Evaluation of Alternative Non-Animal Approaches for the Prediction of Skin Sensitisation Potential of Agrochemicals

Hanjalika William Masinja

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Abstract

Skin sensitisation in humans is an important toxicological effect to be determined should there be frequent and prolonged exposure. Current approaches to assessing skin sensitisation mostly utilise *in vivo* testing, although there is a move to alternatives such as *in silico*, *in chemico* and *in vitro* approaches. The aim of this thesis was to investigate whether these alternative approaches could be utilised for agrochemical active ingredients (AIs) and formulations. Agrochemicals represent a unique problem for risk assessment due to the high level of regulatory compliance required in addition to the intrinsic issues of assessing formulations. Using the *in vivo* experimental results as a benchmark, the overall sensitivity and specificity of the Classification Labelling and Packaging (CLP) threshold calculation method, regardless of agrochemical formulation type, were determined to be 58% and 82% respectively. Thus, for the plant production products (PPPs) assessed, the threshold method had a high probability of accurately predicting non-sensitisers. To supplement the information, the *in vitro* triple pack (Direct Peptide Reactivity Assay, KeratinoSens^{TM} and h-CLAT) was applied using established Integrated Testing Strategies and Defined Approaches. Overall, the triple pack performed poorly for the assessment of AIs and formulations alike. The Genomic Allergen Rapid Detection (GARD) assay for the ten AIs showed a high sensitivity but a low total accuracy; the sensitivity was 0% with the *in vivo* non-sensitisers being predicted as sensitisers in the GARD assay. Eight of the ten PPPs tested in the SENS-IS assay produced results that were in good agreement with the vertebrate study outcomes. Findings from investigations into the Two Dimensional (2D) *in vitro* test methods demonstrated that testing of complex mixtures in those models could not be conducted accurately with current methods. Use of the SENS-IS method allowed for direct application of the PPP to the Reconstructed Human Epidermis (RhE) test system so that good comparison to the products intended and anticipated use can be made. It is envisaged that the work conducted in this thesis will add to the toxicology research conducted on skin sensitisation thus far and be of use primarily for decision making in hazard assessment of agrochemicals.

CONTENTS

List of abbreviations:

3D – Three-Dimensional

AC - Acylation

ACD - Allergic Contact Dermatitis

AI - Active Ingredient

ANVISA - Brazilian Health Regulatory Agency

AO – Adverse Outcome

AOP - Adverse Outcome Pathway

APAC - Asia-Pacific

ARE - Antioxidant/Electrophile Response Element

BA – Buehler Assay

BCOP - Bovine Corneal Opacity Permeability Test

BIT - Benzisothiazolinone

CIS - Commonwealth of Independent States

CLP - Classification Labelling and Packaging

CONCEA - Brazilian National Council for the Control of Animal Experimentation

CRO - Clinical Research Organisation

CS - Capsule Suspension

CV - Coefficient of Variation

CV75 - 75% cell viability

DA - Defined Approaches

DC - Dendritic Cells

DIP - Data Interpretation Procedure

DMSO - Dimethylsulfoxide

DNCB - 2,4-dinitrochlorobenzene

DPD - Dangerous Preparations Directive

DPM - Disintegrations Per Minute

DPRA - Direct Peptide Reactivity Assay

EC - Emulsifiable Concentrate

$EC_{1.5}$ - 1.5-Fold Induction of Luciferase Activity

ECHA – European Chemicals Agency

ECVAM - European Centre for Validation of Alternative Methods

EU – European Union

FCA - Freund's Complete Adjuvant

FN - False Negative

FP - False Positive

GARD - Genomic Allergen Rapid Detection

GCL - Generic Concentration Limit

GHS - Globally Harmonized System

GPMT - Guinea Pig Maximisation Test

GR – Granule

HBA - Hydrogen Bond Acceptors

HBD - Hydrogen Bond Donors

h-CLAT - Human Cell Line Activation Test

HMT - Human Maximisation Test

HRIPT - Human Repeat Insult Patch Test

HPLC - High Performance Liquid Chromatography

IATA - Integrated Approach to Testing and Assessment

IBAMA - Brazilian Institute of Environment and Renewable Natural Resources

ICCVAM - Interagency Coordinating Committee on the Validation of Alternative Methods

ICE - Isolated Chicken Eye test

I_{max} - Mean Maximum Luminescence Induction

ITS - Integrated Testing Strategy

KE – Key Event

Keap1 - Kelch-like ECH-associated protein 1

LC - Langerhans Cells

LD50 - Median Lethal Doses

LLNA - Local Lymph Node Assay

MA - Michael Addition

MAD - Mutual Acceptance of Data

MAPA - Ministry of Agriculture, Livestock and Supply

MCC - Matthews Correlation Coefficient

MHC - Major Histocompatibility Complex

MIE – Molecular Initiating Event

MIT - Minimum Induction Threshold

MW - Molecular Weight

NAM - New Approach Methodology

NC3Rs - National Centre for the 3Rs

NHS – National Health Service

Nrf2 - Nuclear Factor-Erythroid 2-Related Factor 2

OD - Oil Dispersion

OECD - Organisation for Economic Co-operation and Development

PETA - People for the Ethical Treatment of Animals

PI - Propidium Iodide

PMRA - Canadian Pest Management Regulatory Agency

PPE - Personal Protection Equipment

PPP - Plant Protection Products

PPV - Positive Prediction Value

(Q)SAR – (Quantitative) Structure-Activity Relationship

RB - Rotatable Bonds

REACH - Registration Evaluation Authorisation and restriction of Chemicals

RhE - Reconstructed Human Epidermis

RT-PCR - Reverse Transcription Polymerase Chain Reaction

Rv90 - 90% Relative Cell Viability

SC - Suspension Concentrate

SCL - Specific Concentration Limits

SB - Schiff-Base Formation

SDS - Safety Data Sheets

SI - Stimulation Index

SMARTS - Smiles Arbitrary Target Specification (SMARTS)

SMILES - Simplified Molecular Input Line Entry System

S_N2 - Bimolecular Nucleophilic Substitution

S_NAR - Aromatic Nucleophilic Substitution

STS - Sequential Testing Strategy

SVM - Support Vector Machine

TCR - T Cell Receptors

T_C - Cytotoxic $CD8^+$ T-lymphocyte Cells

T_h - T-lymphocyte $CD4^+$ Helper Cells

TN – True Negative

TP - True Positive

TPR - True Positive Rate

UN - United Nations

U-SENS™ - U937 cell line activation test

US EPA - United States Environmental Protection Agency

WoE - Weight of Evidence

WG - Dispersible Granules

1.0 CHAPTER ONE - INTRODUCTION

"*What is there that is not poison? All things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison*" (Grandjean, 2016). As most notably identified by the Renaissance physician, often referred to as the father of toxicology, Paracelsus, all substances have the potential to be poisons at sufficiently high enough doses. Paracelsus reasoned that therapeutic agents at high doses can be harmful, whilst substances generally considered to be toxic may be less harmful, or even beneficial, at lower doses (Tsatsakis et al., 2018). A good understanding of the response to specific doses of a given substance allows for an understanding of the concentration threshold at which a toxic effect can be anticipated. Acute toxicity is the observed adverse effects that occur once the threshold of toxicity has been reached following the administration of a substance once or multiple times during an exposure significantly less than the life cycle, e.g. a 24 hour period for rodents (Manuppello et al., 2020). Depending upon the route of exposure or adverse effect being assessed, acute toxicity is determined by the presence of lethality (for acute oral, dermal or inhalation studies) or the observed unwanted effects at the local site of administration (skin irritation, eye irritation or skin sensitisation). The need to understand the potential hazardous effects associated with acute exposure and the concentrations at which they may occur, has historically driven the hazard evaluation of industrial and agrochemicals products.

This need, to assess short term or local effects, has resulted in the development of an acute testing strategy commonly referred to as the "six pack" (Hamm et al., 2017, Creton et al., 2010). The six pack involves the evaluation of the three systemic acute endpoints i.e., oral, dermal and inhalation acute toxicity (via median lethal dose, LD50 and LC50), and the three local acute endpoints, namely skin irritation, eye irritation and skin sensitisation. At present non-animal alternative methods are being integrated into the safety assessment process, however, this tends to be largely within the initial early-stage screening phase of chemical development. Rowan and Spielmann (2019) previously identified that there was a need for the scientific community to understand that it was possible to use alternative

methods to meet some, or all, regulatory requirements, instead of solely addressing lead chemical prioritisation. The research described in this thesis will focus on the methods used to evaluate skin sensitisation specifically, but always bearing in mind the broader context of risk assessment across a range of endpoints.

The recorded history of method development for the hazard assessment of skin sensitisation begins in 1895 with Jadassohn who first introduced patch testing to identify contact allergy in man (Jadassohn, 1896). Jadassohn developed the patch test through his initial identification that a patient had developed an eczematous reaction to mercury plasters. He termed this initial contact test the "Funktionelle Hautprüfung" (functional skin test) (Lachapelle and Maibach, 2009). Since the development of the first patch test over 125 years ago, there has been appreciation of the value and significance of human testing to provide the most relevant information. Tests on humans have been developed and now include the Human Repeat Insult Patch Test (HRIPT), initially developed by Draize (Bormann and Maibach, 2021, Draize et al., 1944), or Human Maximisation Test (HMT) developed by Kligman (Zaghi and Maibach, 2009, Kligman, 1966). Regulatory guidance documents such as the Regulation (EC) No 1272/2008 for Classification, Labelling and Packaging (ECHA, 2017b) state that classification of a substance can be based on human evidence, such as positive data from patch testing (e.g. HRIPT or the HMT). However, patch tests on humans cannot be carried out for the sole purpose of fulfilling regulatory criteria ((EC), 2009a), rather this source of data is used only when it has been historically generated for other purposes (e.g. clinical studies) and is used as weight of evidence for sensitising potency subcategorisation.

For several years, the chemical industry has attempted to put into place guidance leading towards the use of *in vitro* and/or *in silico* models to determine acute toxicity endpoints. The industry has been led in this endeavour by a combination of the concerns, most notably and recently expressed by the National Centre for the 3Rs (NC3Rs, the 3Rs being the reduction, refinement and replacement (of animal experimentation) a concept original pioneered by William Russell and Rex Burch (Russell and

Burch, 1960)) and also experience with regulations such as the Registration Evaluation Authorisation and Restriction of Chemicals (REACH) and the European Union (EU) Cosmetics Directive. Information on the potential of a chemical to cause sensitisation, along with its potency, must be provided for the classification and labelling of the substance. Specifically, these requirements exist for the classification and labelling of industrial chemicals under REACH Annex VII and VIII ((EU), 2017a), as well as for biocides ((EC), 2012b), pesticides ((EC), 2009a) and cosmetic ingredients ((EC), 2010). Acute toxicity testing is used mainly for the determination of the potential hazard that a given test item (chemical/formulation) may pose following brief exposure via the corresponding routes for the specific endpoints in question. At present, there still remains a substantial reliance on the use of experimental animals for the purpose of hazard identification and risk assessment (Prior et al., 2019).

1.1 What is skin sensitisation?

Skin sensitisation, or allergic contact dermatitis (ACD), is an immunological reaction generated by the body in response to local exposure to specific xenobiotic material on the skin (Brites et al., 2020). One in five people is prone to ACD to at least one contact allergen in their lifetime (Bormann and Maibach, 2021). Symptoms associated with this adverse effect are observed on areas of the body that have been directly exposed to a sufficient amount (a threshold concentration) of the sensitising substance, in an individual who has previously been exposed, and developed a contact allergy, to that, or a closely structurally-related substance. The first exposure to the skin sensitising material initiates a cascade of biological events leading to the induction phase of skin sensitisation (Willett, 2014). The induction phase is not associated with clinical symptoms, however during this phase selective clonal expansion of the allergen-specific T-lymphocytes (T cells) occurs (Basketter and Maxwell, 2007). Upon further exposure to the specific sensitising material, a heightened response in the body may be seen. The heightened response occurs because the immune system is primed to recognise this revisiting allergen and elicit an inflammatory response that can lead to dermal injury (Jaworska et al., 2011). In addition to this, it has been reported that allergens of similar chemical structure can cause sensitised

individuals to experience ACD via cross-reactivity (Basketter and Maxwell, 2007). The symptoms associated with this subsequent contact with the chemical include oedema and the skin becoming itchy, blistered, dry and cracked. In addition, skin redness can be observed in lighter skin, whilst darker skin can become dark brown, purple or grey ((NHS), 2019). As this research aims to investigate new, non-animal alternative methods for hazard assessment of skin sensitisation and to compare them to the *in vivo* methods, it is appropriate that current understanding of the adverse outcome pathway (AOP) and mechanisms are explained further here.

1.2 The skin sensitisation AOP and its relationship to *in chemico* and *in vitro* assays

An AOP organises how the existing mode of action is understood as a series of linkages between measurable key events (KE), and a resulting adverse outcome (AO) seen as an organism's response. The initial KE in an AOP is a molecular initiating event (MIE) (Ankley and Edwards, 2018). The MIE captures the interaction between a chemical and a biological macromolecule, the complex of which then triggers the cascading KE and may potentially lead to an AO (Ankley et al., 2010). The biochemical and cellular events that occur during the cascade of the AOP in skin sensitisation have been well researched and documented (Schultz et al., 2016, Kimber et al., 2018, OECD, 2014). As indicated above, skin sensitisation consists of two major phases. The first is the induction phase, during which an inherently susceptible subject is exposed to an allergen at a specific concentration threshold or greater, priming the immune system (Kimber et al., 2018). This involves the proliferation and differentiation of naïve, T-lymphocyte, helper, $CD4^+$ cells (T_h) to memory and effector $T_{h.}$ In the second phase of skin sensitisation, the elicitation phase, re-exposure (or challenge) of the specific allergen to the previously primed immune system leads to an accelerated and more aggressive secondary immune response that is recognised as ACD in humans or contact hypersensitivity in rodents (Kimber et al., 2018, OECD, 2014). The skin sensitisation AOP details the different processes that need to occur during these two phases for the adverse effect to occur as shown in Figure 1.1.

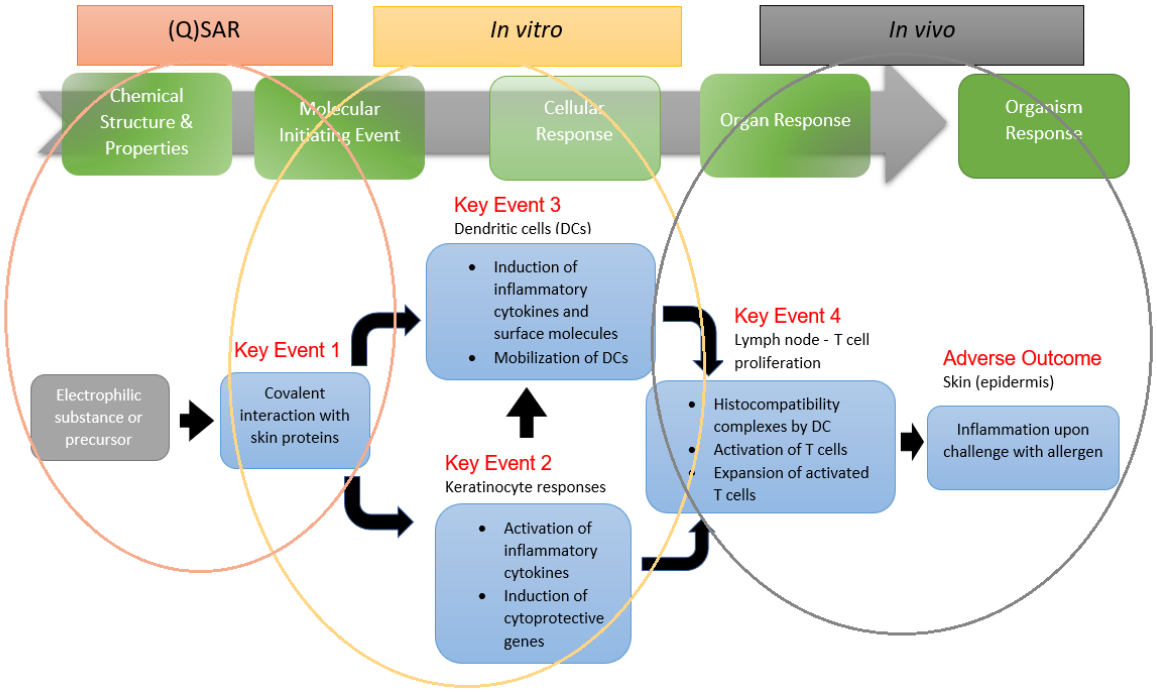


Figure 1.1 The skin sensitisation adverse outcome pathway (Willet, 2014)

During the MIE (i.e. the first key event) of the skin sensitisation AOP, skin sensitising chemicals or haptens come into contact with the stratum corneum and enter into the body via the lipid bilayers, corneocytes or via appendages such as sweat ducts and hair follicles (Maxwell et al., 2014, Potts and Guy, 1992a). Once they have penetrated through the epidermal skin layer, as shown in structure of the skin in Figure 1.2, haptens can form a stable conjugate with endogenous epidermal or dermal molecules. In doing so they modify skin proteins, normally by the electrophilic hapten covalently binding to nucleophilic groups of the protein (Willett, 2014, Rustemeyer et al., 2012), in particular the thiol (i.e., cysteine) and primary amine (i.e., lysine) residues (OECD, 2014). The direct result of an electrophilic substance's interaction with the nucleophilic centre of skin protein is the production of a hapten-protein complex (haptenation) (Basketter and Maxwell, 2007, Maxwell et al., 2014). It is this cysteine/ lysine/ peptide/ hapten complex that is involved in the *in vitro* direct peptide reactivity assay (DPRA), one of the key alternatives to *in vivo* skin sensitisation testing (OECD, 2019a). Prior to this binding, the parent compound may be converted metabolically to protein-reactive derivates (prohaptens) or converted abiotically via oxidation (prehaptens) (Aptula et al., 2007). These processes may also transform an initially reactive parent compound to an inert metabolite.

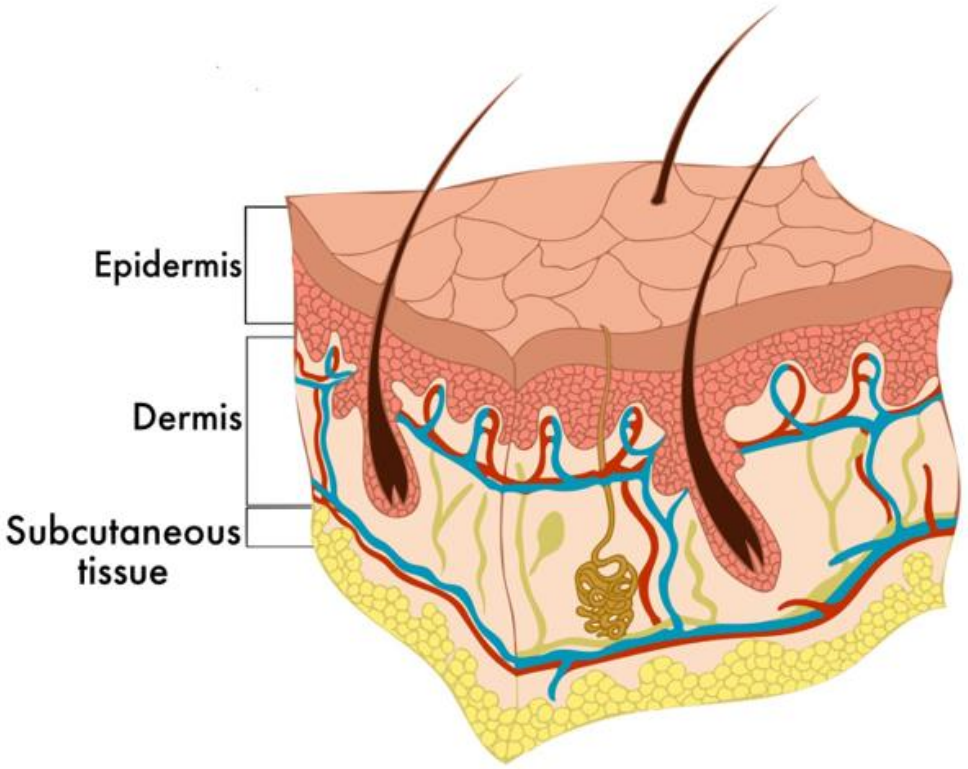


Figure 1.2 A schematic diagram of the structure of the skin (Dijkhoff, 2020)

Figure 1.3 shows the induction phase of skin sensitisation and in the second key event (KE2) of this AOP, haptens react with cell surface proteins and activate response pathways in keratinocytes. These include inflammatory responses and changes in gene expression associated with cell signalling pathways, specifically the antioxidant/electrophile response element (ARE)-dependent pathways (OECD, 2018b). Keratinocytes are highly specialised epithelial cells that, in conjunction with the corneocytes, form the cornified epithelial layer of the epidermis known as the stratum corneum (Basketter and Maxwell, 2007). Keratinocytes are continuously replenished by the basal keratinocytes which divide frequently (Blanpain and Fuchs, 2006). Keratinocyte interaction with sensitising chemicals leads to the induction of ARE-pathways. Specifically the covalent binding of the electrophilic sensitising compound to the sensor protein Keap1 (Kelch-like ECH-associated protein 1) via its nucleophilic cysteine peptide residues, leads to the dissociation of transcriptional regulator Nrf2 (nuclear factor-erythroid 2-related factor 2) from Keap1 (Natsch and Emter, 2008). Nrf2 then accumulates in the nucleus where it activates genes that have an antioxidant response element in

their promotor sequence. Sensitising compound reactivity towards specific cysteine residues therefore triggers a battery of genes mainly coding for phase two detoxifying enzymes (Natsch, 2010). The Keap1-Nrf2-ARE pathway is utilised by the *in vitro* ARE-Nrf2 luciferase assays in determining if a compound has the potential to activate KE2 of the skin sensitisation AOP (OECD, 2018b). The use of this pathway to identify potential sensitisers was further demonstrated by Natsch et al (2009) who determined that 66 of a total 84 tested skin sensitisers induced luciferase gene activity under the control of ARE elements. When investigating those for which human evidence specifically existed, 30 of 34 tested skin sensitisers were correctly identified (Natsch et al., 2009).

The epidermis is also populated by a specialised sub-set of immature dendritic cells (DC) known as Langerhans cells (LC). The LCs maintain a unique dendritic morphology which allows them to project their dendrite arms toward the stratum corneum (Clayton et al., 2017). The LCs and dermal DCs are professional antigen presenting cells that act as "sentinels" of the adaptive immune response (Basketter and Maxwell, 2007). These cells are able to recognise and phagocytose hapten-protein complexes formed via covalent bonding in the AOP molecular initiating event. Following phagocytosis of the hapten-protein complex, the subsequent processing and lysosomal degradation of the allergens takes place. The most resistant peptides (epitopes) are then presented on the major histocompatibility complex (MHC class 2) of the DC plasma membrane for the availability of specific receptors of naive helper $CD4^+$ T cells (Nielsen et al., 2010). Following the DC interaction with the allergen complex, these cells become activated. The maturation of DCs is the significant function that marks key event three (KE3) of the skin sensitisation AOP (Edwards et al., 2018). The activation of immature epidermal LCs and dermal DCs is demonstrated by the increased expression of the DC surface markers, such as CD54, CD80 and CD86 (Humeniuk et al., 2017). Enhanced production of proinflammatory cytokines such as IL-1α, IL-12 and TNF-α occur in parallel to the DC activation.

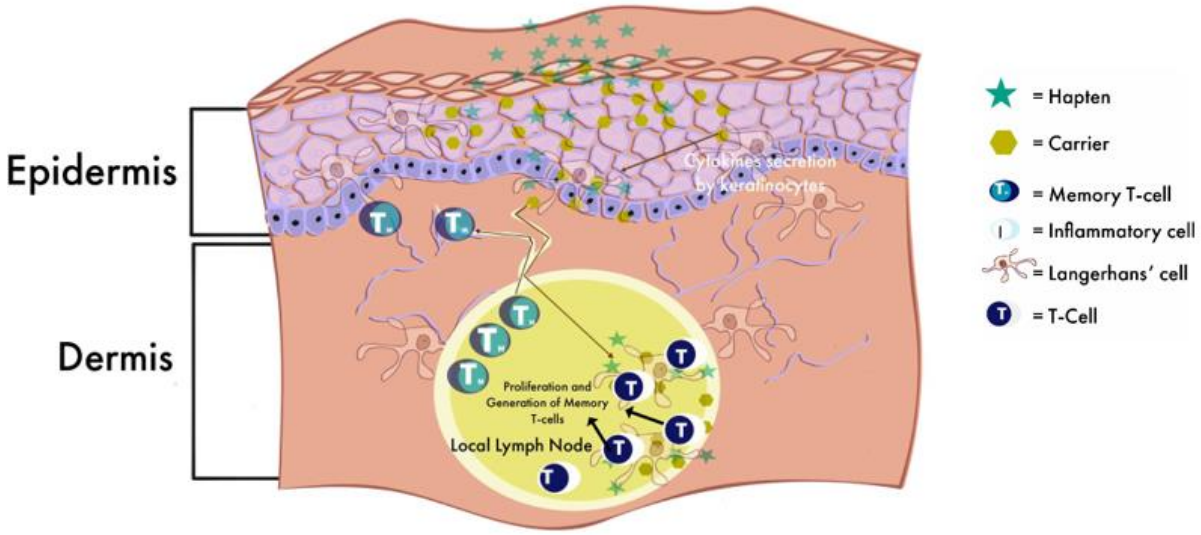


Figure 1.3 A schematic of the skin sensitisation induction phase as presented in OECD, (2014)

The *in vitro* human cell line activation assay (h-CLAT) and the U937 cell line activation test (U-SENS™) use the increase in CD54 and CD86 cell surface markers to identify a substance's potential to activate the KE3 (OECD, 2018a). The induction of the LC activation has been shown to also result in the upregulation of IL-1b and IL-18 expression (Basketter and Maxwell, 2007). Kranzer et al (2004) demonstrated that the stimulation of DCs induced IL-10, IL-12, IL-6 and IL-8 secretion as well as the upregulation of the DC surface co-stimulatory molecules (CD54 and CD86) (Kranzer et al., 2004). A dose-related increase in IL-8 was also demonstrated by Galbiati at al. (2020) following the THP-1 DC line's exposure to allergens (these included α-hexylcinnamaldehyde, hydroxycitronellal and imidazolidinyl urea). It is worth noting that exposure to irritant chemicals failed to induce IL-8 secretion in the THP-1 cell line (Galbiati et al., 2020). THP-1 cells are widely used to investigate the function and regulation of monocytes and macrophages as they resemble them in morphology and differentiation properties (Qin, 2012). The third of the *in vitro* skin sensitisation methods listed in the Organisation for Economic Cooperation and Development (OECD) test guideline 442E, the IL-8 luc assay, utilises the upregulation of IL-8 as a biomarker to indicate the potential of a substance to activate KE3 (OECD, 2018a).

Key event four (KE4) of the AOP involves the migration of the activated, antigen presenting DCs from the epidermis to the T-cell dominant draining lymph nodes via afferent lymphatics (Basketter and Maxwell, 2007). The DCs lose adhesiveness to their surrounding epithelia following their activation and express chemokine receptors to allow them to migrate to the draining lymph nodes in the lymph (Humeniuk et al., 2017). In the lymph nodes the DCs present the protein-hapten complex in conjunction with MHC class II to T cells. Helper $CD4^+$ T-cells (T_h) have unique T cell receptors (TCR) generated by recombination of genomic DNA sequences, during T cell development in the thymus. Each specific TCR is responsible for the specific affinity of each T cell for a particular MHC class II complex (Pennock et al., 2013). Once a naïve T_h cell TCR has interacted with the MHCII of the APC and the peptide presented is recognised as foreign, the T_h cell becomes activated. After this activation the T_h cell proliferates to form effector and memory T_h cells (Willett, 2014).

The memory T_h cells have the same TCR affinity as the parent T_h cell. Memory T_h cells subsequently proliferate and remain in the body for years in preparation for interaction with the same MHC2 peptide complex (Lakkis and Sayegh, 2003). Once the initial bioavailable allergen concentration leading to induction of the T_h cell activation has occurred, in the elicitation phase as shown in Figure 1.4, a lower concentration of the same allergen may trigger a more intense immune response (Lakkis and Sayegh, 2003). The effector T_h cells release cytokines which effectively act as alarm bells by activating B cells via their MHC2 complexes and enact T_h cell dependent activation (Zubler, 2001). The effector T_h also primes cytotoxic $CD8^+$ T cells (T_C). It is the lymph node cell proliferation of KE4 that is the basis for the LLNA (OECD, 2014).

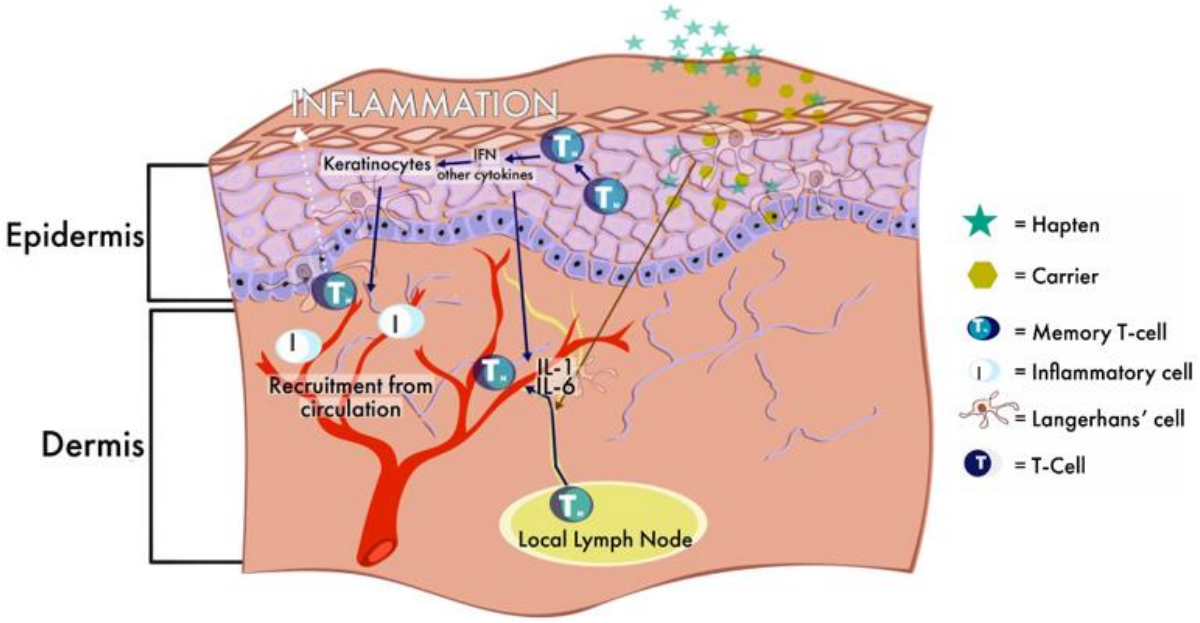


Figure 1.4 A schematic diagram of the skin sensitisation elicitation phase as presented in OECD, 2014

1.2.1 *In Vivo* Tests for Skin Sensitisation

There are three specific *in vivo* animal skin sensitisation tests that have widely been used in the chemical industry. These are the Guinea Pig Maximisation Test (GPMT), the Buehler assay (BA) and the local lymph node assay (LLNA). The Magnusson and Kligman-founded (1969) GPMT uses the elicitation phase of skin sensitisation to identify materials with the potential to cause this adverse effect (Magnusson and Kligman, 1969).

The GPMT combines the use of an initial intradermal induction of the test material in combination with Freund's complete adjuvant (FCA) with a subsequent challenge exposure to the test material via topical application (on day 20 – 22 of the test) (OECD, 1992). Between the initial intradermal induction and the final challenge exposure, there is an additional topical application of test material in an occlusive dressing. The GPMT maximises the possibility of observing an allergic reaction with the use of FCA during the induction phase, allowing the test to detect not only strong, but also weak, skin sensitisers (Momma et al., 1998, Hayes et al 2020). It has been proposed by Nakamura et al (1999) that, with the exception of drugs, chemicals are not usually injected intradermally with an adjuvant and therefore the GPMT may overestimate the skin sensitisation potential of chemicals that would

not readily penetrate the skin (Nakamura et al., 1999). An observed inflammatory response at challenge may not necessarily be allergenicity and rather a false-positive irritant response resulting from an induced hyperirritability (Kligman and Basketter, 1995).

In contrast to the GPMT, the Buehler occluded patch test is a solely topical exposure-based test without the use of an adjuvant (Buehler, 1965, Botham et al., 2005). It is for this reason that agrochemical formulations are generally tested in the Buehler assay instead of the GPMT. This predates the development of the LLNA, during the period when the EU indicated a test preference for the GPMT. This was because the dosing procedure of the GPMT was considered inappropriate for agrochemical formulations that had the final intended product use of being sprayed in crop fields by farmers (Botham et al., 2005). The Buehler assay, like the GPMT, uses a minimum of 30 guinea pigs (20 in the treatment group and 10 in the control group) (OECD, 1992). The Buehler assay employs three 6-hour duration topical applications of a given test material via occlusive patches over an induction period of three weeks (one patch per week) (Frankild et al., 2000, OECD, 1992). Two weeks after induction the test animals are then challenged (elicitation phase) by closed-patch tests for six hours. A nine-application induction version of the Buehler assay, during the initial three-week application period, can also be conducted and has previously been requested by regulatory authorities in Europe that had questioned the sensitivity of the three-induction application assay (Botham et al., 2005). However, in a study conducted by Botham et al (2005) using six reference materials, no significant difference in the ability of the three- and nine-induction variations of the Buehler assay to detect skin sensitisers was shown. The GPMT and the Buehler assay are the tests that comprise the OECD test guideline 406 (OECD, 1992).

The conclusion of skin sensitisation potential of test materials in these two guinea pig tests is made by clinical observational review of all skin reactions and any unusual findings on the guinea pigs treated. This subjective review of these effects on the animal's skin is then graded according to the Magnusson and Kligman grading scale for evaluation of challenge patch test reactions (OECD, 1992). This

qualitative output is considered highly variable in large part due to its subjective nature (Anderson et al., 2011b).

These qualitative guinea pig assays led onto the development of the quantitative murine LLNA. Following explorations into whether it was possible to identify potential sensitisers on the basis of events in the induction phase instead of the elicitation phase, the LLNA was developed and introduced in 1989 (Kimber et al., 1989, Kimber et al., 2002b). The LLNA quantifies the allergen driven T lymphocyte proliferation in the draining lymph nodes during the induction phase of skin sensitisation. Since its introduction, the LLNA has been extensively evaluated nationally and internationally, with an extensive interlaboratory validation conducted (Gerberick et al., 2007). Following this validation, the LLNA was endorsed as a stand-alone test by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in the USA and by the European Centre for Validation of Alternative Methods (ECVAM) (Ulrich and Vohr, 2005). It has been concluded that "*In terms of predictive identification of important skin sensitizers, the LLNA is at least as sensitive as, and much more reliable than, current guinea pig tests*" (Gerberick et al., 1999). In the regulatory validation of the LLNA 126 chemicals tested with reference to the guinea pig tests, produced 88% identical results in the LLNA, with a sensitivity of 90% and a specificity of 82%. The test is characterised by a high positive predictivity of 93% and by a negative predictivity of 76% (Gerberick et al., 2000). However, when comparing to human data, the LLNA has been reported to be approximately 70 – 80% predictive of the human hazard (Kleinstreuer et al., 2018a). Other analyses have also been performed by Urbisch et al comparing the LLNA results of a dataset of 111 compounds to their corresponding human data. They demonstrated that the LLNA had a 91% selectivity, 64% specificity and 82% accuracy (Urbisch et al., 2015). The dataset of 111 individual compounds spanned a variety of industrial domains and are used in products such as fragrances, preservatives, pesticides, solvents, cosmetics, pharmaceuticals and surfactants, amongst others (Urbisch et al., 2015). The ICCVAM test method evaluation report indicated that for pesticide formulations there were no instances of under prediction with the false

negative rate shown at 0% (ICCVAM, 2010). The LLNA method was incorporated into the OECD test guideline 429 formally in April 2002 (OECD, 2010).

Details of the exact method for conducting the LLNA are presented in the OECD test guideline. However, in summary, an initial preliminary test is conducted in mice to identify an appropriate vehicle and the maximum tolerated test material concentration for the main study. Concentrations leading to excessive irritation or systemic toxicity are ruled out (OECD, 2010). After this, mice are given topical applications on the dorsum of both ears at test material concentrations (or a relevant vehicle) according to their assigned groups. The mice receive this dose daily for three days. On day six of the test all mice are injected with tritiated (^{3}H)-methyl thymidine (^{3}H-TdR) via the tail vein. The mice are euthanised five hours after the ^{3}H-TdR administration and the draining auricular lymph nodes of each mouse ear are excised and processed in phosphate buffer solution individually or as a test group in a pooled approach. Following an initial wash and then resuspension in trichloroacetic acid incorporation of ^{3}H-TdR in lymph node cells is measured in scintillation fluid by β-scintillation counting as disintegrations per minute (DPM) for each treatment group (Kimber and Dearman, 2005). A three-fold increase in this DPM value observed in a treatment group in comparison to the vehicle control group is defined as a positive sensitisation reaction. This value of comparison to the control group for each test group is referred to as the Stimulation Index (SI) (i.e. an SI of ≥ 3 indicates a positive skin sensitisation response) (Kimber et al., 2002a).

As shown in Table 1.1 the LLNA provides a quicker *in vivo* test method, with the use of fewer animals and less test material. The LLNA is a more refined method with fewer numbers of test material exposures, no need for an adjuvant and it provides a relevant route of exposure (Kimber and Dearman, 2005). The LLNA also provide a definitive potency value that can be used in risk assessment, whereas the guinea pig assays do not (Basketter, 2016).

Test Method	Minimum number of animals required	Number of test material applications	Length of test
Draize	40 guinea pigs	10	39 days
GPMT	30 guinea pigs	5 (3 initial intradermal injections + 2 topical)	23 days
Buehler assay	30 guinea pigs	4 or 10 (3 or 9 induction respectively)	30 days
LLNA	16 mice	3	6 days

Table 1.1 Overview of *in vivo* skin sensitisation tests

1.2.2 *In Vitro* and *In Silico* Tests for Skin Sensitisation

Numerous non-animal test assays are available for skin sensitisation hazard assessment. The focus of this thesis is to review and assess a number of currently available, non-animal test methods for the assessment of individual agrochemicals and complex mixtures, specifically plant protection products. The *in vitro* methods (for skin sensitisation) that are accepted by the OECD include the direct peptide reactivity assay (DPRA), ARE-Nrf2 Luciferase test methods (specifically focusing on the KeratinoSens^{TM} assay) and the human cell line activation test (h-CLAT). These are summarised below:

The OECD 442C test method– The Direct Peptide Reactivity Assay (DPRA) is an *in chemico* test method which addresses peptide reactivity, postulated to be the MIE (i.e., the first key event) of the skin sensitisation AOP (OECD, 2012). Reactivity is measured by quantifying how much of the test substance being tested does not bind to either cysteine or lysine in a controlled system (OECD, 2019a, ECVAM, 2012).

The OECD 442D test method– The ARE-Nrf2 Luciferase Test Method (KeratinoSens^{TM}) is an *in vitro* test method which addresses keratinocyte induction of a cyto-protective gene pathway linked to skin sensitisation, i.e., the second key event of the skin sensitisation AOP. The test method uses luminescence detection to measure gene expression of the antioxidant/electrophile response element (ARE)-dependent pathway (OECD, 2018b, ECVAM, 2014a).

The OECD 442E test method – The human Cell Line Activation Test (h-CLAT) is an *in vitro* method which addresses the third key event of the skin sensitisation AOP i.e., activation of dendritic cells. The

test measures the upregulation of CD86 and CD54 dendritic cell surface markers in a human monocytic leukaemia cell line by flow cytometry (ECVAM, 2014b, OECD, 2018a).

Throughout this thesis when referring to a combination of the above three *in vitro* and *in chemico* test methods the term "triple pack" will be used. The aim of the combination of these assays is to enable the differentiation between contact sensitising chemicals and those chemicals that do not cause skin sensitisation. Individually, each target a particular KE of the skin sensitisation AOP and, as such, must be used in an integrated approach to testing and assessment (IATA) to achieve a conclusive assessment of skin sensitisation potential (Casati et al., 2018b). There have been 12 defined approaches (DA) initially proposed (Kleinstreuer et al., 2018b) that prescribe how the *in vitro* methods listed above should be used in a fixed data interpretation procedure (DIP) to address the skin sensitisation assessment of a single compound (Casati, 2018). This research focuses on three DAs; the BASF 2 out of 3, the Kao systematic testing strategy and the Kao integrated testing strategy (Kleinstreuer et al., 2018b, OECD, 2017). These three DAs are described in further detail in Chapter Three of this thesis.

Another *in vitro* method that will be evaluated in this thesis is the Genomic Allergen Rapid Detection (GARD) assay. The GARD assay is a method which uses the reverse transcription polymerase chain reaction (RT-PCR) to examine gene expression changes that may occur in dendritic and keratinocyte cell lines (Johansson et al., 2017). The GARD assay utilises the differences in transcriptomic analysis observed in the human myeloid cell line, induced by sensitising chemicals, in comparison to non-sensitising controls. The resulting biomarker signature, which consists of 200 transcripts, is used as an input into a support vector machine (SVM) statistical model to provide a yes or no answer with regard to the sensitising potential of a test item (Johansson et al., 2013). Further research indicates that this assay can also be used to provide a level of potency as signalling pathways have been reported to be triggered differentially depending on the known potency of the subset of chemical reactivity groups (Zeller et al., 2017). In addition to this, Zeller et al (2017) reported that more potent sensitisers are

generally assigned higher GARD SVM values than those of lower potency. This suggests that genes within the signature contribute to potency. There are currently few data on this assay's suitability to accurately determine the skin sensitisation potential of agrochemicals or its potential use for the assessment of complex mixtures.

The SENS-IS assay uses reconstructed human epidermis (RhE) in conjunction with detection of the expression of a panel of genes relevant to the biological process associated with the skin sensitisation AOPs. 38 Genes are observed, including 17 in the REDOX group and 21 in the SENS-IS group. The induction of seven genes of either group indicates a positive reaction, whilst the lowest concentration at which this has occurred is used to identify the test material's potency (Cottrez et al., 2016). This assay claims to discriminate between irritant and sensitising substances, considering the bioavailability of the test item. The use of a three-dimensional (3D) skin cell model enables easy application of multiple forms of test material into the test system without the need for solubility assessments that may be cumbersome when trying to evaluate complex mixtures. The SENS-IS assay's ability to accurately assess the skin sensitisation potential of different types of agrochemical formulations will be investigated in this thesis.

In addition to these *in vitro* methods, it is acknowledged that *in silico* (quantitative) structure-activity relationship ((Q)SAR) models can be used to aid the skin sensitisation hazard assessment process for individual chemicals (Enoch et al., 2008). In particular, by identifying the presence of specific structural alerts within single chemicals that may trigger covalent protein binding and which account for the MIE in the skin sensitisation AOP (Urbisch et al., 2016). Incorporation of (Q)SAR models alongside non-animal methods is integrated into a number of DAs for skin sensitisation (OECD, 2017). *In silico* models such as DEREK Nexus or the OECD QSAR Toolbox can identify the presence of structural alerts for skin sensitisation in single chemical test substances (Wilm et al., 2018, Verheyen et al., 2017). It could be suggested that their use should be common practice, at the very least in early-stage research, to aid in providing confidence in the assessment of potential lead compounds. Currently, however, in the

case of formulations, it is understood that the initial use of a battery of (Q)SAR programmes may not be plausible. This is because presently these *in silico* methods may not be able to account for potential interactions that can occur between different ingredients within a complex mixture. Some of the ingredients are intentionally included to produce these interactions or effects, whilst others may be unexpected and could potentially enhance aspects such as dermal absorption or irritant effects (Lindberg et al., 2020).

1.3 Covalent chemistry for skin sensitisation

There are a number of potential chemical mechanisms of action which are important for determining a chemical's skin sensitisation potential (Enoch et al., 2008). The more commonly observed chemical mechanisms, as suggested by Aptula and co-workers, are Michael addition (MA), Schiff-base formation (SB), acylation (Ac), bimolecular nucleophilic substitution (S_N2) reactions and aromatic nucleophilic substitution (S_NAr) (Fabjan and Hulzebos, 2008, Aptula and Roberts, 2006).

