established HPLC/UPLC-spectrophotometry as a suitable alternative endpoint detection system for measurement of formazan in the MTT-reduction assay of reconstructed human tissue test methods irrespective of the test system involved. This addressed a known limitation for such test methods that use optical density for measurement of formazan and may be incompatible for evaluation of strong MTT reducer and/or coloured chemicals. To build on the original project, Cosmetics Europe has undertaken a second study that focuses on evaluation of chemicals with functionalities relevant to cosmetic products. Such chemicals were primarily identified from the Scientific Committee on Consumer Safety (SCCS) 2010 memorandum (addendum) on the *in vitro* test EpiSkin™ for skin irritation testing. Fifty test items were evaluated in which both standard photometry and HPLC/UPLC-spectrophotometry were used for endpoint detection. The results obtained in this study: 1) provide further support for Within Laboratory Reproducibility of HPLC–UPLC-spectrophotometry for measurement of formazan; 2) demonstrate, through use a case study with Basazol C Blue pr. 8056, that HPLC/UPLC-spectrophotometry enables determination of an *in vitro* classification even when this is not possible using standard photometry and 3) addresses the question raised by SCCS in their 2010 memorandum (addendum) to consider an endpoint detection system not involving optical density quantification in *in vitro* reconstructed human epidermis skin irritation test methods.

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1. Introduction

A number of *in vitro* test methods based on Reconstructed human Tissues (RhT) have been accepted by regulatory authorities for evaluation of skin corrosion/irritation and eye irritation/serious eye damage. Within Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 431 for skin corrosion (OECD, 2015a) these are EpiDerm™ Skin Corrosion Test (EURL ECVAM, 2000; Liebsch et al., 2000; ICCVAM, 2002), EpiSkin™ (EURL ECVAM, 1998; Fentem et al., 1998; Alépée et al., 2014a), SkinEthic™ Reconstructed Human Epidermis (RHE) (EURL ECVAM, 2006; K. Kandárová et al., 2006; Alépée et al., 2014b) and epiCS® (Hoffmann et al., 2005; EURL ECVAM, 2009). For skin irritation within OECD TG 439 (OECD, 2015b) these are EpiDerm™ Skin Irritation Test (Kandárová et al., 2004, 2005, 2009; Spielmann et al., 2007), EpiSkin™ (Cotovio et al., 2007; Spielmann et al., 2007), SkinEthic™ RHE 42bis (H. Kandárová et al., 2006; Alépée et al., 2010; Tornier et al., 2010a, 2010b) and LabCyte EPI-MODEL 24 SIT (Kojima et al., 2012, 2014). According to the United Nations Globally

Abbreviations: CAS RN, Chemical Abstract Service Registry Number; R^2, coefficient of determination; Cat. 1, Category 1; Cat. 2, Category 2; EIT, eye irritation test; DG SANCO, European Commission Directorate General for Health and Consumer Protection; EURL ECVAM, European Union Reference Laboratory for Alternatives to Animal Testing; FDA, Food and Drug Administration; HPLC-UPLC-spectrophotometry, high/ultra high performance liquid chromatography-spectrophotometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt; MSDS, material safety data Sheet; NSC_{Living}, non-specific colour in living tissues; NSC_{killed}, non-specific colour in killed tissues; NSMTT, non-specific MTT reduction; NC, not classified; OD, optical density; OECD, organisation for economic co-operation and development; %, percentage; RhCE, reconstructed human cornea-like epithelium; RHE, reconstructed human epidermis; RhT, reconstructed human tissue; R&I, Research & Innovation; SCCS, scientific committee on consumer safety; SD, standard deviation; SOP, standard operating procedure; TG, test guideline; UN GHS, united nations globally harmonized system of classification and labelling of chemicals; WLR, within laboratory reproducibility.

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Harmonized System of Classification and Labelling of Chemicals (UN GHS) these test methods allow identification of Irritant (Category 2 (Cat. 2))/ Corrosive (Category 1 (Cat. 1)) or Not-Classified (NC). Most recently, the Reconstructed human Cornea-like Epithelium (RhCE) EpiOcular™ Eye Irritation Test (EIT) test method has been adopted by OECD (TG 492) to identify chemicals not requiring classification (NC) and labelling for eye irritation or serious eye damage (Cat. 2/Cat.1) (Freeman et al., 2010; Kaluzhny et al., 2011; Pfannenbecker et al., 2013; EC EURL ECVAM, in preparation; OECD, 2015c).

The endpoint to identify chemical effects in all these RhT test methods is measurement of tissue viability in treated tissues after topical application onto the tissue surface. Tissue viability is determined by enzymatic reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) tetrazolium salt to purple reduced MTT (formazan) (Mosmann, 1983). Formazan is then quantified photometrically using optical density (OD) with the results being expressed as percentage (%) viability of the test item treated tissues relative to negative control treated tissues and converting this to a classification using a prediction model based on a tissue viability cut-off value.