Acylation is the chemical mechanism whereby a carbonyl group is attacked by a nucleophile (Enoch et al., 2011). In the elicitation of skin sensitisation, the carbonyl group is present on a test material while the biological nucleophile is a cysteine or lysine peptide. During this mechanism the carbonyl group is attached to an electronegative leaving group (quite often a halide or carboxyl). This leaving group is expelled from the structure during the reaction, as illustrated in Figure 1.5 (Enoch et al., 2011, Aptula and Roberts, 2006).

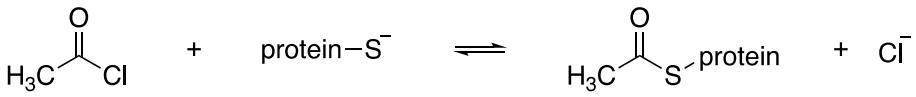


Figure 1.5 Reaction scheme for the acylation reaction

Michael addition is the chemical mechanism of action that occurs when a biological nucleophile is drawn to, and attacks, an electron deficient carbon atom of a polarised electrophilic compound (Enoch et al., 2011). Electronegative substituents can increase the reactivity of a compound as they increase

its polarity and lead to a greater pull of the nucleophilic species towards the electron deficient carbon centre, as shown in Figure 1.6 (Roberts et al., 2007a).

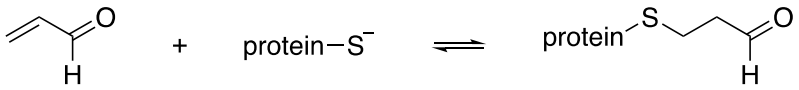


Figure 1.6 Reaction scheme for the Michael addition reaction

A bimolecular nucleophilic substitution reaction (S_N2) involves a biological nucleophile attacking an aliphatic carbon atom (or to a lesser extent nitrogen, sulphur or halogen atoms) to which an electronegative leaving group is attached, as shown in Figure 1.7. This reaction is termed bimolecular as it is a single step reaction and the two molecules (nucleophile and substrate) are both present in the reaction's transition state (Ouellette and Rawn, 2015).

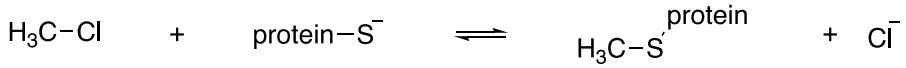


Figure 1.7 Reaction scheme for the S_N2

Aromatic nucleophilic substitution (S_NAr) involves the nucleophilic attack by a cysteine or lysine peptide to an aromatic ring system that has been activated in the presence of electron withdrawing groups, as shown in Figure 1.8. A suitably electronegative leaving group (typically a halogen) must be attached to the carbon for this carbon to be attacked by a nucleophile (Enoch et al., 2012). This results in a new covalent bond being established between the nucleophile and chemical. Two or more activating (electrophilic) groups in the ortho- or para- position on the benzene are also needed to prompt the leaving group (Enoch et al., 2011).

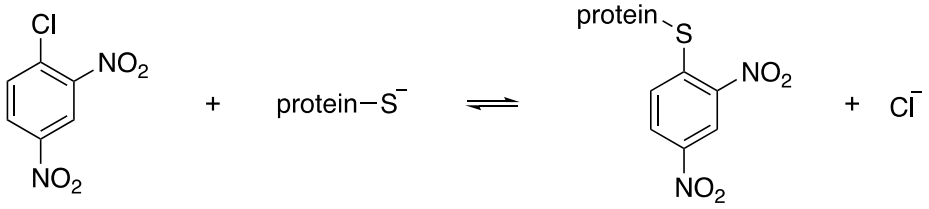


Figure 1.8 Reaction scheme for the S_NAr reaction

Schiff base (SB) formation reactions can only occur with a nitrogen containing nucleophile such as a primary amine (Enoch et al., 2011). Unlike the acylation mechanism, the carbonyl group in the Schiff base formation reaction is not attached to an electronegative leaving group. The SB reaction is a two-step process, with the first involving a biological nucleophilic nitrogen group (e.g., a lysine unit within proteins) attacking the reactive carbonyl centre of a sensitising compound as shown in Figure 1.9. In this first step, protonation occurs forming an intermediate carbinolamine species (Gleeson and Gleeson, 2020). This protonated intermediate species then undergoes a proton transfer leading to a condensation reaction, in which water is eliminated to produce the final Schiff base.

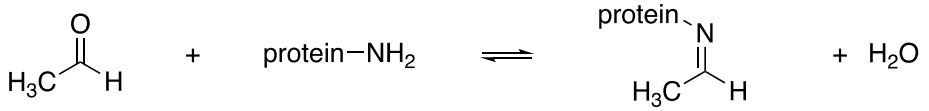


Figure 1.9 Reaction scheme for Schiff base formation

The above chemistry can be encoded as structural alerts, this involves defining the key structural features associated with these chemical mechanisms (which is greater than the simplified outline above) (Enoch 2008, 2011). For example, for the S_NAr reaction shown in Figure 1.8 the relevant structural alert would define the requirement for the presence of the halide leaving group (chlorine) attached to a benzene ring activated by two electron-withdrawing features in the ortho- and para-positions (nitro in the example). The definition of such structural alerts enables a chemical to be assigned into one of these reactivity domains, indicating that it has the ability to stimulate the skin

sensitisation MIE via the formation of a covalent adduct with the biological macromolecule. However, this does not guarantee that the chemical will cause skin sensitisation. Aptula and Roberts (2006) stated that, "*within a reaction mechanistic applicability domain, skin sensitisation potential is a function of reactivity and (usually) hydrophobicity. A compound can be insufficiently reactive or insufficiently hydrophobic to cause sensitisation*". For example, prior to forming a protein-hapten complex, the chemical needs to be able to gain access to the viable epidermis via the stratum corneum. This appears to be an additional factor that is not adequately addressed by assessing potential structural alerts for protein binding alone. Some understanding of the specific location and quantification of chemicals in the epidermal layer, defined as the epidermal disposition of a chemical, would provide further understanding of the potential for interaction with keratinocytes/Langerhans cells (Basketter et al., 2007). Epidermal disposition should not be confused with a substance's ability to penetrate through all layers of the skin and undergo resorption into the vascular system becoming available in systemic circulation (Fitzpatrick et al., 2017). A manually derived expert judgement for the MIE potential of the individual chemical structures should also performed alongside the QSAR prediction.

1.4 Active Ingredients and Formulated Products

Industrial companies must determine the skin sensitisation potential of individual chemicals, be they active ingredients, metabolites, or individual chemical intermediates. This is because users of the consumer product that contains an active ingredient, or workers involved in the manufacture or transportation of quantities of this material, may also encounter the intermediates. While contact might involve minute amounts, the accurate assessment of the skin sensitisation potential of these individual components will enable the correct hazard assessment, and thus labelling, of the container transporting this active ingredient (AI). In addition to the assessment of single chemicals, the assessment of formulations containing numerous co-formulants, (preservatives, bases, surfactants, solvents etc) is also required to enable successful registration, use and transportation. Currently, an

acute toxicity estimate calculation method is being promoted and its use is expected to be seen in the submission of new product formulations by European regulators (ECHA, 2017b). The United States Environmental Protection Agency (US EPA) also favours the use of this calculation in the submission of new formulations (though they are currently assessing reliability compared to known *in vivo* data via a pilot programme collecting data from company submissions (Basketter et al., 2020)). However, regulators in the Asia-pacific (APAC) region are still heavily reliant on the use of *in vivo* methods, in particular skin sensitisation methods for identifying the potential of a formulation or even individual ingredients to lead to an allergic skin reaction.

1.5 Test Guidelines and Current Regulation for Skin Sensitisation

Appropriate guidelines, such as those documented by the OECD, for each validated test method are available, well publicised and should be followed when hazard assessment for test substances is being conducted. However, individual regulatory bodies/global regions have different specific requirements when conducting these tests (i.e. the number of animals used or which methods are generally accepted, such as a preference for the Beuhler test rather than the LLNA in some Asia-Pacific countries) (Daniel et al., 2018b). In Europe, labelling of substances should be in accordance with the Classification Labelling and Packaging (CLP) regulation which has superseded the Dangerous Preparations Directive (DPD). Guidance from the CLP and regulatory bodies encourages the use of non-animal alternative methods to predict acute toxicity. The CLP guidance and Globally Harmonized System (GHS) of Classification acute definitions documents ((EC), 2008, GHS, 2017) report the mathematical formulae used for acute toxicity estimate calculations, which are currently being used to estimate the oral, dermal and inhalation systemic toxicity median lethal doses (LC50) of formulations. There are also predictive calculations for skin and eye irritation, as well as a material percentage threshold method for predicting skin sensitisation using generic or specific concentration thresholds for individual components present within a formulation (Corvaro et al., 2017). These are

all identified in the CLP guidance and all of these methods indicate a strong drive towards alternative testing methods, without the use of animals (ECHA, 2017b).

In order to proceed with REACH registration of an individual chemical, historical data provided by previously performed *in vivo* methods of skin sensitisation testing, specifically the LLNA, or guinea pig maximisation test and/or Beuhler 3 or 9 application induction assay, are required (EU, 2017a). The REACH regulation promotes the sharing of available toxicity data, thus reducing animal usage by eliminating the need for *in vivo* tests to generate new data where data for specific endpoints already exist. In addition, the REACH regulation also promotes the use of alternative non-animal new approach methodologies (NAMs) where available. Registrants must first submit a proposal to the European Chemicals Agency (ECHA) before conducting testing for the purposes of REACH registration. In this process ECHA asks registrants to identify what alternative methods they had considered prior to the submission of these testing proposals (Rowan and Spielmann, 2019). It is recognised that there are now acceptable, validated, non-animal alternative assays available and regulations across the chemical industry promote their use wherever possible (ECHA, 2016b; ECHA, 2017b; EC, 2009a). In the EU, regulatory acceptance of a NAM for deriving information for the hazard assessment of local toxicity and acute or short-term effects has been achieved (Rogiers et al., 2020). However, that is not the case for systemic toxicity effects that are generally observed following longer term exposure, or adequate quantitative risk assessment for acute toxicity (Rogiers, 2019). Legislation states that the development of non-animal test methods should be promoted in order to produce safety data relevant to humans and to replace animal studies currently in use (EC, 2009a). The EU has banned cosmetic ingredient testing on animals (since 11th March 2009) as well as the testing of finished cosmetic products on animals (since 11th September 2004) (Rowan and Spielmann, 2019). In addition, there is a marketing ban prohibiting the sale of finished cosmetics and cosmetic ingredients in the EU that were tested on animals (since 11th March 2013) ((EC), 2021, Rogiers et al., 2020). The framework for this ban on animal testing of cosmetics within the EU was initially phased in via the EU Cosmetics Directive ((EC), 2003). This was further reinforced with the cosmetics regulation containing the same

restrictions (EC, 2009b). The European cosmetic legislation's previous deadline of 2013 for the replacement of animal tests in use of safety assessment of cosmetic ingredients, has spurred on the development of new, OECD validated, *in vitro* sensitisation testing methods. For other endpoints, the use of *in vitro* methods is commonplace, such as the Bovine Corneal Opacity Permeability test (BCOP) and the Isolated Chicken Eye test (ICE) which are used regularly in the cosmetic, biocide and agrochemical industries for risk assessment of acute eye irritation. Recently, where alternative methods are available the REACH regulation and ECHA guidance have been updated to insist that the available *in silico* and *in vitro* test are used for skin sensitisation assessment in the chemical industry, in the specific regions that fall under this regulation (Taylor and Rego Alvarez, 2020).

This thesis will focus on agrochemical active ingredients and agrochemical formulations/final products. Although the regulatory environment is in a constant state of change, Daniel et al (2018) provides an overview of the current skin sensitisation requirements for pesticides across the regulatory landscape of different global regions. These are summarised briefly below.

1.6 Pesticide global regulatory requirements for skin sensitisation

When reviewing, developing, or deciding upon an appropriate skin sensitisation hazard assessment approach, there is a need to understand the requirements of the location at which the final product is intended to be used/sold. Here the different skin sensitisation hazard assessment requirements between the different global regulatory regions are put into context to gain an understanding of where these requirements contrast.

For registration of pesticide formulations in the European Union (EU) the use of the calculation method in accordance with the CLP regulation and United Nations (UN) GHS, needs to be conducted to determine skin sensitisation classification. In order to carry out this method the skin sensitisation potential of the active ingredients and co-formulants within the formulation must be known. This information should be available in the safety data sheets (SDS) of the individual ingredients indicating their skin sensitisation classification, if warranted, and also the method by which this decision was

reached i.e., LLNA, GPMT, Buehler or via read-across. For single compounds the EU currently accepts the use of OECD guideline non-animal *in vitro* and *in chemico* methods. There is an acknowledgment that no single alternative method can be used to fully address this endpoint (OECD, 2017) and, rather, a DA (as indicated above) to provide a weight-of-evidence (WoE) to address this should be conducted (Casati et al., 2018a).

Active ingredients for pesticides or finished pesticide products for use in Brazil are evaluated by three different federal institutions prior to their registration. These institutions are the Brazilian Health Regulatory Agency (ANVISA), the Ministry of Agriculture, Livestock and Supply (MAPA), and the third is the Brazilian Institute of Environment and Renewable Natural resources (IBAMA) (Oliveira et al., 2020). Toxicological review of the products presented for registration is conducted by ANVISA.

The Brazilian Health Regulatory Agency requires hazard assessment relating to skin sensitisation of ingredients and the final product. They recommend using validated and internationally recognised alternative methods where available (Daniel et al., 2018b). All tests used need to be conducted in accordance with internationally accepted guidelines such as the OECD or US EPA. The Mutual Acceptance of Data (MAD) system is in place to bolster the acceptance of data from toxicology studies conducted to OECD test guidelines across different regulatory regions. The MAD system also allows for adherence by OECD non-member countries (Koëter, 2003). The basis for pesticide regulation in Brazil was established by the Brazilian federal law No. 7802 and proceeding Acts 4074/2002 and 5981/2006 (Jardim and Caldas, 2012). For an active ingredient the GPMT or Buehler assay are indicated as the testing methods of choice. However, the Brazilian National Council for the control of animal experimentation (CONCEA) recognise the LLNA, DPRA and the ARE-Nrf2 Luciferase assay (Daniel et al., 2018b). As such these alternatives to the guinea pig assays can be used for skin sensitisation hazard assessment in circumstances where Brazilian registration is necessary for an agrochemical active ingredient. A hazard assessment on the finished pesticide product also needs to

be performed, to appropriately classify it as a sensitiser or not. However, unlike the active ingredient a clear direction on the specific skin sensitisation test method that should be used is not provided.

In Canada, the Pest Management Regulatory Agency (PMRA) of Health Canada is the regulatory body that ensures the correct and appropriate evaluation of toxicity is conducted on finished plant protection products (PPP) or their active ingredients. In accordance with Health Canada regulatory requirements, a skin sensitisation assessment must be conducted ((PMRA), 1984). The preferred test method for this evaluation is the LLNA, although the GPMT and Buehler assay are also accepted (Daniel et al., 2018b). For active ingredients the use of validated non-animal alternative methods conducted to OECD test guidelines is also accepted. However, as no single *in vitro* method has currently been demonstrated to sufficiently evaluate the skin sensitisation, a combination of these methods is required (OECD, 2019a, OECD, 2018a, OECD, 2018b). Canada also accepts a bridging argument in place of an *in vivo* test for the final pesticide product where possible (Corvaro et al., 2016). Bridging of mammalian acute toxicity data is the use of toxicity data available on a formulation of similar composition to fill data gaps for the specific formulation in question. This is a recognised method of hazard assessment for complex mixtures and is accepted in a number of regulatory regions (EC, 2012a).

Chinese regulations require animal tests (Strickland et al., 2019), in particular the Buehler assay for the assessment of skin sensitisation of new pesticide products. This test method is specified in order to provide results that allow for classification of non, weak, light, medium, strong or very strong sensitisers. Only products with test results demonstrating them to be weak or non-sensitising can be accepted for commercial use (Daniel et al., 2018b) in China.

Skin sensitisation assessment on active ingredients and finished product pesticides is required in both Japan and South Korea. Alternative methods are generally accepted however, they are considered in these regions on a case-by-case basis (Daniel et al., 2018b; Strickland et al., 2019).

The US EPA allows for a bridging argument to be used in an evaluation of the skin sensitisation potential of a finished pesticide. If that is not possible a LLNA, GPMT or Buehler assay can be conducted (Strickland et al., 2019). The EPA released an interim science policy on the use of alternative approaches for skin sensitisation as a replacement for laboratory animals in 2018 (EPA, 2018). It expresses that consistent with the organisation's commitment to advancing the implementation of new approach methodology in human risk assessment, the use of defined approaches to skin sensitisation hazard identification with alternative methods is accepted. This is in keeping with the memorandum signed by the EPA Administrator Andrew Wheeler, directing the agency to have completely eliminated their need for animal testing by the year 2035 (EPA, 2019).

Table 1.2 helps to illustrate that although there is strong commitment towards the use and development of non-animal alternative methods for the assessment of skin sensitisation, there is a lack of global regulatory harmonisation on their acceptance. This lack of harmonisation is further demonstrated by the Commonwealth of Independent States (CIS). The CIS is comprises 11 countries, with all still requiring *in vivo* test data for acute toxicity assessment of a pesticide (Ministry of Agriculture of the Russian Federation, 2020).

Region	Material type	Accepted Test Method
Brazil	Active ingredient	*In vitro* alternatives with OECD test guidelines
		LLNA
		Buehler, GPMT
	Pesticide Formulation	LLNA
		Buehler, GPMT
Other Latin American countries	Active ingredient	LLNA
		Buehler, GPMT
	Pesticide Formulation	LLNA
		Buehler, GPMT
		Bridging in some countries
Canada	Active ingredient	*In vitro* alternatives with OECD test guidelines
		LLNA
		Buehler, GPMT
	Pesticide Formulation	Bridging
		LLNA
		Buehler, GPMT
China	Active ingredient	Buehler[a], GPMT
		LLNA
	Pesticide Formulation	Buehler[a], GPMT
		LLNA
CIS countries	Active ingredient	LLNA
		Buehler, GPMT
	Pesticide Formulation	LLNA
		Buehler, GPMT
EU	Active ingredient	*In vitro* alternatives with OECD test guidelines
		LLNA
	Pesticide Formulation	Bridging
		CLP/GHS calculation method
		LLNA
US	Active ingredient	*In vitro* alternatives with OECD test guidelines
		LLNA
	Pesticide Formulation	LLNA[a]
		Buehler, GPMT
Japan/South Korea	Active ingredient	Buehler, GPMT
		LLNA
		In vitro methods considered case-by-case
	Pesticide Formulation	LLNA
		Buehler, GPMT
Australia/New Zealand	Active ingredient	*In vitro* alternatives with OECD test guidelines
		LLNA
	Pesticide Formulation	Bridging
		CLP/GHS calculation method
		LLNA

Table 1.2 Current understanding of the regional regulatory requirements for skin sensitisation testing (Basketter et al., 2020; Strickland et al., 2019; Daniel et al., 2018a) [a] preferred assay

1.7 Research aims of this thesis

The overall aim of this thesis was to evaluate the ability of a selection of non-animal, alternative skin sensitisation approaches to accurately predict the skin sensitisation potential and, where possible, the potency of agrochemical active ingredients and formulated plant protection products. The focus was to address areas of regulatory application where there could be a greater uptake of alternative methods. LLNA data will be used as the *in vivo* benchmark against which to compare these alternative methods. Where LLNA data are unavailable, data on previously conducted guinea pig assays will be used for comparison. The discussion of this endpoint for complex agrochemical mixtures is presented in Chapters Two, Three and Five. The prediction of this endpoint will be investigated and discussed for single chemicals in Chapters Three and Four.

The specific objectives of the thesis were:

- To evaluate the CLP/UN GHS threshold calculation method for skin sensitisation classification.
    - This involved identifying suitable agrochemical formulations of varying types that had data from previously conducted *in vivo* skin sensitisation tests (the results and methodology outlined in Chapter Two).
- To investigate if the DPRA, KeratinoSens™ and h-CLAT assays could be effective and suitable in the prediction of skin sensitisation potential of agrochemical formulations.
    - This involved understanding previous research in this area, the concentration levels leading to cytotoxicity that impeded accurate results and the most appropriate solvent for use for each test material and assay (the results and methodology outlined in Chapter Three).
- To evaluate the integrated testing strategy, the systematic testing strategy and the “two out of three” Defined Approaches in order to understand the benefits, limitations and general differences in accuracy of these approaches when assessing skin sensitisation of agrochemical active ingredients.

- Appropriate compounds with previous *in vivo* skin sensitisation data were identified and used in the assays and *in silico* models in accordance with the Defined Approaches. An appropriate performance evaluation of the approaches was conducted (results outlined in Chapter Three).

- To evaluate the GARD assay's ability to predict the skin sensitisation potential of agrochemical active ingredients accurately, using the *in vivo* data as a benchmark for this evaluation.
    - The same, or similar, active ingredients used in Chapter Three were evaluated. The predictions were reviewed and the need for further investigative work into the assay's assessment of formulations and also potency was determined (results in Chapter four).
- To investigate if the use of the 3D SENS-IS assay provided a more robust test model for the evaluation of both sensitising potential and potency of agrochemical formulations.
    - This required the investigation of the irritation potential of the test materials, to ensure that *in vivo skin sensitisation* data were available for the test materials to provide a benchmark from which to evaluate the assay results (results outlined in Chapter Five).

2.0 CHAPTER TWO - EVALUATION OF THE SKIN SENSITISATION THRESHOLD CALCULATION METHOD FOR THE HAZARD ASSESSMENT OF AGROCHEMICAL FORMULATIONS

Hazard assessment methods used to evaluate the potential of a single chemical, or a complex mixture, to cause an allergic skin reaction have been established, and gradually refined over the years (Ibrahim et al., 2017, Doe and Botham, 2019). In conjunction with the efforts and work of various organisations such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in the UK and People for the Ethical Treatment of Animals (PETA), changes in regulatory acceptability of *in vivo* mammalian test methods across industries (such as those identified in the pesticide global regulatory requirements for skin sensitisation section of Chapter One) have continued to drive the development of non-animal alternative methods including *in silico* approaches.

The previously established *in vivo* approaches to determine xenobiotic skin sensitisation potential, such as the GPMT and Buehler assay (OECD, 1992), aimed principally to utilise observed clinical effects on the skin to assess potential hazard. Further development of hazard assessment approaches for skin sensitisation has led to methods that target changes in specific biomarkers (Koppes et al., 2017), focusing on different key events of the agreed skin sensitisation AOP and their relationship to *in chemico* and *in vitro* assays section of Chapter One (OECD, 2014). In so doing, the adaptation of alternative assay methods has moved away from *in vivo* approaches towards *in vitro* tests and potentially an accepted synergistic output of results from *in silico* and *in vitro* methods (Johnson et al., 2020). This shift is in tandem with the ideal of reducing, refining and ultimately replacing animal use, particularly in this case for skin sensitisation hazard assessment.

Whilst alternative methods to assess skin sensitisation potential are validated for single substances, there are currently no regulatory accepted *in vitro* skin sensitisation methods that have been validated for the assessment of complex mixtures. The OECD test guidelines for the Direct Peptide Reactivity

Assay (DPRA), KeratinoSens™ and the human Cell Line Activation Test (h-CLAT) (all three are defined in more depth in Chapter Three and will be referred to as the *in vitro* triple pack from hereon) indicate that limited information on the applicability of these test methods to mixtures is available and that where a test substance is not clearly within the applicability domain, "*upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically*."(OECD, 2019a, OECD, 2018a, OECD, 2018b).

The threshold calculation method allows for the classification of complex mixtures for acute human health toxicological effects, without the *in vivo* testing of the mixture in question. This chapter aimed to evaluate the calculation method's efficiency to accurately classify the skin sensitisation potential of plant protection products (PPP). The calculation method for skin sensitisation was conducted using the reported skin sensitisation potency of the individual components present within the complex mixture in question. These data were predominantly taken from the safety data sheets (SDS) of each of the components. A SDS may indicate if a component is classified according to CLP regulation (EC, 2008). If the material in question is classified as a skin sensitiser, it will have been assigned the hazard phrase "H317" in accordance with EU CLP (ECHA, 2017b) and the Globally Harmonised System (GHS) (GHS, 2017). Alongside identification in the SDS of a substance as a skin sensitiser (H317), the category, and hence potency, of the material as a skin sensitiser is also given. A Generic Concentration Limit (GCL) is associated with the 1, 1A or 1B skin sensitisation sub-classifications provided in the SDS. There are certain individual chemicals for which these GCLs do not apply. These chemicals have Specific Concentration Limits (SCL) associated with them that can lead to the overall classification of the complex mixture at lower concentrations of a particular component. SCLs are assigned to chemicals in accordance with CLP (ECHA, 2017b) where the GCLs are not considered to be sufficiently protective. SCLs are set where there is adequate and reliable evidence indicating that the specific hazard (in this case skin sensitisation) is below the GCL for classification. Reported human data from occupational

exposure, such as workplace studies, are considered reliable data for the assignment of a chemical's SCL.

These SCLs are recorded and can be obtained from the European Chemical Agency (ECHA) website (https://echa.europa.eu/). An apparent limitation of the skin sensitisation calculation method is for chemicals that do not achieve the Registration Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation tonnage requirements and hence do not require a chemical safety report to be submitted (EU, 2017a). As such, and as indicated by Corvaro et al (2017), the absence of a skin sensitisation classification in the SDS of a chemical may not always indicate that the material is a non-sensitiser. Instead, there may be occasions where the absence of classification and mention of skin sensitisation testing in the SDS is an indication that no testing for the skin sensitisation potential has been performed. This may be the case where a chemical is manufactured or imported at less than 10 metric tons a year (Rudén and Hansson, 2010). Another concern may be if the data present were derived from *in vitro* testing alone and, thus, assessment of potency and subsequent sub-categorisation (category 1A or category 1B) cannot be indicated in the SDS (ECHA, 2016b). Either of these scenarios could potentially lead to an underestimation of the skin sensitisation potential of the formulation via the threshold calculation method. The impact of these possible limitations on the accuracy of the skin sensitisation calculation method on agrochemical PPP is discussed below.

In a previously conducted evaluation of the calculation method (Corvaro et al., 2017), 54% agreement between the calculation and *in vivo* studies was determined. A later study by Kurth et al. (Kurth et al., 2019) found a slightly improved agreement of 65%, when *in vivo* classified products were considered only. These results have to be placed in the context of the OECD guidance on the validation and international acceptance of new or updated test methods for hazard assessment, which states "*for a test method to be considered as a replacement, it should offer advantages over the accepted method.*"(OECD, 2005). These advantages include a reduction in animal numbers used and their overall suffering. However, the OECD guidance also states that validation exercises need to demonstrate unambiguously that the new method meets, or exceeds, the performance of the

previous method. To evaluate performance, accuracy has been defined by The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as determinations of concordance, sensitivity, specificity, predictivity (positive and negative) and false positive and negative rates (OECD, 2005). In an assessment of the validity of the LLNA for pesticide formulations by the National Toxicology Program (NIH, 2010), the sensitivity of the LLNA against human data was shown to be 72%, specificity was 67%, accuracy was 72%, whilst the false positive and false negative rates were 33% and 28% respectively (n=72). However, the assessment compared the LLNA to guinea pig tests (Buehler and GPMT) and produced slightly higher values of 87% for sensitivity, 82% for specificity, 86% for accuracy and for false positive and false negative results 18% and 13% respectively.

At present, the threshold calculation method is the only non-animal alternative method for determining skin sensitisation of complex mixtures accepted by European regulatory authorities (Daniel et al., 2018a). This method is identified in the United Nations Globally Harmonised System of classification and labelling of chemicals and also in the European Chemical Agency's guidance on the application of the CLP criteria (GHS, 2017, ECHA, 2017b). There are several pieces of legislation that propelled the development of non-animal alternative methods to testing for PPP. These include Regulation (EC) No. 1107/2009, concerning the placing of PPP on the market. Reduction of animal testing is a requirement of this regulation as it refers specifically PPP and their active substances, safeners, co-formulants, synergists and adjuvants. (EC) No. 1107/2009 outlines the acceptable rules in which PPP can be authorised for their placement on the market. In paragraph 40 of the regulatory document it states that, where available, suitable non-animal test methods should be used and that if animal testing is undertaken as a last resort, justification for the steps to avoid testing and prevent duplication of testing should be provided ((EC), 2009a). The alternative method should be fit for purpose, i.e. reproducible and produce reliable predictions of adverse effects that may occur in humans (Kurth et al., 2019). Leading on from regulation (EC) No 1107/2009, regulation (EC) 1272/2008 (CLP) outlines suitable non-animal methods to predict the acute toxicity of complex mixtures using data available on the individual components of the formulation (EC, 2008). As such in accordance

regulation (EC) No 1107/2009 the acute toxicity hazard assessment of PPP is for European submission is conducted using regulatory accepted NAMs wherever possible. Though in other sectors different requirements may be needed as identified in Table 1.2.

Regulation (EC) No. 1107/2009 officially began to apply from 14 June 2011 and is also accompanied by the EC 1223/2009. This regulation prohibits the use of mammalian testing of ingredients for inclusion in cosmetic products and also the testing of the products themselves within the EU (EC, 2009b). A postponement of the cosmetic marketing ban on animal tested products was implemented, whilst a search for alternative methods for three endpoints (toxicokinetics, repeated dose and reproductive toxicity) was conducted. In an EC-funded report on the progress of this research (Taylor et al., 2011, European Commission, 2011), it was observed that not only could timelines for the availability of suitable new alternatives for the three endpoints not be provided, but an additional two endpoints (skin sensitisation and carcinogenicity) had been added to the list (Taylor and Rego Alvarez, 2020). Finally in an impact assessment on the cosmetics industry it was revealed that the proportion of new ingredients entering the market that would ultimately be affected by the ban was relatively low (4% of a company's portfolio) (EC, 2013a). Leading on from that the EC decided not to extend the animal testing ban and it fully came into force on 11 March 2013. In addition there is the REACH 1907/2006 regulation ((EU), 2017a) and the European directive 2010/63 which cover the use of animals for scientific purposes (EU, 2010). The EC No 1907/2006 regulation in accordance with the objective of promoting non-animal testing and the replacement, reduction or refinement of animal testing states that, "use of animals should be avoided by recourse to alternative methods validated by the Commission or international bodies, or recognised by the Commission or the Agency as appropriate to meet the information requirements under this Regulation"(EC), 2013b). In addition, it also requires that where data are already available, no additional animal testing should be conducted which may duplicate these data. This promotes the sharing of this toxicity data and improving the data available to conduct calculations as per the methods outlined in CLP and UN GHS guideline documents mentioned above. The REACH regulation specifically mentions the use of alternative test methods on

an international and national level, including qualitative or quantitative structure-activity relationship models or from information from structurally related substances (grouping or read-across), *in vitro* methodologies and other relevant methodologies. Whilst outlining these approaches it is also stated that these methods shall be regularly reviewed and improved with a view to reducing testing on vertebrate animals and the number of animals involved. In keeping with that this chapter aims to review the CLP threshold calculation method.

2.1.1 History of skin sensitisation methods

The Draize Test was developed as an *in vivo* test for the assessment of the skin sensitisation potential of individual, or mixtures of, chemicals. It is one of the first predictive skin sensitisation tests in guinea pigs to be described, with the original description of the method going back to 1944 (Botham et al., 1991). It was also the first to be included in a test guideline in 1959 (Maurer et al., 1994). The Draize Test was the first attempt to standardise predictive sensitisation testing and was designed to identify potent skin sensitisers. Similar to the guinea pig test, the Draize Test used guinea pigs as the test species and had two test phases i.e., an induction phase and a challenge phase. As indicated in Table 1.1 the Draize Test has been reported to need approximately 39 days for completion (Draize et al., 1944) and was a non-adjuvant test that required ten intradermal applications of the test substance during the induction. This was followed by a single challenge injection of half the test material volume used in each of the induction injections, two weeks after the intradermal injections were completed (Johnson and Goodwin, 1985). A noted disadvantage of this assay is that false negative results have been recorded for known human skin sensitisers, such as nickel sulphate, benzocaine, neomycin and mercaptobenzothiazole (Botham et al., 1991, Maurer et al., 1975). This limitation of the Draize test drove the development of the alternative guinea pig skin sensitisation testing options. Although the Draize method was included in an older 1981 OECD 406 skin sensitisation test guideline alongside seven other methods, it was later removed from the revised OECD test guideline (OECD, 1992) in

favour of two methods (GPMT and the Buehler assay) that were considered to be more sensitive and more commonly used across different regulatory regions (Maurer et al., 1994a).

Following on from the Draize method, the OECD guideline for testing of chemicals (OECD 406) was released and described two skin sensitisation methods also using the guinea pig. The non-adjuvant Buehler assay has been used since 1965 in addition to the GPMT of Magnusson and Kligman (1970). The GPMT and Buehler guinea pig assays measure skin sensitisation by focusing on the dermal inflammation caused by the challenge phase of their testing methods. There are, however, limitations associated with these tests. In particular, although a scale is available for the evaluation of challenge patch test reactions (OECD, 1992), judgment of the severity of any observed dermal inflammation following elicitation can be considered subjective. As such, results may differ dependent on the individual recording the animal results. Consequently, and in line with a clearer understanding of the cellular and molecular events following exposure to contact allergens, research into a more robust method led to the development of the LLNA. More detail on the specifics of these *in vivo* skin sensitisation tests and how the data is used are given in Section 1.2.1.

The LLNA, which uses the mouse as the test animal, is viewed at present as the "gold standard" (Anderson et al., 2011a), for skin sensitisation determination for most regulatory sectors. The availability of a substantial amount of published literature on the LLNA makes it an appropriate benchmark against which new alternative methods are evaluated and validated. Although considered the gold standard and used as a benchmark in this fashion, it should be acknowledged that along with the LLNA's strengths (the assay's reproducibility, the reduction in animal numbers and the refinement leading to a single dose to animal to produce a quantitative result, rather than the use of clinical signs to reach a result that might be considered subjective as it is open to the interpretation of the study director), as is common with most predictive tests, the LLNA also has its own limitations. Specific limitations associated with the LLNA include:

- The LLNA's susceptibility to variability due the use of different vehicles (Basketter et al., 2009) (i.e., bioavailability can be altered by the vehicle used and the maximum concentration at which a suitable homogenous testing solution can be formed may be altered depending on the vehicle).
- The occurrence of false positive (observed for some strong irritants and unsaturated fatty acids (Roberts et al., 2016, Kreiling et al., 2008)) or false negative results (potentially for weak sensitisers or metal salts (Sailstad et al., 2001))
- Potential interspecies differences especially between the mouse and man (Basketter et al., 2009).

Whilst historical data may be obtained from the Draize test, important for Chapter Two are the results from the Buehler, GMPT and most recently the LLNA. These three test methods are appropriate for the assessment of complex mixtures and test result data on the chosen agrochemical test formulations is more readily available from tests previously conducted according to these methods.

In an ICCVAM evaluation it was acknowledged that where discordance between the LLNA and guinea pig assays was observed, the LLNA was a better predictor of the human response (Sailstad et al., 2001). As such, where LLNA data are available these results will be used as the comparative benchmark to evaluate against in this chapter, with regard to understanding the success, or otherwise, of the threshold calculation method.

In Europe as of June 2011, the use of acceptable non-animal alternative methods for acute toxicity hazard assessment became a regulatory requirement for agrochemical mixtures, as per Commission Regulation (EU) No 545/2011 (Heppner, 2019). Consequently, the use of calculation methods to perform the hazard assessment of acute toxicity endpoints for complex mixtures, using data from each of the individual components present within a formulation, is an accepted and widely used method in the EU. For skin sensitisation, generic and specific concentration limits for potential sensitising components within a given formulation are now used to determine the sensitising potential of an

agrochemical mixture within Europe (ECHA, 2017b, GHS, 2017). It is worth noting that there are two separate calculation methods to perform acute local toxicity hazard assessments to provide classifications for plant protection products under (EC) No. 1272/2008 (Draisci, 2011). For skin and eye irritation, the additivity calculation is used. As previously stated, the threshold calculation is used for sensitisation. As their names imply, the two methods are very different. The additivity calculation conducts its evaluation of irritation by providing a summation of the irritation potential of all ingredients (active ingredients and co-formulants) present in a specific mixture (Corvaro et al., 2017). In contrast, the threshold calculation examines each component of the formulated mixture for its own potential to cause an allergic skin sensitisation response. This is in line with the respective AOPs, as each sensitising chemical leads to the proliferation of allergen specific memory T cells (OECD, 2014). If a mixture contains two sensitising compounds that are both below their concentration triggering threshold (or concentration limit), the mixture is considered a non-sensitiser. Unlike the additivity calculation, in a scenario such as that just described, the two components are not combined to provide a concentration value above their individual triggering concentrations. Whilst these calculation methods are commonly used, there is no consensus as to their relative level of accuracy or sensitivity.

2.2 Aim of Chapter Two

The aim of this chapter was to evaluate the accuracy and appropriateness of the threshold calculation method for determining skin sensitisation potential of agrochemical formulations. This enables understanding of how pesticide products are categorised for skin sensitisation via this method and identifies whether the pesticide formulation type has any impact on the results of the threshold calculation. The threshold calculations of several Syngenta agrochemical formulations of varying types were conducted. These calculation results were compared to the experimental results of previously conducted *in vivo* skin sensitisation tests. The comparison was conducted using statistical parameters to allow for criteria against which the threshold method's performance could be evaluated.

2.3 Materials and Methods

2.3.1 Selection of agrochemical formulations

64 agrochemical formulations for consideration were selected based on the availability of results on the formulations from previously conducted mammalian skin sensitisation tests i.e., GPMT, Buehler assay or LLNA study results. In addition, formulations were selected in order to provide a diverse set of agrochemical formulation types. These formulation types include capsule suspension (CS), emulsifiable concentrate (EC), granule (GR), oil dispersion (OD) and seven other agrochemical formulation types as shown in Table 2.1.

Type	Description	Major Use	Number of formulations
CS	Capsule suspension (CS)	For mixing and spraying with water (W)	1
DT	Tablet for direct application	Tablets to be applied individually and directly in the field, and/or bodies of water	1
EC	Emulsifiable concentrate	W	10
FS	Flowable concentrate for seed treatment	Seed treatments (S)	13
GR	Granule	Dry (D)	2
OD	Oil dispersion	W	1
SC	Suspension concentrate	W	20
SE	Suspo-emulsion	W	2
SL	Soluble concentrate	W	5
WG	Water dispersible granules	W	7
ZC	A mixed formulation of CS and SC	W	2

Table 2.1. The number and type of agrochemical formulations assessed in this investigation

2.3.2 Determining skin sensitisation potential

The *in vivo* data from previously conducted studies were obtained by performing a search on each of the individual formulations through the Syngenta internal database. The study type, study result and corresponding sub-categorisation, where available, were recorded in a spreadsheet. Each formulation was assigned an identification number for the purposes of this investigation, which was recorded alongside the product's formulation type. Once details of all the formulations and their information were collected, each formulation's sensitisation potential was assessed using the threshold calculation method and recorded.

The threshold calculation was conducted in accordance with the methods outlined by both the UN GHS and EU CLP (EC, 2009a; ECHA, 2017b), using generic concentration limits (GCLs) or/and specific concentration limits (SCLs) where appropriate. According to both regulatory documents, where ingredients are present in the formulation above their GCL or SCL, the formulation should be classified as a Category 1 skin sensitiser. This threshold calculation does not allow for sub-categorisation of the formulation, it simply provides a binary, sensitiser or non-sensitiser, result.

The formulation composition (i.e., individual ingredients and concentrations at which they were present) was recorded to enable the threshold calculation to be conducted (this is proprietary information and has not been included here). The Safety Data Sheets (SDS) of each ingredient were reviewed in order to identify any skin sensitisation classifications that may be present in Section two of the SDS or recorded skin sensitisation study data that would be recorded in Section 11 of the SDS, the Toxicology section. In addition to the CAS number, the chemical name of the ingredient was entered into the European Chemical Agency (ECHA) chemical database finder in order to identify if the chemical in question had a SCL associated with it (ECHA, 2020).

Once the formulation composition was collected, the skin sensitisation potential/classifications of the individual ingredients and the corresponding GCL or SCLs were identified, the threshold calculation was conducted to determine the skin sensitisation classification of the formulation.

For GCLs, the criteria for classification as outlined in Table 2.2 were followed. For example, where an individual ingredient was classified as a category 1A skin sensitiser; if it was present at above or equal to 0.1%, the entire formulation should be classified as a skin sensitiser. The same principle was applied to the other two classification categories.

Skin sensitisation classification of component	**Concentration triggering category 1 mixture classification**
Category 1	≥1%
Category 1A	≥0.1%
Category 1B	≥1%

Table 2.2. Generic concentration limits of components of a mixture classified as a skin sensitiser (ECHA, 2017b)

Where an ingredient in the formulation was identified to have a SCL, this was used to determine if the concentration at which that ingredient was present in the formulation would trigger the classification of the whole mixture. Three specific ingredients and their SCLs that were identified via the ECHA website, and were present among the evaluated formulations, were benzisothiazolinone (BIT), 5-chloro-2-methyl-2*H*-isothiazol-3-one and 2-methyl-2*H*-isothiazol-3-one (3:1) (kathon) and clodinafop-propargyl as shown in Table 2.3. These SCL thresholds were noticeably lower than the GCLs and where a mixture within the formulation might contain ingredients with separate SCLs or GCLs, these were considered separately and not, for example, as a mean calculated threshold. The concentration of any classified skin sensitiser ingredient that was present in a diluted mixture should be recorded at the diluted concentration and not that of the whole mixture. For example, if a preservative mixture is present at 10% of product formulation, and 0.25% BIT was present in that preservative mixture. It should be calculated that 0.025% of BIT was present in the formulation as a whole and not 10% and is below the SCL of BIT and does not lead to the classification of the formulation.

Chemical	Harmonized skin sensitisation classification	Concentration triggering category 1 mixture classification
benzisothiazolinone	category 1	≥0.05%
clodinafop-propargyl	category 1A	≥0.001%
Kathon	category 1	≥0.0015%

Table 2.3. Examples skin sensitisation specific concentration limits for three substances (ECHA, 2020)

Using the GCLs and SCLs, the calculated formulation skin sensitisation classifications were collected and compared to the *in vivo* experimental data to determine performance and reliability of this threshold method in relation to the animal methods. The calculated classifications were compared to the *in vivo* results using the performance criteria as described below. Examples of how the CLP threshold calculation is performed are given in Appendix 1.

2.3.3 Performance criteria

In order to assess the performance of the threshold calculations, the results of the calculations were compared to those of the *in vivo* experiments. The results were entered into a 2x2 confusion matrix, alongside the recorded results from the previously conducted *in vivo* skin sensitisation experiments. In order to evaluate the predictive performance of the threshold calculation, the following statistical parameters were calculated: sensitivity, specificity, total accuracy, positive and negative predictivity, Cohen's kappa coefficient and Matthews Correlation Coefficient (MCC). These performance criteria were calculated as per the method indicated by Modi et al (2012) and are defined below.

2.3.3.1 Sensitivity

This parameter reflects the proportion of formulations correctly predicted as skin sensitisers in relation to those determined by the *in vivo* experiments.

$$\text{Sensitivity} = (\text{True Positive (TP)} / (\text{TP} + \text{False Negative (FN)})) \times 100$$

A true positive being a skin sensitisation prediction by the threshold method that agreed with the observed result from the *in vivo* test conducted on that specific formulation. Whilst a false negative was a non-sensitiser prediction from the threshold method that disagreed with the observed result of an *in vivo* skin sensitisation test indicating that the given formulation was a skin sensitiser.

2.3.3.2 Specificity

This aspect of the performance criteria demonstrates the proportion of formulations evaluated that have been correctly predicted by the threshold calculation to not be skin sensitisers, relative to all of the formulations experimentally determined to not be skin sensitisers (Cotter and Peipert, 2005).

$$\text{Specificity} = (\text{True Negative (TN)} / (\text{TN} + \text{False Positive (FP)})) \times 100$$

For this evaluation a true negative was a non-sensitising prediction generated by the threshold method that agreed with the observed result from the *in vivo* test conducted on that specific formulation. Conversely, a false positive was a positive skin sensitiser prediction from the threshold method that disagreed with the observed result of the *in vivo* skin sensitisation test indicating that the given formulation was not a skin sensitiser.

2.3.3.3 Total Accuracy

This represents the number of formulations accurately predicted by the threshold calculation as either a skin sensitiser or a non-skin sensitiser relative to the number of total predictions.

$$\text{Total accuracy} = ((\text{TP} + \text{TN}) / (\text{TP} + \text{FP} + \text{TN} + \text{FN})) \times 100$$

2.3.3.4 Positive Predictivity

This reflects the number of formulations correctly predicted to be skin sensitisers relative to all those that produced confirmed skin sensitisation results in the *in vivo* experiments.

$$\text{Positive predictivity} = (\text{TP} / (\text{TP} + \text{FP})) \times 100$$

2.3.3.5 Negative Predictivity

This calculated value represents the proportion of formulations correctly predicted by the threshold calculation as non-sensitisers relative to the non-sensitiser *in vivo* experimental outcomes.

$$\text{Negative predictivity} = (TN / (TN + FN)) \times 100$$

2.3.3.6 Cohen's Kappa Coefficient

The Cohen's kappa coefficient was chosen to indicate the extent of agreement between the frequencies of the two sets of data. Cohen's kappa coefficient is commonly used to estimate interrater reliability (Yu, 2005). This allows understanding of what proportion of values not expected to be in agreement (by chance) actually are in agreement (DeVellis, 2005).

$$\text{K-value} = (\text{observed agreement} - \text{expected agreement}) / (1 - \text{expected agreement})$$

When two measurements demonstrate agreement by chance, the kappa value (k-value) is zero. When the two measurements agree perfectly the k-value is 1.0 (Ensrud and Taylor, 2013). A k-value of 0 – 0.20 indicates a slight agreement between measurements, 0.21 – 0.40 indicates fair agreement, 0.41 – 0.60 for moderate agreement, 0.61 – 0.80 a substantial agreement and 0.81 – 1.0 almost perfect agreement. In the event that a negative k-value is calculated (less than 0, theoretically as low as – 1), this indicates the observed agreement was worse than chance agreement (McGee, 2018, Modi et al., 2012)

2.3.3.7 Matthews Correlation Coefficient (MCC)

As the two data sets were balanced, MCC was been used alongside the Cohen's kappa coefficient for classification accuracy (Zhu, 2020). The MCC represents the correlation between the experimental and predicted classifications and was calculated from the confusion matrix values. An MCC of +1 indicated a perfect prediction, while an MCC value of -1 demonstrated total disagreement between predicted and experimental results. An MCC value of zero meant that this was no better than a random prediction (Tharwat, 2018).

MCC = (TP X TN) – (FP X FN) / √(TP + FP)(TP + FN)(TN + FP)(TN + FN)

= (TP/N) – (TPR x PPV) / √PPV x TPR (1 – TPR)(1 – PPV)

Positive prediction value (PPV)

True Positive Rate (TPR) or sensitivity

Negative class (N)

2.4 Results

The distribution of 64 agrochemical formulations from the recorded i*n vivo* experimental results was 41% sensitisers and 59% skin non-sensitisers. The 64 formulations were a mixture of various agrochemical formulation types and distributed as identified in Table 2.1 above.

The results of the threshold calculation were tabulated against those of the *in vivo* experimental studies in Table 2.4. To enable the threshold calculation to be conducted the composition of the individual formulations (i.e., the percentage at which each of the co-formulants or active ingredients of the mixture were present) was recorded, however this is Syngenta proprietary information and has not been included here. The threshold calculation method agreed with the experimental *in vivo* data for 46 of the 64 reviewed agrochemical formulations.

N	*Pred. sensitiser*	*Pred. non sensitiser*
Obs. sensitiser	15 (58%)	11 (42%)
Obs. non sensitiser	7 (18%)	31 (82%)

Table 2.4 Confusion matrix of predicted results for the classification of skin sensitisation using the threshold calculation approach against the experimental results (number and percentage agreement)

Obs. – observed experimental result
Pred. – predicted threshold calculation result

The percentage agreement between the experimental and predicted data indicated that 58% of skin sensitising formulations and 82% of non-sensitising formulations were correctly assigned by the threshold calculation.

The results of statistical parameters that were calculated to provide criteria to evaluate the performance of the threshold method are shown in Table 2.5. The positive predictivity was 68%, demonstrating that among the formulations identified to be skin sensitisers in the *in vivo* assays, the calculation method had the probability of predicting 68% of them as sensitisers. Statistical measurements demonstrated that the calculation method had a higher probability of predicting formulations shown to be non-sensitisers in the *in vivo* experiments, as non-sensitisers, with the negative prediction value of 74%. The sensitivity value is 58% and this represents the proportion of the test formulations correctly predicted to be skin sensitisers. Demonstrating that this method would correctly identify only 58% of formulations as sensitisers, whilst misclassifying 42% of formulations as non-sensitisers.

Positive predictivity	68.2%
Negative predictivity	73.8%
Sensitivity	57.7%
Specificity	81.6%
Total Success/Accuracy	71.9%
*kappa-value	0.40
MCC	0.43

Table 2.5 Statistical parameters used for evaluation of the threshold calculation predictions of the agrochemical formulation test set results versus the *in vivo* experimental skin sensitisation results

***Kappa-value: < 0.20 poor, 0.21 - 0.40 fair, 0.41 - 0.60 moderate, 0.61 - 0.80 substantial, 0.81 - 1.00 almost perfect agreement**

The statistics for the performance of the threshold calculation method are shown diagrammatically in Figure 2.1, where the specificity of the calculation method is 82%. This demonstrates the methods ability to correctly identify non-sensitising formulations. Only 18% of skin sensitising formulations were predicted as non-sensitisers. The total accuracy has been calculated as 72% and the Cohen's kappa coefficient value was 0.40. This kappa-value demonstrates that the threshold calculation prediction provides a fair level of agreement with the *in vivo* experimental results.

The four most represented formulation types among the test set (SC, FS, EC and WG formulations as per Table 2.1) were further evaluated by comparing the predicted results of these specific formulation

types to the experimental results. This was again performed by including the results in 2x2 confusion matrices (presented in the appendices) and the calculated statistical parameters are as shown in the radar plots in Figures 2.2 and 2.3 below.

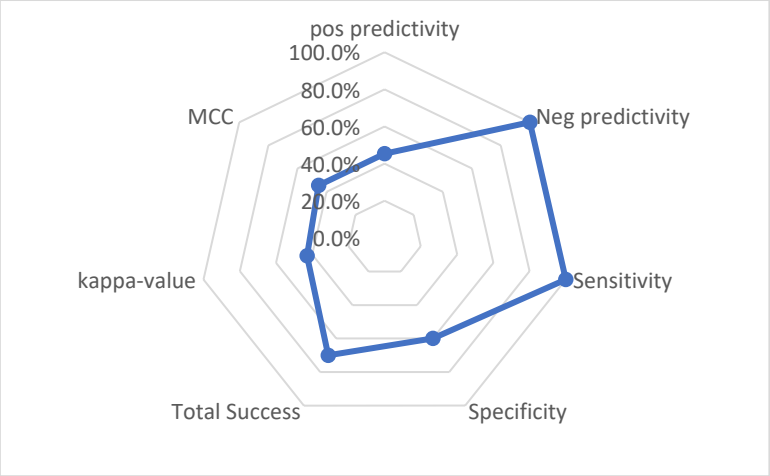


Figure 2.1 Statistical parameters used for evaluation of the threshold calculation predictions on the SC agrochemical formulation test set results against the *in vivo* experimental skin sensitisation results

Of the SC agrochemical formulations that were observed to be skin sensitisers in the *in vivo* experimental studies, none were predicted as non-sensitising by the calculation method. The calculation showed agreement with 100% of experimental results for skin sensitisers as shown in Figure 2.1. The negative predictivity of the threshold calculation for SC formulations was 100%, indicating that all formulations it predicted as non-sensitisers were demonstrated in the *in vivo* studies to be non-sensitising. However, the positive predictivity for the SC formulations was much lower at 46%. Sensitivity was 100%, while the specificity and total success were calculated to be low at 60 and 70% respectively. The kappa-value demonstrated a moderate level of agreement between the calculation method and the experimental data with a value of 0.43.

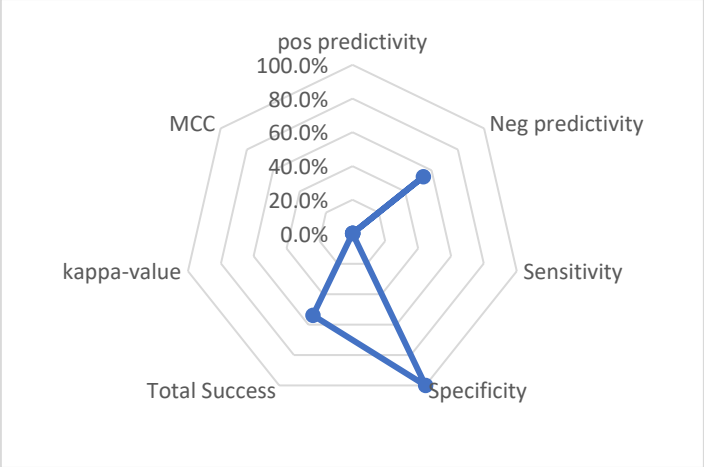


Figure 2.2 Statistical parameters used for evaluation of the threshold calculation predictions on the FS agrochemical formulation test set results against the *in vivo* experimental skin sensitisation results

The negative predictivity of the threshold calculation for FS formulations was 54%, indicating that the method had a probability of predicting formulations demonstrated in the *in vivo* studies to be non-sensitising, as non-sensitiser, a little more than half of the time. However, the positive predictivity for the FS formulations was shown to be 0% as all *in vivo* experimental sensitisers were predicted to be non-sensitising. Sensitivity as shown in Figure 2.2 as also 0%, whilst the specificity was shown to be 100% and total success 54%. The kappa-value demonstrated a poor level of agreement between the calculation method and the experimental data with a value of 0.

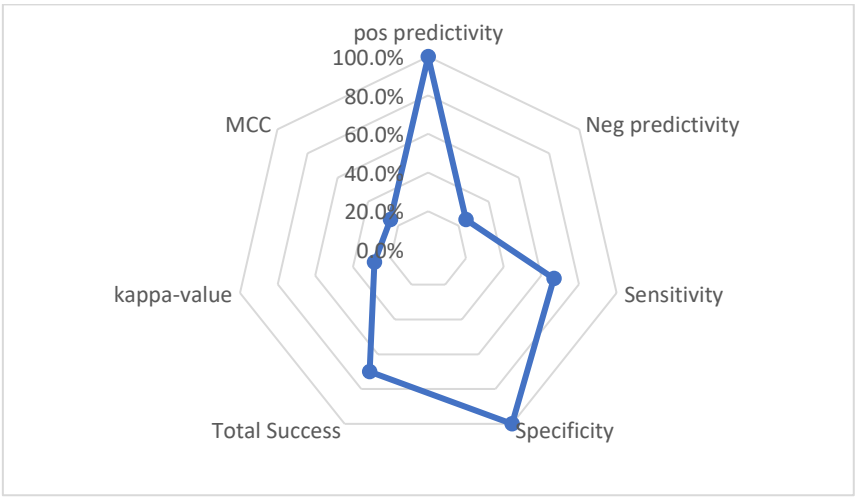


Figure 2.3 Statistical parameters used for evaluation of the threshold calculation predictions on the EC agrochemical formulation test set results against the *in vivo* experimental skin sensitisation results

Of the EC formulations that were observed to be skin sensitisers in the *in vivo* experimental studies, 33% were predicted as non-sensitising by the calculation method. The calculation showed 67% agreement with the experimental results for skin sensitisers as shown in the radar plot in Figure 2.3. The positive predictivity of the threshold calculation for EC formulations was 100%, indicating that all formulations predicted as skin sensitisers were demonstrated in the *in vivo* studies to be skin sensitising. However, the negative predictivity for the SC formulations was much lower at 25%. Sensitivity as shown in Figure 2.3 was 67%, whereas the specificity and total success were 100% and 70% respectively. The kappa-value demonstrated a fair level of agreement between the calculation method and the *in vivo* experimental data with a value of 0.29.

7 WG formulations	*Pred. sensitiser*	*Pred. non sensitiser*
Obs. sensitiser	2 (100%)	0
Obs. non sensitiser	0	5 (100%)

In vivo dataset distribution	Value (%)
sensitiser	29%
non sensitiser	71%

Table 2.6 WG formulation type threshold predictions against the *in vivo* study results

As the number of WG formulations evaluated was considered relatively low the same statistical analysis as performed in the previous formulation types was not performed. However, Table 2.6 shows that the calculation predictions for WG formulations were in agreement with the experimental results for both skin sensitising and non-sensitising formulations of this type.

2.5 Discussion

The aim of this Chapter was to investigate the suitability of the currently used threshold calculation method to determine the skin sensitisation potential of agrochemical complex mixtures. Predicted thresholds for 64 different agrochemical formulations were compared to the experimental results of previously conducted *in vivo* skin sensitisation tests. An effort was made to ensure that a number of different agrochemical formulation types were included amongst the 64 to allow for an observation

of any specific trends in the results of the predictions from the calculation method that might be correlated with the formulation type.

An assessment of the threshold method prediction results for all 64 formulations, regardless of formulation type, was conducted. From the *in vivo* experimental data, 41% of the 64 formulations were evaluated as skin sensitisers and the remaining 59% as non-sensitisers. The threshold calculation predictions were in 58% agreement with the formulations shown to be skin sensitisers in the *in vivo* experiments. These predictions showed much greater agreement with experimental non-sensitisers, with a prediction accuracy of 82%. In terms of risk assessment, it may be preferable to see a reverse in these trends, such that the more conservative result is obtained. This approach of awarding a greater weight of confidence to positive skin sensitisation predictions than the negative predictions is in agreement with the previously identified reasoning indicated by Corvaro et al (2017). Where a positive result is indicated by the analysis of the product composition, it can be accepted without any further testing, with the appropriate Personal Protection Equipment (PPE) being indicated via labelling (Corvaro et al., 2017). Corvaro et al., 2017 recommended that negative results obtained from the calculation approach should be verified via other methods such as *in vitro* tests. However, at present, there are no validated *in vitro* or *in vivo* methods for the evaluation of skin sensitisation potential of complex mixtures. The use of *in vitro* methods currently accepted for the evaluation of single chemicals (specifically the *in vitro* triple pack) and also a newly developed *in vitro* method, the SENS-IS assay (Cottrez et al., 2017), will be investigated further in the proceeding chapters of this thesis for their use in the assessment of the skin sensitisation potential of complex mixtures.

As noted above, the threshold calculations showed good specificity but poor sensitivity. The availability of human data for the skin sensitisation of these agrochemical formulations, and specifically the lack of such data, is a particular limitation of the analysis in this investigation in terms of hazard identification. However, the findings of a previously conducted evaluation of LLNA to human data from the US National Institute of Health (NIH, 2010) allow comparison with values obtained from

the performance criteria evaluation in this research, with an understanding of the inter-species variability. The sensitivity of the calculation method, in comparison to the *in vivo* methods, was 14% lower than the LLNA compared to the human data. The specificity showed an improvement of 15%, while total accuracy was matched at 72%. With the decreased sensitivity, without taking into account the formulation type, the calculation method had a lower proportion of correctly predicted skin sensitisers than observed in the *in vivo* methods. Although the specificity is relatively high, the sensitivity value does indicate that non-sensitiser calculation results need further verification to be accepted confidently. The calculated K-value reinforces this hypothesis with a value of 0.40, indicating the agreement between the calculation method and *in vivo* experimental results was only reasonable.

Looking, more specifically, at the results of the individual agrochemical formulation type evaluation, it can be determined whether or not there is a trend in the calculation classifications. Formulation types with at least seven different test formulations were further evaluated SC formulation results were investigated (20), FS formulations (13), EC formulations (10) and WG formulations (7). Of the 20 suspension concentrate formulations, five were sensitisers in *in vivo* skin sensitisation studies and 15 were shown to be non-sensitisers. When assessing the performance of the sensitivity of the calculation method in comparison to the mammalian skin sensitisation experiments of these SC formulations was 100%. The calculation identified all five of the skin sensitising SC formulations as category 1 skin sensitisers. However, the observed specificity of the calculation was much lower at 60%. This demonstrates that the calculation method was less accurate at predicting experimentally non-sensitising formulations. The overall accuracy for SC was 70%, while the K-value showed moderate levels of agreement between the calculation predictions and the experimental results. Perfect agreement between methods is an unrealistic expectation when looking at the interrater reliability between two methods of measurement/hazard assessment. There is never 100% agreement between research results (Marusteri and Bacarea, 2010), especially where the species for which the hazard assessment is being performed is not that being compared to. Nonetheless, as discussed by McHugh et al (2012), although low K-values are often accepted in research, a low level of interrater

reliability is not acceptable for health care or clinical research. It has been suggested that Cohen's proposed interpretation may be too lenient (with a value of 0.41) as it could be considered acceptable. McHugh et al suggested a reinterpretation of the levels that should be used when a kappa value of less than 0.60 (0.40 – 0.59) is considered to give a weak level of agreement (McHugh, 2012). If one uses this interpretation alongside the percentage total accuracy values, this indicates that for the SC formulation type the calculation approach is over predictive and could be considered insufficient as the sole method to determine skin sensitisation potential.

Thirteen FS formulations were included in the evaluation of the skin sensitisation calculation method. Six were observed to be skin sensitisers in the *in vivo* experiments and the remaining seven were non-sensitising. The predicted results from the calculation method for the FS formulation type, when compared to the experimental results, demonstrated the calculation was more likely to underestimate the skin sensitisation potential. The calculation method gave a positive predictivity value of 0%, as it failed to identify any of the skin sensitising FS formulations as category 1 skin sensitisers. In addition, the sensitivity value mirrored these results at 0%, reflecting the proportion of formulations correctly predicted as skin sensitisers. In contrast, the calculation method showed direct agreement with the prediction of the non-sensitising *in vivo* experimental data with specificity at 100%. Principally the calculation method predicted all of the FS formulations to be negative as it failed to identify sensitising FS formulations. This is contrary to the general premise associated with these calculation methods. This is a premise that indicates that the calculation method is at worst, a conservative approach to hazard assessment that may overestimate the potential for adverse effects more commonly than underestimate it (Corvaro et al., 2017). This further indicates that the calculation method should be used as an initial starting point for assessment and then should be followed up with more investigative methods to confirm the initial findings.

Analysis of the calculation method's performance on EC formulations showed a similar trend to that seen in the FS formulations, but not to the same extent. Thus, the EC formulations provided a

sensitivity of 67% with six of the nine *in vivo* experimentally skin sensitisers being correctly predicted by the calculation method. Specificity was 100%, however only one of the formulations was observed to be non-sensitising *in vivo* and an increase in the number of EC formulations (and other formulation types) would be required to allow for better evaluation and understanding of this performance criterion. Although the total accuracy seen for this formulation type was below that observed with the LLNA vs human assessment (70% EC calculation prediction, 72% LLNA (NIH, 2010)), the K-value was very low at 0.29 (McGee, 2018). If the standard interpretation of this value by Cohen (Modi et al., 2012) (fair, 0.21-0.40) or McHugh's re-interpretation (McHugh, 2012) is used (minimal, 0.21-0.39), this level of agreement with the *in vivo* results is not considered acceptable.

The poor performance, at times, of the calculation method indicates that more data and understanding is required. Further work could include an increase in the number of formulations being used in the evaluation of the calculation approach. This would also allow for the inclusion of additional formulation types (Table 2.1) in the individual analysis. Whilst the calculation method demonstrated good agreement with the *in vivo* results of the seven WG formulations, the number of WG formulations was low. As such, conducting the performance criteria evaluation as done with the previous formulation types, would not have provided information that would assist in further understanding the calculation prediction's suitability for this formulation type. At present the data generated here indicate that the calculation method is appropriate as an initial step in the assessment of the WG formulation type. These results have shown that understanding what direction (over-estimation or under-prediction) the calculation method has the higher probability to tend toward for each formulation type can provide useful information in forming a strategy for skin sensitisation assessment. This could allow for appropriate counteraction by exploring an alternative method of measurement when using the calculation method in a weight of evidence approach, instead of the stand-alone evidence for hazard assessment of the skins sensitisation endpoint as it is currently used.

There were a number of formulations for which the skin sensitisation calculation provided predictions that under-estimated the skin sensitisation potential. To improve the specificity of the method, identifying potential reasons for these false negative predictions is necessary. A number of potential reasons have been identified previously (Van Cott et al., 2018, Corvaro et al., 2017). When evaluating the individual ingredient safety data sheet information there are a number of coformulants that do not have a skin sensitisation classification and are assumed to have no skin sensitisation potential, these should be included in deriving the calculation prediction. However, it should be confirmed whether this absence of a skin sensitisation classification (with a globally harmonised classification hazard phrase of H317) in the SDS is due to an observed absence of skin sensitisation effects in an accepted test method. If there is no information regarding a skin sensitisation test conducted on the ingredient in question in the Toxicology Section 11 of the SDS, there is a possibility that the testing has not been conducted. For some specialty ingredients, if produced at a low tonnage, a skin sensitisation assessment would not have been a REACH requirement under current guidelines ((EU), 2017a). In these scenarios there are no study data from which to derive a GCL or SCL to include in the calculation method and consequently this may be a cause for the false negative predictions observed. There is an overarching need to develop *in silico* methods that are fit for purpose. In order to accomplish this, methods such as the skin sensitisation calculation method need to be evaluated and scrutinised well by scientists from academia, industry and the regulatory bodies to whom the results of these assessments will ultimately end up with. A model is only as reliable as the data used to create it (Benfenati et al., 2019). The availability, abundance and quality of toxicology data play a crucial role in determining if a particular *in silico* model will be able to be considered reliable for that particular assessment. This is demonstrated with the skin sensitisation threshold method in instances where data on the co-formulants within the formulation being evaluated is absent. Where a scarce data set is available for formulations being evaluated, this draws into question the reliability of the predictions based on that information, especially where a prediction indicates the skin sensitisation hazard is not of concern. It is reasonable to see why a weight of evidence evaluation would be required to accept a

non-sensitiser prediction, where the data being used to reach that conclusion are scarce or their quality is in question. Thus, the data available need to be of good quality. Some information may be obtained from private data resources, where study reports detailing methods, good laboratory practice compliance, quality assurance and any deviations that occurred during the study are available for review. Such information can be shared in accordance with the REACH regulation, where the tonnage applies (EU, 2017a). Nonetheless, drawing on publicly available data still requires an expert's review of its reliability, e.g. the Klimisch score (Klimisch et al., 1997), before its inclusion into a model such as the skin sensitisation threshold calculation. This is consistent with the views of (Cronin et al., 2019) who stated that "*Appreciation of data quality will enable higher confidence in models and understanding of their limitations*".

An additional possibility that could explain the false negative predictions is the that the GCL assigned may have been too high and that the *in vivo* study to which the prediction is being compared, achieved a skin sensitisation response at a lower threshold concentration. This may be the case when comparing the predictions from the GCL to the results of an *in vivo* guinea pig challenge test, conducted at much lower concentration (OECD, 1992). This would agree with the high false negative observed by Corvaro et al (2017) when the comparing calculation methods to the results of the Buehler assay. Dumont et al. (Dumont et al., 2016) showed that when the solvent is not taken into account, the percentage of chemicals with discordant LLNA studies increased to 32% from 22% for a dichotomous hazard classification scheme (POS/NEG). It was demonstrated that when considering this dichotomous scheme, LLNA studies resulting in negative classifications showed a tendency to be less reliable than positive results. When considering the solvent there was a 35% chance for studies that had initially produced a negative result to then produce a positive result. When the solvent was not considered this increased to 50% (Dumont et al., 2016). Understanding the limitations of the reference data is important when using them to assess the performance of new test methods. The reproducibility of the *in vivo* methods must be understood when using them for the purpose of assessment. The

performance of LLNA on a compound using multiple vehicles can lead to different overall results on skin sensitisation potential and potency. Jowsey et al (Jowsey et al., 2008) demonstrated that vehicle differences do impact on the induction of skin sensitisation in the LLNA. Identifying that an underestimation of potency (EC3) can be associated with predominantly aqueous vehicles. Predicting where more substantial effects can be anticipated than is demonstrated by a skin sensitisation test is difficult. However, in this study where multiple *in vivo* skin sensitisation results were available for individual components of a formulation, the most potent value was used to derive the threshold prediction.

An understanding of potential interactions between formulation components is required when conducting hazard assessments of complex mixtures, utilising any method. The calculation method fails to account for toxicodynamic interactions that may occur (intentionally designed or not), and the potential for these to happen only increases with the increased complexity of a formulation. Acute toxicity estimate calculations, as described in the EU CLP and GHS guidance documents, have been discussed by Van Cott et al. (Van Cott et al., 2018). The authors indicate that these methods do not take into account interactions between formulation ingredients that may alter the otherwise expected outcome of the components, via simple additivity. Effects caused by these interactions that are not taken into account by these calculations include changes in solubility of the toxic components, alteration of the absorption characteristics of toxic components due to surface tension and pH. In addition, some interactions are purposefully projected by the manufacturers, to lead to the formation of physical structures which reduce exposure to formulation components. Specifically, in the agrochemical industry the formation of structures such as urea capsule walls to aid in encapsulation of the formulation components, is performed to alter their bioavailability. Consequently, the effects of formulation types such as capsule suspensions (CS) may be over-estimated. This was not observed for CS formulation type in this research; however, only one of the 64 reviewed formulations was a CS formulation and as such further review of this formulation type would need to performed to explore

this possibility. As the calculation does not take these possibilities into account, the results presented here confirm that this does lead to over- or under- estimation of the skin sensitisation potential when compared to the *in vivo* experimental results.

In addition, the current calculation method does not account for any alteration in particle size of components that may occur i.e., formation of granules. Changes in particle size can have an effect on reactivity of the substances because of the surface area availability, which is not taken into account by the calculation. However, from the results of our WG formulation type, although low in sample size, the potential for over or under estimation due to the particle size was not observed in this formulation type.

A further interaction that the skin sensitisation calculation does not take into consideration is the potential for disruption of the dermal layer by materials of the formulation with irritant properties. This may lead to increased exposure (bioavailability) of materials in the formulation with skin sensitising potential. It could be that in such cases the thresholds need to be altered, specifically that a reduction of the corresponding GCL or SCL should be considered. It may be that this calculation is not appropriate for all formulation types, specifically those where interactions between the co-formulants are pre-engineered. In those cases, alternative approaches, whether *in silico, in vitro* or *in vivo* may be more appropriate to derive an accurate hazard assessment. This calls for the development of more investigative non-animal approaches that can be used to assess the skin sensitisation potential of complex mixtures.

2.6 Conclusions

This Chapter has investigated the threshold calculation method as a means of assessing the hazard of pesticide formulation through comparison with existing *in vivo* data. The threshold calculation method was shown to be in better agreement for non-sensitising plant protection products, in comparison to experimental data, when the formulation type was not specified. The threshold method's ability to

accurately identify sensitising plant protection products of unspecified agrochemical formulation types was, however, found to be less reliable. When the threshold calculation was further scrutinised by examining its predictions on specific formulation types, differences in the levels of specificity, sensitivity and accuracy were observed. The calculation predictions for the SC and EC agrochemical formulation types produced identical levels of accuracy, whilst the sensitivity was higher for the SC formulation predictions, indicating that the threshold method had identified sensitisers more efficiently for this formulation type. The lowest threshold calculation accuracy was observed for FS formulation predictions. These findings should be considered with a degree of caution, as a larger number of formulations for each product type may be required to build confidence in the performance criteria results. However, they do indicate that the general premise that the CLP/GHS calculation method is, at worst, conservative (Kurth et al., 2019) was not true for all complex mixture types. In addition, the k-values showed that the level of agreement between the calculation predictions and the *in vivo* experimental results was, at best, moderate. As such it can be concluded that a weight-of-evidence approach using this method, in conjunction with other non-animal methods, needs to be performed to reach a confident assessment of skin sensitisation. As described, there are currently no non-animal methods for the assessment of skin sensitisation that have been validated for the testing of complex mixtures. In Chapters Three and Five of this thesis the capability of various potential *in vitro* assays to evaluate this hazard for agrochemical formulations will be examined in an attempt to address this issue.

3.0 CHAPTER THREE – SKIN SENSITISATION *IN VITRO* TRIPLE PACK AND *IN SILICO* ASSESSMENT

3.1 Introduction

Chapter Two describes the details of the threshold calculation method and how it is used for the skin sensitisation hazard assessment of complex mixtures. However, one of the main conclusions of Chapter Two is that there are instances in which these calculations may be limited by presence of chemicals for which no test data on skin sensitisation potential exists. Examples of these may be compounds in early development or those that have not have previously met the required tonnage necessary to deem this hazard data necessary for REACH (EC, 2017). There is, therefore, a need to develop new test methods and refine available alternative non-animal test methods that can provide a reliable hazard assessment of single compounds and also potentially complex mixtures. Three OECD test guidelines detail accepted *in vitro* methods for the assessment of skin sensitisation potential. These are the OECD 442C, D and E test guidelines which provide methods and criteria to conduct the direct Peptide Reactivity Assay (DPRA) (OECD, 2019a), the KeratinoSens™ assay (OECD, 2018b) and the human cell line activation test (h-CLAT) (OECD, 2018a) respectively. Each of these three assays has undergone evaluation by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (Silvia et al., 2013, Silvia and Maurice, 2015, Silvia et al., 2014) or a ring study involving different laboratories to confirm transferability and reproducibility of their protocols (Ashikaga et al., 2008). As mentioned in Chapter One, when referring to the combination of these three test methods in Defined Approaches (DA) such as the two out of three approach, the term triple pack will be used.

Section 1.2.2 provides a detailed overview of the three non-animal assays that comprise the triple pack. Briefly these are the DPRA, KeratinoSens™ and h-CLAT. The DPRA is an *in chemico* assay used in conjunction with the aforementioned *in vitro* assays, to determine the skin sensitisation potential of a given test substance. The majority of chemical allergens are electrophilic and react with nucleophilic amino acids such as lysine and cysteine (Gerberick et al., 2004). The DPRA uses this interaction to determine a test substance's potential to haptenate proteins and peptides. The assay evaluates a test

material's reactivity towards synthetic cysteine and lysine peptides by recording peptide depletion after exposure to the test substance for a set period. The higher the peptide depletion, as observed using high performance liquid chromatography (HPLC), the more reactive a test substance is considered to be (Hoffmann et al., 2018). Gerberick et al (2004) demonstrated that cysteine was the peptide with higher sensitivity for measuring this skin sensitising peptide reactivity. Although indicative of the skin sensitisation reaction, it has been reported that chemical respiratory allergens do show a selective affinity for interaction with the lysine peptide (Kimber et al., 2018). As such it could be considered that an additional advantage of this *in chemico* method, as it allows for the specific evaluation of the interaction with cysteine.

The KeratinoSens™ assay is an *in vitro* test method that utilises the Keap1-Nrf2-antioxidant response element pathway to quantify the luciferase gene induction of a test substance in an immortalized adherent reporter cell line (Natsch, 2018). An electrophilic test substance with skin sensitisation potential is anticipated to bind to the Keap 1 protein and, in the binding process, dissociate the Nrf2 transcription factor. A build-up of Nrf2 in the cytoplasm leads to the translocation of Nrf2 into the nucleus, where it binds with and activates the antioxidant response element (ARE) triggering the luciferase gene (Natsch, 2010). The luciferase gene induction is monitored in the KeratinoSens™ assay by quantitative luminescence measurement. The predictivity, transferability and reproducibility of the KeratinoSens™ assay has been studied in detail (Natsch et al., 2011) and assessed by ECVAM prior to OECD test guideline acceptance (OECD, 2018b). Nonetheless, this assay does have limitations and there are a number of false negative (e.g. 2-methoxy-4-methylphenol, 3-aminophenol) and false positive results (e.g. tween 80) for materials that have been reported by this assay (Kolle et al., 2019). Among the false negatives are pro-haptens, which are not expected to elicit this outcome, as all of the *in vitro* assays mentioned here lack metabolic capabilities. Cytochrome P450 enzymatic mediated oxidation is required to transform these pro-haptens to reactive species (Emter et al., 2013). Tween 80, which is not electrophilic and is considered to be a relatively inert compound has previously been identified as a false positive in the KeratinoSens™ assay (Kolle et al., 2019). Sodium dodecyl sulphate

has also been identified as generating a false positive result in the KeratinoSens™ as it has also been reported to induce luciferase at low cellular viability (Emter et al., 2013).

The h-CLAT assay operates on the principle of monitoring the third KE of the skin sensitisation AOP (OECD, 2014). Upon activation of dendritic cells, which occurs through haptenisation with an electrophilic/sensitising compound, phenotypic and functional changes take place. These include changes such as the upregulation of the expression of major histocompatibility complex (MHC) class two molecules, co-stimulatory molecules (e.g. CD80, CD86 and CD54) and cytokines on the dendritic cell surface (Galbiati et al., 2020). Following their activation, the matured dendritic cells migrate to the lymph node where specific T-cells are activated. The h-CLAT assay provides a yes or no answer as to whether or not a test substance has dendritic cell activation potential or not. The assay does so by reporting the flow cytometry results of any changes in the expression of dendritic cell surface markers CD86 and CD54. A human monocytic leukaemia cell line is used to achieve this goal (Kim et al., 2018).

The h-CLAT assay is used alongside the KeratinoSens™ and DPRA assays in an integrated approach to testing and assessment (IATA) of skin sensitisation potential of chemicals. Depending on the DA used (12 are reported (Kleinstreuer et al., 2018b)), the incorporation of an *in silico* method such as the OECD QSAR Toolbox (here after referred to as the Toolbox) or DEREK Nexus (as indicated in the Kao sequential testing strategy (OECD, 2017)) alongside these 2D *in vitro* assay methods, provides the potential for potency prediction alongside the initial hazard identification. This chapter will explore the use of some of these DAs.

3.1.1 Aim of This Chapter

An acknowledged limitation of *in vitro* assays is that there is little understanding of how they can be applied to assess the skin sensitisation potential of complex mixtures (Settivari et al., 2015a). The overarching aim of this chapter was to investigate and potentially address the issue of assessing the skin sensitisation potential of complex mixtures through three specific objectives:

- The first objective was to gain further understanding of how the *in vitro* triple pack test methods can be used to evaluate the skin sensitisation potential of complex mixtures, with specific evaluation of Plant Protection Products (PPP).
- The second objective was to determine if any changes are needed to the standardised methods (if any) of the triple pack *in vitro* skin sensitisation assays, for these assays to be considered suitable for the assessment of complex mixtures. Whilst striving to achieve the first objective, this chapter also investigated the use of a combination of *in silico* methods (the CLP threshold method, DEREK Nexus and the Toolbox) alongside the *in vitro* methods. This was to investigate if these approaches could provide accompanying evidence to the results and could be considered as a potential new DA for skin sensitisation hazard assessment of complex mixtures.
- The third objective of this chapter was to investigate the suitability of published DA to skin sensitisation of single compounds (OECD, 2017).

In order to accomplish the overall aim, the *in vitro* triple pack methods were applied to selected agrochemicals' Active Ingredients (AIs). This allowed for the production of case studies to evaluate the chosen DA from analysis of the results for skin sensitisation for the 10 AIs. Attention was paid to the approaches that allow incorporation of *in silico* methods, specifically, the two out three weight of evidence (WoE) approach, the Kao ITS v1 and Kao ITS v2 approaches (OECD, 2017). Ten PPP formulations representing the AIs were also assessed in the *in vitro* triple pack. Once triple pack *in vitro* testing of the ten PPP was completed, potential integration of the CLP threshold method (ECHA, 2017b) was evaluated. All *in vitro* assay results produced were assessed in comparison to the results of previously conducted *in vivo* experiments for the individual AIs and the PPP accordingly.

3.2 Materials and Methods

3.2.1 Selection of Substances for Testing and Analysis

A third-party Clinical Research Organisation (CRO) was contracted to conduct the *in vitro* triple pack skin sensitisation tests on the agrochemical AIs and formulations listed in Tables 3.1 and 3.2 below. The details of the experiments conducted by the CRO, Gentronix Ltd (Macclesfield, UK), are provided in this materials and methods section with further in the appendix 3, 4 and 6.

3.2.2 Active Ingredients

Ten agrochemical active ingredients were chosen for testing in the *in silico* and *in vitro* triple pack 2D cell assays. The critical criterion for selecting these ten active ingredients was to ensure that study data from previously conducted mammalian *in vivo* skin sensitisation tests were available. In addition, any globally harmonised skin sensitisation classifications were also recorded. The AIs tested and their existing data and information are reported in Table 3.1. Following identification and selection of the ten AIs, permission for their use was obtained from Syngenta.

Active Ingredient	**CAS Number**	**Indication (F, H, I) ***	***In Vivo* Study**	***In Vivo* Study Result**	**Skin Sensitisation Harmonised Classification Labelling and Packaging (CLP) Category**
Acetamiprid	135410-20-7	I	GPMT	Non-sensitiser	Not classified (EPA, 2002, FAO, 2005)
Acibenzolar-S-methyl	135158-54-2	F	GPMT	Sensitiser	Skin Sens. 1, H317 (EFSA, 2014a)
Benzovindiflupyr	1072957-71-1	F	LLNA	Non-sensitiser	Not classified (EFSA, 2015)
Chlorantraniliprole	736994-63-1	I	GPMT LLNA	Non-sensitiser	Not classified (EFSA, 2013a)
Chlorothalonil	1897-45-6	F	Buehler	Sensitiser	Skin Sens. 1, H317 (EPA, 2011, FAO, 2015)
Cyantraniliprole	736994-63-1	I	LLNA	Non-sensitiser	Not classified (EFSA, 2014b)
Dicamba	1918-00-9	H	LLNA	Non-sensitiser	Not classified (EFSA, 2011a, Harp, 2010, EPA, 2006, ECHA, 2008)
Mesotrione	104206-82-8	H	GPMT	Non-sensitiser	Not classified (EFSA, 2016)
Pinoxaden	243973-20-8	H	LLNA	Sensitiser	Skin Sens. 1A, H317 (ECHA, 2016a, EFSA, 2013b, FAO, 2016)
**AI1		I	LLNA	Sensitiser	No harmonised classification

Table 3.1 Selected agrochemical AIs for skin sensitisation triple pack evaluation

*F= fungicide, H= herbicide, I= insecticide

** This AI is in the early stage of research and as such has remained confidential

3.2.3 Agrochemical Formulations

The applicability of the three OECD test guideline *in vitro* assays to evaluate the skin sensitisation of complex mixtures (specifically PPPs) was investigated using ten agrochemical formulations: four suspension concentrates [SC], two emulsifiable concentrates [EC], two flowable concentrates for seed treatment [FS] and two water dispersible [WG] formulations. The ten agrochemical formulations chosen for testing in the *in chemico/in vitro* triple pack assays are listed in Table 3.2 below. Of the ten agrochemical formulations selected, six had previously yielded positive results in *in vivo* skin sensitisation tests, whilst four had delivered negative results and were therefore considered non-sensitisers. These formulations were supplied by Syngenta Ltd for the purpose of this research.

Formulation Number	**Formulation Type**	**Active ingredient**	***In Vivo* Study**	***In Vivo* Study Result***	**CLP Skin Sensitisation Calculation Result**
SYN 1	EC	Difenoconazole/ Benzovindiflupyr	LLNA	Sensitiser	Not classified
SYN 2	FS	Acibenzolar-S-methyl	LLNA	Sensitiser	Not classified
SYN 3	SC	Cyantraniliprole/ Diafenthiuron	LLNA	Non-sensitiser	Not classified
SYN 4	FS	Metcamifen	LLNA	Non-sensitiser	Not classified
SYN 5	WG	Mesotrione/Dicamba/ Nicosulfuron	LLNA	Non-sensitiser	Not classified
SYN 6	SC	Chlorothalonil	Buehler test	Sensitiser	Sensitiser
SYN 7	EC	Pinoxaden/ Cloquintocet-mexyl	Buehler test	Sensitiser	Sensitiser
SYN 8	SC	AI1	LLNA	Sensitiser	Sensitiser
SYN 9	SC	Chlorantraniliprole	LLNA	Non-sensitiser	Sensitiser
SYN 10	WG	Acetamiprid/AI1	LLNA	Sensitiser	Sensitiser

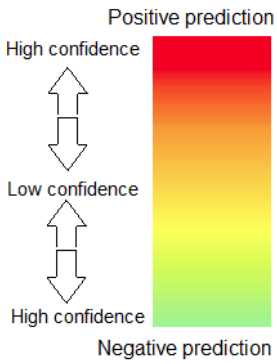
*As indicated in Syngenta Ltd records

Table 3.2 Selected agrochemical formulations for evaluation of the applicability of the OECD in vitro skin sensitisation methods

3.2.4 Prediction of Skin Sensitisation Potential Using *In Silico* Approaches

The ten active ingredients were evaluated for their skin sensitisation potential by identification of any structural alerts for skin sensitisation that were present in their chemical structure. This was achieved by entering their Simplified Molecular Input Line Entry System (SMILES) strings into DEREK Nexus (version 2.3) and the OECD QSAR Toolbox (version 4.4.1) (referred to as the "Toolbox") *in silico* QSAR tools. In addition, a manually derived expert judgement for the electrophilic activity associated with skin sensitisation MIE potential of the individual chemical structures was undertaken. During the expert review identification of any possible steric hinderance that may impede the potential for activity through the protein binding structural alerts identified by the *in silico* tools was conducted.

DEREK Nexus indicated the presence or absence of a structural alert that can be associated with the endpoint in question. Alongside this, a measure of confidence, termed the likelihood measure, is provided by the model. The likelihood measure uses the following terms; certain, probable, plausible, equivocal, doubted, improbable and impossible as defined in Figure 3.1 (Judson et al., 2013). In this evaluation DEREK NEXUS results with likelihood terms of plausible, probable and certain were considered a positive prediction for protein reactivity. The terms doubted, improbable and impossible when associated with alerts were taken to not be indicative of skin sensitisation.



Likelihood measure	Formal definition
Certain	Proven to be true
Probable	There is at least 1 strong argument that the proposition is true and there are no arguments against
Plausible	The weight of evidence supports the proposition
Equivocal	Equal weight of evidence for and against a proposition
Doubted	The weight of evidence opposes the proposition
Improbable	There is evidence to show the proposition to be false and none indicating it to be true
Impossible	Proven to be false

Figure 3.1 DEREK Nexus Likelihood measurements (adapted from Judson et al., 2013)

In the evaluation of protein reactivity in the Toolbox, the standardised workflow reported by Yordanova et al (2019) was used to obtain binary predictions for the ten AIs. The Toolbox is able to

assign a category associated with skin sensitisation potency, however a simple binary result predicting if the compound was or was not a skin sensitiser was adequate for this study. The Toolbox was used to evaluate whether the ten AI structures contained an electrophilic moiety that could lead to covalent binding to the skin proteins via one of the mechanistic domains mentioned earlier (e.g. acylation, Michael addition, nucleophilic substitution as defined fully in Section 1.3) (Aptula and Roberts, 2006, Enoch et al., 2011). The following Toolbox profilers were applied to identify structural features associated with protein binding and / or skin sensitisation potential:

- Protein binding alerts for skin sensitisation by OASIS
- Protein binding alerts for skin sensitisation according to GHS
- Protein binding alerts for skin sensitisation by OASIS following autoxidation
- Protein binding alerts for skin sensitisation according to GHS following autoxidation
- Protein binding alerts for skin sensitisation by OASIS following skin metabolism
- Protein binding alerts for skin sensitisation according to GHS skin metabolism

Many skin sensitisers are activated via metabolism. Where a structural alert was not initially identified from the parent AI compound, the autoxidation profiler in the Toolbox was applied. In the absence of a metabolite with an alert being identified from this profiler, the skin metabolism profiler was then also applied to identify any potential metabolites. All metabolites identified by the metabolism profilers applied were run through the protein binding profilers as listed above using the same Toolbox workflow as previously identified (Yordanova et al., 2019). A compound was considered to be associated with an alert representing skin sensitisation if the OASIS/GHS profiler gave an outcome of 1A or 1B for the parent structure, or if the OASIS with skin metabolism profiler gave a 1A result. A conclusion of non-sensitiser was made if the OASIS/GHS profiler identified no alert, or if the OASIS/GHS with skin metabolism profiler gave a 1B result (Masinja et al., 2021).

3.2.5 *In vitro* methods

In vitro skin sensitisation testing using the triple pack of the agrochemical AIs was undertaken before that of the agrochemical formulations.

3.2.5.1 Direct Peptide Reactivity Assay (DPRA)

The DPRA was conducted at the Gentronix Laboratory (Alderley Park, Macclesfield, UK) on all ten agrochemical AIs listed in Table 3.1 and subsequently on the ten agrochemical formulations listed in Table 3.2. For the AIs, DPRA testing was conducted in accordance with OECD Test Guideline 442C (OECD, 2019a) and the ECVAM DB-ALM protocol 154 (ECVAM, 2012), with an initial preliminary solubility assessment conducted on all ten test materials. Table 3.3 below lists the solvents used for each AI test material and the maximum concentration achieved for each.