A known limitation of the photometric MTT-reduction assay is possible interference of coloured chemicals with the absorbance measurement of formazan (McNamee et al., 2009). This limitation has been addressed in a project completed by Cosmetics Europe in which the use High/Ultra High Performance Liquid Chromatography Performance (HPLC-UPLC)-spectrophotometry for endpoint detection of formazan was established (Alépée et al., 2015). In this project, using the approach recommended by the United States Food and Drug Administration (FDA) guidance for validation of bio-analytical methods (FDA, 2001), three independent laboratories established and qualified their HPLC/UPLC-spectrophotometry systems to reproducibly measure formazan from tissue extracts. This was followed by testing of up to 26 chemicals in three RhT test systems for skin corrosion/irritation and eye irritation/serious eye damage. The chemicals set was selected to achieve a balanced representation of coloured chemicals anticipated to produce colour interference of formazan measurement using photometry, coloured chemicals not anticipated to produce colour interference of formazan measurement using photometry and non-coloured chemicals. The resulting formazan samples were measured by the three participating laboratories using both OD and HPLC-UPLC-spectrophotometry. Results identified that for non-coloured chemicals, use of HPLC/UPLC-spectrophotometry for measurement of formazan yielded the same tissue viability measurements as did use of OD for endpoint detection. For colour interfering chemicals, use of HPLC/UPLC-spectrophotometry was capable of measuring formazan resulting in a tissue viability measurement when this was not possible with OD measurement of formazan. Furthermore, HPLC-UPLC-spectrophotometry, was shown to be highly reproducible between different laboratories. Based on these findings, it was concluded that HPLC/UPLC-spectrophotometry as an analytical technique for measurement of formazan is relevant to all *in vitro* RhT test methods irrespective of the test system and test method and can be applied to any of the other RhT test systems within the relevant OECD TGs. Moreover, any HPLC/UPLC-spectrophotometry system could be considered for such analysis once the system has been qualified as described in the original project (Alépée et al., 2015). Finally, use of HPLC/UPLC-spectrophotometry for measurement of formazan has now been incorporated into the revised OECD TGs 431 (skin corrosion) and 439 (skin irritation) and the newly adopted 492 (eye irritation/serious eye damage) (OECD, 2015a, 2015b, 2015c).

It was acknowledged, ahead of the original project, that the known limitation of possible interference of coloured chemicals with the absorbance measurement of formazan in the photometric MTT-reduction assay was recognized by the European Commission Directorate General for Health and Consumer Protection (DG SANCO) Scientific Committee on Consumer Safety (SCCS) which provides scientific advice to the European Commission on issues related to non-food topics. In 2010, the SCCS published a "Memorandum (addendum) on the *in vitro* test EpiSkin™ for skin irritation testing" (SCCS, 2010) in which they identified that for coloured substances, a different endpoint, not involving optical density quantification, should be envisaged to address this limitation. Analytical methods such as HPLC/UPLC might be more appropriate to detect formazan in the *in vitro* assay (McNamee et al., 2009)".

With the use of HPLC/UPLC-spectrophotometry established and adopted in the relevant OECD TGs, Cosmetics Europe undertook a second project with focus on chemicals with functionalities relevant to cosmetic products. To do this, the SCCS 2010 memorandum (addendum) was used to identify such chemicals, namely ultraviolet filters, preservatives, skin conditioning agents and hair dyes/coloured chemicals) as well as the general chemicals identified therein to be tested in the EpiSkin™ *in vitro* skin irritation test method.

As such, the current paper describes evaluation of test items in the EpiSkin™ skin irritation test in which both standard photometry (OD) and HPLC/UPLC-spectrophotometry are used as endpoint detection systems for measurement of formazan thereby enabling a direct comparison with all of the commercially available chemicals with functionalities relevant to cosmetic products identified within the SCCS 2010 Memorandum (addendum).

2. Materials and methods

2.1. Chemicals selection

The fifty test items evaluated were composed of 46 individual chemicals tested neat and/or in dilution. Within this set, the majority (42) of the chemicals were identified from those included in the SCCS 2010 memorandum (addendum) (SCCS, 2010) which could be obtained commercially or obtained from proprietary sources. Four chemicals were additionally selected by Cosmetics Europe for inclusion in the final chemicals set. Three of the 4 Cosmetics Europe selected chemicals were anticipated to produce MTT interference and have been identified as classified (Cat. 2 (skin irritant) (#40) or Cat. 1 (skin corrosive) (#49, #50)) *in vivo*. Details of all the test items including chemical name, Chemical Abstract Service Registry Number (CAS RN) number, physical form, colour functionalities relevant to cosmetic products and *in vivo* classification for skin irritation/corrosion according to the UN GHS classification system (UN, 2013) are provided in Table 1.

2.2. Test methods

All of the chemicals identified from the SCCS 2010 memorandum (addendum) were evaluated in the *in vitro* skin irritation test method EpiSkin™ consistent with how these chemicals were originally tested. As such, 46 individual chemicals were evaluated in this test method. Within the overall chemical set, since 2 of the 4 chemicals selected by Cosmetics Europe are classified *in vivo* as UN GHS Cat. 1 (skin corrosive) (#49, #50), the *in vitro* test method for skin corrosion EpiSkin™ was conducted for these chemicals to correlate with the existing *in vivo* data. For all of the testing, the *in vitro* test methods were performed using Standard Operating Procedures (SOP) in accordance with the relevant OECD TGs. For skin irritation, this was OECD TG 439: "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method" (EpiSkin™ SOP, 2009; OECD, 2015b) and for skin corrosion this was TG 431: "*In vitro* skin corrosion: Reconstructed Human Epidermis test method" (EpiSkin™ test method SOP, 2011; OECD, 2015a).

For the chemicals that were identified in the pre-checks as direct MTT reducers or coloured, adapted controls for Non-Specific MTT reduction (% NSMTT) or Non-Specific Colour (% NSC_{living}) were included in the experimental phase. Adapted controls for Non-Specific Colour obtained from killed tissues (% NSC_{killed}) were also included when both of the other adapted controls were required (colour interfering and MTT reducing chemicals). Pre-checks for colour interference and direct MTT reduction were conducted in accordance with the *in vitro* test method SOPs for the two RhT test methods.

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