Active ingredient	Solvent(s) selected	Maximum AI Concentration (mM)
Acetamiprid	Acetonitrile	100
Acibenzolar-s-methyl	Acetonitrile	80
Benzovindiflupyr	Acetonitrile	100
Chlorantraniliprole	Acetone: acetonitrile 1:1	6.25*
Chlorothalonil	Acetonitrile	50
Cyantraniliprole	Acetone: acetonitrile 1:1	6.25*
Dicamba	Water	100
Mesotrione	Acetonitrile	100
Pinoxaden	Acetonitrile	100
AI1	Acetonitrile	100

Table 3.3 Selected solvents for the ten Active Ingredients and maximum concentration achieved

*Both cyantraniliprole and chlorantraniliprole dropped out of the solution readily. In order to maintain their dissolution both materials were sonicated prior to sampling for peptide HPLC analysis runs.

The DPRA test guideline indicates that the test chemical should be pre-weighed and dissolved in an appropriate solvent to achieve a 100 mM solution before testing in the peptide solution (OECD, 2019a, ECVAM, 2012). However, to achieve this for multi-constituent mixtures without a singular defined Molecular Weight (MW), such as the ten agrochemical formulations being tested, a calculation of average MW for each formulation was required. A single average MW for each formulation was calculated by considering the MWs of each component (including active/non-active ingredients) and

their individual proportions. This resulted in MWs for testing ranging from 177 to 2260 Da (1M solution equating to the calculated average MW in grams per litre) as shown in Table 3.4. This approach was previously described by Settivari et al (2015) in the KeratinoSens™ assay (Settivari et al., 2015a). Polymers present in the formulation without a clearly defined MW were excluded from the calculation.

Formulation number	Average MW excluding water	% of polymers with unknown MW
SYN 1	232.71	0.02
SYN 2	177.25	4.2
SYN 3	578.92	0
SYN 4	483.61	1.3
SYN 5	254.40	0
SYN 6	232.98	1.3
SYN 7	332.39	0
SYN 8	1008.41	4.2
SYN 9	319.26	4.5
SYN 10	2259.37	0

Table 3.4. The calculated average MWs and percentage of polymers with unknown MW for the ten agrochemical test formulations tested.

Following solubility assessment, five of the ten formulations demonstrated acceptable solubility in acetonitrile:DMSO (1:1). The remaining five were insoluble in all Test Guideline recommended solvents and, as a result, further assessment using these remaining five formulations did not proceed. Table 3.5 shows the five formulations solubilised in acetonitrile:DMSO and the concentrations at which they were added to the peptide test system.

Formulation number	Concentration added to peptide (mM)	Solubility observations
SYN 2	100	clear yellow solution at the start and end of incubation
SYN 4	25	opaque white precipitate clearing by the end of incubation
SYN 6	100	clear solution forming a murky solution by the end of incubation
SYN 8	50	moderate opaque at start to a clear solution with particles on base of vial
SYN 9	50	slightly cloudy at start to a clear solution by the end of incubation

Table 3.5 DPRA formulation test concentrations

During the preliminary solubility assessment cysteine and lysine peptides were incubated with the test materials and observations of precipitation were made and recorded in Table 3.6. The test AI material concentration that the peptides were incubated with was the maximum test concentration recommended by the OECD Test Guideline 442C (5mM for incubation with cysteine and 25 mM for incubation with the lysine peptide).

Active ingredient	**Observation in cysteine buffer**	**Observation in lysine buffer**	**Observation in cysteine buffer after 22-26 hours incubation**	**Observation in lysine buffer after 22-26 hours incubation**
Acetamiprid	no precipitation observed	no precipitation observed	no precipitation observed	no precipitation observed
Acibenzolar-s-methyl	precipitation observed	precipitation observed	precipitation observed	precipitation observed
Benzovindiflupyr	precipitation observed	precipitation observed	precipitation observed	precipitation observed
Chlorantraniliprole	precipitation observed	precipitation observed	precipitation observed	precipitation observed
Chlorothalonil	precipitation observed	precipitation observed	precipitation observed	precipitation observed
Cyantraniliprole	precipitation observed	precipitation observed	no precipitation observed	no precipitation observed
Dicamba	no precipitation observed	no precipitation observed	no precipitation observed	no precipitation observed
Mesotrione	no precipitation observed	no precipitation observed	no precipitation observed	no precipitation observed
Pinoxaden	no precipitation observed	no precipitation observed	no precipitation observed	no precipitation observed
AI1	no precipitation observed	precipitation observed	no precipitation observed	no precipitation observed

Table 3.6 AI solubility assessment following addition to peptide buffers

The ten AIs were administered in the selected solvents at the highest test concentrations listed in Table 3.3 (±10% as per OECD test guideline 442C). Once formulated, a sample from each AI formulation was transferred from Gentronix Ltd to Alderley Analytical Ltd. under appropriate storage conditions for the HPLC analysis phase. All relevant reagents and solutions were prepared freshly for each independent HPLC run, where appropriate. The DRPA positive and solvent experimental controls used were as indicated in Appendix 3.

Cysteine and lysine peptide depletion prediction models

The mean percentage cysteine and lysine peptide depletion models that were used to determine a DPRA reactivity class based on the peptide depletion results are outlined in the ECVAM DB-ALM protocol 154 and OECD Test Guideline for this assay (OECD, 2019a). Tables 3.7 and 3.8 outline the cysteine 1:10/lysine 1:50 prediction model and the cysteine 1:10 prediction model respectively. The cysteine 1:10 prediction model was only used if cysteine depletion alone result was produced from the DPRA. This may have occurred if AI co-elution with the lysine peptide was observed.

Mean of cysteine and lysine % depletion	Reactivity class	DPRA prediction
0% ≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

Table 3.7 Cysteine 1:10/lysine 1:50 prediction model (OECD, 2019)

Mean of cysteine (cys) % depletion	Reactivity class	DPRA prediction
0% ≤ cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < cys % depletion ≤ 100%	High reactivity	

Table 3.8 Cysteine 1:10 prediction model (OECD, 2019)

3.2.5.2 KeratinoSens™ assay

The ten AIs and ten agrochemical formulations listed in Tables 3.1 and 3.2 respectively were tested in the KeratinoSens™ assay. A pre-test solubility assessment was conducted and, based upon outcome of that testing, the DMSO solvent was selected for use with the AI test materials and formulations. The highest concentration of each test material that could be formulated in DMSO is listed in Table 3.9 for the AIs and Table 3.10 for the agrochemical formulations. Upon administration of the test material in DMSO to the KeratinoSens™ exposure medium, any colour changes or formation of precipitate was recorded. The concentrations of the test materials used for this pre-test solubility assessment on a microplate are also given in Tables 8.8 and 8.9 in Appendix 4.

KeratinoSens™ assay acceptance criteria

All the requirements listed below must have been met for a KeratinoSens™ experimental run to be considered valid, in accordance with EURL ECVAM DB-ALM number protocol 155 (ECVAM, 2014a) and the OECD Test Guideline 442D(OECD, 2018b). Acceptance criteria for the KeratinoSens™ assay are as follows:

- The positive control, cinnamic aldehyde, must induce a statistically significant response above the threshold of 1.5 for at least one dose.
- The Imax and EC1.5 for cinnamic aldehyde must meet at least one of the following criteria:
    - The average induction in three replicates for cinnamic aldehyde at 64 μM should be between 2 and 8.
    - The EC1.5 value of the positive control should be within two standard deviations of the Gentronix laboratory historical mean.
    - At least one of these criteria must be met, otherwise the run will be discarded. If only one of the criteria was fulfilled, then the dose response for cinnamic aldehyde was visually inspected to decide on acceptability.
- For acceptance of the test, the average variability for the 3 x 6 solvent control wells (6 wells across 3 luminescence measurement microplates per experimental run) should be below 20%. If variability is higher, then the run should be discarded.

Prediction model

To derive a prediction, at least two valid (i.e., all acceptance criteria met) repetitions of the full experiment were needed if concordant results were obtained. If the first two calls were not-concordant, then at least three valid repetitions of the full experiment were required. Based on the individual run results a positive KeratinoSens™ conclusion using the prediction model criteria outlined below, was made:

- The Imax is > 1.5-fold and statistically significantly different compared to the negative control (as determined by a two-tailed, unpaired Student's t-test).
- At the lowest concentration with a gene induction > 1.5-fold (i.e., at the EC1.5 determining value), the cell viability was > 70%.
- The EC1.5 value was < 1000 µM (or < 200 µg/mL for test item without MW).
- There was an apparent overall dose-response for luciferase induction (or a reproducible biphasic response).

Where the test item was not soluble (or did not form a stable dispersion) at 1000 µM, the following applied:

- If the test item induced luciferase at a lower, non-cytotoxic concentration, where it was still soluble, this was accepted as a positive result.
- If the test item did induce cytotoxicity (viability <70%) at the maximal soluble concentration but did not induce luciferase up to the maximal soluble concentration, this was accepted as a negative result.
- If a test item did not cause cytotoxicity or luciferase induction at the maximal soluble concentration, which was <1000 µM, this was be considered as an inconclusive result.

3.2.5.3 h-CLAT

A pre-test solubility assessment was initially performed on the ten AIs and ten formulations. This identified nine of the AIs to be soluble in DMSO solvent, whilst dicamba was solubilised in saline. The initial concentrations of AI test material solubilisation in vehicle are shown in Table 8.22 and Table 8.23 (Appendix 7) for the formulations.

Pre-test solubility assessment identified saline as the appropriate vehicle for eight of test formulations, whilst cell media was identified for SYN5 and DMSO for the SYN8 formulation. For the SYN8 formulation there was very poor solubility observed in DMSO, saline and media. Full solubility

was not achieved at 5 mg/ml in any solvent, however DMSO was the better of the solvents and therefore selected for this formulation.

Following the initial pre-test solubilisation in the solvents listed above, the AI test formulations were dissolved in cell culture medium to initially identify if any precipitation would arise that may render a negative result inconclusive. Concentrations for the cell culture medium were chosen as a result of this.

A master stock of the THP-1 human monocytic leukaemia cell line was created and stored in liquid nitrogen at the Gentronix laboratory (Alderley Park, UK). The initial growing THP-1 cell culture was obtained from Public Health England culture collections (Porton Down, UK) whilst the h-CLAT test material exposure and cell reading were performed in the Gentronix laboratory.

Prior to the initial cytotoxicity assessment on the test material's concentrations, a pre-test analysis of the THP-1 cells was conducted. Initially a comparison of the doubling time of the THP-1 cells was conducted to confirm that they fall within the historic data control doubling number range for that laboratory. After this a positive and negative reactivity check, using 2,4-dinitrochlorobenzene (DNCB) or nickel sulphate as a positive control and lactic acid as the negative control, was conducted. This reactivity check was conducted to confirm that the results fell within defined acceptance criteria as outlined in the OECD Test Guideline 442E (OECD, 2018a).

A 75% cell viability (CV75) dose finding assay was conducted. The dose finding assay was performed using the highest soluble concentrations of the different test material formulations and seven subsequent doses, using a 2-fold dilution series. This dose range concentration for each AI enabled the determination of the concentration leading to a CV75 compared to the cell viability present, following exposure to medium/solvent control. The individual CV75 concentrations for each of the test materials were then used to determine a range of concentrations of the test materials to be used in the h-CLAT for measurement of CD86/CD54 expression. If determined using CV75, the highest test material concentration was 1.2 x CV75 concentration, however for test materials where no

cytotoxicity was observed, the highest soluble concentration previously determined at the initiation of this h-CLAT assay was used. Agrochemical formulation and AI test material formulation as well as the specifics of the test item administration of the CV75 dose finding assay for this h-CLAT experiment are provided in Appendix 6.

h-CLAT Assay acceptance criteria

All the requirements listed below had to have been met for a h-CLAT run to have been accepted. If these requirements were not met, then the run was discarded:

- Cell viability of medium and solvent controls should exceed 90%
- In the positive control (DNCB), RFI values of both CD86 and CD54 should exceed the positive criteria (CD86 ≥ 150%, CD54 ≥ 200%) and cell viability should be more than 50%
- In the solvent control, RFI values compared to the medium control of both CD86 and CD54 should not have exceeded the positive criteria
- For both medium and solvent controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%

If the majority of runs led to a negative outcome, the general outcome of the h-CLAT assay was considered negative if the following criteria applied:

- Maximum tested concentration (CV75 x 1.2) leads to a viability < 90%
- Or, when 5000 µg/mL in saline or 1000 µg/mL in DMSO or the highest soluble concentration is used as the maximal test concentration instead of CV75-based dose, even if the cell viability is above 90%

Otherwise, the outcome will be considered inconclusive.

Prediction model

Based on the results of individual runs a h-CLAT conclusion using the prediction model illustrated in Figure 3.2 and applying the criteria outlined below, was made:

- If the first two runs were both positive for CD86 and/or were both positive for CD54, the h-CLAT prediction for the give test material was considered positive and a third run was not conducted.
- If the first two runs were negative for both markers, the h-CLAT prediction was considered negative (with consideration given of the highest-tested dose conditions) without the need for a third run.
- If, however, the first two runs were not concordant for at least one of the markers (CD54 or CD86), a third run was needed and the final prediction was based on the majority result of the three individual runs (i.e. 2 out of 3). In this respect, it should be noted that if two independent runs were conducted and only one was positive for CD86 and the other was only positive for CD54, a third run was required. If this third run was negative for both markers the h-CLAT prediction is considered negative. However, if the third run is positive for either marker or for both markers, the h-CLAT prediction is considered positive.

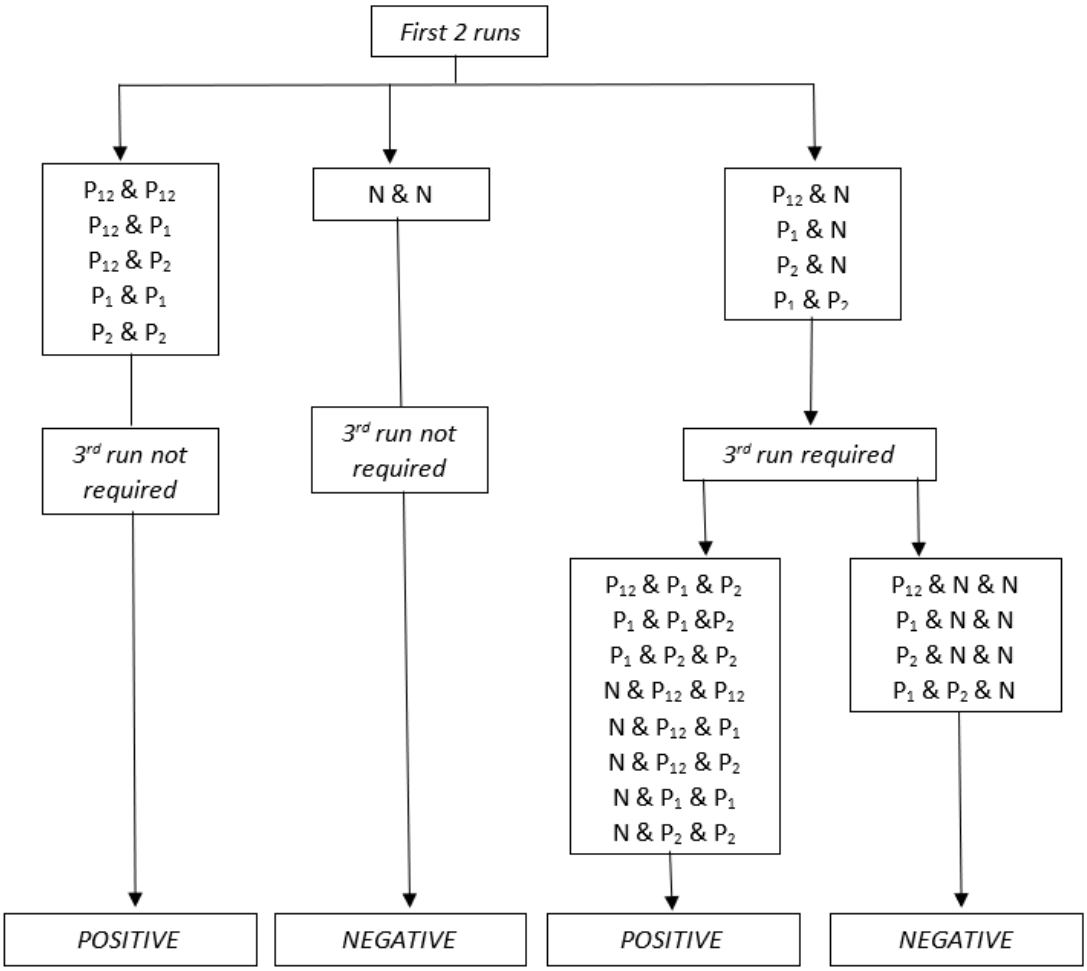


Figure 3.2 h-CLAT Prediction model (OECD, 2018a)

P_1: run with only CD86 positive; P_2: run with only CD54 positive; P_{12}: run with both CD86 and CD54 positive; N: run with neither CD86 nor CD54 positive. The figure shows the relevant combinations of results from the two/three successive runs, but does not reflect the order in which they may all be obtained.

3.2.6 Defined approach (DA) evaluation

The previously mentioned skin sensitisation testing techniques in this chapter were integrated to evaluate different DAs to testing of skin sensitisation. The *in silico* prediction was used in combination with the *in vitro* experimental data. This was done to provide further understanding for the potential of each of the chemicals to trigger the initial MIE and the ensuing key events in the AOP (OECD, 2014). Using the results from the triple pack *in vitro* tests, the three following DAs were investigated.

3.2.6.1 Kao Integrated Testing Strategy

The Kao Integrated Testing Strategy (ITS) versions one and two (Takenouchi et al., 2015), Sequential Testing Strategy and the two out of three DAs (OECD, 2017) were evaluated using the results of the *in silico* and *in vitro* methods.

In the ITS based on the quantitative outcomes from *in vitro* assays (h-CLAT and the DPRA), a score of 0 to 3 is awarded to the test chemical, while in the *in silico* method used (DEREK Nexus for version 1 or the Toolbox for version 2 of this ITS) is awarded a score of 0 in the absence of an alert or one if an alert is reported as present (Kleinstreuer et al., 2018b). The summed score of the ITS combination of methods is used to predict the skin sensitisation potential and potency as shown in Table 3.9.

DPRA assay outcome	**Assigned ITS score**
High reactivity	3
Moderate reactivity	2
Low reactivity	1
No or minimal reactivity	0
h-CLAT minimum induction threshold (MIT)	
≤10 µg/mL	3
>10, ≤150 µg/mL	2
>150, ≤5000 µg/mL	1
not calculated	0
DEREK Nexus (ITSv1)	
Alert	1
No Alert	0
OECD Toolbox (ITSv2)	
Sensitiser	1
Non-sensitiser	0
Potency Total battery score	Strong (GHS category 1A): 7 Weak (GHS category 1B): 2 - 6 Not classified: 0 -1

Table 3.9 Schematic of the Kao ITS (version 1 and 2) (Kleinstreuer et al., 2018b)

Rather than strictly following the ITS scoring system, the evaluation of the agrochemical AI test set incorporated a manual assessment by expert judgement on the results of *in silico* tools before using them in the scheme indicated in Table 3.9.

3.2.6.2 Kao Sequential Testing Strategy

For evaluation of the Kao sequential testing strategy (STS) as shown in Figure 3.3, the approach developed by Kao (Nukada et al., 2013, Takenouchi et al., 2015) was followed without adaptation or alteration. Unlike the ITS the Kao STS does not integrate *in silico* models into its assessment of the skin sensitisation potential of single compounds. The STS uses the results of the DPRA and h-CLAT assays in a decision tree that allows for both hazard and potency evaluation of the given test material.

The Kao STS assesses the results of the h-CLAT study in a tiered approach. Where a positive result has been produced in the h-CLAT assay (i.e., CD54 and/or CD86 dendritic cell surface markers have been induced by 200% or 150% respectively) a positive skin sensitisation result is accepted. The minimum induction threshold (MIT) value (i.e., the lowest test material concentration to lead to the dendritic cell marker positive response) is then used to identify a sensitisation potency classification. An MIT value of equal to or greater than 10 µg/ml leads to the test material being classified as a strong sensitiser while a MIT value of greater than 10µg/ml warrants a weak skin sensitiser classification for the test material. In the event that the h-CLAT results are negative, the DPRA assay results are reviewed. Individual peptide depletion values are not required for this evaluation. A simple positive or negative result from the DPRA assay allows for the classification of the test material to a weak skin sensitiser or for the material to not be classified as a skin sensitiser (Nukada et al., 2013).

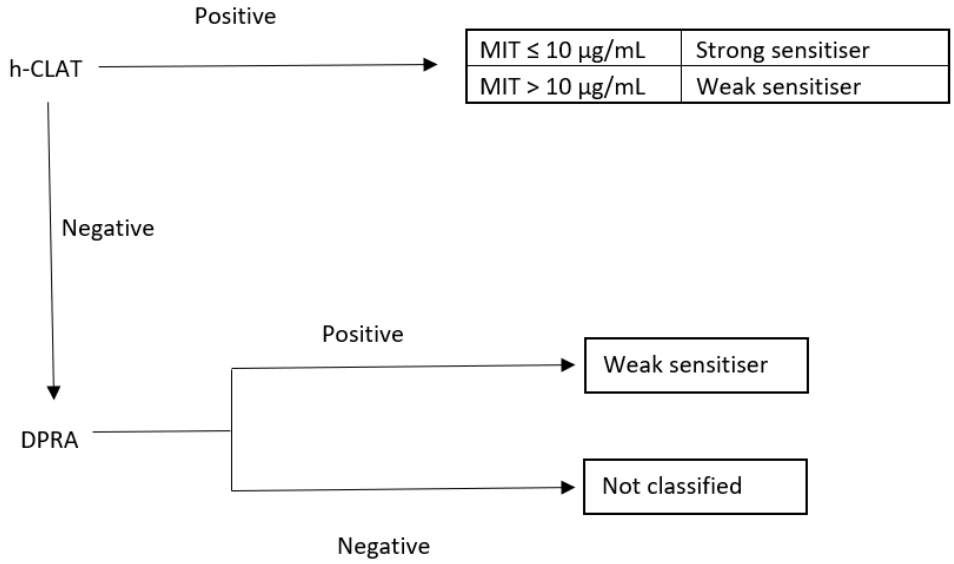


Figure 3.3 Schematic of the Kao STS defined approach (Nukada et al., 2013)

3.2.6.3 Two out of three Defined Approach

The two out of three DA uses a weight of evidence method to determine if a test material is a skin sensitiser (Urbisch et al., 2015). This chapter compared the two out of three approach to the other two previously described DA methods. This comparison was done by using the results of the DPRA, KeratinoSens™ and the h-CLAT *in vitro* and *in chemico* skin sensitisation assays on the ten agrochemical AI case studies.

3.3 Results

3.3.1 *In silico* results

The ten active ingredients were entered into the DEREK Nexus model and the Toolbox using the compounds' specific SMILES strings. Using the procedure as outlined in the methods and materials section, the skin sensitisation potential of the AIs was predicted/assessed by recording:

- The DEREK alerts to measure the likelihood of the compound in leading to skin sensitisation and
- The identification of possible mechanistic domains that may lead to covalent binding to skin proteins from the Toolbox.

The predictions from the two models for all ten compounds are reported in Table 3.10. The DEREK Nexus likelihood measure relates to the possibility of a compound being a skin sensitiser.

Chemical name	Log P*	DEREK alerts - likelihood measure	OECD toolbox mechanistic domains
acetamiprid	0.79	No alerts – impossible	Parent – none Skin metabolite – Schiff base formation
acibenzolar-s-methyl	2.17	Thioester - plausible	Parent – none Skin metabolite – nucleophilic substitution (S_N2)
benzovindiflupyr	4.3	No alerts – impossible	Parent – none Skin metabolite 1 – Schiff base formation Skin metabolite 2 & 4 – acylation
chlorantraniliprole	2.76	No alerts – impossible	Parent - acylation
chlorothalonil	2.94	Activated benzene - plausible	Parent – Nucleophilic aromatic substitution (S_NAr)
cyantraniliprole	1.94	No alerts – impossible	Parent – none Skin metabolite 1 – Schiff base formation Skin metabolites 3,6, 7 & 8 – acylation
dicamba	2.24	No alerts – impossible	Parent – none Skin metabolite – Schiff base formation
mesotrione	0.73	1,3-Diketone - plausible Nitrobenzene - plausible	Parent –S_NAr & Schiff base formation
pinoxaden	3.2	Hydrazine or precursor - equivocal	Parent - acylation
AI1	3.3	No alerts – impossible	Parent - acylation

Table 3.10 Predictions of log P, skin sensitisation and possible mechanisms of action from Derek Nexus and Toolbox chemical for the ten AIs

*CAESAR (version 1.1.4) predicted AlogP values which were used to calculate log P in accordance with the previous work by Guziałowska-Tic, 2017.

3.3.2 *In vitro* results

3.3.2.1 DPRA Results for the AIs

The ten agrochemical AIs were initially evaluated for cysteine/lysine peptide reactivity in the DPRA *in chemico* assay. The mean percentage peptide depletion values for lysine and cysteine following AI incubation are reported in Table 3.11 below.

Active ingredient	Mean % peptide depletion		Standard deviation		Mean Cysteine and Lysine % peptide depletion
	Cysteine	Lysine	Cysteine	Lysine	
acetamiprid	14.6	8.51	3.37	3.38	11.6
acibenzolar-s-methyl	Could not be determined				
benzovindiflupyr	13.8	14.2	2.33	1.94	14.0
chlorantraniliprole	2.48	4.45	1.99	2.84	3.47
chlorothalonil	97.9	3.18	0.10	2.77	50.5
cyantraniliprole	1.38	2.38	0.783	1.36	1.88
dicamba	12.7	9.64	1.91	1.29	11.2
mesotrione	95.1	12.3	0.289	0.757	53.7
pinoxaden	100	9.42	0.00	3.11	54.7
AI1	95.7	0.379	42.2	1.97	48.0

Table 3.11 Percentage peptide depletion (from the DPRA assay) for the ten AIs

The DPRA prediction model (OECD, 2018b) was used to determine the reactivity class of the active ingredients based upon the mean percentage cysteine and lysine peptide depletion values. As a percentage of peptide depletion was observed for both cysteine and lysine in all AI test materials, the cysteine 1:10/lysine 1:50 prediction model was used. Due to co-elution effects and an inability to meet acceptance criteria for the DPRA test peptide depletion for acibenzolar-s-methyl could not be determined. The peptide reactivity class was determined from the DPRA experiment for nine of ten AIs and reported in Table 3.12.

Active ingredient	Mean Cysteine and Lysine % peptide depletion	Prediction model used	DPRA prediction
acetamiprid	11.6	Cys 1:10/Lys 1:50	Positive – Low Reactivity
acibenzolar-s-methyl	Could not be determined		
benzovindiflupyr	14.0	Cys 1:10/Lys 1:50	Positive – Low Reactivity*
chlorantraniliprole	3.47	Cys 1:10/Lys 1:50	Negative - No or Minimal Reactivity*
chlorothalonil	50.5	Cys 1:10/Lys 1:50	Positive – High Reactivity*
cyantraniliprole	1.88	Cys 1:10/Lys 1:50	Negative - No or Minimal Reactivity*
dicamba	11.2	Cys 1:10/Lys 1:50	Positive – Low Reactivity
mesotrione	53.7	Cys 1:10/Lys 1:50	Positive – High Reactivity
pinoxaden	54.7	Cys 1:10/Lys 1:50	Positive – High Reactivity
AI1	48.0	Cys 1:10/Lys 1:50	Positive – Moderate Reactivity*

Table 3.12 Reactivity class of Ten Active Ingredients as Sensitisers or Non-Sensitisers in the DPRA

*Exceptions to the prediction model(s) occurred with these test items – specifically the presence of precipitate upon test material addition to the peptide buffer.

3.3.2.2 DPRA Results for the test formulations

For the agrochemical test formulations, the reference control C in the acetonitrile:DMSO vehicle mixture showed a large depletion of cysteine and according to the guideline would give non-conclusive results with respect to the formulations. For the agrochemical test formulation reference control C in DMSO:Acetonitirle (1:1), the Coefficient of variation (CV) of peptide peak areas for the nine reference controls C in acetonitrile should be <0.15. The agrochemical formulations reference C control achieved a CV of 0.242. The mean peptide concentration of the reference control C was 0.073 mM. This was also below the acceptance criteria (0.45 – 0.55 mM). Therefore, the test guideline outlined criteria as outlined by the OECD Test Guideline 442C for the DPRA was not met for the five tested agrochemical formulations.

The peptide depletion results of the agrochemical test formulations following DPRA testing are reported in Table 3.13 below.

Formulation number	Solubility observations	% Cysteine Depletion*	DPRA prediction
SYN 2	clear yellow solution at the start and end of incubation	97%	Positive
SYN 4	opaque white precipitate clearing by the end of incubation	Interference	Inconclusive
SYN 6	clear solution forming a murky solution by the end of incubation	11.8%	Positive
SYN 8	murky at start to a clear solution with particles on base of vial	90.1%	Positive
SYN 9	little cloudy at start to a clear solution by the end of incubation	Interference	Inconclusive

Table 3.13 Percentage peptide depletion (from the DPRA assay) for five agrochemical formulations

**The 1:1 acetonitrile:DMSO solvent used as vehicle demonstrated extensive (85.4%) cysteine depletion in reference control C. Further cysteine depletion caused by formulation, relative to cysteine control has been reported here*

Following a preliminary assessment of formulation solubility, five formulations remained insoluble in all OECD recommended solvents, however, five of the formulations achieved solubility (100mM–25mM) in acetonitrile:DMSO (1:1). Although 1:1 acetonitrile:DMSO is a suggested solvent for DPRA, it caused extensive (85.4%) cysteine depletion leading to a failed run (OECD, 2019a). Despite this, three of the formulations that were classified as sensitisers *in vivo* exhibited cysteine depletion indicative of a sensitiser in this *in vitro* assay as defined by the appropriate prediction model (Table 9). Assay interference was observed for the two non-sensitising formulations. However, as no formulation co-eluted at or near the retention time of the cysteine peptide, this interference appeared to occur through the two formulations' stabilising the cysteine peptide. As such, a negative peptide reactivity result could not be concluded for formulations SYN4 and SYN9. Confirming a negative result for the non-sensitising formulations was problematic due to solubility/cytotoxicity issues impacting the running of the assays to recommended guidelines and therefore formulations SYN4 and SYN9 were interpreted as inconclusive.

3.3.2.3 KeratinoSens™ results for the AIs

The ten agrochemical AIs and ten PPP formulations were tested in the KeratinoSens™ assay.

The raw data from the KeratinoSens™ tests on the AIs mentioned here is presented in appendix 5. The results for the luciferase determinations for all ten AIs are shown in Table 3.14, the luciferase determinations, expressed as I_{max} values, indicated the maximum induction over the tested concentration range of for each AI. Table 8.17 shows the AI $EC_{1.5}$ values for all ten AIs. Cell viability at all of the reported $EC_{1.5}$ concentrations was ≥70%. The IC_{50} values determined in this assessment are also shown in Table 8.18.

Table 8.16 also presented in appendix 5 shows that the average induction (I_{max}) in the replicate plates for the positive control at 64 µM was calculated to be between 2 and 8. The mean $EC_{1.5}$ value was also within two standard deviations of the Gentronix laboratory's historical mean, as shown in Table 8.20. These criteria were met in all runs except replicate five, where the induction of cinnamic aldehyde at 64 µM was above the required range at 9.82. As the former criterion was not fulfilled in that run, the dose-response of cinnamic aldehyde was carefully checked, and the run was accepted as there was a clear dose-response with increasing luciferase activity at increasing concentrations for the positive control. All concentrations of cinnamic aldehyde tested produced cell viability results of > 70%.
As per the OECD test guideline for the KeratinoSens™ evaluation the vehicle control variability of the 18 vehicle control wells within one experimental run must be below 20%. This criterion has been met as shown in table 8.21 to demonstrate acceptable variation that would not impact final results of the assay. Solvent variation from 12.11% to 15.19% was observed keeping within the KeratinoSens™ criteria (OECD, 2018).

As all of the required acceptance criteria for this experiment have been fulfilled, the predictions derived from the luciferase inductions observed alongside the cytotoxicity evaluation at the 12 concentrations of each AI were accepted. These assessments are listed in Table 3.14 and illustrated graphically in Figures 3.5-3.14.

Active ingredient	KeratinoSens™ assessment
acetamiprid	negative
acibenzolar-s-methyl	inconclusive
benzovindiflupyr	positive
chlorantraniliprole	inconclusive
chlorothalonil	positive
cyantraniliprole	negative
dicamba	negative
mesotrione	positive
pinoxaden	positive
AI1	positive

Table 3.14 Summary of the KeratinoSens™ assay predictions in the ten agrochemical AIs

Fig 3.4. Dose response curve for acetamiprid

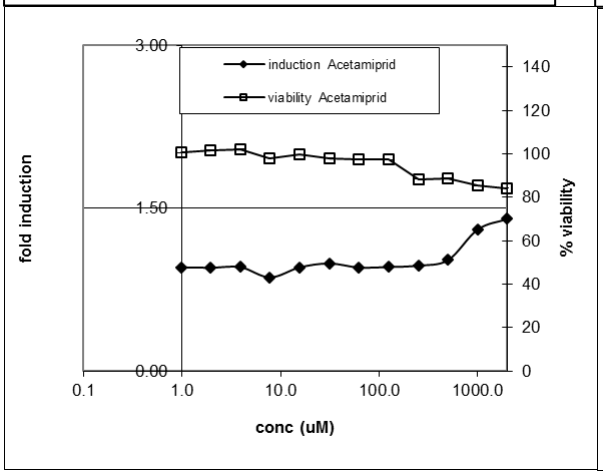


Fig 3.5. Dose response curve for cyantraniliprole

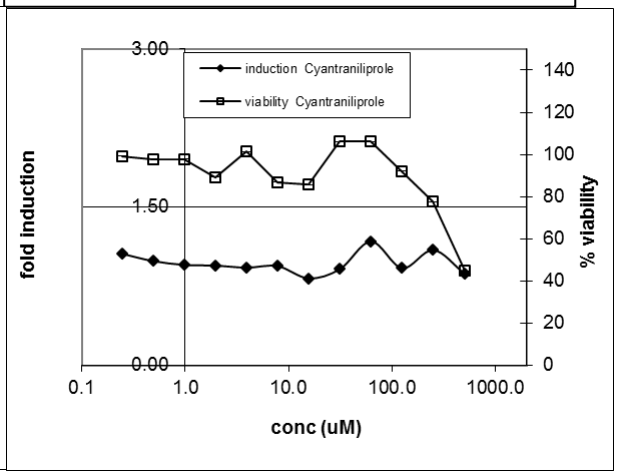


Fig 3.6. Dose response curve for dicamba

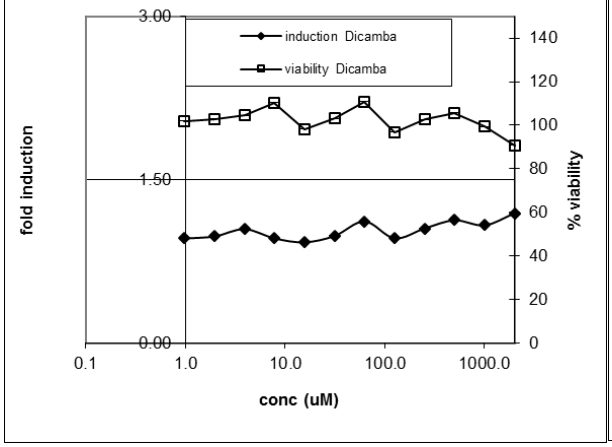


Fig 3.7. Dose response curve for benzovindiflupyr

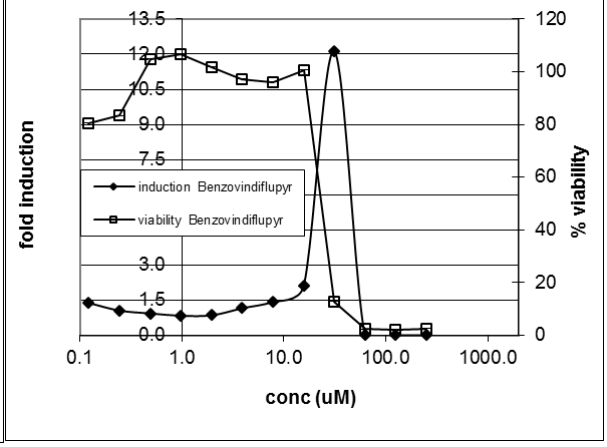


Fig 3.8. Dose response curve for chlorothalonil

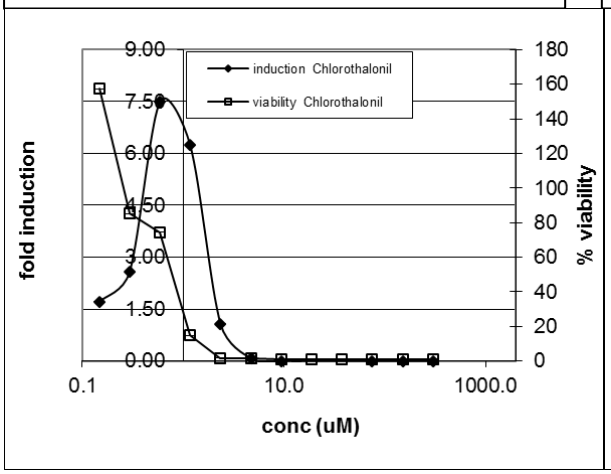


Fig 3.9. Dose response curve for mesotrione

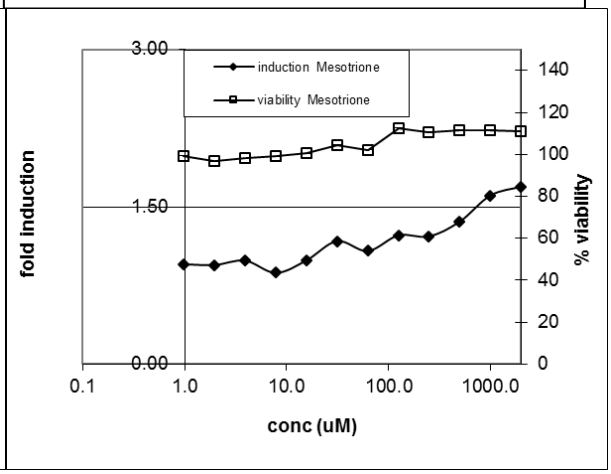


Fig 3.10. Dose response curve for pinoxaden

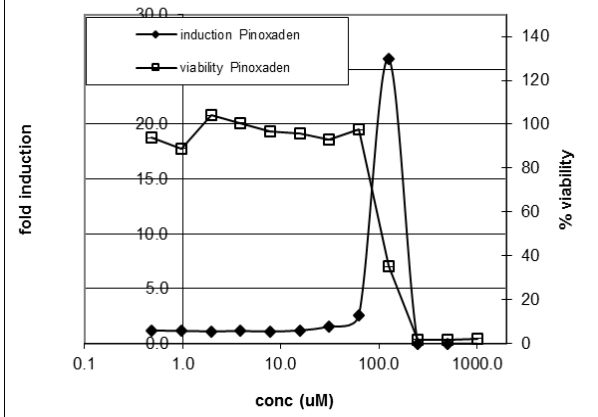


Fig 3.11. Dose response curve for AI1

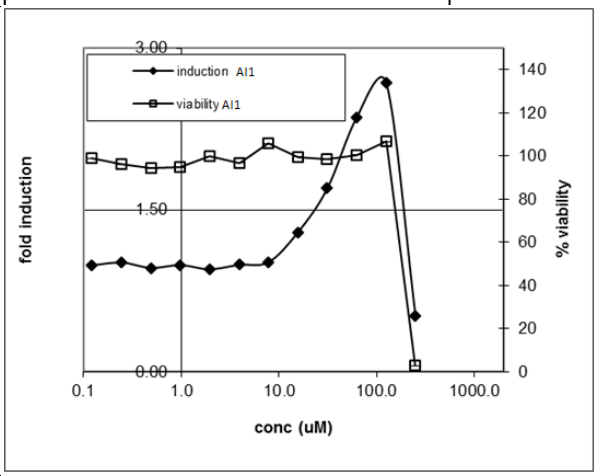


Fig 3.12. Dose response curve for acibenzolar-s-methyl

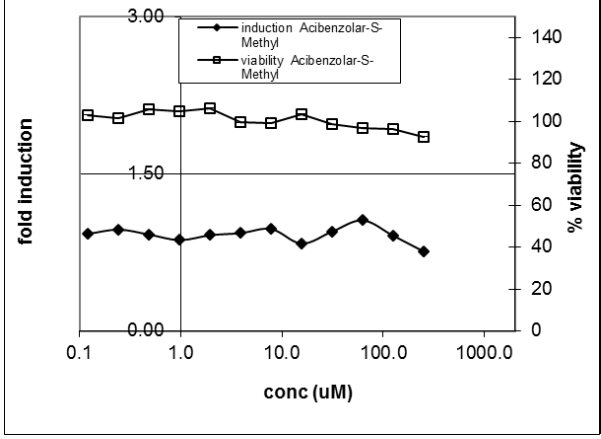
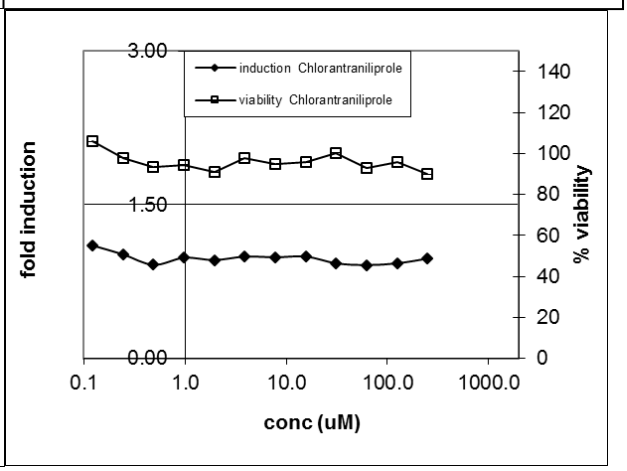


Fig 3.13. Dose response curve for chlorantraniliprole



Figures 3.4 – 3.13 Active ingredient KeratinoSens™ assay dose response curves

Luminescence measurement following treatment with dicamba did not produce luminescence induction of over 1.5-fold in any of the 12 concentrations. The MTT cytotoxicity test indicated that cell viability of >70% was observed in all 12 concentrations as shown in Figure 3.6.

The results of the KeratinoSens™ assay also gave positive predictions for five of the ten AIs tested. These five AIs are indicated in Table 3.14, being namely benzovindiflupyr, chlorothalonil, mesotrione, pinoxaden and AI1.

3.3.2.4 KeratinoSens™ results for the test formulations

Solvent selection and maximal soluble concentration for each of the ten agrochemical formulations were determined in the initial step of the KeratinoSens™ assay. Subsequently, cytotoxicity was evaluated using the MTT assay and formulation doses adjusted to achieve >70% cell viability across as much of the dose response curve as possible. As such, starting doses of 1000mM to 2mM and 1.25 to 1.5-fold dilution steps, were applied depending on solubility and cytotoxicity of the formulation. The results of the KeratinoSens™ assay on the agrochemical formulations are shown in Table 3.15.

Agrochemical formulation number	Concentration range tested (µM)			EC1.5	IC50	Imax	Test outcome
	min	max	fold dilution				
SYN 1	26.8	312.5	1.25	91.7	196.60	3.96	Positive
SYN 2	28.8	335.5	1.25	-	319.91	1.00	Negative
SYN 3	85.9	1000	1.25	297.7	>1000	2.56	Positive
SYN 4	0.5	44	1.50	-	>44	1.26	Inconclusive
SYN 5	0.3	26.6	1.50	-	>26.6	1.20	Inconclusive
SYN 6	0.2	2.2	1.25	0.38	1.95	7.90	Positive
SYN 7	1.9	21.6	1.25	-	>21.64	1.10	Inconclusive
SYN 8	1.5	132.2	1.50	-	>132.2	1.30	Inconclusive
SYN 9	68.7	800	1.25	-	>800	1.04	Inconclusive
SYN 10	9.2	800	1.50	11.5	>228	2.96	Positive

Table 3.15 Results of the KeratinoSens™ assay for the ten agrochemical formulations tested

EC1.5: *Concentration leading to a 1.5 -fold induction of the luciferase activity.*
IC50: *Concentration effecting a reduction of cellular viability by 50%.*
Imax*: the maximum induction factor of luciferase activity compared to the solvent (negative) control measured at any test chemical concentration*

Figure 3.14. Dose response curve for SYN1

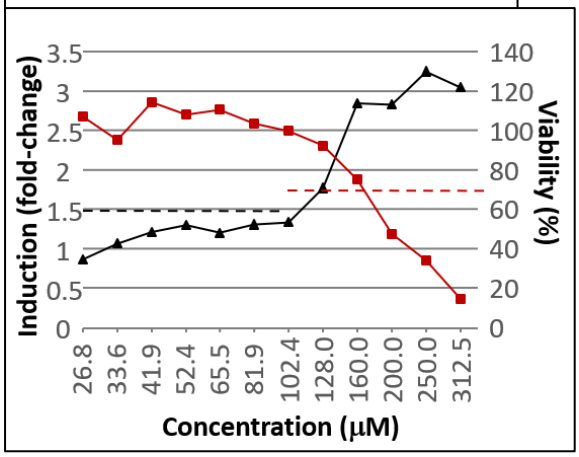


Figure 3.15. Dose response curve for SYN2

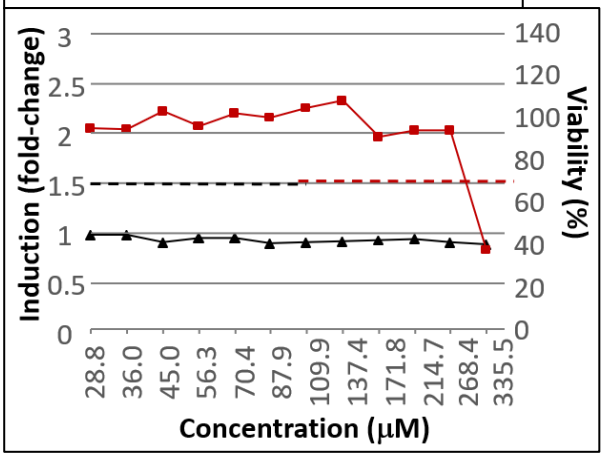


Figure 3.16. Dose response curve for SYN3

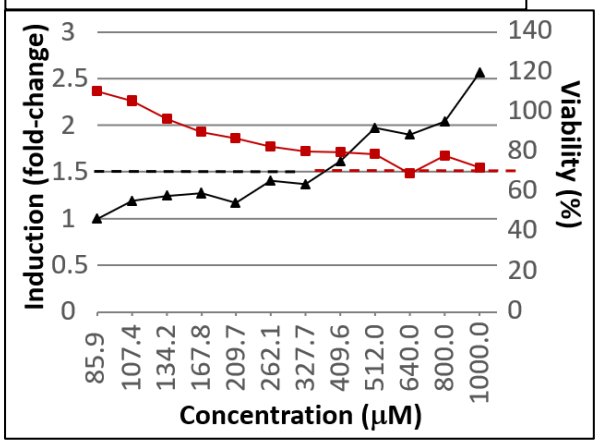


Figure 3.17. Dose response curve for SYN4

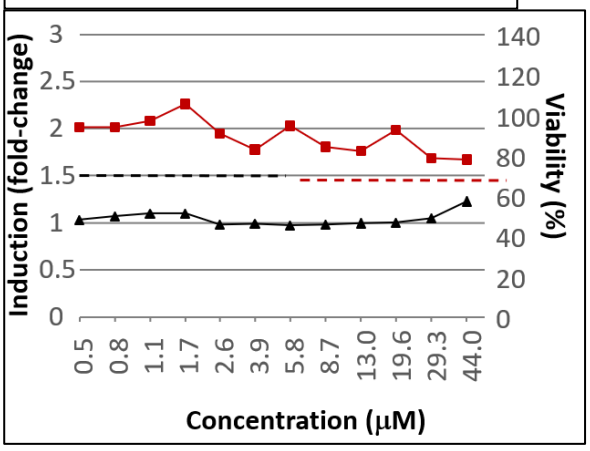


Figure 3.18. Dose response curve for SYN5

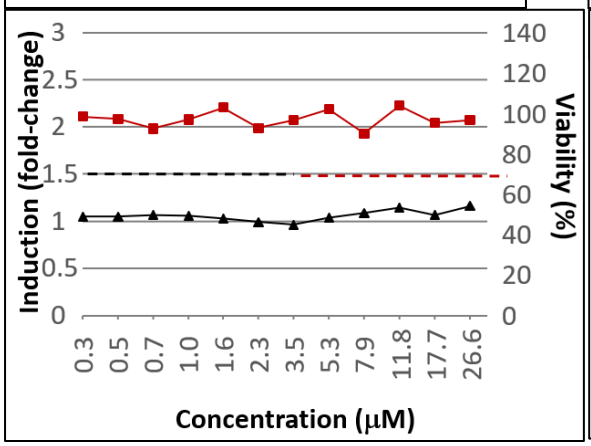
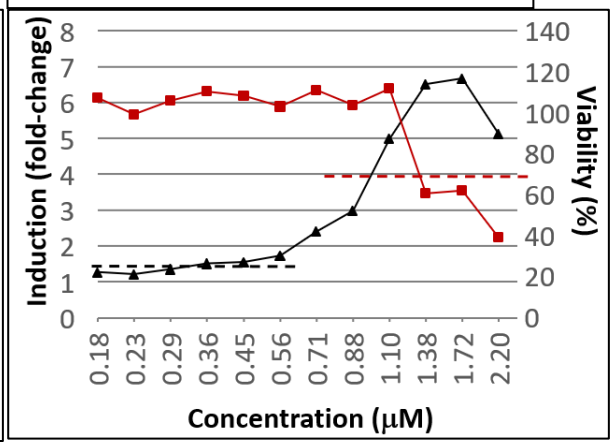


Figure 3.19. Dose response curve for SYN6



Luciferase induction (▲) and cell viability (■)

Figure 3.20. Dose response curve for SYN7

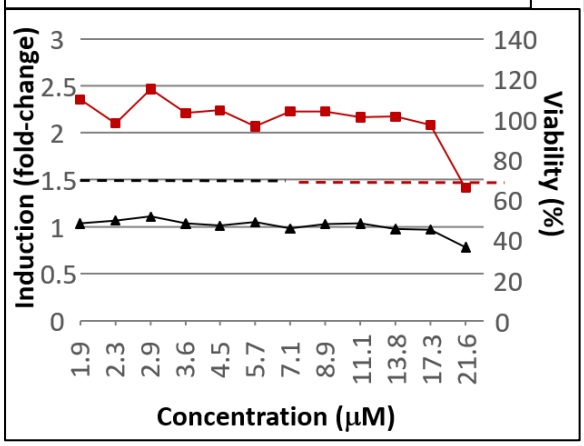


Figure 3.21. Dose response curve for SYN8

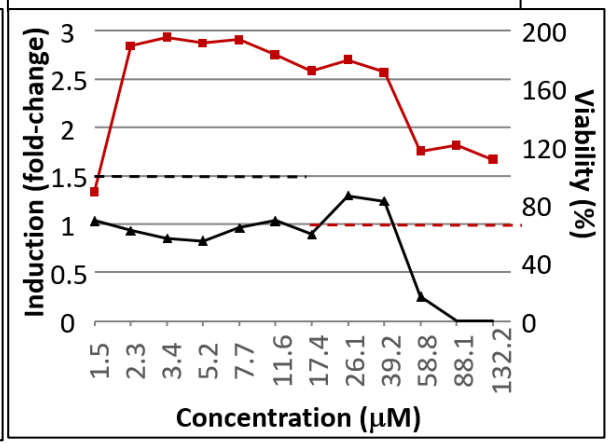


Figure 3.22. Dose response curve for SYN9

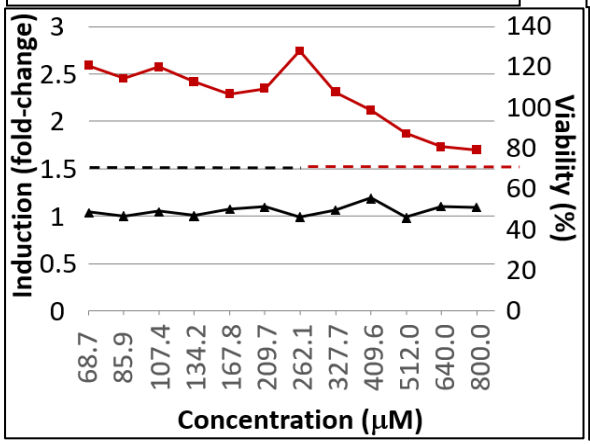
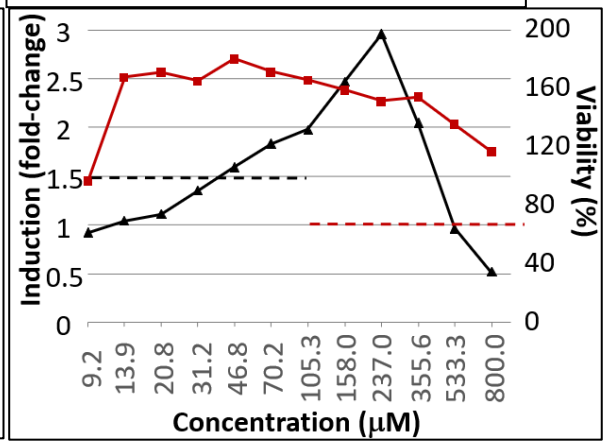


Figure 3.23. Dose response curve for SYN10



Luciferase induction (▲) and cell viability (■)

Figure 3.14 – 3.23 Agrochemical test formulations KeratinoSens™ assay dose response curves

The mean I_{max} determined for SYN1 was 3.96, while the mean EC1.5 concentration was 91.7 µM. Cell viability of >70% was observed at the lowest dose with luminescence induction >1.5 when compared to the solvent control and at least two concentrations tested gave rise to viability >70%. A clear dose response for luminescence was also observed, as shown in Figure 3.14.

Luminescence measurement following treatment with SYN2 did not produce luminescence induction of over 1.5-fold in any of the concentrations tested. The MTT cytotoxicity test indicated that cell viability of >70% was observed in all concentrations with the exception of the maximum concentration tested, as shown in Figure 16. Similar results were also seen following treatment with SYN7 as shown in Figure 3.15.

Following cell treatment with the SYN3, the luminescence measurement allowed for the determination of a mean I_{max} of 2.56. The mean EC1.5 concentration was 297.70 µM. Cell viability of >70% was observed at the lowest dose with luminescence induction >1.5 when compared to the solvent control and at least two concentrations tested gave rise to viability >70%. The calculated mean for the $EC_{1.5}$ was <1000 µM and a clear dose response for luminescence was also observed as shown in Figure 3.16.

Luminescence measurements following treatment with SYN4, SYN5, SYN8 and SYN9 did not produce luminescence induction of over 1.5-fold in any of the concentrations tested. Cell viability of >70% was observed in all concentrations tested as shown in Figures 3.18, 3.19, 3.22 and 3.23 respectively.

The mean I_{max} determined for SYN6 was 7.9, while the mean EC1.5 concentration was 0.38 µM. Cell viability of >70% was observed at the lowest dose with luminescence induction >1.5 when compared to the solvent control and at least two concentrations tested gave rise to viability >70%. A clear dose response for luminescence was also observed as shown in Figure 3.19.

The mean I_{max} determined for SYN10 was 2.96, while the mean EC1.5 concentration was 11.5 µM. Cell viability of >70% was observed at the lowest dose with luminescence induction >1.5 when compared to the solvent control. A clear dose response for luminescence was also seen and is shown in Figure 3.23.

3.3.2.5 Human Cell Line Activation Test (h-CLAT) results for the AIs

The h-CLAT assay was performed on all ten AIs and agrochemical formulations. To begin with the ten AIs were initially tested, and data reviewed. After the initial work on the AIs the h-CLAT evaluation proceeded to testing the agrochemical formulations. In the positive h-CLAT control (DNCB), RFI values of both CD86 and CD54 exceeded the positive criteria (CD86 ≥ 150%, CD54 ≥ 200%) and cell viability was more than 50%, thus this was deemed acceptable.

In the solvent control, RFI values compared to the medium control of both CD86 and CD54 did not exceed the positive criteria (CD86 ≥ 150%, CD54 ≥ 200%) and were also considered to be acceptable.

For both medium and solvent controls, the MFI ratio of both CD86 and CD54 to isotype control was shown to be > 105% in each experimental run and concluded to be acceptable.

The results of the 75% cell viability (CV75) dose range finding assay which was conducted for the ten AIs are detailed in Table 3.16.

Active ingredient	**Highest test concentration on the plate *(µg/mL)***	**Rep 1 CV75**	**Cell viability at highest dose tested (%)**	**Rep 2 CV75**	**Cell viability at highest dose tested (%)**	**Mean CV75**
acetamiprid	800.0	no cyto	84.22	no cyto	96.20	N/A
acibenzolar-s-methyl	30.0	no cyto	95.75	no cyto	98.32	N/A
benzovindiflupyr	15.6	no cyto	83.74	no cyto	83.82	N/A
chlorantraniliprole	62.5	no cyto	95.82	no cyto	96.67	N/A
chlorothalonil	20.0	8.03	14.01	0.909	16.04	4.47
cyantraniliprole	200.0	no cyto	96.76	no cyto	97.61	N/A
dicamba	5000	no cyto	86.72	no cyto	87.62	N/A
mesotrione	600.0	no cyto	94.49	no cyto	97.39	N/A
pinoxaden	150.0	no cyto	94.54	no cyto	97.69	N/A
AI1	75.0	no cyto	96.76	no cyto	94.20	N/A

Table 3.16 CV75 Dose-Finding Assay Results for the Ten AIs

No Cyto = No reduction in cell viability to 75% was observed at the test concentrations used in this study.

N/A = A mean CV75 could not be calculated as no cytotoxicity was observed for these test items during the CV75 dose-finding assay.

The CD86 and CD54 dendritic cell surface marker expression as measured in all of the AI h-CLAT runs are reported in Tables 3.17 and 3.18 respectively.

Active ingredient	**Highest test concentration on the plate *(µg/mL)***	**Rep 1**	**Rep 2**	**Rep 3**	**EC150[b]**
acetamiprid	800.0[a]	no EC	no EC	Not required	N/A
acibenzolar-s-methyl	30.0	no EC	no EC	Not required	N/A
benzovindiflupyr	15.6	no EC	no EC	Not required	N/A
chlorantraniliprole	62.5	no EC	no EC	Not required	N/A
chlorothalonil	5.3	no EC	no EC	Not required	N/A
cyantraniliprole	200.0	no EC	no EC	Not required	N/A
dicamba	5000	no EC	no EC	no EC	N/A
mesotrione	600.0	no EC	no EC	Not required	N/A
pinoxaden	150.0	no EC	no EC	Not required	N/A
AI1	75.0	no EC	24.72	no EC	N/A

Table 3.17 The EC150 (CD86) values for the ten AIs

[a] = The highest concentration determined for chlorothalonil as cytotoxicity was observed in CV75 dose-finding assay. Top test concentrations for the 9 remaining test items were determined by solubility results as no cytotoxicity was observed during the CV75 dose-finding assay.

[b] = EC values were defined as the median of the values calculated in all three independent experiments (or in instances where only two of the three runs were positive, the highest of the two concentrations used is considered the EC value)

Active ingredient	Highest test concentration on the plate *(µg/mL)*	Rep 1	Rep 2	Rep 3	EC200*
acetamiprid	800.0	686.10	670.20	789.50	686.10
acibenzolar-s-methyl	30.0	no EC	no EC	Not required	N/A
benzovindiflupyr	15.6	no EC	no EC	Not required	N/A
chlorantraniliprole	62.5	no EC	no EC	Not required	N/A
chlorothalonil	5.3	no EC	no EC	Not required	N/A
cyantraniliprole	200.0	no EC	no EC	Not required	N/A
dicamba	5000	no EC	4167	1424	4167
mesotrione	600.0	no EC	no EC	Not required	N/A
pinoxaden	150.0	no EC	no EC	Not required	N/A
AI1	75.0	36.17	28.22	no EC	36.17

Table 3.18 The EC200 (CD54) values for the ten AIs

**EC200 values were defined as the median of the values calculated in all three independent experiment runs (or in instances where only two of the three runs were positive, the highest of the two concentrations used is considered the EC value).*

The final h-CLAT predictions for the individual AIs as an outcome of the test results indicated above are listed in Table 3.19.

Active ingredient	h-CLAT prediction
acetamiprid	positive
acibenzolar-s-methyl	negative
benzovindiflupyr	negative
chlorantraniliprole	negative
chlorothalonil	negative
cyantraniliprole	negative
dicamba	positive
mesotrione	negative
pinoxaden	negative
AI1	positive

Table 3.19 Summary of predictions in the h-CLAT for the ten AIs

Using the h-CLAT prediction model outlined in figure 3.2, three of the ten AIs (acetamiprid, dicamba and AI1) were classified as positive and seven of the ten AIs (acibenzolar-s-methyl, chlorothalonil,

cyantraniliprole, mesotrione, pinoxaden, benzovindiflupyr and chlorantraniliprole) were classified as negative under the test conditions used in the h-CLAT experimental runs.

3.3.2.6 Human Cell Line Activation Test (h-CLAT) results for the test formulations

The results of the 75% cell viability dose range finding assay initially conducted for the ten agrochemical formulations are detailed in Table 3.20.

Agrochemical formulation number	Highest test concentration on the plate *(µg/mL)*	Rep 1 CV75	Cell viability at highest dose tested (%)	Rep 2 CV75	Cell viability at highest dose tested (%)	Mean CV75
SYN 1	78.13*	79.12	75.21	59.58	67.32	69.35
SYN 2	78.13	no cyto	95.89	no cyto	92.41	N/A
SYN 3	250.00	no cyto	89.49	no cyto	95.47	N/A
SYN 4	875.00	no cyto	95.89	no cyto	92.03	N/A
SYN 5	39.06	no cyto	96.80	no cyto	97.20	N/A
SYN 6	3.91	1.49	56.30	3.31	72.85	2.40
SYN 7	78.13*	73.10	69.94	76.01	72.14	74.56
SYN 8	10.00	no cyto	96.90	no cyto	93.90	N/A
SYN 9	500.00	no cyto	95.01	no cyto	92.21	N/A
SYN 10	156.30	112.9	60.50	132.3	69.20	122.60

Table 3.20 CV75 Dose-Finding Assay Results for the Ten agrochemical formulations

**Due to high cytotoxicity observed in the initial run of the CV75 test, following of the CV75 tests were tested from a lower top test concentration (78.13 µg/ml).*
No Cyto = No reduction in cell viability to 75% was observed at the test concentrations used in this study.
N/A = A mean CV75 could not be calculated as no cytotoxicity was observed for these test items during the CV75 dose-finding assay.

The CD86 and CD54 dendritic cell surface marker expression as measured in all of the agrochemical formulation h-CLAT runs are reported in Tables 3.21 and 3.22 respectively.

Agrochemical formulation number	Highest test concentration on the plate *(µg/mL)*	Rep 1	Rep 2	Rep 3	EC150[a]
SYN 1	83.22	69.28	no EC	no EC	N/A
SYN 2	78.13	no EC	no EC	Not required	N/A
SYN 3	250.00	no EC	no EC	no EC	N/A
SYN 4	875.00	no EC	no EC	no EC	N/A
SYN 5	39.06	no EC	no EC	Not required	N/A
SYN 6	2.87	1.16	0.69	0.53	0.69
SYN 7	89.47	no EC	86.50	no EC	N/A
SYN 8	10.00	no EC	no EC	Not required	N/A
SYN 9	500.00	no EC	no EC	No EC	N/A
SYN 10	147.10	no EC	no EC	No EC	N/A

Table 3.21 The EC150 (CD86) values for the ten agrochemical formulations

[a] = EC values were defined as the median of the values calculated in all three independent experiments (or in instances where only two of the three runs were positive, the highest of the two concentrations used is considered the EC value)

Agrochemical formulation number	Highest test concentration on the plate *(µg/mL)*	Rep 1	Rep 2	Rep 3	EC200*
SYN 1	83.22	no EC	no EC	no EC	N/A
SYN 2	78.13	no EC	no EC	Not required	N/A
SYN 3	250.00	61.43	84.73	58.76	61.43
SYN 4	875.00	313.50	57.87	461.50	313.50
SYN 5	39.06	no EC	no EC	Not required	N/A
SYN 6	2.87	0.82	0.70	0.75	0.70
SYN 7	89.47	no EC	no EC	no EC	N/A
SYN 8	10.00	no EC	no EC	Not required	N/A
SYN 9	500.00	298.60	254.10	485.80	298.60
SYN 10	147.10	117.90	45.67	No EC	117.90

Table 3.22 The EC200 (CD54) values for the ten agrochemical formulations

**EC200 values were defined as the median of the values calculated in all three independent experiment runs (or in instances where only two of the three runs were positive, the highest of the two concentrations used is considered the EC value).*

The final h-CLAT predictions for the individual agrochemical formulations as an outcome of the test results indicated above are listed in Table 3.23.

Agrochemical formulation number	h-CLAT prediction
SYN 1	negative
SYN 2	negative
SYN 3	positive
SYN 4	positive
SYN 5	negative
SYN 6	positive
SYN 7	negative
SYN 8	negative
SYN 9	positive
SYN 10	positive

Table 3.23 Summary of predictions in the h-CLAT for the ten agrochemical formulations

3.3.2.7 Summary of *in vitro* results and final assessments

Tables 3.24 and 3.25 summarise the results of all *in vitro* and *in silico* predictions carried out on the AIs and agrochemical formulations, with the results from the previously conducted *in vivo* skin sensitisation tests.

Active ingredient	*In vitro* tests			*In silico* prediction		*In vivo test conclusions*
	DPRA	KeratinoSens™	h-CLAT	DEREK	Toolbox	
acetamiprid	positive	negative	positive	negative	positive (skin metabolite – 1a)	non-sensitiser
acibenzolar-s-methyl	inconclusive	inconclusive	negative	positive	negative (skin metabolite – 1b)	sensitiser
benzovindiflupyr	positive	positive	negative	negative	positive (skin metabolite – 1a)	non-sensitiser
chlorantraniliprole	inconclusive	inconclusive	negative	negative	negative	non-sensitiser
chlorothalonil	positive	positive	negative	positive	positive (parent – 1a)	sensitiser
cyantraniliprole	inconclusive	negative	negative	negative	positive (skin metabolite – 1a)	non-sensitiser
dicamba	positive	negative	positive	negative	positive (skin metabolite – 1a)	non-sensitiser
mesotrione	positive	positive	negative	positive	positive (parent – 1b)	non-sensitiser
pinoxaden	positive	positive	negative	equivocal	negative	sensitiser
AI1	positive	positive	positive	negative	positive (skin metabolite – 1a)	sensitiser

Table 3.24 Summary of all skin sensitisation tests performed on the ten AIs
Red – disagreement with *in vivo* conclusion, Green – agreement with *in vivo* conclusion

Agrochemical formulations	*In vitro* tests			CLP Skin Sensitisation calculation result	*In vivo* test conclusions
	DPRA	KeratinoSens™	h-CLAT		
SYN 1	not conducted	positive	negative	non-sensitiser	sensitiser
SYN 2	positive	inconclusive	negative	non-sensitiser	sensitiser
SYN 3	not conducted	positive	positive	non-sensitiser	non-sensitiser
SYN 4	inconclusive	inconclusive	positive	non-sensitiser	non-sensitiser
SYN 5	not conducted	inconclusive	negative	non-sensitiser	non-sensitiser
SYN 6	positive	positive	positive	sensitiser	sensitiser
SYN 7	not conducted	inconclusive	negative	sensitiser	sensitiser
SYN 8	positive	inconclusive	negative	sensitiser	sensitiser
SYN 9	inconclusive	inconclusive	positive	sensitiser	non-sensitiser
SYN 10	not conducted	positive	positive	sensitiser	sensitiser

Table 3.25 Summary of all skin sensitisation tests performed on the ten agrochemical formulations
Red – disagreement with *in vivo* conclusion, Green – agreement with *in vivo* conclusion

3.3.2.8 Defined approach predictions

Review of Kao STS results

The predicted outcomes for all AI test compounds from the Kao STS were determined and are shown in comparison to their *in vivo* outcomes in Table 3.26.

Active ingredient	h-CLAT result	DPRA result	KAO STS prediction	*In vivo* conclusion
acetamiprid	positive (MIT = 800 µg/mL)	positive	weak sensitiser	non-sensitiser
acibenzolar-s-methyl	negative	inconclusive	inconclusive	sensitiser
benzovindiflupyr	negative	positive	weak sensitiser	non-sensitiser
chlorantraniliprole	negative	inconclusive	inconclusive	non-sensitiser
chlorothalonil	negative	positive	weak sensitiser	sensitiser
cyantraniliprole	negative	inconclusive	inconclusive	non-sensitiser
dicamba	Positive (MIT = 1674 µg/mL)	positive	weak sensitiser	non-sensitiser
mesotrione	negative	positive	weak sensitiser	non-sensitiser
pinoxaden	negative	positive	weak sensitiser	sensitiser
AI1	positive (MIT = 30.14 µg/mL)	positive	weak sensitiser	sensitiser

Table 3.26 Skin sensitisation classifications of the ten AIs according to the Kao STS
Red – disagreement with *in vivo* conclusion, Green – agreement with *in vivo* conclusion

Results from the Kao Integrated Testing System (version 1 & 2)

The results for the ten AIs from the Kao ITS (1 and 2) are shown in Table 3.27.

Active ingredient	h-CLAT result	DPRA result	KAO ITS 1 prediction	KAO ITS 2 prediction
acetamiprid	positive (mit = 800 µg/ml)	positive – low reactivity	weak sensitiser	weak sensitiser
acibenzolar-s-methyl	negative	inconclusive	not classified	not classified
benzovindiflupyr	negative	positive – high reactivity	weak sensitiser	weak sensitiser
chlorantraniliprole	negative	inconclusive	not classified	not classified
chlorothalonil	negative	positive – low reactivity	weak sensitiser	weak sensitiser
cyantraniliprole	negative	inconclusive	not classified	not classified
dicamba	positive (mit = 1674 µg/ml)	positive – low reactivity	weak sensitiser	weak sensitiser
mesotrione	negative	positive – high reactivity	weak sensitiser	weak sensitiser
pinoxaden	negative	positive – high reactivity	weak sensitiser	weak sensitiser
AI1	positive (mit = 30.14 µg/ml)	positive – moderate reactivity	weak sensitiser	weak sensitiser

Table 3.27 Skin sensitisation classifications of the ten AIs according to the KAO ITS (1&2)

Two out of three defined approach results

The predictions of skin sensitisation from the two out of three DA for the AI materials tested are given in Table 3.28.

Active ingredient	*In vitro* tests			2/3 skin sensitisation prediction
	DPRA	KeratinoSens™	h-CLAT	
acetamiprid	positive	negative	positive	sensitiser
acibenzolar-s-methyl	inconclusive	inconclusive	negative	inconclusive
benzovindiflupyr	positive	positive	negative	sensitiser
chlorantraniliprole	inconclusive	inconclusive	negative	inconclusive
chlorothalonil	positive	positive	negative	sensitiser
cyantraniliprole	inconclusive	negative	negative	non-sensitiser
dicamba	positive	negative	positive	sensitiser
mesotrione	positive	positive	negative	sensitiser
pinoxaden	positive	positive	negative	sensitiser
AI1	positive	positive	positive	sensitiser

Table 3.28 Skin sensitisation predictions for the ten AIs according to the 2/3 WoE defined approach

The skin sensitisation predictions derived from the two out of three DA (incorporating the CLP skin sensitisation calculation) for the ten agrochemical formulations are given in Table 3.29.

Agrochemical formulations	*In vitro* tests			CLP Skin Sensitisation calculation result	2/3 skin sensitisation prediction
	DPRA	KeratinoSens™	h-CLAT		
[A]**SYN 1**	not conducted	positive	negative	**non-sensitiser***	non-sensitiser
[A]**SYN 2**	positive	negative	negative	non-sensitiser	non-sensitiser
[B]**SYN 3**	not conducted	positive	positive	non-sensitiser	sensitiser
[B]**SYN 4**	inconclusive	inconclusive	positive	non-sensitiser	inconclusive
[B]**SYN 5**	not conducted	inconclusive	negative	**non-sensitiser***	non-sensitiser
[A]**SYN 6**	positive	positive	positive	sensitiser	sensitiser
[A]**SYN 7**	not conducted	inconclusive	negative	sensitiser	inconclusive
[A]**SYN 8**	positive	inconclusive	negative	**sensitiser***	sensitiser
[B]**SYN 9**	inconclusive	inconclusive	positive	**sensitiser***	sensitiser
[A]**SYN 10**	not conducted	positive	positive	sensitiser	sensitiser

Table 3.29 Skin sensitisation predictions for the ten formulations according to the 2/3 WoE defined approach

**CLP Calculation result substituted into 2/3 approach in place of inconclusive in vitro result*

[A] skin sensitiser result via in vivo experiment

[B] non-skin sensitiser results via in vivo experiment

The results in Table 3.29 indicate that six of the tested formulations were identified as skin sensitisers and four as non-sensitisers *in vivo* experiments. Three of the sensitisers identified experimentally *in vivo* were predicted to be skin sensitisers in the 2/3 approach above. Two were predicted as non-sensitisers, whilst a conclusive prediction for the SYN7 formulation could not be made as the only two definitive results available were divergent. Only one of the four formulations identified as non-sensitising in vivo (SYN 5) was predicted as a non-sensitiser via the 2/3 approach. In contrast to their *in vivo* results, both SYN3 and SYN9 were predicted to be skin sensitisers, with positive KeratinoSens™ and h-CLAT test results, whilst an inconclusive prediction was derived for the SYN4 formulation.

3.4 Discussion

This chapter examined the use of the DPRA, KeratinoSens™ and h-CLAT 2D *in vitro* triple pack test methods and their combination with additional *in silico* tools in defined approaches for the skin sensitisation hazard assessment of agrochemical active ingredients. After this initial evaluation of the methods focusing on the AIs, the research moved on to gain an understanding of the triple pack 2D *in vitro* methods' suitability for the skin sensitisation hazard assessment of complex mixtures.

3.4.1 DPRA Active Ingredient Assessment

The ten agrochemical AIs identified in Table 3.1 were all tested in the skin sensitisation *in vitro* triple pack, beginning with their assessment in the DPRA test. In the DPRA of the ten AIs, the peptide reactivity could not be determined for one substance, acibenzolar-s-methyl. This was due to co-elution of acibenzolar-s-methyl with lysine from the HPLC column. In addition to that, the acibenzolar-s-methyl experimental runs failed to meet acceptance criteria. Therefore, it was not possible to obtain valid peptide depletion values for this test item in the DPRA.

Seven of the of the ten AIs produced experimental peptide depletion values that were considered positive in accordance with the DPRA prediction model. The seven AIs were pinoxaden, dicamba, mesotrione, acetamiprid, AI1, chlorothalonil and benzovindiflupyr. The positive results indicate that these AIs have the potential to cause skin sensitisation as they may trigger the first key event in the skin sensitisation AOP.

Chlorantraniliprole and cyantraniliprole produced mean cysteine and lysine percentage peptide depletion results of 3.47% and 1.88% respectively. In accordance with the DPRA prediction model these results indicate no, or minimal, reactivity and are thus classed as a negative result. However, observations of both chlorantraniliprole and cyantraniliprole following their incubation with the peptide buffers showed precipitation. It is possible that the results of the DPRA for these two compounds may be an underestimation of the peptide depletion. Therefore, a conclusion on the lack of reactivity could not be drawn with sufficient confidence in the case of the negative DPRA result for

these two AIs. In addition to this, chlorantraniliprole and cyantraniliprole were tested at concentrations lower than the recommended maximum concentration (OECD, 2019a, ECVAM, 2012) of 100mM. This is also considered an exception to the prediction model where a negative result is obtained (i.e., a sensitising threshold may not have been achieved) which could further indicate a lack of confidence in the negative result obtained for these two compounds. As such, the results for chlorantraniliprole and cyantraniliprole in this DPRA study are considered inconclusive.

Chlorothalonil was also tested at concentrations below the recommended maximum concentration, however the mean peptide depletion for chlorothalonil was 50.5%, giving a positive result that indicated that the material is highly reactive. Consequently, there is no underprediction to be considered for this material. In the DPRA evaluation of benzovindiflupyr, precipitation was observed immediately after application of the material to the cysteine and lysine buffers and also observed again following the 22-26-hour incubation period. This indicates that although a positive result was obtained in the DPRA study for benzovindiflupyr (14% mean peptide depletion), the corresponding reactivity may have been underestimated due to the precipitate formation.

3.4.2 KeratinoSens™ Active Ingredient assessment

The KeratinoSens™ Assay was undertaken to gain an insight into the potential of the AIs to cause keratinocyte activation in the second key event of the skin sensitisation AOP. A positive result was concluded from the assay and the test substance identified as a potential skin sensitiser if the maximal average fold induction observed at any concentration tested (I_{max}) was statistically significantly higher than 1.5-fold (as compared to the basal luciferase activity and the interpolated concentration for which there is a 1.5 fold induction of luciferase activity ($EC_{1.5}$) value was below 1000 µM in at least two repetitions). In addition, at the lowest concentration where a gene induction of ≥1.5-fold was observed, the cellular viability must be above 70%.

Of the ten AIs, three produced negative predictions in the KeratinoSens™ assay. This indicates that they did not lead to Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes

in the second key event of the skin sensitisation AOP. The three AIs were acetamiprid, cyantraniliprole and dicamba.

Acetamiprid was tested at the highest concentration of 2000 µM as per the OECD 442D Test Guideline. At the maximal concentration and those below it, acetamiprid did not give rise to the positive result triggering luminescence induction of over 1.5-fold. In addition to this, at all 12 tested concentrations the MTT cytotoxicity test produced cell viability results of >70% as shown in Figure 3.4. Consequently, the negative prediction was accepted as per the prediction model.

Cyantraniliprole was dosed at the maximal concentration of 500 µM. Luminescence measurement following its exposure did not give rise to luminescence induction of over 1.5 fold at any of the 12 concentrations tested. Although the maximal dose tested for this AI was below the OECD guideline and ECVAM SOP recommended maximum concentration, cytotoxicity was observed at the highest of the 12 concentrations (cell viability <70%) in two out of three repetitions. Therefore, as an interaction between the AI and the test cell system has been observed, the KeratinoSens™ negative prediction can be accepted.

Benzovindiflupyr was tested and, as shown in Figure 3.7, cell viability of >70% was observed at the lowest AI concentration to produce a luminescence induction value of > 1.5 in comparison to the solvent control. There is a clear dose response observed as an increase in luminescence induction can be seen as the AI concentration increases. A dose response can also be seen with cytotoxicity evaluation, with a steep drop in viability observed at 31.25 µM. The > 1.5-fold induction is achieved at a benzovindiflupyr concentration lower than that triggering the cell viability of < 70%. As the $EC_{1.5}$ was < 1000 µM and a clear dose response was observed the positive KeratinoSens™ prediction for benzovindiflupyr can be accepted.

The mean maximum luminescence induction observed (I_{max}) in the KeratinoSens™ assay for chlorothalonil was 12.67 (above the 1.5 fold positive threshold). The MTT cytotoxicity test indicated a steep decrease in cell viability as the chlorothalonil test concentration increased. Figure 3.8 shows a

cell viability of >70% at the lowest concentration, however every increased concentration following that led to a cell viability of <70%. Luminescence induction of >1.5, when compared to the solvent control, was observed at the concentration with >70% cell viability. The calculated geometric mean for the $EC_{1.5}$ was <1000 µM, a clear dose response for luminescence was also observed and was comparable in both repetitions. Therefore, chlorothalonil was classed as positive in the KeratinoSens™ assay.

Mesotrione was tested at a concentration range of 2000 µM to 0.98 µM and a mean I_{max} value of 1.95 was determined. Cell viability of >70% was observed at the lowest concentration causing luminescence induction of >1.5 when compared to the solvent control. The calculated mean for the EC1.5 was <1000 µM and a clear dose response for luminescence was also observed as illustrated in Figure 3.9. Therefore, mesotrione was classed as positive in the KeratinoSens™ assay.

Following cell treatment with pinoxaden, a mean I_{max} luminescence measurement of 23.7 was determined. The mean EC1.5 concentration result was 28.6 µM. Cell viability of >70% was observed at the lowest dose with luminescence induction >1.5 when compared to the solvent control and at least two concentrations tested gave rise to viability >70%. The calculated mean for the $EC_{1.5}$ was <1000 µM and a clear dose response for luminescence was also observed as shown in Figure 3.10. As such, pinoxaden was classed as positive in the KeratinoSens™ assay and was considered to activate the Nrf2 transcription factor.

The mean I_{max} determined for AI1 was 2.87, while the mean EC1.5 concentration was 28.79 µM. Cell viability of >70% was observed at the lowest dose with luminescence induction >1.5 when compared to the solvent control and at least two concentrations tested gave rise to viability >70%. The calculated mean for the $EC_{1.5}$ was <1000 µM and a clear dose response for luminescence was also observed as shown in Figure 3.11

Due to solubility issues with the solvent, acibenzolar-s-methyl and chlorantraniliprole were both tested at maximal concentrations below the guideline recommended concentrations. Luminescence

measurement did not give rise to an increase in luminescence induction over 1.5-fold in either AI. However, as cell viability below 70% was not observed at any of the concentrations tested in the 12 dose range of either of these AIs, a negative prediction could not be accepted and instead the KeratinoSens™ predictions for these AIs are considered to be inconclusive.

3.4.3 h-CLAT Active Ingredient assessment

The ten agrochemical active ingredients were tested in the *in vitro* h-CLAT assay. This was performed to understand the potential for each of the ten AIs to successfully activate the third key event of the skin sensitisation AOP. This is the activation process in which dendritic cells change from antigen processing to antigen presenting cells. This activation process involves the modulation of the expression of dendritic cell surface phenotypic markers CD54 and CD86. The expression of CD54 and CD86 protein markers on the surface of the human monocytic leukaemia cell line, following exposure to the ten AIs was measured.

Based on results of solubility and CV75 dose-finding tests, the AIs were formulated in a selected solvent (DMSO or saline as shown in appendix 7) to final concentrations of between 2.665 mg/mL and 500.0 mg/mL (between 5.330 µg/mL and 5000 µg/mL final top test concentration on the 24-well plate). The AIs were identified as potential skin sensitisers if the RFI for CD54 was ≥ 200% (cell viability must be > 50%) and/or if the RFI for CD86 was ≥ 150% (cell viability must be > 50%) with concordant results in at least two independent h-CLAT runs.

In the h-CLAT evaluation five AIs produced results that were in agreement with their *in vivo* animal skin sensitisation test outcomes. These five AIs were benzovindiflupyr, chlorantraniliprole, cyantraniliprole, mesotrione and AI1. In relation to the comparative *in vivo* results, the only true positive that was identified by the h-CLAT assay was AI1. Two experimental runs identified concentrations of AI1 leading to an increase in the CD54 cell surface marker considered positive. The remaining *in vivo* skin sensitising AIs (acibenzolar-s-methyl, chlorothalonil and pinoxaden)

produced false negative results in the h-CLAT assay. In h-CLAT assay two repetitions were conducted for each of these three AIs. In the experimental runs acibenzolar-s-methyl, chlorothalonil and pinoxaden failed to produce a single repetition with a CD86 or CD54 cell surface marker increase at the relative fluorescence intensity values considered positive.

In a study by Takenouchi et al (2013) the predictive performance of the h-CLAT assay was evaluated for 112 water soluble chemicals with a log P of < 3.5 (Takenouchi et al., 2013). The sensitivity, specificity and overall accuracy reported by Takenouchi et al (2013) for the h-CLAT was 94%, 74% and 88%, respectively. As indicated in table 3.10, all ten of the AIs tested had calculated log P values < 3.5. However, the predictive performance results (presented in appendix 7) generated from the testing of these agrochemicals produced lower values than those reported by Takenouchi et al (2013). Takenouchi et al (2013) tested 143 chemicals in the h-CLAT of which 31 had log P values above 3.5. The results showed that the h-CLAT has low sensitivity to chemicals in this log P range, with false negative results being identified for 13 of the 31 chemicals. In addition to the issue of log P domain, poor solubility was reported by Takenouchi et al (2013) with precipitation or oil droplet formation in the culture medium at the concentrations tested. Due to these issues, any negative results from the h-CLAT assay on test substances with a log P > 3.5 are considered inconclusive. The sensitivity, specificity and accuracy of the h-CLAT assay for the ten agrochemical AIs tested were 25%, 67% and 50% respectively. It could be proposed that a greater number of chemicals identified as skin sensitisers *in vivo* could have provided greater confidence in the predictive performance results seen here, in particular low sensitivity result. However, it is also worth noting that the high cytotoxicity observed from tested AIs such as chlorothalonil led to a relatively low maximum test concentration being used in the h-CLAT test (i.e. 5.3 µg/mL). As such it could be considered that a higher concentration of the test material may lead to induction, however the h-CLAT test criteria prevent exploration into the more cytotoxic concentrations.

3.4.4 Active ingredient assessment of the skin sensitisation defined approaches

3.4.4.1 Kao Sequential Testing Strategy

The results for the Kao sequential testing strategy skin sensitisation/non skin sensitisation classifications of the ten agrochemical AIs in table 3.26 indicate that only three of the ten evaluated predictions agreed with the *in vivo* experimental results for those AIs. The three correctly predicted AIs were chlorothalonil, pinoxaden and AI1.

Chlorothalonil was determined to be a skin sensitiser in both the *in vivo* testing and by the Kao STS DA. The Kao STS DA also allows for an assessment of skin sensitisation potency of test materials. As the initial point of decision making in the Kao STS (as shown in Figure 3.3) is the review of h-CLAT assay results, a negative chlorothalonil result in the h-CLAT assay indicated that the highest potency value assigned by the STS was that of "weak sensitiser". The positive DPRA result for chlorothalonil confirmed this weak sensitiser classification by the Kao STS. These chlorothalonil results demonstrate a limitation of the Kao STS categorisation strategy. The strategy relies upon the sensitivity and accuracy of the h-CLAT assay in order to correctly differentiate strong from weak skin sensitisers. If a false negative test result is obtained from the h-CLAT assay, a misclassification through the Kao STS can be expected. This limitation is also seen with the pinoxaden AI. Pinoxaden has a CLP skin sensitisation category 1A classification as shown in table 1 and the DPRA and KeratinoSens™ for this material were both positive, showing agreement with the *in vivo* sensitisation. However, the h-CLAT results for this material were negative and as such its overall potency is underpredicted by the Kao STS classification.

There are a number of sources of existing experimental data for chlorothalonil. The United Nations Food and Agriculture Organisation (UN, 2015) reported that the majority of recorded *in vivo* study results for chlorothalonil indicate that it is a skin sensitiser. A guinea pig test conducted in China gave a "faint sensitiser" conclusion for chlorothalonil (UN, 2015). Another two reported skin sensitisation studies on chlorothalonil performed according to OECD Test Guideline 406 (guinea pig maximisation

test (GPMT)) provided conclusive evidence that the chlorothalonil is a sensitiser. However, it is also worth noting that the first GPMT study gave equivocal results due to the use of a chlorothalonil concentration that led to irritation during the GPMT challenge phase. In the second GPMT study on chlorothalonil, nine out of ten animals produced evidence of skin sensitisation. This second GPMT study was deemed to be more credible than the first as appropriate chlorothalonil concentrations were used throughout (UN, 2015). However, contradicting these results, a Buehler study on chlorothalonil by the US EPA (EPA, 2011) reported that none of the tested animals demonstrated any dermal reactions after the chlorothalonil challenge phase. Published case reports on human exposure (UN, 2015) also contradicted the EPA Buehler study, finding that chlorothalonil was a skin sensitiser. In addition, Boman et al (2000) (Boman et al., 2000) reported the findings of a LLNA which determined that chlorothalonil is an "extremely potent sensitiser".

Given the available published weight of evidence, chlorothalonil is currently considered to be an extremely potent skin sensitiser. A published peer review on chlorothalonil by European Food Safety Authority (EFSA) (EFSA et al., 2018) considered a category 1A skin sensitisation classification to be appropriate for this chemical. As such although it correctly identified chlorothalonil as a sensitiser, the Kao STS underpredicted the potency of chlorothalonil due to the h-CLAT indicating an inability for chlorothalonil to activate the third key event in the skin sensitisation AOP.

The Kao STS DA gave a classification of weak sensitiser for pinoxaden based on the *in vitro* and *in chemico* results. The classification of pinoxaden as a sensitiser from the Kao STS was in agreement with the *in vivo* data (WHO, 2019). Results from *in vivo* studies are presented in an ECHA harmonised classification and labelling (CLH) report on pinoxaden (ECHA, 2016a). This report mentions two skin sensitisation tests conducted on pinoxaden. The first was a GPMT in which, at the maximal concentration of 50%, there was no indication of a dermal reaction in all 19 tested animals following challenge test (1 animal died). The second *in vivo* test reported in the CLH report was for the LLNA which produced an EC3 value of 0.43%, leading to the conclusion that pinoxaden was a strong

sensitiser under the conditions of that assay. Although no indication of skin sensitisation was in the GPMT, this may potentially have been as a result of alternative vehicles used in the two tests (carboxymethyl cellulose and Tween 80 used in the GPMT, whilst N,N-dimethylformamide was used in the LLNA) or a species specific effect observed as has been previously reported to occur with oleic, linoleic and linolenic acid (Roberts et al., 2016, Kreiling et al., 2008). With that being stated, a single human case of skin sensitisation was reported (ECHA, 2016a) in which pinoxaden was identified as the cause based on the exclusion of other potential causative agents by skin patch testing. Based on the result of the LLNA test, pinoxaden has an agreed harmonised classification as a category 1A strong skin sensitiser (EFSA, 2013a). As the weight of evidence indicates that pinoxaden is a strong skin sensitiser, the Kao STS classification has underestimated its potency. As observed with chlorothalonil, the underestimation of potency by the Kao STS has been driven by the negative result obtained from the h-CLAT result on pinoxaden.

A further four AIs showed conflicting results between the Kao STS and the reported *in vivo* conclusions. These four materials, namely acetamiprid, benzovindiflupyr, dicamba and mesotrione, were predicted to be weak sensitisers by the Kao STS whilst their *in vivo* data indicated no skin sensitisation potential. In a joint review of acetamiprid by FAO/WHO a negative GPMT skin sensitisation result was reported (Banasiak et al., 2012) and, as such, the material was considered not to be a skin sensitiser. However, the positive h-CLAT and DPRA result lead to acetamiprid being classified as a skin sensitiser by the Kao STS. Specifically, based on the minimum induction threshold (MIT) of 800 µg/mL in the h-CLAT assay, acetamiprid was classified as a weak sensitiser. A similar outcome was observed for dicamba, with the h-CLAT and DPRA results leading to a classification of weak sensitiser by the Kao STS. For both acetamiprid and dicamba the KeratinoSens™ assay was not taken into consideration by the Kao STS which gave a negative result for the activation of keratinocytes. The accuracy of the Kao STS was limited due to the fact it did not consider the KeratinoSens™ data, which related to the second KE of the skin sensitisation AOP.

It is also possible that the *in vivo* results are an underprediction of the AIs skin sensitisation potential. For underprediction it is possible that in the GPMT, Buehler assay or LLNA *in vivo* test the sensitisation threshold concentration required to cause an effect may not have been achieved. There is a greater possibility of this occurring with dicamba than with acetamiprid, as the latter does not have irritant properties that would limit its *in vivo* study concentration. Whilst In contrast, dicamba has been classified as an irritant (EFSA, 2011a) and may, therefore, have had reduced concentrations in the GPMT. In addition to that limitation, these *in vivo* studies have been performed on test species that are not the ultimate target species of concern. With that in mind there were no reported cases of human sensitisation reactions associated with the use of dicamba from 1982 to 2006 according the Pesticide Handler Database (O'Malley, 2010). As such, the weight of evidence indicates an over estimation by the Kao STS rather than an underprediction by the *in vivo* tests for dicamba and acetamiprid.

The Kao STS was unable to provide a prediction for three of the AI materials tested, namely acibenzolar-s-methyl, chlorantraniliprole and cyantraniliprole. Inconclusive outcomes were obtained for these three AIs due to the inability to provide results for these AIs from the DPRA, one of the two assays used in the Kao STS DA. With regard to the DPRA assay, peptide co-elution was observed with acibenzolar-s-methyl. However, peptide depletion could not be determined for acibenzolar-s-methyl as its reaction with the lysine peptide led to co-elution from the HPLC column, resulting in excessively high peak areas. For cysteine, the peptide depletion generated for acibenzolar-s-methyl showed a continuing decrease over the time of the HPLC run, which resulted in its peak area coefficient variability not meeting the acceptance criteria of the assay (OECD, 2019a). Therefore, it was not possible to obtain valid peptide depletion values for acibenzolar-s-methyl and an inconclusive result was the result of this DPRA test. In the absence of a conclusive DPRA result and without a positive h-CLAT result, the Kao STS does not allow for a prediction to be made on the skin sensitisation potential of a test material, leaving an inconclusive overall outcome using this DA for acibenzolar-s-methyl.

Chlorantraniliprole and cyantraniliprole produced mean cysteine and lysine peptide depletion results of 3.47 and 1.88% respectively. In accordance with the DPRA prediction model, these results indicate no or minimal reactivity which is interpreted as a negative result. In the absence of any confounding factors, these negative DPRA results, alongside the negative h-CLAT results obtained for both AIs, would in accordance with the Kao STS (OECD, 2017, Nukada et al., 2013) lead to a negative assessment for skin sensitisation potential. However, observations of both chlorantraniliprole and cyantraniliprole following their incubation with the peptide buffers showed the formation of precipitation. As such, it is possible that the DPRA results for these two compounds may be an underestimation of the peptide depletion. Therefore, a conclusion on the lack of reactivity could not be drawn with sufficient confidence for these two AIs on the basis of the DPRA results. In addition to this, chlorantraniliprole and cyantraniliprole were tested at concentrations lower than the recommended maximal concentration (OECD, 2019a, ECVAM, 2012) of 100mM. This is also considered an exception to the prediction model, where a negative result is obtained (i.e. a sensitising threshold may not have been achieved) and further indicates a lack of confidence in the negative result obtained for these two compounds. As such, the results for chlorantraniliprole and cyantraniliprole in this DPRA study were considered inconclusive. This led to an overall prediction of the skin sensitisation from the Kao STS as being inconclusive. At present, the Kao DA does not allow for the use of alternative assays where a method may not be suitable for a test material, as has been observed here. Use of an alternative assay such as the KeratinoSens^{TM} or integration of the results from an *in silico* method in circumstances such as these may allow for a complete evaluation of a given test material avoiding inconclusive conclusions.

As indicated in the Methods Section, in order to perform the 2D *in vitro* skin sensitisation tests on the agrochemical formulations, there was a need to calculate a theoretical MW for each formulation in order to achieve a defined test item concentration in a solvent for testing (Settivari et al., 2015a). A limitation observed with this was that calculating an average MW for each of the formulations was only accurate to a limited degree. There are many possible sources of inaccuracy in the calculation of

theoretical molecule weight, but in particular this was due to the presence of large polymer ingredients in the formulations that had no defined MW. As indicated in Table 3.4, the maximum percentage of polymers with unknown MW was 4.5%. A potential effect of this is that there could be an incorrect calculation of the amount of test material required to achieve the 100mM solution in vehicle, possibly leading to solubility issues (as was frequently observed throughout the formulation testing) or, at worst, an underprediction of skin sensitisation potential due to lower actual test concentration ranges being tested than indicated by calculation. In addition to this, published test method guidelines for the KeratinoSens™ and h-CLAT assays document limitations associated with log P ranges. Specifically, for the h-CLAT assay, substances with a log P of greater than 3.5 have a tendency to produce false negative results, as stated in section 3.4.3. A similar limitation has been reported with the KeratinoSens™ assay, where substances with a log P above 7 are insoluble in the exposure medium. However, for this assay, if test material with a log P value > 7 is able to demonstrate solubility or a stable dispersion can be obtained, testing can be conducted and results accepted accordingly (OECD, 2018b). As indicated by the reported log P values in Table 3.13, this physicochemical property does not raise any issues with the interpretation of data for the AIs. However, accounting for solubility is not so straightforward for the agrochemical formulations. Whilst work by Duman et al (2014) and Dordick (1989) was considered while attempting to develop a method to determine the log P of the ten agrochemical formulations, it became clear that being able to calculate the log P for these test formulations was not possible (Dordick, 1989, Duman et al., 2014). Placing the formulation into the octanol:water system would lead to the different ingredients of the formulation separating out at different levels as they each have their own individual log P values, and as such deriving a octanol:water partition-coefficient for the formulation as a whole is not an option. Taking that into account, use of the test guideline prescribed solvents, such as acetonitrile, to form a fully dissolved test solution will lead to the individual components of the formulations (such as the AIs or preservative materials) being separated in the test mixture. This presents the test systems of the individual assays with a form of the agrochemical formulations that could be considered to not be truly representative

of the agrochemical products as they would appear in the field. As such the conclusions drawn from these tests may perhaps not be considered suitable for true hazard assessment of this test material type. As an example of other possible vehicles, pluronic is used frequently for testing agrochemical formulations in the LLNA. Subsequently a 1% pluronic PE 9200 vehicle was explored during this research, however, the viscosity of the mixture did not allow for accurate testing and therefore this was not pursued further.

3.4.4.2 Kao Integrated Testing Strategy (version one and two)

The skin sensitisation predictions from the Kao integrated testing strategies (ITS) (one and two) for the ten AIs were examined and the results are in Table 3.27. Kao ITS predictions for five of the ten AIs were contradictory to their *in vivo* results. Acibenzolar-s-methyl was predicted not to have any skin sensitising potential by the Kao ITS, whilst the *in vivo* GPMT was positive (UN, 2016, European Food Safety, 2014). The other four AIs incorrectly assessed, namely acetamiprid, benzovindiflupyr, dicamba and mesotrione were predicted to be weak skin sensitisers by the Kao ITS in contrast to their negative *in vivo* test results (EFSA, 2015, EFSA, 2011a, European Food Safety, 2016, European Food Safety, 2015).

Kao ITS predictions for four of the remaining five AIs were in agreement with the skin sensitisation potential identified by the *in vivo* test results. Amongst these four, cyantraniliprole and chlorantraniliprole were identified by the Kao ITS as materials that should not be classified for skin sensitisation. Overall, only three of the ten AIs were predicted by Kao ITS as non-sensitisers, whilst six of the ten AIs were identified as non-sensitisers by *in vivo* testing. However, the Kao ITS predictions for acibenzolar-s-methyl, cyantraniliprole and chlorantraniliprole may be considered to inconclusive rather than negative for skin sensitisation. This is because of the results obtained from the *in vitro* assays, specifically the DPRA result. The DPRA assay produced inconclusive results for these three AIs and, as such, a clear negative skin sensitisation prediction should not be derived using the Kao ITS.

This is because a third of the data required for the WoE assessment for this DA strategy is not available to make a complete conclusion.

The DEREK Nexus skin sensitisation likelihood prediction obtained for the pinoxaden AI was equivocal. As indicated in Figure 3.1, an equivocal DEREK Nexus prediction is of low confidence on the prediction scale described by (Judson et al., 2013). As such, a lack of certainty score of 0 was assigned to the DEREK Nexus assessment in the Kao ITS version one for pinoxaden. This approach is as conducted by (Macmillan and Chilton, 2019) where a reasoning level of equivocal obtained from DEREK Nexus, or a negative prediction of non-sensitiser with mis/un-classified features, was not considered further in the DA. The *in vitro* assay results from the testing of pinoxaden were such that the equivocal DEREK Nexus result did not have an impact on the overall classification assigned in accordance with the Kao ITS potency total battery score. Specifically, the DPRA assay outcome for pinoxaden indicated high reactivity and, as such, it was assigned a Kao ITS score of three. In contrast, the h-CLAT result for pinoxaden was negative and thus assigned a score of zero. The overall ITS score for pinoxaden was three, placing it in the weak skin sensitiser (GHS category 1B) 2-6 score category for the Kao ITS version 1 DA. As such, the presence or absence of an additional score from the *in silico* evaluation did not have a deciding impact on the overall categorisation of this AI. However, where an equivocal result is obtained from DEREK Nexus, it may be considered more appropriate to take a conservative approach and assign a score of one rather than zero. This approach would be appropriate in instances where this additional point would alter the final skin sensitisation categorisation of the material being tested.

When consideration is given for the Kao ITS potency assessment of the four AIs demonstrated to be skin sensitisers *in vivo,* there appears to be a demonstrable underestimation of skin sensitisation potential. Pinoxaden and AI1 have LLNA EC3 values of < 1% (Australian Government, 2019, ECHA, 2016a) and, according to the GHS and CLP classification criteria, an EC3 value of less than 2% supports classification as a category 1A (strong sensitiser) (ECHA, 2017b, GHS, 2017). In addition, an expert peer review in the EFSA journal on chlorothalonil considered that a category 1A classification for skin

sensitisation was appropriate (EFSA et al., 2018). The Kao ITS potency predictions for pinoxaden, AI1 and chlorothalonil were the same for all three, namely assessing them as weak sensitisers. This was an underprediction of the potency of these AIs in comparison to the *in vivo* results. As stated previously, the Kao ITS DA failed to identify acibenzolar-s-methyl as a skin sensitiser and, as such, a potency evaluation was not conducted. Predictions of skin sensitisation were not matched consistently between the two *in silico* models used (OECD QSAR Toolbox and DEREK Nexus) when evaluating all ten AIs. However, this did not lead to any differences in the final skin sensitisation predictions obtained from the Kao ITS version one or version two.

3.4.4.3 Two out of three defined approach

In the two out of three approach, as initially described by (Bauch et al., 2012), skin sensitisation can be predicted using up to three accepted non-animal OECD Test Guideline test methods (e.g. DPRA, KeratinoSens™ or LuSens and h-CLAT or U-Sens™). In this investigation, sequential testing was performed using the DPRA, KeratinoSens™ and the h-CLAT assays in no defined order. The two out of three defined approach (2/3 DA) uses the weight of evidence of the three *in vitro* assays to provide a positive or negative prediction of skin sensitisation. This DA allows for skin sensitisation potential of single chemicals to be predicted but does not extend to predicting skin sensitisation potency (OECD, 2017). Upon reviewing the predictions using the 2/3 DA in this investigation, based on the *in vitro* assay results of the ten AIs, four AIs had predictions that were in agreement with the *in vivo* test conclusions, these results are shown Table 3.28. These four AIs were chlorothalonil, pinoxaden, AI1 (all predicted to be skin sensitisers) and cyantraniliprole (predicted non-sensitiser). There were four predictions from the 2/3 DA for AIs that did not match the *in vivo* skin sensitisation test results. These were for acetamiprid, benzovindiflupyr, dicamba and mesotrione. These four AIs had results from *in vivo* tests indicating that none of them were skin sensitisers. The 2/3 DA, however, indicated that all four of these AIs were skin sensitisers with at least two of the three *in vitro* tests producing a positive result for each of these AIs. The *in chemico* DPRA results were positive for all four AIs predicted by the 2/3 DA to be skin sensitisers, contrary to the *in vivo* results showing them to be non-sensitisers. A

hypothesis that can be derived from this is that key event one, i.e. the MIE, of the skin sensitisation AOP was being passed by acetamiprid, benzovindiflupyr, dicamba and mesotrione. Or that the DPRA *in chemico* method was over predictive for these AIs leading to an over-estimation by the 2/3 DA.

None of the AIs were predicted negative by the DPRA. Seven of the ten AIs produced positive results from the DPRA, with the remaining three (acibenzolar-s-methyl, chlorantraniliprole and cyantraniliprole) giving inconclusive results. For acibenzolar-s-methyl and chlorantraniliprole, this led to an overall inconclusive prediction by the 2/3 DA. Both acibenzolar-s-methyl and chlorantraniliprole also had inconclusive KeratinoSens™ results alongside negative h-CLAT results, leading to the overall inability of the 2/3 DA to make a final prediction for these two AIs. Due to solubility issues with the solvent in the testing of acibenzolar-s-methyl and chlorantraniliprole, both were tested at maximal concentrations below the guideline recommended maximum concentrations for the KeratinoSens™. The measurement of luminescence did not give rise to an increase in luminescence induction of more than 1.5-fold in either AI. However, as cell viability below 70% was not observed at any of the concentrations tested in the 12 dose range of either of these AIs, a negative prediction could not be accepted. This leaves an inconclusive KeratinoSens™ result for acibenzolar-s-methyl and chlorantraniliprole.

Overall, the 2/3 DA identified three of the four *in vivo* skin sensitisers. However, the approach only identified one of six *in vivo* non skin sensitising AIs, whilst four of the six as sensitisers and one giving an inconclusive result. Removing the two inconclusive 2/3 DA predictions (acibenzolar-s-methyl and chlorantraniliprole), leaving a test sample of eight, the specificity observed from this DA is low at 20%. One negative prediction was provided by this DA, and since it was in agreement with the *in vivo* result for that AI, the negative predictivity for the 2/3 DA based on the AIs tested was 100% although this is somewhat misleading due to there only being one compound. The overall performance of this DA based upon the results of the eight definitive predictions for the ten agrochemical active ingredients tested, as compared to the *in vivo* test results was 50%. When the inconclusive 2/3 DA predictions

(recorded as predicted neutral) were included in the results evaluation, in a 3x3 confusion matrix (included in appendix 8), the total success was lower at 40%. In the evaluation of the 2/3 DA predictions, both including and excluding the inconclusive predictions, a Cohens Kappa value of less than 0.2 was observed indicating a poor level of agreement with the *in vivo* results (Modi et al., 2012, McGee, 2018).

3.4.5 Analysis of the results for Agrochemical formulation *in vitro* triple pack testing

After completing the *in vitro* triple pack testing of the ten AIs and reviewing the results, this investigation moved on to pursue the next objectives. Specifically, how the *in vitro* triple pack test methods can be used to evaluate the skin sensitisation potential of the complex mixtures and if any changes could be made to the triple pack standardised methods for the assessment of PPP formulations.

3.4.5.1 DPRA test formulation assessment

Solubility issues limited the number of formulations that could be tested in the DPRA test. Five (SYN2, SYN4, SYN6, SYN8 and SYN9) of the ten agrochemical formulations were tested in the DPRA. Various methods and solvent combinations were unable to produce acceptable testing solutions for the remaining five formulations (SYN1, SYN3, SYN5, SYN7 and SYN10). This inability to produce acceptable testing solutions was observed through either poor/no solubility and/or because the solvent combinations led to substantial peptide depletion profiles, limiting the dynamic range of the test system. Test formulations SYN2, SYN6 and SYN8 produced positive DPRA results indicating that they have the potential for the skin sensitisation MIE. Due to the impact on the cysteine peptide depletion caused by the acetonitrile:DMSO solvent, the use of the cysteine prediction model (Table 3.8) to provide a reactivity class has been excluded. However comparing the concentration levels with the DPRA control C containing the acetonitrile:DMSO mix it appears that formulations SYN2 and SYN8 have high peptide reactivity. The cysteine/lysine prediction model (Table 3.7) places materials that lead to a mean peptide depletion in the range of 42.47% to 100% in the high reactivity class. As such

this places the tested formulations SYN2 and SYN8 within the high reactivity DPRA class. As the observed cysteine depletion caused by the DMSO:Acetonitrile solvent alone was 0.073 mM (85.4%) (shown in appendix 3), the measured high peptide depletion levels for formulations SYN2 (97%) and SYN8 (90%) can be confidently accepted as true DPRA positives. Though it is acknowledged that their reactivity may be overpredicted.

Formulations SYN4 and SYN9 showed significant interference in the DPRA assay through physicochemical means of stabilising the cysteine peptide. This interference was potentially via oxidation of the peptide leading to dimerisation, as proposed by Akimoto et al (2020). Akimoto et al (2020) demonstrated that although DMSO is known to be an excellent solvent, it is also known to promote the oxidation of thiol groups. Their report shows a DMSO dose dependent decrease of residue levels of cysteine peptide due to oxidation in the DPRA (Akimoto et al., 2020). The use of 5% DMSO in acetonitrile resulted in a decrease in the mean cysteine peptide levels that were still within acceptable range, as per the test guideline (OECD, 2019a). However, the report states this oxidation of thiol groups by DMSO still presents a high likelihood that some individual measurements will still fall outside of the test guideline acceptable range. In addition, precipitation was observed during their solubility assessment. It is therefore very difficult to make assumptions regarding these two formulations and thus an inconclusive DPRA result for both formulations has been reached.

3.4.5.2 KeratinoSens™ test formulation assessment

Of the six formulations identified as skin sensitisers through *in vivo* experiments, three were demonstrated to have keratinocyte induction potential in the KeratinoSens™ assay. These three formulations were SYN1, SYN6 and SYN10. They produced Imax values of 3.96, 7.90 and 2.96 respectively, with a low EC1.5 value of 0.38 µM for the SYN6 formulation. It should be acknowledged that the KeratinoSens™ test has the limitation of not being able to determine potency. Nonetheless, the low EC1.5 value produced from formulation SYN6 gives an indication that this formulation is more reactive in the second key event of the skin sensitisation AOP than the other tested formulations.

The KeratinoSens™ results for SYN3 were contrary to the formulation's non-sensitising result from the LLNA. SYN3 produced a positive KeratinoSens™ result with the highest EC1.5 of the four positive formulation results and three times that of its nearest comparable formulation, SYN1. This indicates a much greater concentration of the SYN3 formulation is required to trigger key event two than for the other formulations. This contrasts with the lower intensity (with an Imax of 2.56) at which SYN3 triggered key event two, which was lower than the positive results of the other three formulations tested at lower maximum concentrations.

The *in vivo* experimental results are being considered the benchmark against which the *in vitro* results are being assessed. As such the KeratinoSens™ assay was able to produce comparable positive results for the remaining three formulations identified as skin sensitisers *in vivo.* Formulations, SYN7 and SYN8 produced inconclusive results in the KeratinoSens™. Whilst formulation SYN2 produced a negative result conflicting with the *in vivo* result. Following initially solubility testing, the SYN2 formulation was shown to be soluble in DMSO at the top concentration of 335.5 µM. Concurrent cytotoxicity testing of the SYN2 formulation using MTT demonstrated that the maximum concentration induced cell viability of <70% (approximately 40% as shown in Figure 3.15), whilst luciferase induction remained below the 1.5 threshold at all 12 tested concentrations. Luciferase induction activity may potentially have been inhibited at the maximum SYN2 concentration, due to the observed steep cytotoxicity at the high end of the SYN2 test concentration range. An argument against this hypothesis is that the absence of a luciferase induction dose response from the SYN2 concentration ranges tested indicates an absence of an increase in keratinocyte activation potential in the SYN2 formulation. This negative KE2 interpretation is aligned with the current OECD 442D (2018) KeratinoSens™ test guideline. The test guideline states that, "if cytotoxicity (<70% viability) is reached at a maximal soluble test concentration <1000 µM, criteria for negativity can still be applied" (OECD, 2018b). OECD (2018b) was an update from the previous version of the 2015 OECD KeratinoSens™ test guideline, which stated that negative results from concentrations of <1000 µM should be considered inconclusive (OECD, 2015b).

The use of the current KeratinoSens™ acceptance criteria has led to the results of remaining formulations tested in the assay being determined as inconclusive. All five remaining formulations (SYN4, SYN5, SYN7, SYN8 and SYN9) did not produce an Imax equal to or greater than the 1.5 threshold through any of the formulations tested concentration ranges. Alongside that they were unable to produce cell viability of <70% at their maximum test concentrations, failing to demonstrate keratinocyte test material interaction for these five formulations in the KeratinoSens™ assay.

3.4.5.3 h-CLAT test formulation assessment

All ten agrochemical formulations were also tested in the h-CLAT assay. Of those ten only three produced h-CLAT results that agreed with the *in vivo* results. These three concordant results were for the SYN5 (negative), SYN6 (positive) and SYN10 (positive) formulations. The overall performance of the h-CLAT on these formulations compared to the *in vivo* experimental results was 30%. Of the four *in vivo* non-sensitising formulations, three (SYN3, SYN4 and SYN9) were identified as positive in the h-CLAT alongside SYN6 and SYN10. These positive h-CLAT results given a sensitivity value of 33.3%, indicating a high potential for false positive h-CLAT predictions for agrochemical formulations.

The negative predictivity of the h-CLAT assay for these ten formulations was 20%. This was as only one of the five negative h-CLAT predictions was in agreement with the *in vivo* experimental results, as shown in Table 3.23. This led to a relatively low specificity of 25%, reflecting a low proportion of formulations correctly predicted as being non skin sensitisers.

Although the number of tested formulations could be considered low, a Kappa coefficient value was calculated to indicate the extent of agreement between the *in vivo* and *in vitro* experimental results. The calculated kappa value was negative, -0.40, and according to McGee et al (2018), this indicates that any observed agreement was worse than a chance agreement (McGee, 2018). Due to the relatively low number of formulations tested, future investigative work using a larger data set may provide greater confidence in the kappa value obtained.

3.4.5.4 Final Weight of Evidence Evaluation of non-animal skin sensitisation test results on agrochemical test formulations

A weight of evidence (WoE) approach was applied to produce a final prediction of the skin sensitisation potential of the ten formulations. A two out of three WoE approach used the three *in vitro* results. Where there was an inconclusive test prediction or inability to conduct a test (due to solubility or cytotoxicity issues), the results from the CLP threshold calculation have been substituted into the WoE approach.

Using the WoE approach, four of the ten formulations produced skin sensitisation predictions that were in agreement with their *in vivo* experimental results, as shown in Table 3.29. Amongst these formulations, SYN5 and SYN8 required the inclusion of threshold calculation results in place of the inconclusive KeratinoSens™ predictions. SYN5 was predicted to be non-sensitising, whilst the SYN6, SYN8 and SYN10 formulations were predicted as sensitising formulations.

Two of the ten formulations still produced inconclusive final skin sensitisation predictions through the WoE approach. This was observed in formulations SYN4 and SYN7, as definitive results could not be obtained from the DPRA or KeratinoSens™ tests and results from the CLP threshold calculation and h-CLAT were disconcordant.

The WoE predictions for the SYN1, SYN2, SYN3 and SYN9 formulations were not in agreement with the *in vivo* experimental results. Of these four formulations, two had their WoE predictions based solely on two concordant *in vitro* assays. For both SYN2 and SYN3 concordant KeratinoSens™ and h-CLAT assay results led to WoE predictions that were in disagreement with the *in vivo* results. Omitting the inconclusive WoE results, the sensitivity of this approach was calculated to be 60% whilst the specificity and total success were 33% and 50% respectively. These performance criteria values are relatively low and do not indicate that a good degree of confidence can be given to the WoE approach predictions for these agrochemical formulations.

A prominent limitation of using these 2D *in vitro* skin sensitisation assays has been the inability to complete the tests for several of the ten formulations. As identified with the DPRA testing performed here, the need to completely dissolve the test material in a solvent may not always be possible. In formulations where it is possible, the dissolved test formulation does not represent the agrochemical formulation as workers or bystanders may be exposed to it in the field. As such the use of these 2D *in vitro* cell models to conduct a skin sensitisation hazard assessment for complex mixtures, such as the agrochemical formulations is not appropriate. In addition to that, the results obtained indicate poor predictivity for these complex mixture test substances. As such, further investigation into methods that allow for the testing of these formulations without disruption/transformation of their intended form needs to be done. In Chapter Five this thesis moves to investigate the SENS-IS 3D *in vitro* model that uses the human reconstructed human epidermis. This 3D *in vitro* assay will be investigated to understand if this adaptation in the test system exposure improves the skin sensitisation predictivity for agrochemical formulations.

3.5 Conclusion

In a final conclusive review of the four DAs used to assess the skin sensitisation potential of the 10 agrochemical AIs, it can be seen that the Kao ITS 1 and 2 DAs were able to assess all 10 AIs. Six of the 10 AIs were correctly identified agreement with the *in vivo* test results. Whilst the remaining four AIs had false positive predictions generated through the Kao ITS 1 and 2. The 2 out of 3 skin sensitisation prediction DA was unable to produce a clear prediction for two of the 10 AIs, giving inconclusive predictions. While the Kao STS DA provided inconclusive predictions for 3 of the 10 AIs as shown in Table 3.30. For the AIs that all four DAs were all able to provide a prediction on, the DAs were all in agreement with each other. Whether that be to provide predictions that showed agreement with the *in vivo* tests, or predictions that were false positives. With that being said, when the DAs capable of providing a potency prediction (i.e., Kao STS, Kao ITS 1 and 2) generated a false positive, it was for indication as weak sensitiser rather than a larger over estimation as a strong sensitiser. There is some

reassurance in observing that these methods provided an overestimation of the sensitising potential of these AIs and not an underestimation.

Active ingredient	2/3 skin sensitisation prediction	KAO ITS 1 prediction	KAO ITS 2 prediction	KAO STS prediction	*In vivo* conclusion
Acetamiprid	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Non-Sensitiser
Acibenzolar-S-methyl	Inconclusive	Not Classified	Not Classified	Inconclusive	Sensitiser
Benzovindiflupyr	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Non-Sensitiser
Chlorantraniliprole	Inconclusive	Not Classified	Not Classified	Inconclusive	Non-Sensitiser
Chlorothalonil	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Sensitiser
Cyantraniliprole	Non-Sensitiser	Not Classified	Not Classified	Inconclusive	Non-Sensitiser
Dicamba	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Non-Sensitiser
Mesotrione	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Non-Sensitiser
Pinoxaden	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Sensitiser
AI1	Sensitiser	Weak Sensitiser	Weak Sensitiser	Weak Sensitiser	Sensitiser

Table 3.30 Conclusive summary of the evaluated defined approach predictions for the 10 active ingredients against the comparative benchmark in vivo skin sensitisation test results.
Red – disagreement with *in vivo* conclusion, Green – agreement with *in vivo* conclusion

The results of this chapter demonstrating the specific DAs' inability to complete assessment on the full list of ten AI test materials leads us to identify the Kao ITS as the most efficient of the evaluated DAs. The Kao ITS allows for fewer *in vitro* tests to be conducted, reducing the time and resource required to complete the skin sensitisation hazard assessment. Incorporation of the *in silico* models in Kao ITS also increases confidence in the findings which the 2 out of 3 DA does not.

When considering the combined use of these *in chemico/ in vitro* methods to provide a skin sensitisation prediction for the ten PPPs, fewer than half showed predictions that were in agreement with their *in vivo* benchmark results. This is effectively less predictive than making a random guess. As such, when considering their use to provide information to replace *in vivo* skin sensitisation tests for complex mixtures, at present these three *in chemico/in vitro* methods are not suitable.

4.0 CHAPTER FOUR - COMPARISON OF THE PREDICTIVE NATURE OF THE GENOMIC ALLERGEN RAPID DETECTION (GARD) ASSAY WITH MAMMALIAN ASSAYS IN DETERMINING THE SKIN SENSITISATION POTENTIAL OF AGROCHEMICAL ACTIVE INGREDIENTS.

4.1 Introduction

The research in this chapter has been published and the information presented here is based on that publication (Masinja et al., 2020). Recently attempts have been made to identify non-animal test methods with good predictive power for chemical hazard identification in a bid to reduce laboratory animal use (Alloul-Ramdhani et al., 2014, Doe and Botham, 2019, Reisinger et al., 2015, Ivan de Ávila et al., 2019). In accordance with Article 62 of the European Regulation (EC) No. 1107/2009, concerning the placing of plant protection products on the market; the use of *in vivo* mammalian test methods should only be used as a last resort. Where available non-animal test methods should be used and promoted ((EC), 2009a), several such *in vitro* assays have been developed for skin sensitisation. In this thesis the triple pack approach is initially introduced and described in Section 1.2.2. Chapter Three gave an overall evaluation of the function of the triple pack. Another, as yet relatively unproven, approach is the Genomic Allergen Rapid Detection (GARD) assay. The GARD assay's potential for evaluating the skin sensitisation potential of agrochemicals is unknown, hence it was selected for evaluation in this chapter.

The GARD assay is a cell-based, *in vitro* alternative to animal testing which assesses skin sensitisation by measuring the biomarker signature in chemical-stimulated, human MUTZ-3 cells (Johansson et al., 2011). The MUTZ-3 cell line serves as a surrogate for dendritic cells (DC) and changes in transcription in the genes can be linked to processes involved in skin sensitisation (Rovida et al., 2013, Masterson et al., 2002). The GARD assay was first described in 2011 and measures transcriptional changes in 200 genes associated with sensitisation (Johansson et al., 2011). The 200 gene biomarker signature includes transcripts involved in oxidative stress, dendritic cell maturation and cytokine responses

(Johansson et al., 2011). The results are placed into a support vector machine (SVM) model trained on a set of reference chemicals (Forreryd et al., 2016). In particular, genes in pathways involved in dendritic cell maturation and activation, associated with key event three of the skin sensitisation adverse outcome pathway (AOP), which is also measured by the h-CLAT assay (OECD 2014, OECD 2018a), are included in the GARD assay. The Nrf-2 mediated oxidative response (Uruno and Motohashi, 2011), which is also the pathway measured in the KeratinoSens™ and LuSens™ assays (OECD 2018b, DB-ALM (INVITTOX), 2013) is included in the GARD assay. Test materials of higher sensitising potency are assigned higher GARD SVM values than those of weaker sensitisers (Stevenson et al., 2019).

During the validation process of alternative methods for skin sensitisation, a wide array of test materials from different industrial sectors have been tested using the GARD (Johansson et al., 2019), and other, assays (OECD, 2018a). This has aided in ascertaining limitations and, more specifically, chemical types that do not fall within the applicability domain of each method. The GARD assay consistently reports accuracies of close to 90 to 95% compared to *in vivo* data (Johansson et al., 2017, Johansson et al., 2014, Johansson et al., 2013; Zeller et al., 2017). The evaluation of the GARD assay in a blind study using cosmetics ingredients (from Cosmetics Europe) demonstrated a predictive performance of 83% (Johansson et al., 2017). Whilst the GARD assay has shown good performance in evaluation studies, it is worth noting that *in vitro* assays for skin sensitisation are not intrinsically standalone assays and none of them are perfectly predictive. However, they can be used as part of a weight of evidence approach, as such, knowledge about the chemical and property domain in which an assay works is crucial.

The aim of this chapter was to assess the *in vitro* GARD assay's skin sensitisation predictivity in comparison with mammalian skin sensitisation tests on agrochemical active ingredients. To achieve this, agrochemical compounds for which sensitising potential had been previously established through GLP *in vivo* studies (OECD 429 murine local lymph node assays, OECD 406 guinea pig maximisation test

and Buehler assays) were tested in the GARD assay. As a weight of evidence approach is advocated by the ECHA when using *in vitro* data for the purpose of classification (ECHA 2017), a QSAR analysis of each of the test materials was also performed. Human data are available elsewhere for some of the active ingredients however for the purposes of this evaluation these were not included as the comparison was with the available animal data. The mammalian studies are considered to be an appropriate standardised data set for comparison purposes.

4.2 Materials and Methods

4.2.1 GARD assay cell line

The GARD assays were conducted by Senzagen (Lund, Sweden) on behalf of Syngenta according to the protocol as described in Forreryd et al., 2016 and Johansson et al., 2013. The human myeloid leukemia-derived cell line SenzaCell (available through American Type Culture Collection (ATCC)) was used. This was maintained in α-minimum essential medium (Thermo Scientific Hyclone, United States) supplemented with 20% (volume/volume) foetal calf serum (Life Technologies, US) and 40 ng/ml recombinant human Granulocyte Macrophage Colony Stimulating Factor (rhGM-CSF) (Miltenyi Biotec, Germany). A medium change during cell expansion was performed every three to four days. Working stocks of cultures were grown for a maximum of 16 passages or two months after thawing. The chemically exposed cells were incubated for 24h at 37°C, 5% CO_2 and 95% humidity.

4.2.2 GARD Assay

Active ingredient test substances shown in Table 4.1 were dissolved in dimethylsulfoxide (DMSO) or water, based on physico-chemical properties. The cytotoxic effects of test substances were monitored, as a concentration leading to 90% relative cell viability (Rv90) demonstrating the test substance's toxicity, was used in the assay.

Agrochemical Active Ingredient**	**Indication (F, H, I) ***	***In Vivo* Outcome**	***In Vivo* Study**	**Skin Sensitisation Harmonised Classification Labelling and Packaging (CLP) category**
Benzovindiflupyr	F	Negative	LLNA	Not classified (EFSA, 2015, FAO, 2013)
Chlorothalonil	F	Positive	Buehler	Skin Sens. 1, H317 (EFSA et al., 2018)
Clodinafop-propargyl	H	Positive	GPMT	Skin Sens. 1, H317 (EFSA et al., 2020)
Cyantraniliprole	I	Negative	LLNA	Not classified (EFSA, 2014, FAO, 2013)
Dicamba	H	Negative	LLNA	Not classified (EFSA, 2011a, Harp, 2010, ECHA, 2008, EPA, 2006)
Difenoconazole	F	Negative	Buehler	Not classified (EFSA, 2011b)
Pinoxaden	H	Negative Positive (EC3 =0.43%)	GPMT LLNA***	Skin Sens. 1A, H317 (EFSA, 2013b, FAO, 2016)
AI1	I	Positive (EC3 =0.13%)	LLNA	No harmonised classification
AI2	I	Positive (EC3 =1.1%)	LLNA	No harmonised classification
AI3	I	Positive	Reduced Local lymph node assay (rLLNA)	No harmonised classification
AI4	I	Negative	rLLNA	No harmonised classification
AI5	I	Positive	rLLNA	No harmonised classification

Table 4.1 The active ingredients, agrochemical use and in vivo skin sensitisation outcomes of agrochemical compounds tested in the GARD assay

*F: fungicide, H: herbicide, I: insecticide

**AI1 - AI5: anonymised agrochemical active ingredients

*** The result corresponding with the harmonised classification (LLNA) has been used for the purposes of comparison. These studies were considered OECD & GLP compliant

The assayed test substances were titrated to concentrations ranging from 1 µM to the maximum soluble concentration in cell media. For freely soluble test substances, 500 µM was set as the upper limit of the titration range. For test substances dissolved in DMSO, the in-well concentration of DMSO was 0.1%. After incubation with the test substance for 24 hours, harvested cells were stained with the viability marker Propidium Iodide (PI) (BD Bioscience, USA) and analysed by flow cytometry. For non-toxic test substances, a concentration of 500 µM was used, if possible. When test substances were poorly dissolved in cell medium or insoluble at the 500 µM concentration, the highest soluble concentration was assessed and used. The concentration to be used for any given chemical was termed the 'GARD input concentration', shown in Table 4.3.

Once the input concentration had been established, the cells were exposed solely to this concentration. A set of positive and negative controls were included as reference and quality controls. The test substances and controls were assayed in biological triplicates, performed at different timepoints, and using different cell cultures. After incubation for 24h at 37°C, 5% CO_2 and 95% humidity, the cell cultures were lysed in TRIzol reagent (Life Technologies, Carlsbad, California, US) and stored at -20°C until RNA had been extracted. In parallel, stimulated cells were propidium iodide (PI) stained and analysed using flow cytometry to verify the expected relative viability (Johansson et al., 2019).

4.2.3 Performance criteria

In order to understand the GARD assay's predictive power and the accuracy of its performance in comparison with the *in vivo* laboratory animal test data, statistical parameters were calculated between *in vivo* experimental and GARD *in vitro* assay result data. Sensitivity, specificity, total success/accuracy, positive and negative predictivity as well as the Cohen's kappa coefficient were calculated to evaluate the performance of the GARD assay. These parameters were all calculated using the method described by Modi et al (2012) and also described fully and used for the evaluation of results in Chapter Two.

The differences in chemical characteristics between the GARD training set and agrochemical AI test set were also examined. This was performed by assessing the MW, logarithm of the octanol-water partition coefficient (log P) and numbers of hydrogen bond donors (HBD), hydrogen bond acceptors (HBA) and rotatable bonds (RB) present in each compound in the two chemical sets. AlogP was used to calculate log P in accordance with the previous work by Guziałowska-Tic (Guziałowska-Tic, 2017) who demonstrated that AlogP provided the optimum conformity for this chemical property. It should be noted that Kathon CG/ICP is present in the GARD training set. In order to best capture the physicochemical properties of this preservative mixture, its two active components (Methylisothiazolinone and Methylchloroisothiazolinone) were entered individually into the data set for this evaluation.

4.2.4 Evaluation of structural alerts for protein binding and skin sensitisation

Structural alerts for protein binding and skin sensitisation (Aptula and Roberts, 2006, Enoch et al., 2011) were identified from the OECD QSAR Toolbox version 4.3 for the chemicals in both the training and test sets. The following profilers were applied:

- Protein binding alerts for skin sensitisation by OASIS
- Protein binding alerts for skin sensitisation by OASIS with skin metabolism
- Protein binding alerts for skin sensitisation according to GHS
- Protein binding alerts for skin sensitisation according to GHS with skin metabolism

The alerts were assessed for their association with *in vivo* skin sensitisation. A compound was considered to be identified as a skin sensitiser if the OASIS/GHS profiler gave an outcome of 1A or 1B, or if the OASIS with skin metabolism profiler gave a 1A result. A non-sensitiser was concluded if the OASIS/GHS profiler identified no alert or if the OASIS/GHS with skin metabolism profiler gave a 1B result. The GARD assay does not encompass the metabolic system, consequently this was the rationale for rating OASIS with metabolism 1B as a non-sensitiser. This evaluation scheme is shown in Table 4.2 below.

OECD QSAR Toolbox prediction scheme	
Sensitiser	OASIS GHS profiler - 1A or 1B OASIS w/metabolism profiler - 1A
Non-sensitiser	OASIS GHS profiler - No alert OASIS GHS w/metabolism profiler - 1B

Table 4.2. OECD QSAR Toolbox prediction scheme

4.3 Results

4.3.1 GARD Assay

The aim of this research was to compare the results of the GARD assay to the available *in vivo* skin sensitisation study outcomes for twelve agrochemical AIs. The results from the GARD assay are summarised in Table 4.3.

Test material	Structural alert**	*in vivo* study	*in vivo* study result	Rv90***	GARD input concentration	GARD Decision Value (Mean ± SD)	GARD skin result
benzovindiflupyr	AC/SB	LLNA	negative	40 µM	40 µM	6.0±0.9	positive
chlorothalonil	SNAr	Buehler	positive	0.5 µM	0.5 µM	4.6±1.4	positive
clodinafop-propargyl	No alert	GPMT	positive	-	100 µM	6.1±0.9	positive
cyantraniliprole	AC/SB	LLNA	negative	-	100 µM	3.4±0.7	positive
dicamba	SB	LLNA	negative	-	500 µM	0.0±0.8	positive
difenoconazole	No alert	Buehler	negative	50 µM	50 µM	6.3±0.9	positive
pinoxaden	No alert	LLNA	positive (EC3 =0.43%)	-	500 µM	-0.5±0.5	negative
AI1	SB/NA	LLNA	positive (EC3 =0.13%)	-	100 µM	1.1±0.7	positive
AI2	No alert	LLNA	positive (EC3 =1.1%)	140 µM	140 µM	6.9±0.4	positive
AI3	MA	rLLNA	positive	-	100 µM	3.4±0.6	positive
AI4	No alert	rLLNA	negative	250 µM	250 µM	6.4±0.4	positive
AI5	SNAr	LLNA	positive (EC3 = 0.9%)	50 µM	50 µM	4.4±0.5	positive

Table 4.3 Protein binding alerts*, in vivo study results, Rv90**, GARD input concentration, GARD skin results, GARD decision values

* AC, Acylation; MA, Michael addition; NA, Nucleophilic addition; SB, Schiff base formation; SNAr, Aromatic nucleophilic substitution;

Reaction domains were assigned based on expert judgment using the chemistry defined in Enoch et al (2011)*Rv90 - concentration of test substance inducing 90% relative viability.

Positive control p-phenylendiamine,

Negative control dimethylsulfoxide

4.3.2 Analysis of Applicability Domain of GARD Assay and Agrochemical AIs Tested

Table 4.4 details the *in vivo* assay predictions compared to those of the GARD assay for this study's test set. The GARD assay correctly predicted the six sensitisers, however, the negative predictivity of the GARD assay for the test set was not concordant with that of the *in vivo* results.

	GARD Positive	GARD Negative
In vivo Positive	6	1
In vivo Negative	5	0

Table 4.4 Summary of the concordance of test results of agrochemical test set in vivo skin sensitisation results versus the GARD assay results

Performance analysis of these data using the statistical parameters was conducted as shown in Table 4.5 and illustrated in Figure 4.1. When compared to the *in vivo* results, the negative predictivity of the of the GARD assay was mainly nonconcordant for this test set, with a positive predictivity of 55%, sensitivity of 86% and a total accuracy of 50%. Cohen's Kappa coefficient provided a statistical measure of inter-rater agreement for categorical items (sensitiser/non-sensitiser) and the value for Cohen's Kappa value was -0.16 indicating poor agreement between sensitisers and non-sensitisers.

Positive predictivity	54.5%
Negative predictivity	0.0%
Sensitivity	85.7%
Specificity	0.0%
Total Success/Accuracy	50.0%
*kappa-value	-0.16
MCC	-0.14

Table 4.5 Statistical parameters used for evaluation of the GARD assay predictions of the agrochemical test set results versus the in vivo skin sensitisation assay results

*kappa-value: < 0.20 poor, 0.21 - 0.40 fair, 0.41 - 0.60 moderate, 0.61 - 0.80 substantial

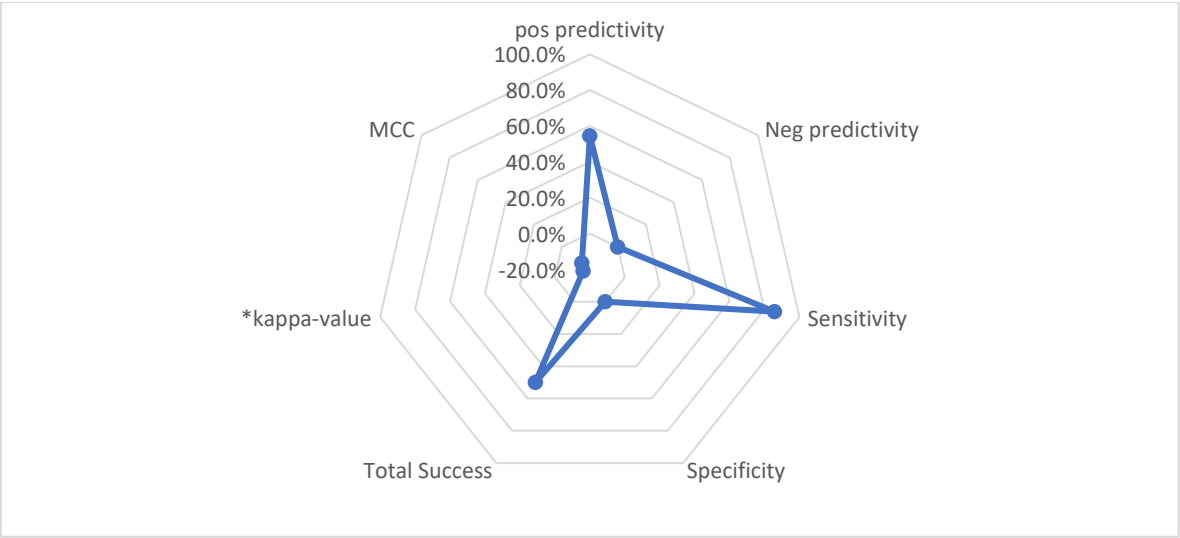


Figure 4.1 Various statistical parameters adopted to evaluate prediction of skin sensitisation potential by the GARD assay conducted on the test set (12 agrochemical active ingredients)

Matthews Correlation Coefficient (MCC)

In order to determine possible reasons for the nonconcordant results between the GARD assay and *in vivo* test results for the agrochemical AI test set, the ranges of physicochemical properties of the GARD training set and the AIs tested were compared. A broad overview of the range relative physicochemical properties which may affect solubility and uptake is provided as a plot in Figure 4.2. A range of physicochemical properties (i.e. calculated log P (AlogP) against molecular weight) associated with the AIs were plotted against the published training set of the GARD assay.

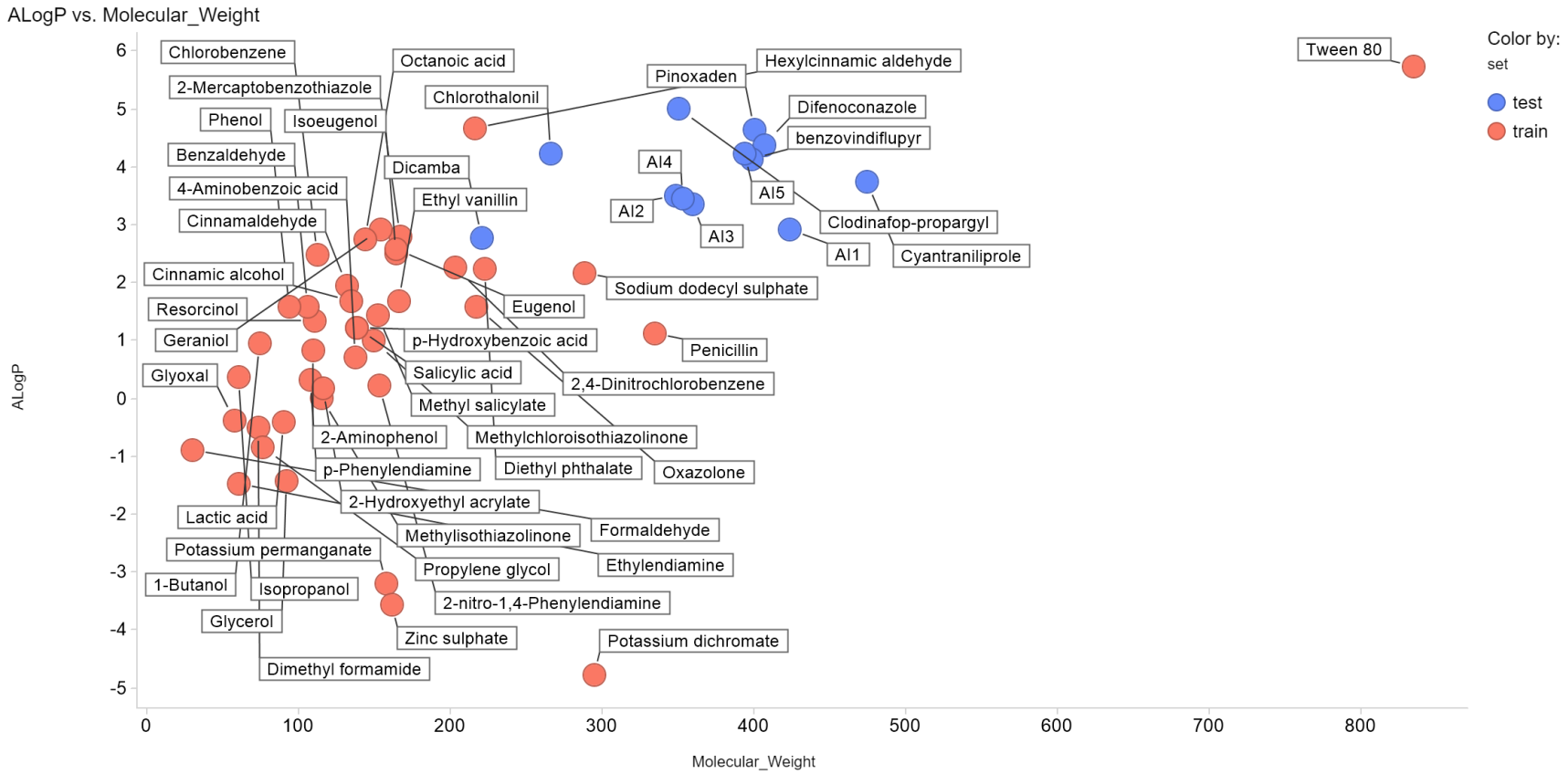
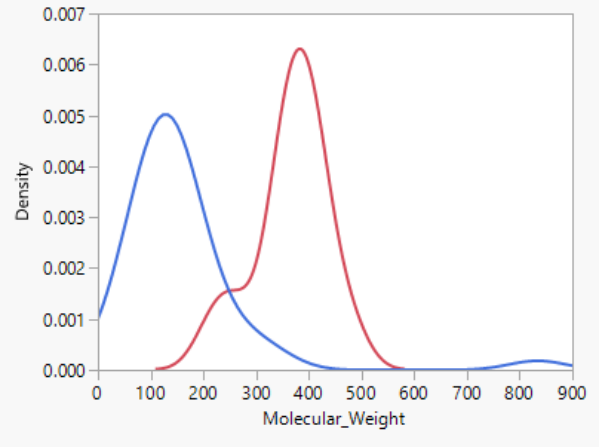


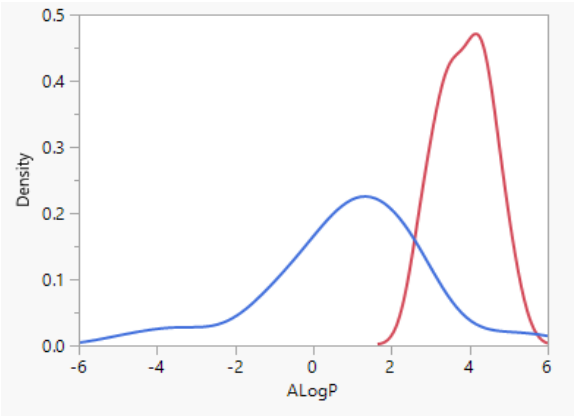
Figure 4.2 The molecular weight and Log P values of both the GARD training set (Forreryd et al., 2018) and agrochemical AIs tested

Figure 4.3 shows the comparison of the distribution of molecular weight for the GARD training set and the agrochemical AIs tested. The chemicals in the GARD training set had molecular weights of approximately 150Da and only two had molecular weights above 300Da. The molecular weights of the agrochemical AIs tested were higher, with many approximately 400Da and only two agrochemicals (dicamba and chlorothalonil) with molecular weights below 300Da. A comparison of the distribution of AlogP for the GARD training set and the agrochemical AIs tested is given in Figure 4.4. The majority of the chemicals in the GARD training set have AlogP values in the range of -2 to 4, and only two were above 4. In contrast, most of the agrochemicals tested had an AlogP of approximately 4 and none had an AlogP value below 2. In terms of the ranges of the two physicochemical properties considered, there is a difference between those of the GARD training set and the AIs tested.



Red	Agrochemical test set
Blue	GARD training set

Figure 4.3 The distribution of molecular weights of the GARD training set of compounds (Forreryd et al., 2018) and the agrochemicals tested



Red	Agrochemical test set
Blue	GARD training set

Figure 4.4 The distribution of log P values of the GARD training set of compounds and the agrochemicals tested

Following the conformal prediction analysis, the GARD assay result for dicamba was changed from being a skin sensitiser to a non-sensitiser as shown in Table 4.6. This was due to the derived Pnon-sens value of 0.16, indicating that dicamba had non-conformity to the non-sensitising group of 84% as opposed to the 86% it demonstrated for the sensitising group.

Test material	*in vivo* Study result	GARD skin prediction	Psens*	Pnon-sens**	Conformal Prediction
benzovindiflupyr	negative	positive	0.84	0.02	sensitiser
chlorothalonil	positive	positive	0.76	0.05	sensitiser
clodinafop-propargyl	positive	positive	0.85	0.02	sensitiser
cyantraniliprole	negative	positive	0.63	0.05	sensitiser
dicamba	negative	positive	0.14	0.16	non-sensitiser
difenoconazole	negative	positive	0.85	0	sensitiser
pinoxaden	positive	negative	0.11	0.4	non-sensitiser
AI1	positive	positive	0.31	0.07	sensitiser
AI2	positive	positive	0.85	0	sensitiser
AI3	positive	positive	0.63	0.05	sensitiser
AI4	negative	positive	0.85	0	sensitiser
AI5	positive	positive	0.72	0.05	sensitiser

Table 4.6 GARD assay conformal predictions of the test items

*A measure of the Test Item non-conformity compared to the calibration set. If the p-value is below the error level 0.15 the Test Item is strange compared to calibration sensitisers. A value of greater than 0.15 indicates that it belongs to the group sensitisers with 85% confidence.

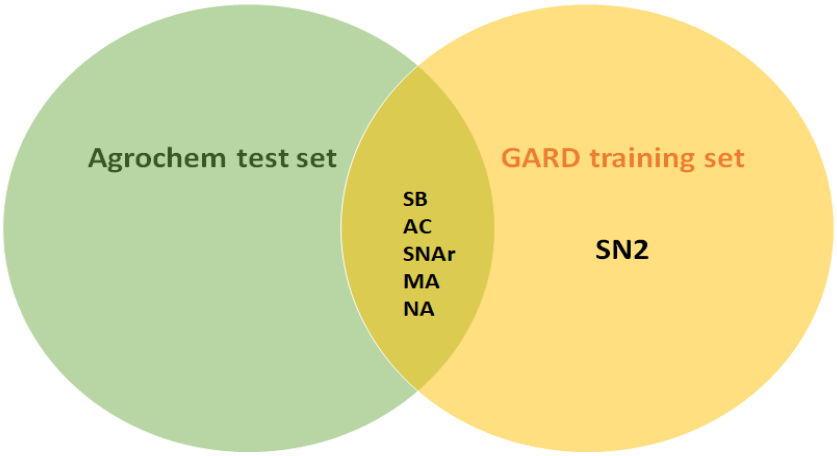
**A measure of the Test Item non-conformity compared to the calibration set. If the p-value is below the error level 0.15 the Test Item is strange compared to calibration non-sensitisers. A value above 0.15 is therefore proof that it belongs to the group non-sensitisers with a confidence of 85%.

An evaluation of the physico-chemical similarities between the GARD training set (Forreryd et al., 2018) and the test set of 12 agrochemical AIs was performed, and the results shown in Table 4.7. The evaluation focused upon the test materials' log P, MW, HBA, HBD and RB, which are molecular descriptors often associated with membrane permeability and included in the defined rules for pesticide likeness (Avram et al., 2014). The HBA and RB also demonstrated a noticeable difference in recorded median values with little difference between the two sets seen in HBD. Table 4.7 shows the differences between physicochemical values of the training and test set.

	Training set				Test set			
Properties	**Range**	**1st Quartile**	**Median**	**3rd Quartile**	**Range**	**1st Quartile**	**Median**	**3rd Quartile**
MW	30 to 834	106	138	164	221 to 473	349	377	402
Log P	-4.77 to 5.74	0.18	1.12	2.17	2.78 to 5.02	3.44	3.94	4.28
HBA	0 to 12	1	2	3	2 to 6	4.5	5	5
HBD	0 to 3	0	1	2	0 to 2	0	0	0.25
RB	0 to 19	0	1	3	0 to 7	3.75	6	7

Table 4.7 Test set versus training set molecular properties

An assessment of the chemical domains covered by both sets of chemicals was performed. The Venn diagram shown in Figure 4.5 indicates that the training set covers all the chemical domains identified in the agrochemical AI test set and also covers bimolecular nucleophilic substitution (S_N2) which was not present in the test set.



MA	Michael addition
SB	Schiff base
NA	Nucleophilic addition
AC	Acylation
SNAr	Aromatic nucleophilic substitution
SN2	Bimolecular nucleophilic substitution

Figure 4.5 The Venn diagram of the chemical domains identified in the two chemical sets i.e. the GARD training set and the agrochemicals. Training data set as indicated in Forreryd et al., 2018

4.3.3 *In Silico* Evaluation

Further evaluation using the OECD QSAR Toolbox comparing the *in vivo* study experimental results against the *in silico* profiling of both the agrochemical AI test set and GARD assay training set was performed. The structural alerts in the profilers were predictive of the skin sensitisation *in vivo* experimental outcome for the training set (Table 4.8).

Number of compounds in each class	Training set	Predicted Sensitiser	Predicted non-sensitiser
20	Exp-sens	85%	15%
20	Exp-NS	20%	80%
40			

Number of compounds in each class	Test set	Predicted Sensitiser	Predicted non-sensitiser
7	Exp-sens	71%	29%
5	Exp-NS	60%	40%
12			

Table 4.8 The OECD QSAR Toolbox prediction for skin sensitisation against the *in vivo* experimental results of the test and training sets

Exp-sens – demonstrated to have skin sensitiser potential in *in vivo* experimental studies.
Exp-NS – demonstrated to have no skin sensitiser potential in *in vivo* experimental studies.

4.4 Discussion

This study compared the predictions of the GARD assay to the results of previously conducted *in vivo* animal assays testing the skin sensitisation potential of 12 agrochemical AIs. The GARD assay identified ten of the test materials as skin sensitisers and two as non-sensitisers. The results from the GARD assay were not in agreement with the *in vivo* data for five of the 12 agrochemical AI materials tested. In order to ensure the veracity of the outcome of the GARD assay, conformal prediction analysis was performed, and this changed the outcome of the GARD assay for dicamba from being a sensitiser to a non-sensitiser.

4.4.1 *In Silico* Evaluation – Mechanistic Chemistry and Physicochemical Property Domains

In order to understand the performance of the GARD Assay compared to *in vivo* results for the AIs, their coverage in terms of mechanistic and chemical applicability domains was examined. First, an assessment was undertaken to comprehend the change in sensitisation outcome for dicamba using conformal prediction analysis (as shown in Table 4.6) and whether this could give an insight into domains. In this instance, it appears that this conformity exercise does not necessarily indicate if a chemical was within the appropriate applicability domain of the GARD assay, but rather it indicates using the model's own training set, within which of the two groups of potential outcomes the test compound is most likely to fall. Thus, the conformal method would not necessarily be able to indicate how appropriate the GARD assay is for a chemical that falls outside of the chemical space of the training set used. As such it can be determined that the use of conformal predictions is not an appropriate method to ascertain whether the agrochemical AI test materials in this study fall within the current applicability domain of the GARD assay.

To determine the possible role of mechanistic chemistry with regard to domain alerts flagged by the OECD QSAR Toolbox, each set of chemicals were investigated (note structural alerts are discussed in more detail below). No significant differences between the GARD assay training set and the agrochemical AI test set were observed during our evaluation. All of the chemical mechanisms of action important for skin sensitisation *(*Aptula and Roberts, 2006, Enoch et al., 2011) have been identified in the GARD assay training set. Thus, differences in the responses from the GARD assay and *in vivo* rodent skin sensitisation test results for the AIs tested are not as a result of any specific chemical mechanism of action for skin sensitisation being absent in the GARD training set, as it encompassed all those identified in the agrochemical AI test set. Therefore, in order to further understand why the difference in results between the GARD assay and *in vivo* experimental tests were observed, the physico-chemical properties of the chemicals in the GARD training set and the agrochemical test set were examined.

The physicochemical property domains of the GARD training set and the 12 AIs tested were compared to provide further understanding of the differences observed between the predicted GARD results and the *in vivo* experimental results. The purpose of this analysis was to determine if the sets represented different areas of chemical space, as defined by the physicochemical properties considered. Such properties are a key component of the "applicability domain" of a test assay or QSAR and other components of the applicability domain (where relevant) include structural similarity, mechanism of action, metabolism, reactivity and toxicokinetics (Dimitrov et al., 2005, Netzeva et al., 2005, van der Laan et al., 2012). The purpose of this analysis was not a full determination of the applicability domains of the AI test set and GARD assay training set. For skin sensitisation, a full analysis of applicability domain would include an analysis of the mechanistic reactivity domain associated with each chemical (Aptula et al., 2005, Aptula and Roberts, 2006, Roberts et al., 2007b). However, definition and consideration of the physico-chemical property ranges, such as compound solubility, is a key step in the assessment of technical limitations to assist in the evaluation and ultimate validation of an *in vitro* assay (Bruner et al., 1996, Worth and Balls, 2004) and assists in its correct usage. Following the evaluation of molecular descriptors of the test and training set chemicals, an apparent difference in molecular weights was observed between the test and training set indicating that a higher MW range is present in the agrochemical AI test set compared to that seen in the training set. Whilst the training set contained molecules with a MW of predominantly 50-200Da, one further compound, Tween 80 with a MW 833Da, was included in the training set. This compound is, however, benign, a non-sensitiser and is used regularly as a vehicle in toxicity studies. Thus, the inclusion of Tween 80 in the training set has expanded the MW range of this set and this range may not be representative of all the compounds contained within it. This is demonstrated by the median of the training set.

There is a substantial difference between the MW of Tween 80 and the nearest training set neighbour (penicillin). This indicated that the MW of the test set of agrochemicals was not adequately represented within the GARD training set however it is acknowledged that these were well within the

limits of absorption and skin penetration (Lipsinki et al., 2001). This means that there is a domain of chemical reactivity unaccounted for concerning the MW of the penicillin compound in the training set. As many of the agrochemicals fall within this domain, confidence in the accuracy with which the GARD assay will be able to give the correct prediction may not be strong.

In addition to the difference in MW there is also a separation between the training set log P (with a range of -4.77 to 5.74 and median of 1.12) and the test set log P values (2.78 to 5.02 with a median of 3.94). In this context the initial GARD predictions cannot be considered robust based on the current test data used in the assay. It is well reported that MW and log P have an influence on the rate of dermal absorption of chemicals (Potts and Guy, 1992b). These chemical parameters have not been used in this study to aid in the evaluation of skin sensitisation potential, instead they have been used here to identify potential differences in the chemical space between the two sets of chemicals. It has been previously reported that the most marked difference in physico-chemical properties between pharmaceuticals and agrochemicals is the lower number of hydrogen bond donors (Clarke and Delaney, 2003, Tice, 2001). Consequently the HBDs, HBAs and RBs in the training and test set groups have been compared (Clarke and Delaney, 2003). The addition of these three physico-chemical properties to this study's evaluation enabled the complete comparison of the chemical sets in accordance with Lipinski's "rule of five" and Hao and coworkers' rules for pesticide likeness (Avram et al., 2014, Clarke and Delaney, 2003, Barret, 2018, Lipinski et al., 2001, Hao et al., 2011). A clear difference in distribution can be observed in four of the five physico-chemical properties of the chemical sets that have been reviewed here. Thus, at this time there is insufficient evidence to suggest the GARD training set offers the width in range necessary to capture the agrochemical AI test set properties.

4.4.2 Review of Structural Alerts

The assessment of the presence of structural alerts for skin sensitisation, as identified from the OECD QSAR Toolbox, in the chemical structures of the GARD assay training set and agrochemical AI test set

also provided predictions that, in comparison to the *in vivo* experimental data, overestimated the skin sensitisation potential of the test set. For the test set, there was 71% agreement between experimental sensitisation and predicted sensitisation. However, only 60% of the test set agrochemical AIs with *in vivo* non-sensitising results, were associated with structural alerts for protein binding (and hence skin sensitisation) by the OECD QSAR Toolbox. This overestimation of the sensitisation potential of the agrochemical AI test set is largely in keeping with the trend observed with the GARD assay results. It should be remembered that structural alerts for protein binding (related to skin sensitisation) in the OECD QSAR Toolbox have been developed from many sources including historical skin sensitisation data. For instance, Enoch et al (2008) developed a set of structural alerts for skin sensitisation based on historical LLNA data compiled by Gerberick et al (2005). These data, and a subsequent expanded LLNA data set (Kern et al 2010), are predominantly for small, low MW compounds, the majority of which are relevant as cosmetics ingredients or represent the chemical of cosmetic ingredient space with few, or no compounds representative of agrochemicals.

The results seen in the OECD QSAR Toolbox profiling suggest that differences in chemical space can also influence skin sensitisation outcome. It may be hypothesised that the structural alerts are more informative of the skin sensitisation potential of low MW, cosmetic-like compounds than the potential for adverse outcomes in agrochemicals and specifically for our test set. In addition, the shift towards increased hydrophobicity and MW in the agrochemical AIs compared to the training set values, indicates a potential for lower skin penetration which is not accounted for. This is in line with a previous publication by Basketter et al (1992) suggesting that an important factor governing the skin sensitisation potential of halogenated chemicals, such as bromoalkanes, is their skin penetration rate (Basketter et al., 1992). To attain a more predictive set of structural alerts for agrochemicals these additional physicochemical factors and skin penetration need to be accounted for, or a factor may need to be applied to account for the dermal absorption differences. This is also an important consideration for all *in vitro* assays for skin sensitisation and is often accommodated within the weight of evidence or as part of the risk assessment.

To illustrate the issue of the assessment of halogenated compounds, dicamba is a chlorinated benzoic acid that has been used widely on a variety of crops as an effective herbicide for more than 50 years (Wang et al., 2016, Yao et al., 2015). Whilst some acids are included in the training set e.g., salicylic acid, lactic acid, benzoic acid, the GARD assay was unable to make an accurate prediction for dicamba. The GARD assay predicted dicamba to be a skin sensitiser, whilst the *in vivo* study and ECHA harmonised classification have not classified it as such. The acids present in the training set were not halogenated and the only compound present in the GARD training set that was halogenated was methylchloroisothiazolinone. As expected with agrochemicals (Jeschke, 2010), nine of the 12 compounds in the agrochemical AI test set were halogenated. This further indicates the difference between the chemistry of the two chemical sets evaluated in this study. In particular, there was discordance between the GARD and *in vivo* results for pinoxaden, which had an identified EC3 value from a previously conducted LLNA corresponding to a harmonised classification skin sensitisation Category 1A, H317 (ECHA, 2015, EFSA, 2013). This compound is outside of the applicability domain; however, this does not fully explain why this assay was unsuccessful at predicting a potent sensitiser. A potential limitation of the *in vivo* methods may also have been a factor in the differences in results seen between the GARD assay and *in vivo* methods results. The highest test material dose that could be selected for the guinea pig or LLNA skin sensitisation tests is the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation (OECD, 1992, OECD, 2010). Where observed toxicity of a given test material may have limited the highest concentration that could be tested in the *in vivo* experiments, the GARD assay was still able to use the high concentrations and investigate skin sensitisation potential at these levels. For these test substances, solubility and cytotoxicity were not limiting factors in the methodology as the maximum exposure concentration was used for negative outcomes.

The chemical space disparity that has been identified between the GARD training set and the agrochemical AI test set may have occurred because the predictive model is a machine learning classifier (a support vector machine model) that has been trained on gene signals mainly for

compounds used as, or similar to, cosmetics ingredients. The gene signal in relation to cosmetic ingredients has been learned by the model and chemicals of all domains are classified in this way. This gives each chemical a biological fingerprint relevant to cosmetics but not to agrochemicals.

4.4.3 Future work and opportunities for further improvement

The results from both the *in silico* work and the GARD assay indicate that the biological fingerprint (i.e. the changes in transcription in the genes in the Mutz-3 cells (surrogates for dendritic cells)) for skin sensitisation is not consistent across all chemicals. The GARD assay performed in the manner expected of it, in that it provided predictions of skin sensitisation potential for the agrochemical AI test set using the machine learned, biological fingerprint provided by its training set. In an attempt to improve skin sensitisation predictivity for agrochemical compounds in the GARD assay in the future, additional compounds should be added to the GARD training set with molecular weights between 300- 800Da and ALogP values of 3-5. Halogenation has not been identified as a cause of sensitisation; however unlike cosmetics, agrochemicals are intentionally biologically active and frequently halogenated. This may skew or change the biological fingerprint of this chemical set in a manner that affects the prediction produced by the GARD assay in comparison to the *in vivo* experimental results. As noted above, halogenation of compounds is important when considering skin sensitisation (Basketter et al., 1992), thus addition of halogenated compounds to the GARD assay training set may improve its capability to predict skin sensitisation of agrochemicals. The compounds added to the training set should include an increased number of different chemistries i.e., biocides and agrochemicals and this may aid to further investigate this hypothesis.

The design of the training set for machine learning in the GARD assay is a key component to adequately establish an understanding of biological outputs and how they apply to the individual domains of the AIs tested. The application of structural alerts delivers a clear understanding of the applicability domain and is required to be able to identify limitations to mechanistic chemistry in the *in vitro* assay being evaluated. However, it can be observed in this study that all reactivity domains present in the

test set are covered by the training set, and yet a nonconcordant result is observed between the *in vitro* and *in vivo* test methods. A hypothesis can be made that the physico-chemical parameters of the test and training chemical sets examined in this study also play a role in the setting of an applicability domain. This is in line with the previously made assumption that similar predictivity can be achieved by substances that are similar to those in the training set and that the applicability domain of a model would depend on the structural, physico-chemical and response information in the data used for training a model (Wilm et al., 2018). It is noteworthy that 12 compounds is a small test set to evaluate the GARD assay, the lack of overlap between the test and training set additional work needs to be conducted to address false positive and negative outcomes.

Whilst the GARD assay is not an approved OECD test guideline, it may have the potential to replace mammalian testing in a number of different chemistries as part of a weight of evidence. However, this research has demonstrated the need for the GARD training set to be expanded, in particular to include agrochemical compounds that occupy a different chemical space in terms of size and hydrophobicity. Additional confidence needs to be demonstrated or limitations to the assay identified, before the GARD assay can be considered for use as a replacement to animal testing. Its appropriate use would be in conjunction with test methods that focus on other key events of the skin sensitisation AOP. Validation of new alternative methods using different chemistries to ensure robustness of *in vitro* assays and scientifically reliable results across chemical domains is crucial.

5.0 CHAPTER FIVE - USE OF SENS-IS IN DETERMINING THE SKIN SENSITISATION OF AGROCHEMICAL FORMULATIONS

5.1 Introduction

The previous chapters have explored the use of *in vitro* methods (DPRA, KeratinoSens™ and h-CLAT) to determine the skin sensitisation potential of agrochemical formulations. The *in vitro* approaches investigated were 2D cell-based models which resulted in the identification of an apparent limitation in each of the methods, namely that the 2D cell model methodology used in each of the assays required the agrochemical products to form a homogenous solution with a solvent in order to be tested. However, it was not always possible to form that homogenous solution and the lack of it may have led to a disruption of the composition of the agrochemical formulation. As such, these *in vitro* systems did not give a true representation of the formulation used or applied in real life application. In keeping with these observations, this research examined the use of a 3D *in vitro* model that allowed for the direct application of the agrochemical formulation in its intended manufactured form. The hypothesis of this chapter is that the use of the SENS-IS assay should allow for the direct application of the agrochemical formulations to the test system and, as such, provide comparative results to *in vivo* vertebrate skin sensitisation studies.

The SENS-IS assay is an *in vitro* method that utilises a three-dimensional Reconstructed Human Epidermis (RHE) model, known as the EpiSkin™, to predict the skin sensitisation potential and, where positive, potency of a given test material (Cottrez et al., 2015). SENS-IS is one of several reconstructed 3D cultured human skin models which are available and used as alternatives to human skin hazard assessment experiments. For example, epiCS™ (Henkel), EpiDerm™ (MatTek) and EpiSkin™ (skin ethic laboratories, St. Philippe, France) are used in the assessment of dermal irritation, permeability and corrosivity potential of chemical compounds. These are widely accepted (e.g. in industry) and are listed as test systems in the OECD Test Guidelines 439 and 431 (OECD, 2019b, OECD, 2019c).

The EpiSkin™ model used in the SENS-IS assay is comprised of non-transformed, adult, human-derived epidermal keratinocytes, which have been cultured on a collagen substrate at the air-liquid interface

(OECD, 2019c) in conditions which permit terminal differentiation and the reconstruction of an epidermis with a functional stratum corneum. This multi-layered, highly differentiated model of the human epidermis exists at different stages of maturity. The model is histologically similar to the *in vivo* human epidermis (Netzlaff et al., 2007), though structural differences between RHE models (including the EpiSkin™) have been reported and investigated (Kano et al., 2011). Kano et al (2011) concluded that histological observations suggested that structural differences could be reasons for differences in the skin permeation of compounds among the different RHE models. They suggested a combination of histological analysis and RHE model assay results may be the most effective way to utilise a 3D skin model in the assessment of test substance effects on the skin.

Whilst widely used, two dimensional skin cell cultures such as those used in models discussed in earlier chapters (i.e. the GARD assay, KeratinoSens, h-CLAT) have "an overt lack of physiological relevance" (Klicks et al., 2017). These 2D cultures omit the principal functions of the skin such as cell sheeting and layering, barrier function, immune function, and blood perfusion. However, the 3D EpiSkin™ model consists of a stratum corneum containing intracellular lamellar lipid layers, an organised basal spinous and granular layers to represent those found *in vivo* (OECD, 2019c). The human keratinocytes cultured for the development of the EpiSkin™ test system are obtained from healthy consenting donors during plastic surgery. The Human Immunodeficiency Virus along with hepatitis B and C tests are performed on donor bloods as well as the bacterial and fungal sterility verification of the cells and absence of *Mycoplasma* (Cottrez et al., 2017).

A noted disadvantage of the use of 3D skin models is that there tends to be a high batch-to-batch variability which can make result replication difficult (Kano et al., 2011). Specifically, in the SENS-IS assay some quality controls are performed by the EpiSkin™ provider and are required to fulfil the following criteria before assay use:

- Histology scoring (Haematoxylin eosin and safran (HES) stained vertical paraffin section, n=6) ≥ 19.5.

- IC50 determination (sodium lauryl sulfate concentration, MTT test, n=14) ≥ 1 mg/ml. This is the lower limit for an EpiSkin™ batch. With the upper limit, as reported in the OECD *in vitro* skin irritation test guideline, being IC50 = 3.0 mg/ml (OECD, 2019c).

Once the test criteria have been applied to the EpiSkin™ test system for the required amount of time (as indicated in Materials and Methods), evaluation criteria are used to determine the test materials' potential to cause skin sensitisation or irritation, with sensitisation being the ultimate endpoint. The SENS-IS assay uses a genomic signature to distinguish sensitisers from non-sensitisers. A test material is considered an irritant if it induces the overexpression of at least 15 genes of a group of 24 genes referred to as the "IRRITANT" gene set for this assay (Cottrez et al., 2017). The sensitisation endpoint focuses on two groups of gene sets. Specifically, the "SENS-IS" group, which contains 21 genes and the "redox" group which contains 17 genes. The test substance is considered a sensitiser if it induces the over expression of at least 7 genes in either or both of those gene groups i.e., a greater than 1.25-fold increase in comparison to the mean value of the phosphate buffer saline solution and olive oil negative controls. The expression of these biomarkers is measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) (Cottrez et al., 2016). In order to also evaluate potency, the lowest test concentration that produces a positive response by inducing the required gene fold increase in the "SENS-IS" or "redox" groups, is used to allocate a potency classification for the tested material. The majority of *in vitro* skin sensitisation tests lack a quantitative aspect and are not able to deliver the information necessary to estimate safe exposure levels to allow for a quantitative risk assessment to be conducted (Cottrez et al., 2020).

The identity of the irritation group of genes used in the SENS-IS assay has been published (Cottrez et al., 2016) however the identity of the genes that make up the "SENS-IS" and "redox" groups has only been disclosed in the assay patent (Mehling et al., 2019). The redox gene group consists of 17 genes that have Anti-oxidant Response Element (ARE) in their protective signals induced through the

interaction with sensitising compounds binding to cysteine amino acids of the keap1-NRF2 complex (Petry et al., 2018, Cottrez et al., 2016). The keap1-NRF2 system plays a vital role in dealing with various stressors. It concomitantly regulates many genes that contribute to the response to intrinsic and extrinsic stressors. These include oxidants and xenobiotics that may have been metabolised to electrophilic intermediates by cytochrome P450 enzymes. The keap1-NRF2 ligand binding interaction is also utilised in the KeratinoSens™ assays evaluation of the second key event in the skin sensitisation adverse outcome pathway (OECD, 2018b, ECVAM, 2014a, Settivari et al., 2015b).

Electrophilic compounds are thought to attack the Keap1-Nrf complex causing the dissociation of the Nrf2 (nuclear factor-erythroid 2-related factor 2) compound from Keap1. This leads to a nuclear accumulation of Nrf2 in an induced state. The induced Nrf2 compound forms a heterodimeric partnership with a small Maf proteins, as the Maf protein confers a unique feature on the Antioxidant Response Element (ARE) sequence. It is this Nrf2-maf-ARE combination that is responsible for the induction of genes encoding detoxifying enzymes to counteract the electrophilic compounds that triggered the initial dissociation of Nrf2 from Keap1 (Uruno and Motohashi, 2011). The ARE Nrf2 interaction is utilised in both the SENS-IS and Keratinosens assays (Settivari et al., 2015b, Cottrez et al., 2020).

The SENS-IS gene group consists of 21 genes. These genes are involved in inflammation, danger signals and cell migration which address the activation of dendritic cells by sensitising chemicals through a complex cascade of events (Cottrez et al., 2016). The focus on these particular genetic biomarkers enables the SENS-IS assay to determine if the KE3 of the skin sensitisation AOP (as outlined in Chapter One) is being activated by the test material in question. The specific genes markers monitored by the SENS-IS assay are confidential to Immunosearch and as such have not been reported in this thesis.

The aim of this chapter is to explore the use of the SENS-IS 3D *in vitro* model, in order to understand if it can with good confidence accurately predict the skin sensitisation potential and potency of complex mixtures, specifically here agrochemical formulations.

5.2 Materials and Methods

A third-party CRO was contracted to conduct the *in vitro* SENS-IS assay tests on the ten agrochemical formulations listed in Table 5.1. The details of the experiments conducted by the CRO, Immunosearch (Grasse, France), are provided in this materials and methods section with further details in the Appendix 9.

5.2.1 Test Materials

Ten agrochemical formulations were used as the test materials. They are as indicated in Table 4.1 and were provided by Syngenta Ltd, UK. The ten formulations selected had previously been tested through *in vivo* methods for skin sensitisation (in the LLNA or Buehler assay) and irritation (Draize skin irritation - OECD test guideline 404). The results of these *in vivo* tests were provided by Syngenta ltd and are given in Table 4.1, however the specific study report details remain proprietary information and as such are not reported here. The test materials were dissolved at 0.1%, 1%, 10% or 50% in PBS, DMSO or olive oil dependent upon the results of the solubility assessment. A solubility assessment was initially conducted to determine which of these three vehicles was the most suitable for each test material.

5.2.2 Solubility assessment

Each test material was diluted at two concentrations, 10% and 50%, in three separate vehicles, PBS, DMSO and olive oil. A solubility check was conducted based on the visual homogeneity observed in the three vehicles at these two concentrations. If multiple vehicles provided a homogenous solution deemed suitable for the assay, the corresponding test material/vehicle solutions were both tested in the SENS-IS assay at 10%. The vehicle solution that provided the highest gene induction response was used for the main test. The assay was continued with the selected vehicle at different concentrations, above or below 10% depending on the first analysis outcome. Positive results led to a reduced concentration for testing and a negative result led to an increase in the concentration used. Specific

details of the vehicles, positive and negative control materials used in this SENS-IS assay research are captured in Appendix 9.

Thirty µL of each formulated test material solution was applied to the top layer of the reconstituted human epidermis (Episkin™ model) with a micropipette. This application was approximately 26 µL/cm^2 on the Episkin™ layer and was applied using a positive displacement pipette and gently spread evenly to ensure it covers the top layer.

The test material was allowed to rest on the test system surface layer for 15 minutes at room temperature. After the 15 minutes exposure, the Episkin™ unit was rinsed with PBS and incubated at 37°C, 5% CO_2 for 6 hours.

After the 6-hour incubation, the complete epidermis was transferred into a vial to be snap frozen in liquid nitrogen. The epidermis model was then briefly placed in a 1ml RNAzol solution and homogenized to isolate the total RNA using two steel beads with a TissueLyser (Qiagen). Following centrifugation, the supernatant was collected, 0.2 ml of bromochloropropane was added and the mixture was vortexed. The vortexed mixture was centrifuged at 12000g for 15 minutes at 4°C. After vortexing the aqueous phase was collected and 1mL ethanol was combined with it. This mixture was immediately mixed by pipetting and loaded onto a RNeasy spin column and placed in a 2ml collection tube. The total RNA was extracted in concordance with the manufacturer's instructions (Cottrez et al., 2016).

After reverse transcription, quantitative gene expression was measured by RT-PCR using a SYBR green real-time PCR master mix buffer with 0.4µM of each oligonucleotide primer in a total volume of 10µL. The reaction was conducted inside of a rapid high-throughput, plate-based real-time PCR amplification and detection instrument (Roche's LightCycler® 480 system, France). Amplification followed and the detection instrument was conducted at 95°C with a 5-minute hold, after which 40 amplification cycles were carried out (at 95°C for 10 seconds and annealing at 60°C for 10 seconds) and this was completed with a 72°C cycle for 10 seconds.

The relative amount of each transcript was normalised to the amount of the mean expression levels of 3 house-keeping genes transcripts (Glucuronidase ß, ß2 microglobuline, and “non-POU domain containing, octamer-binding” (NONO)) (Cottrez et al., 2020).

For each analysis three negative controls (PBS, olive oil or DMSO treated skin), a positive irritation control (5% SLS) and a positive sensitisation control (TNBS at 1%) were included. The test product and the controls were tested in at least two experiments (using different batches of Episkin models). Further experiments were conducted if invalid results were obtained in the previous experiments.

5.2.3 Acceptance criteria

As indicated previously, the initial integrity of the Episkin™ test system must be fit for the purposes of this assay prior to study initiation. The IC50 acceptance criteria of the Episkin units must be ≥1.2 mg/mL. The purpose of the irritant gene set in the SENS-IS assay evaluation criteria is to take into account genes that are not thought to be associated with skin sensitisation, but that are demonstrated to be overexpressed after exposure to a test compound, i.e. the irritation group takes into account non-specific gene expression due to cell stress (Petry et al., 2018). Amongst the irritation group of genes measured by the SENS-IS assay is the HSPAA1 gene. The expression of the HSPAA1 gene is used by the assay to measure tissue destruction. Following test material exposure to the epidermis, if the expression of the HSPAA1 gene is shown to be above 10% of that shown in the tissues exposed to the negative controls, the acceptance criteria for that run are not considered to have been met.

In addition, tissue damage is also measured in the SENS-IS assay if greater than the irritation gene expression. If more than 20 genes are overexpressed from the irritant gene group, the result of the SENS-IS run is deemed to be inconclusive and the test substance is reanalysed at a lower concentration (Petry et al., 2018). This process of understanding the highest concentration that may be tolerated by

the assay or test system is similarly performed in the preliminary phases of other skin sensitisation tests, such as the local lymph node assay and guinea pig assays (OECD, 1992, OECD, 2010). The h-CLAT assay, which focuses on dendritic cell activation for biomarkers, uses a dose range finding assay prior to the main test itself to understand the test material concentration that will result in 75% cell viability (OECD, 2018a). The test material cytotoxicity is taken into account by the assays listed here when trying to evaluate skin sensitisation potential of a test material. This could be considered an essential phase of these assays to address potential for misinterpretation of irritation for sensitisation, though it may not necessarily address the potential for irritation to potentiate sensitisation potential of a complex mixture (Corvaro et al., 2016).

Potency classifications for the test material identified as positive skin sensitisers by the SENS-IS assay are assigned based upon the lowest tested concentration to produce a positive response. A chemical is classified as an extreme, strong, moderate, or weak skin sensitiser if it produces a positive response at 0.1%, 1%, 10% or 50%, respectively. If negative at all concentrations, then the tested material is considered not to be a skin sensitiser (Cottrez et al., 2016).

5.3 Results

The SENS-IS assay was conducted on ten selected agrochemical formulations. These ten formulations had had previously been tested in vivo, for skin sensitisation through the LLNA or Buehler assay and also for skin irritation in the rabbit using OECD test guideline 404 (OECD, 2015a). The results of the SENS-IS assay previously conducted *in vivo* experiments are summarised in Table 5.1 and explained in more detail below. The raw data from the triplicate experimental runs is for all ten of the test formulations are presented in Appendix 10.

Formulation number	Formulation type*	Active ingredient	LLNA/Buehler result	EC3**	SENS-IS sensitisation result	*In vivo* irritation result	SENS-IS irritation result
SYN 1	EC	Difenoconazole/ Benzovindiflupyr	Sensitiser	64.1%	Positive Moderate sensitiser	Non-irritant	Irritant
SYN 2	FS	Acibenzolar-S-methyl	Sensitiser	37.0%	Positive Moderate sensitiser	Non-irritant	non-irritant
SYN 3	SC	Cyantraniliprole/Diafenthiuron	Non-sensitiser		Non-sensitiser at up to 100%	Non-irritant	non-irritant
SYN 4	FS	Metcamifen	Non-sensitiser		Positive Weak sensitiser	Non-irritant	non-irritant
SYN 5	WG	Mesotrione/Dicamba/Nicosulfuron	Non-sensitiser		Non-sensitiser at up to 100%	Mild irritant	non-irritant
SYN 6	SC	Chlorothalonil	Buehler study - sensitiser		Positive Extreme sensitiser	Mild irritant	non-irritant
SYN 7	EC	Pinoxaden/Cloquintocet-mexyl	Buehler study - sensitiser		Non-sensitiser at up to 100%	Moderate irritant	non-irritant
SYN 8	SC	AI1	Sensitiser	0.79%	Positive Strong sensitiser	Non-irritant	non-irritant
SYN 9	SC	Chlorantraniliprole	Non-sensitiser		Non-sensitiser at up to 100%	Non-irritant	Irritant
SYN 10	WG	Acetamiprid/AI1	Sensitiser	0.23%	Positive Strong sensitiser	Non-irritant	non-irritant

Table 5.1 Summary of the main outcomes of the SENS-IS assay in addition to existing *in vivo* skin sensitisation and irritation data.

*EC-emulsifiable concentrate, FS-flowable concentrate for seed treatment, SC-suspension concentrate, WG- water dispersible granules

**EC3 is a potency value that indicates the estimated concentration of test substance that induces a stimulation index of 3.0 in the LLNA

5.3.1 SYN1 Formulation

SYN1 is an Emulsifiable Concentrate (EC) formulation with two main active ingredients, difenoconazole and benzovindiflupyr. An *in vivo* skin irritation study had also been previously conducted on the SYN1 formulation in accordance with OECD Test Guideline 404 (OECD, 2015a). A non-irritant result was obtained from that study. In addition to the irritation study, a LLNA (OECD, 2010) study was previously conducted using concentrations of 25%, 50% and 100% of SYN1. Both *in vivo* animal tests were previously conducted by Syngenta Ltd and the results of these tests are captured in Table 5.1. The formulation was demonstrated to be skin sensitiser in the LLNA study, with an EC3 value of 64.1% equating to a skin sensitisation category 1B classification in accordance with CLP and UN GHS regulations (ECHA, 2017b, GHS, 2017). Table 5.2 summarises the number of genes expressed in the SENS-IS assay following exposure to the different concentrations of SYN1 in DMSO and PBS.

SYN1 formulation **Number of overexpressed genes**	**1%** **DMSO**	**10%** **PBS**	**10%** **DMSO**	**50%** **DMSO**
Irritation	6	14	16	23
SENS-IS	3	7	3	4
REDOX	3	7	7	8
Irritation Outcome	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	POSITIVE	POSITIVE

Table 5.2 Summary of the results of the SENS-IS assay for the SYN1 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Red cell = positive gene induction criteria achieved

Irritation indication

The SYN1 test material produced an irritant result at the 50% and 10% concentrations in DMSO, with more than 15 genes overexpressed in the irritation gene group. At 1% SYN1 concentration < 7 genes of the irritation gene group were induced.

Sensitisation potential

1% SYN1 in DMSO did not lead to an over-induction of ≥ 7 genes in the SENS-IS or REDOX groups. However, the application of the two 10% SYN1 formulations (in PBS and DMSO) resulted in an over-induction of ≥ 7 genes in the SENS-IS and/or Redox groups. This ≥ 7 gene expression was also seen at the 50% SYN1 concentration tested. Under the experimental conditions of this study and in accordance with the potency classification scheme for the SENS-IS assay (Cottrez et al., 2020), the SYN1 formulation tested would be placed into the moderate skin sensitiser category.

5.3.2 SYN2 Formulation

Formulation SYN2 is a Flowable concentrate for a Seed treatment (FS) agrochemical product. The main active ingredient present in this formulation is acibenzolar-s-methyl. A previously conducted *in vivo* skin irritation study demonstrated the SYN2 formulation to be non-irritant to skin. In addition to the irritation study, a LLNA was previously performed on this formulation at concentrations of 25%, 50% and 100% as captured in Table 4.1. The results of the LLNA on this formulation demonstrated it to be a skin sensitiser to the mouse, with an EC3 value of 37% equating to a skin sensitisation category 1B classification in accordance with CLP and UN GHS regulations.

SYN2 was applied to the epidermis model (Episkin^{TM}) at 50%, 10% and 1% dissolved in PBS. Table 5.3 provides a summary of the number of genes expressed in the SENS-IS assay following exposure to SYN2 at the three concentrations.

SYN2 formulation Number of overexpressed genes	1% PBS	10% PBS	50% PBS
Irritation	4	12	13
SENS-IS	5	10	10
REDOX	6	12	15
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	POSITIVE

Table 5.3 Summary of the results of the SENS-IS assay for the SYN2 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

Fewer than 15 genes of the irritation gene group expressed at up to 50% of the SYN2 FS formulation. Therefore, it can be considered that the SYN2 test material did not produce an irritant result at the any of the concentrations tested.

Sensitisation potential

1% SYN2 in PBS did not lead to an induction of 7 or more genes in the SENS-IS or REDOX groups. However, the application of 10% and 50% of the SYN2 formulation to the EpiDerm model led to an over-induction of more than 7 genes in the SENS-IS and Redox groups. Under the experimental conditions of this study and in accordance with the potency classification scheme for the SENS-IS assay (Cottrez et al., 2020), as the lowest concentration of with more than 7 genes overexpressed was 10%, the SYN2 formulation tested would be placed into the moderate skin sensitiser category.

5.3.3 SYN3 Formulation

SYN3 is a Suspension Concentrate (SC) agrochemical formulation with two main active ingredients, cyantraniliprole and diafenthiuron. An *in vivo* skin irritation study had also been previously conducted on the SYN3 formulation in accordance with OECD Test Guideline 404. A non-irritant result was obtained from that study. In addition to the irritation study, a LLNA was previously performed on this

formulation at concentrations of 0.25%, 0.5% and 1%. The results of that *in vivo* skin sensitisation assay demonstrated that the SYN 3 formulation was not a skin sensitiser in the mouse.

In the assessment of the SENS-IS assay the skin sensitisation potential of the SYN3 formulation was evaluated. SYN3 was applied to the Episkin™ model undiluted (100%) and at 50% and 10% dissolved in PBS. The resulting gene expression following exposure to the SYN3 concentrations is shown in Table 5.4.

SYN3 formulation **Number of overexpressed genes**	**10%** **PBS**	**50%** **PBS**	**PURE**
Irritation	0	1	3
SENS-IS	6	3	2
REDOX	4	1	2
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE

Table 5.4 Summary of the results of the SENS-IS assay for the SYN3 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

Fewer than 15 genes of the irritation gene group expressed at up to 100% of the SYN3 SC formulation. Therefore, the SYN3 test material did not produce an irritant result at the any of the concentrations tested.

Sensitisation potential

At all concentrations of SYN3 that the Episkin™ model was exposed to, fewer than 7 genes were over-expressed in either of the skin sensitisation gene groups. The SYN 3 formulation was not considered to be a skin sensitiser under the conditions of the SENS-IS assay.

5.3.4 SYN4 Formulation

Formulation SYN4 is a Flowable concentrate for Seed treatment (FS) agrochemical product. The main active ingredient present in this formulation is Metcamifen. A previously conducted *in vivo* skin irritation study (OECD Test Guideline 404) demonstrated the SYN2 formulation to be non-irritant to skin. A LLNA had also been previously conducted to assess this formulation's skin sensitisation potential. It was performed on this formulation at concentrations of 25%, 50% and 100%. The results of that *in vivo* skin sensitisation assay demonstrated that the SYN 4 formulation was not a skin sensitiser in the mouse.

SYN4 was applied to the epidermis model (Episkin^{TM}) pure (100%), at 50% and 10% in PBS and at 10% in DMSO. Table 5.5 provides a summary of the number of genes expressed in the SENS-IS assay following exposure to SYN4 at the three concentrations.

SYN4 formulation **Number of overexpressed genes**	**10%** **DMSO**	**10%** **PBS**	**50%** **PBS**	**PURE**
Irritation	4	7	1	14
SENS-IS	5	3	1	11
REDOX	3	2	5	11
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE

Table 5.5 Summary of the results of the SENS-IS assay for the SYN4 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

The SYN4 test material did not produce an irritant result at the any of the concentrations tested. Fewer than 15 genes of the irritation gene group expressed at up to 100% of the SYN4 FS formulation.

Sensitisation potential

10% and 50% concentrations of the SYN4 formulation did not lead to an over-induction of more than 7 genes in the SENS-IS or REDOX groups. The application of 50% of the SYN4 formulation to the EpiDerm model led to an over-induction of 11 genes in each of the SENS-IS and Redox groups.

Under the experimental conditions of this study and in accordance with the potency classification scheme for the SENS-IS assay the SYN4 formulation is considered to be a skin sensitiser with the lowest concentration triggering the overexpression of more than 7 genes was 50%, the SYN4 formulation is considered to be a weak skin sensitiser.

5.3.5 SYN5 Formulation

SYN5 is a water dispersible granules (WG) agrochemical formulation with three active ingredients, mesotrione, dicamba and nicosulfuron. The previously conducted *in vivo* skin irritation study on the SYN5 formulation produced a mild irritant result. In addition to the irritation study, a LLNA was previously performed on this formulation at concentrations of 10%, 25% and 50%. The results of that *in vivo* skin sensitisation assay demonstrated that the SYN 5 formulation was not a skin sensitiser in the mouse. In the assessment of the SENS-IS assay the skin sensitisation potential of the SYN5 formulation was evaluated. SYN5 was applied to the Episkin™ model undiluted (100%), at 50% and 10% dissolved in PBS and 10% in DMSO. Table 5.6 captures the gene expression in the SENS-IS assay following exposure to the SYN5 concentrations.

SYN5 formulation **Number of overexpressed genes**	**10%** **DMSO**	**10%** **PBS**	**50%** **PBS**	**Pure**
Irritation	4	7	13	14
SENS-IS	3	1	5	6
REDOX	5	6	6	5
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

Table 5.6 Summary of the results of the SENS-IS assay for the SYN5 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

Fewer than 15 genes of the irritation gene group were over-expressed at up to 100% of the SYN5 WG The SYN5 test material did not produce an irritant result at any of the concentrations tested. formulation.

Sensitisation potential

At all concentrations of SYN5 that the Episkin™ model was exposed to, fewer than 7 genes were overexpressed in either of the skin sensitisation gene groups. The SYN 5 formulation was not considered to be a skin sensitiser under the conditions of the SENS-IS assay.

5.3.6 SYN6 Formulation

Formulation SYN6 is a Suspension Concentrate (SC) agrochemical product. The main active ingredient present in this formulation is chlorothalonil. The previously conducted *in vivo* skin irritation study on the SYN6 formulation produced a mild irritant result. In addition to the irritation study, a Buehler guinea pig skin sensitisation test (OECD, 1992) was previously conducted on this formulation. The induction phase of the Buehler assay was conducted with 100% concentration of the SYN6 formulation and the challenge phase was conducted with 35%. The result of that Buehler study showed that the SYN6 formulation was a skin sensitiser in the guinea pig.

SYN6 was applied to the epidermis model (Episkin™) at 50%, 10%, 1% and 0.1% dissolved in PBS. Table 5.7 summarises the number of genes expressed in the SENS-IS assay following exposure to the four different concentrations of SYN6 in PBS.

SYN6 formulation Number of overexpressed genes	0.1% PBS	1% PBS	10% PBS	50% PBS
Irritation	4	10	14	14
SENS-IS	8	5	10	5
REDOX	7	13	8	8
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	POSITIVE	POSITIVE	POSITIVE	POSITIVE

Table 5.7 Summary of the results of the SENS-IS assay for the SYN6 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

The SYN6 test material did not produce an irritant result at the any of the concentrations tested. Less than 15 genes of the irritation gene group expressed at up to 50% of the SYN6 SC formulation.

Sensitisation potential

All concentrations of the SYN6 formulation tested in this assay (50 – 0.1%) lead to an overexpression of ≥ 7 genes in the SENS-IS and/or Redox groups. Under the experimental conditions of this study and in accordance with the potency classification scheme for the SENS-IS assay, as the lowest concentration of ≥ 7 gene overexpression was 0.1%, the SYN6 formulation tested was considered to be an extreme skin sensitiser.

5.3.7 SYN7 Formulation

SYN7 is an Emulsifiable Concentrate (EC) agrochemical formulation with two active ingredients, pinoxaden and cloquintocet. The previously conducted *in vivo* skin irritation study on the SYN7 formulation produced a moderate irritant result. In addition to the irritation study, a 9 induction Buehler guinea pig skin sensitisation assay was previously conducted on this formulation. The Buehler assay was conducted with 5% of the SYN7 in induction phase, with 0.1 and 0.01% used in the challenge

phase. The result of that Buehler assay was that the SYN7 formulation was demonstrated to be a skin sensitiser in the guinea pig.

For the SENS-IS assay the SYN7 formulation was applied to the Episkin™ model undiluted (100%), at 50% and at 10% dissolved in PBS. The number of genes expressed in the SENS-IS assay following exposure to the three different concentrations of SYN7 are presented in table 5.8.

SYN7 formulation **Number of overexpressed genes**	**10%** **PBS**	**50%** **PBS**	**PURE**
Irritation	6	1	3
SENS-IS	4	3	1
REDOX	4	3	3
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE

Table 5.8 Summary of the results of the SENS-IS assay for the SYN7 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

The SYN7 test material did not produce an irritant result at any of the concentrations tested. Fewer than 15 genes of the irritation gene group were expressed at up to 100% of the SYN7 EC formulation.

Sensitisation potential

At all concentrations of SYN7 that the Episkin™ model was exposed to, less than 7 genes were over-expressed in either of the skin sensitisation gene groups. The SYN7 formulation was not considered to be a skin sensitiser under the conditions of the SENS-IS assay.

5.3.8 SYN8 Formulation

Formulation SYN8 is a Suspension Concentrate (SC) agrochemical product. The main active ingredient present in this formulation is AI1. The previously conducted *in vivo* skin irritation study on the SYN8 formulation produced a non-irritant result. In addition to the irritation study, a LLNA was previously performed on this formulation at concentrations of 1%, 2.5%, 10% and 25%. The results of that *in vivo*

skin sensitisation assay demonstrated that the SYN 8 formulation is a skin sensitiser in the mouse, with an EC3 value of 0.79% equating to a skin sensitisation category 1A classification in accordance with CLP and UN GHS regulations.

SYN8 was applied to the epidermis model (Episkin™) at 50%, 10%, 1% and 0.1% dissolved in PBS. The number of genes expressed in the SENS-IS assay following exposure to the four different concentrations of SYN8 are presented in table 5.9.

SYN8 formulation **Number of overexpressed genes**	**0.1%** **PBS**	**1%** **PBS**	**10%** **PBS**	**50%** **PBS**
Irritation	1	2	6	13
SENS-IS	4	3	7	1
REDOX	4	9	8	7
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	POSITIVE	POSITIVE

Table 5.9 Summary of the results of the SENS-IS assay for the SYN8 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

Fewer than 15 genes of the irritation gene group expressed at up to 50% of the SYN8 SC formulation. Therefore, the SYN8 test material did not produce an irritant result at the any of the concentrations tested.

Sensitisation potential

0.1% SYN8 did not lead to an over-induction of ≥ 7 genes in the SENS-IS or REDOX groups. The application of 1%, 10% and 50% of the SYN8 formulation to the EpiDerm model lead to an over-induction of ≥ 7 genes in the SENS-IS and/or Redox groups.

Under the experimental conditions of this study and in accordance with the potency classification scheme for the SENS-IS assay, as the lowest concentration of ≥ 7 gene overexpression was 1%, the SYN8 formulation tested was placed into the strong skin sensitiser category.

5.3.9 SYN9 Formulation

SYN9 is a Suspension Concentrate (SC) agrochemical formulation. The main active ingredient present in this formulation is chlorantraniliprole. The previously conducted *in vivo* skin irritation study on the SYN9 formulation produced a non-irritant result. In addition to the irritation study, a LLNA was previously performed on this formulation at concentrations of 25%, 50%, and 100%. The results of that *in vivo* skin sensitisation assay demonstrated that the SYN 9 formulation did not cause skin sensitisation in the mouse under the conditions of the LLNA study.

SYN9 was applied to the epidermis model (Episkin^{TM}) at 100%, 50% and 10% dissolved in PBS and DMSO as indicated in the table below. Table 5.10 summarises the number of genes expressed in the SENS-IS assay following exposure to the different concentrations of SYN9 in PBS and DMSO.

SYN9 formulation **Number of overexpressed genes**	**10%** **DMSO**	**10%** **PBS**	**50%** **PBS**	**100%** **PBS**
Irritation	8	6	17	19
SENS-IS	4	0	4	4
REDOX	**4**	**5**	**2**	**5**
Irritation Outcome	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

Table 5.10 Summary of the results of the SENS-IS assay for the SYN9 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

The SYN9 test material produced an irritant result at the 100% and 50% concentrations in both DMSO and PBS vehicles, with more than 15 genes expressed in the irritation gene group. At 10% SYN9 concentration fewer than 7 genes of the irritation gene group were induced.

Sensitisation potential

At all concentrations of SYN9 that the Episkin™ model was exposed to, less than 7 genes were over-expressed in either of the skin sensitisation gene groups. The SYN 9 formulation was not considered to be a skin sensitiser under the conditions of the SENS-IS assay.

5.3.10 SYN10 Formulation

Formulation SYN10 is a WG agrochemical product. The two main active ingredients present in this formulation are acetamiprid and AI1. The previously conducted *in vivo* skin irritation study on the SYN10 formulation produced a non-irritant result. In addition to the irritation study, a LLNA was previously performed on this formulation at concentrations of 0.1%, 1%, 10% and 50%. The results of that in vivo skin sensitisation assay demonstrated that the SYN 10 formulation is a skin sensitiser in the mouse, with an EC3 value of 0.23% equating to a skin sensitisation category 1A classification in accordance with CLP and UN GHS regulations.

SYN10 was applied to the epidermis model (Episkin™) at 10%, 1% and 0.1% dissolved in PBS and DMSO as shown in the table below. Table 5.11 provides a summary of the number of genes expressed in the SENS-IS assay following exposure to SYN10 at the three concentrations.

SYN10 formulation **Number of overexpressed genes**	**0.1%** **DMSO**	**1%** **DMSO**	**10%** **DMSO**	**10%** **PBS**
Irritation	6	3	12	13
SENS-IS	2	3	1	7
REDOX	3	11	8	9
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	POSITIVE	POSITIVE

Table 5.11 Summary of the results of the SENS-IS assay for the SYN10 formulation, specifically the number of genes overexpressed and the overall outcome for irritation and skin sensitisation

Irritation indication

The SYN10 test material did not produce an irritant result at the any of the concentrations tested. Less than 15 genes of the irritation gene group expressed at up to 10% of the SYN10 WG formulation.

Sensitisation potential

0.1% SYN10 did not lead to an over-induction of ≥ 7 genes in the SENS-IS or REDOX groups. The application of 1% and 10% of the SYN10 formulation to the epiderm model led to an over-induction of ≥ 7 genes in the SENS-IS and/or Redox groups. Specifically, the 1 and 10% SYN10 in DMSO lead to ≥ 7 gene over-expression in the REDOX group. The 10% SYN10 in PBS lead to ≥ 7 gene over-expression in both the REDOX and SENS-IS gene groups.

Therefore, under the experimental conditions of this study and in accordance with the potency classification scheme for the SENS-IS assay, as the lowest concentration of ≥ 7 gene overexpression was 1%, the SYN10 formulation tested was placed into the strong skin sensitiser category.

Formulation number	***In vivo* experimental result**	**SENS-IS sensitisation result**	***In vivo* vs SENS-IS result comparison**
SYN 1	Sensitiser	Sensitiser	Agreement
SYN 2	Sensitiser	Sensitiser	Agreement
SYN 3	Non-sensitiser	Non-sensitiser	Agreement
SYN 4	Non-sensitiser	Sensitiser	Disagreement
SYN 5	Non-sensitiser	Non-sensitiser	Agreement
SYN 6	Sensitiser	Sensitiser	Agreement
SYN 7	Sensitiser	Non-sensitiser	Disagreement
SYN 8	Sensitiser	Sensitiser	Agreement
SYN 9	Non-sensitiser	Non-sensitiser	Agreement
SYN 10	Sensitiser	Sensitiser	Agreement

Table 5.12 Complete comparative summary of the *in vivo* and SENS-IS skin sensitisation experimental results for all ten tested agrochemical formulations

5.4 Discussion

The aim of the chapter was to evaluate the ability of the SENS-IS assay to determine skin sensitisation potential of agrochemical complex mixtures/products. With Syngenta's consent, ten of their agrochemical formulations were selected for this evaluation.

In this evaluation of the SENS-IS assay, its ability to correctly characterise the skin sensitisation potential of agrochemical products was assessed by comparing its results to those of vertebrate *in vivo* skin sensitisation tests. The basis for evaluating the SENS-IS assay result is not a direct comparison with human response (such as the human repeat insult patch test (Basketter, 2009) or reported in clinical data). As such, it should be acknowledged that the LLNA and Buehler assays are not a like-for-like prediction of the human response.

The comparisons between the vertebrate animal tests and human data were based on individual compounds, whilst this SENS-IS evaluation has been conducted on complex pesticide mixtures. Thus, it should not be assumed that the LLNA and Buehler assay performance will be the same in the assessment of pesticide formulations. An independent peer review provided a comparison between the available *in vivo* methods and human data on the skin sensitisation response of different chemicals. It also noted that there was an absence of human data on pesticide products to compare to the *in vivo* results (NIH, 1999). However, in the absence of human data, SENS-IS results were compared to the Buehler assay and "gold standard" (Basketter et al., 2009) LLNA data. As mentioned in Chapter One these two *in vivo* test methods are considered acceptable across global regulatory regions, as such in the absence of human skin sensitisation data on these ten test formulations the LLNA and Buehler assay results were used as benchmarks from which to assess the SENS-IS performance.

In the comparative Table 5.12 it is shown that of the ten tested formulations only two SENS-IS results did not produce a skin sensitisation prediction that was in agreement with the previously conducted animal experiments. The data in Table 5.1 indicate that six of the ten formulations tested in the SENS-

IS assay have been shown to be skin sensitisers in the *in vivo* assays, whilst the remaining four were non-sensitisers. Two of the six positive *in vivo* sensitisers (SYN6 and 7) were tested in the Buehler assay whilst all of the remaining eight formulations were previously tested in the LLNA. The SENS-IS skin sensitisation results for the ten formulations were in concordance with all but two of the *in vivo* results. The two discordant results were for formulations SYN4 and SYN7. SYN4, an FS agrochemical product, had previously been tested in the LLNA and demonstrated to be a non-sensitiser at up to 100% concentration. In the SENS-IS assay this formulation was tested up to 100% and produced a result indicating skin sensitisation potential. At the two lower concentrations of the SYN4 formulation tested in this assay (50% and 10%), the gene induction threshold was not reached in either gene groups for skin sensitisation. At the highest concentration of SYN4 both the SENS-IS and REDOX gene groups demonstrated an overexpression of 11 genes, well over the greater than the seven gene threshold. As the SYN4 formulation was only demonstrated to be positive in the SENS-IS assay at 100%, it is placed in the weak sensitiser category for the SENS-IS assay.

The irritation identification parameters of the SENS-IS assay allow us to rule out the potential for misclassifying irritant potential as sensitising potential. Amongst the acceptance criteria for the SENS-IS assay is the criterion that if more than 20 genes are overexpressed in the irritation gene group with an over-expression of more than seven genes in the two gene groups at the same test material concentration, then the results at that concentration must be considered as being inconclusive. This is done to remove bias from any non-specific gene expression that may be caused by cell stress (Reisinger et al., 2015). The SYN4 formulation tested at 100% over-expressed 14 of the irritation gene set. This was just below the ≥15 genes required to classify it as an irritant in this assay. This non-irritant result is in agreement with the *in vivo* non-irritant result for this formulation and allows us to rule out the difference between the SENS-IS and LLNA result as being a consequence of irritation.

The difference in the construction of the epidermis layer between rodents and man may be a potential reason for the observed conflicting sensitisation potential in different assays. Skin from experimental

animals has been reported to have a different structure from the human skin and to also be more permeable (Basketter et al., 2007). This potential species difference may have led to a possible higher bioavailability in the skin, and hence greater access to immunoproteins of the compounds within the SYN4 formulation that may have sensitising potential. Although occupational toxicology data are currently not available for this thesis, comparison of the results of the SENS-IS assay for SYN4 to human exposure data from occupational toxicology reports may also help to provide evidence of whether the result of the SENS-IS assay is a true representation of the human response to this formulation. However, within the remit of this investigation, it can be concluded that the SENS-IS assay did not produce a skin sensitisation potential result for the SYN4 formulation that was predictive of the *in vivo* LLNA outcome. In order to understand if this outcome is specific to the FS pesticide formulation type or is attributed to specific ingredients, more formulations of an FS type need to be examined, as only two were used in this study.

The second formulation tested which produced a SENS-IS result that was not in agreement with its *in vivo* result was SYN7. SYN7 is an EC pesticide formulation that, when tested in the Buehler assay, produced a positive result for skin sensitisation. However, when tested at up to 100% in the SENS-IS assay the SYN7 formulation it produced a non-sensitiser result. Upon initial testing in the SENS-IS assay SYN7 was tested at 100%, 50% and 10%. Phosphate buffered saline was initially used as the vehicle for concentrations at 50% and 10% SYN7. Following the initial review of the results obtained for this formulation, in which over-expression of the two sensitisation gene groups was below 7 at all concentrations, a re-run of this formulation in the SENS-IS assay was conducted. This was conducted using olive oil as the vehicle to allow us to determine if the vehicle had been a factor in the generation of sensitisation potential through means of test material delivery to the skin proteins. In particular, the Buehler assay on SYN7 had been conducted at very low concentrations (5% induction, 0.1% and 0.01% challenge phase) and produced a positive result as a skin sensitiser. Conducted at 50% in olive oil, the SYN7 formulation still produced a result in the SENS-IS indicating it is a non-sensitiser. As such it can be ruled out that PBS or the olive oil vehicles had an impact on the outcome of the SENS-IS assay

for this formulation. The initial irritation screen performed in the Buehler assay on SYN7 led to 10% SYN7 being used for the induction phase, as the higher concentrations caused excessive irritation. Irritation potential of the SYN7 formulation did not limit the test concentrations used in the SENS-IS assay. At 100% SYN7 exposure, irritation was not indicated in the SENS-IS assay, as the number irritation genes expressed were below the threshold. Therefore, test material concentration was not a limiting factor for the assessment of the SYN7 formulation in the SENS-IS assay. There may potentially have been prohaptens present in the formulation requiring metabolic activation. That would provide logical reasoning as to why the *in vivo* sensitising result was not reflected in the *in vitro* SENS-IS assay that lacks metabolic capability.

All 8 of the other formulations tested in the SENS-IS assay produced results that were in good agreement with the vertebrate study outcomes. In addition to being in concordance with the eight *in vivo* sensitisation results, the *in vitro* SENS-IS assay also generated potency predictions that fell in line with the EC3 values for the four skin sensitising formulations (SYN1, 2, 8 and 10) tested in the LLNA as shown in Table 5.1 and 5.12.

The research conducted demonstrates the potential for the SENS-IS assay to replace the need to conduct vertebrate *in vivo* skin sensitisation assays such as the LLNA to determine potency of a complex mixture, such as those tested in this evaluation, or a single chemical. At present regulatory guidance documents (ECHA, 2017a, (EU), 2017b) allow for the use of the LLNA to determine potency of a test material that has been predicted as positive by the available and regulatory accepted *in vitro* skin sensitisation test methods. This research has been able to demonstrate that the SENS-IS assay is an *in vitro* method suitable for identifying both the skin sensitisation potential and potency of an agrochemical formulation to allow for suitable hazard identification.

5.5 Conclusion

The ten formulations were selected based on their different formulation types, the data available on each of them from previously conducted *in vivo* studies and the fact that they have been used to

investigate other *in vitro* assays in previous chapters. This selection should allow for the comparison between all of the methods investigating skin sensitisation of formulations in future assessment. The SENS-IS assay measured the level of expression of the two separate gene sets at a given time point after application of the formulation as compared to an internal negative, irritant (sodium lauryl sulphate) and positive sensitiser (2,4,6-trinitrobenzene sulfonic acid) controls. The SENS-IS assay displayed good agreement with the *in vivo* skin sensitisation results, correctly predicting five out of six of the previously identified formulations with skin sensitising potential. In addition, the assay demonstrated agreement with the previous potency evaluations for these formulations. The SENS-IS assay also correctly identified three out of four formulations previously shown to be non-skin sensitisers. In conclusion, this chapter indicates that the SENS-IS assay provides an *in vitro* testing option for accurate hazard assessment of skin sensitisation potential for agrochemical formulations.

6.0 CONCLUSION

This thesis has focused on providing novel research on the use of non-animal approaches for the acceptable hazard assessment of skin sensitisation, focusing in on active ingredients and complex mixtures used to create plant protection products within the agrochemical industry. During this research attention was paid to recognising the regulatory accepted and test guideline established *in vivo* methods that have been used regularly in different chemical industries, as outlined in Chapter One. Varying material types have been tested in these *in vivo* methods, some which may not necessarily have been suitable given their intended product use. An example of this misappropriate use has been the assessment of PPP intended for field spray undergoing skin sensitisation hazard assessment in the GPMT. Early animal methods lacked more intricate quantitative details of the risk that the identified hazard may cause. However, through the refinement of these initial methods and the development of approaches such as the LLNA, more tangible quantitative data from these assessments have been provided. Specifically, the updated methods have allowed the evaluation of potency of those materials identified as sensitisers, information which can then be further used for risk assessment.

It must also be said that as these previously established methods and approaches were refined and improved upon, there was conscious shift to reduce the number of animals they required. Ultimately these tests have been conducted to gain some knowledge of the potential for an allergic contact dermatitis event to occur to a human. Methods such as the HRIPT and HMT allowed for direct species comparison, however there is a clear ethical dilemma, and it is considered unsound practice to fill the skin sensitisation knowledge gap of a formulation or single material through exposing it directly to human skin. In addition, human patch tests cannot be carried out for the sole purpose of fulfilling regulatory criteria (EC., 2009a). These issues provide some clarity as to why *in vivo* animal studies have been used as the benchmark to provide a potential comparative response, for materials of unknown skin sensitisation potential.

Throughout this thesis alternative skin sensitisation hazard assessment approaches have been explored. This has been undertaken with an interest in seeking out where potential limitations within some of these methods may lie, where synergy between methods may provide a better option and to further understand how new alternative methods that are currently at the forefront of development/acceptance can potentially address complications. One of the specific complications of interest that was explored in this research has been identifying a non-animal experimental method that can be used to provide confident predictions for skin sensitisation potential of complex mixtures. In addition to that, it's worth acknowledging the prospect of using the non-animal experimental approaches for the screening of lead compounds in early-stage product development.

Before diving into the laboratory bench based non-animal experimental methods, the approach was taken in this thesis to understand how suitable any currently accepted non-animal methods for determining skin sensitisation of complex mixtures were for agrochemical formulations. As such, the established CLP skin sensitisation threshold calculation approach was investigated in Chapter Two. The results of the evaluation in Chapter Two, alongside the results from all other evaluated methods, are provided in Tables 6.1 and 6.2. It was shown that with sufficient information regarding the composition of the formulation, the binary threshold calculation method had the potential to provide a quick and useful understanding of what the skin sensitisation potential of a formulation might be. However, when insufficient test material information was available for the ingredients present within a formulation being evaluated, the accuracy of the skin sensitisation classification by this method was limited. The calculated accuracy of the threshold calculation method when comparing against GPMT/ Buehler or LLNA as a benchmark was 72%. This is mediocre at best and for a hazard assessment unlikely to give an acceptable level of confidence. However, Chapter Two showed that the performance of the threshold calculation method varied dependent on the agrochemical formulation type.

Agrochemical AI	*In vivo* Study result	GARD skin prediction	DPRA	KeratinoSens™	h-CLAT	DEREK	Toolbox
Acetamiprid	Non-sensitiser	Not tested	Sensitiser	Non-sensitiser	Sensitiser	Non-sensitiser	Sensitiser
Acibenzolar-s-methyl	Sensitiser	Not tested	Inconclusive	Inconclusive	Non-sensitiser	Sensitiser	Non-sensitiser
Benzovindiflupyr	Non-sensitiser	Sensitiser	Sensitiser	Sensitiser	Non-sensitiser	Non-sensitiser	Sensitiser
Chlorantraniliprole	Non-sensitiser	Not tested	Inconclusive	Inconclusive	Non-sensitiser	Non-sensitiser	Non-sensitiser
Chlorothalonil	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Non-sensitiser	Sensitiser	Sensitiser
Clodinafop-propargyl	Sensitiser	Sensitiser	Not tested	Not tested	Not tested	Sensitiser	Sensitiser
Cyantraniliprole	Non-sensitiser	Sensitiser	Inconclusive	Non-sensitiser	Non-sensitiser	Non-sensitiser	Sensitiser
Dicamba	Non-sensitiser	Sensitiser	Sensitiser	Non-sensitiser	Sensitiser	Non-sensitiser	Sensitiser
Difenoconazole	Non-sensitiser	Sensitiser	Not tested	Not tested	Not tested	Non-sensitiser	Non-sensitiser
Mesotrione	Non-sensitiser	Not tested	Sensitiser	Sensitiser	Non-sensitiser	Sensitiser	Sensitiser
Pinoxaden	Sensitiser	Non-sensitiser	Sensitiser	Sensitiser	Non-sensitiser	Equivocal	Non-sensitiser
AI1	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Sensitiser
AI2	Sensitiser	Sensitiser	Not tested	Not tested	Not tested	Sensitiser	Non-sensitiser
AI3	Sensitiser	Sensitiser	Not tested	Not tested	Not tested	Sensitiser	Sensitiser
AI4	Non-sensitiser	Sensitiser	Not tested	Not tested	Not tested	Sensitiser	Non-sensitiser
AI5	Sensitiser	Sensitiser	Not tested	Not tested	Not tested	Sensitiser	Sensitiser

Table 6.1Final summary of the skin sensitisation in vivo test results and the evaluated NAM test results for the ten agrochemical AIs

Formulation number	*In vivo* experimental result	SENS-IS sensitisation result	DPRA	KeratinoSens™	h-CLAT	CLP calculation result
SYN 1	Sensitiser	Sensitiser	Not conducted	Sensitiser	Non-sensitiser	Non-sensitiser
SYN 2	Sensitiser	Sensitiser	Sensitiser	Non-sensitiser	Non-sensitiser	Non-sensitiser
SYN 3	Non-sensitiser	Non-sensitiser	Not conducted	Sensitiser	Sensitiser	Non-sensitiser
SYN 4	Non-sensitiser	Sensitiser	inconclusive	Inconclusive	Sensitiser	Non-sensitiser
SYN 5	Non-sensitiser	Non-sensitiser	Not conducted	Inconclusive	Non-sensitiser	Non-sensitiser
SYN 6	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Sensitiser	sensitiser
SYN 7	Sensitiser	Non-sensitiser	Not conducted	Inconclusive	Non-sensitiser	sensitiser
SYN 8	Sensitiser	Sensitiser	Sensitiser	Inconclusive	Non-sensitiser	sensitiser
SYN 9	Non-sensitiser	Non-sensitiser	inconclusive	Inconclusive	Sensitiser	sensitiser
SYN 10	Sensitiser	Sensitiser	Not conducted	Sensitiser	Sensitiser	sensitiser

Table 6.2 Final summary of the skin sensitisation in vivo test results and the evaluated NAM test results for the ten agrochemical formulations

A particular concern identified from the performance review of the CLP threshold calculation on different agrochemical formulation types was the potential for false negative results. The results for the FS agrochemical formulations as given in Chapter two and summarised in Table 6.2 demonstrate the potential of the CLP threshold calculation methods to predict false negatives. With low accuracy and potential for misclassification, Chapter Two indicates that this CLP threshold method should not be used as a standalone approach. Instead, the threshold calculation was proposed to be used in conjunction with another method that may/may not corroborate its binary result and, as such, provide a more robust final skin sensitisation classification of the formulation.

In Chapter Three the CLP threshold method was used in combination with the *in vitro* triple pack in order to provide a WoE for a final skin sensitisation assessment on the chosen agrochemical formulations. The observed limitations associated with testing complex mixtures in the 2D cell assays of the triple pack hindered the triple pack's ability to accurately determine the skin sensitisation potential of the PPPs. Although testing of individual AIs was performed successfully, one of the specific limitations identified with testing the agrochemical formulations in the triple pack assays was the need to be able to form a homogenous solution with a solvent in order to be tested. This experimental requirement is difficult to achieve for complex mixtures, as shown in the DPRA testing where only five

of the ten formulations were successfully placed in suspension. In addition, where certain materials in a formulation (such as preservatives) are intentionally encapsulated, the need for a homogenous solution can lead to the disruption of these capsules and result in direct exposure of the in vitro test system to the encapsulated material. Furthermore, attempting to evenly disperse agrochemical formulations into a solvent led to separation of the components of the designed formulations. As such, the test system was exposed to the ingredients of the PPP in a manner that would not represent potential exposure in real life field application. It was apparent that the use of these *in vitro* triple pack methods for the assessment of skin sensitisation potential of agrochemical formulations is not suitable in the method's current design.

Further investigation into two gene expression based *in vitro* methods was conducted in Chapters Four and Five. Research into the GARD assay was conducted first with the AIs, with the intention to proceed to test formulations once the research was able to demonstrate good concordance with the *in vivo* experimental results of the AIs. However, the GARD assay results showed poor agreement with the AI *in vivo* experimental results. Following review of the differences in physicochemical properties between the GARD assay training set and AI test set, it was hypothesised that the chemical space disparity between the two chemical sets led to a chemical domain that was not applicable to the biologically active agrochemicals. The GARD training set is currently highly populated with commonly used cosmetic materials, expanding it to include more biologically active agrochemicals may improve classifications from the machine learning classifier of the GARD assay by identifying specific gene signalling associated with sensitising and non-sensitising biologically active agrochemicals. As the results of the GARD assay evaluation did not demonstrate good agreement with the *in vivo* experimental results used as benchmarks, further investigation with complex mixtures was not pursued with this assay. Instead, using the findings from investigations into the 2D *in vitro* test methods, the decision was made to explore the use of the 3D *in vitro* SENS-IS assay. This assay allows for direct application of the PPP to the RhE test system so that good comparison to the products intended and anticipated use can be made. Good performance was demonstrated by the SENS-IS assay

in predicting both skin sensitisation potential and potency of the PPP. As such of the methods tested in this thesis, the SENS-IS assay has been shown to be the most reliable for skin sensitisation hazard assessment of agrochemical formulations. It could be used in conjunction with the CLP threshold method to provide accompanying supporting evidence or to verify the result of the initial calculation classification.

A gold standard benchmark for which to compare the results of the *in vitro* assays to would be recorded human data. However, these were not available for the AI or formulations that were tested and so it should be acknowledged that using the *in vivo* animal assay results to determine non-animal alternative methods proficiency for hazard assessment of skin sensitisation when considering human use has a limitation. In addition to the *in vivo* animal studies being a combination of both GPMT and LLNA, going forward in order to reduce variability in comparative performance analysis, in absence of human data a preference of a single *in vivo* method type and not multiple would be considered primarily. Although the five different *in chemico* and *in vitro* test methods investigated in this thesis each targets a particular KE of the skin sensitisation AOP, there is scarce, if any, integration of data on the dermal disposition of test material in the skin layers proposed by any current defined approaches. Potential future research can investigate gaining a greater understanding of how dermal absorption data (specifically percentages present in the epidermis) can be used in conjunction with the *in vitro* skin sensitisation hazard assessment data, with the aim to provide an accurate risk assessment. In addition to that, further research to understand exactly how certain co-formulants (e.g., adjuvants, surfactants) and AIs may interact and impact skin sensitisation potential should be performed. This may potentially occur through changing dermal absorption or through potential damage to the stratum corneum, ultimately again altering absorption. It is anticipated that greater understanding of this would aid in the development of agrochemical formulations with better skin sensitisation profiles.

This thesis has evaluated *in chemico, in vitro* and *in silico* hazard assessment methods for the skin sensitisation adverse effect. The aim being to identify effective and appropriate methods for the

assessment of skin sensitisation potential from agrochemical active ingredients and PPP. The protection goal of the skin sensitisation hazard assessment is to minimise the toxicological risk to humans with regards to this adverse effect. This goal has been pursued whilst understanding the intention of science in 21st Century toxicology to be a move towards decision making based on a predominantly predictive science that targets specific mechanism based biological observations (National Toxicology Program, 2004). The overall intention is to generate the information necessary to make a risk assessment decision whilst striving to reduce animal use. The purpose of the new approaches in toxicology is, in part at least, to move away from the selection of test methods based on what might have been considered a regulatory box ticking exercise, towards focussing resources on the key questions that will affect a risk assessment. It must be acknowledged that although skin sensitisation assessments began with human tests (as stated in Chapter One) or, where available, occupational toxicology or clinical reports, testing on humans is not conducted for the sole purpose of identifying chemical hazard. As such a move to using *in vivo* tests as the surrogates for human data was the first shift seen. The drive in 21st Century toxicology is to shift away from the classical *in vivo* tests that have formed the basis for decisions related to product safety and prioritisation of chemicals in early-stage research. The move away from *in vivo* animal testing and to the use of NAMs as surrogates for these classical *in vivo* animal tests is now being undertaken. Although this has resulted in a reduction in animal use, a noted limitation of how NAMs are being evaluated is that animal tests are regularly being used (especially in the case of agrochemicals) as the benchmark to which the performance of these skin sensitisation NAMs are assessed. It has been demonstrated that these animal methods are not 100% representative of an expected human response and as such where solely animal tests have been used as a benchmark for validation, a potential limitation of the NAM in question should be acknowledged.

In the recently published OECD 497 guideline (OECD, 2021) on the use of skin sensitisation DAs, the integration of the *in silico* methods mentioned in Chapter Three have been outlined and accepted. This demonstrates the move by regulatory bodies to begin accepting the inclusion of the predictive

data generated by these *in silico* models in the regulatory submission of active ingredients. This *in silico* data are, however, considered alongside data produced from other NAMs in a WoE approach, to reinforce a final decision based on these outcomes together. In that context the use of the AOP for skin sensitisation has allowed the outlining of strategies using test methods that focus on the cascading steps required to lead to the adverse biological effect. In doing this the science has been able to move away from the *in vivo* tests that focus on the observation of the final outcome, and instead utilise and develop non animal NAMs. These NAMs focus on measuring biological interactions, specific biomarkers (*in chemico/in vitro)* or identifying chemical characteristics (*in silico)* that are essential in signifying the occurrence or activation of the events identified in the AOPs. In addition to the use of (Q)SAR models through WoE approaches in a regulatory context (OECD, 2021), these *in silico* approaches can be and have been used on their own as an initial screen on lead compounds. In early-stage research (Q)SAR models are used to focus resource on specific compounds by providing an initial indication of potential adverse effects. That indication of potential effects can then be further addressed through the selection of test methods to produce data to make decisions on those highlighted adverse effects in question.

With the ultimate goal being to eliminate the need for animal testing and with a schedule such as the US EPA's mandate to eliminate it by 2035 (EPA, 2019), it needs to be recognised that for us to achieve this, certain test material types require more focused attention to develop methods applicable to them. This is one of the issues addressed in this thesis. There are non-animal methods currently accepted for skin sensitisation testing of single chemicals. However, the research done here and specifically in Chapter Three on the use of triple pack methods, shows that these currently accepted NAMs were not appropriate for the skin sensitisation assessment of PPP. Demonstrating the need to focus efforts on identifying and developing alternative approaches suitable for test material types that would require risk assessment.

We have been able to demonstrate that the potential future direction for the hazard assessment of skin sensitisation of agrochemical complex mixtures lies in 3D *in vitro* reconstructed human skin models and identifying the genomic signature associated with skin sensitisation. It can be projected that the future incorporation of the test methods evaluated in this thesis will also need to include exposure data in order to provide a balanced risk assessment that does not assume 100% test substance interaction with the test system. Ultimately, the final barrier for the global elimination of vertebrate testing is being able to achieve harmonised acceptance of available NAMs across the global regulatory landscape.

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8.0 APPENDICES

Appendix 1. Examples of CLP threshold calculations

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Appendix 9. SENS-IS solvent material list

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Appendix 13. Copy of published work

8.1 Appendix 1. Examples of CLP threshold calculations

Formulation example 1

Coformulant Material Type	%	CLP Skin Sensitisation Classification	GCL/SCL
Active Ingredient A	11	Not classified	NA
Active Ingredient B	7	Not classified	NA
Solvent	31.5	Not classified	NA
Solvent	10	Not classified	NA
Surfactant	25	Not classified	NA
Antifoaming agent	0.5	Not classified	NA
Adjuvant	8	Not classified	NA
Antifreeze	7	Not classified	NA

In vivo test result for example formulation 1:

Positive skin sensitiser

Skin sensitisation classification derived from CLP threshold calculation:

As all coformulant materials are not reported to have skin sensitisation potential in accordance with the threshold method the formulation would be determined not to have skin sensitisation potential.

Formulation example 2

Coformulant material type	%	CLP skin sensitisation classification	GCL/SCL
Active Ingredient A	20	Category 1	GCL -1%
Antifreeze	15	Not Classified	NA
Dispersing agent	2	Not Classified	NA
Wetting agent	1	Not Classified	NA
Catalyst	0.5	Not Classified	NA
Stabilising agent	1	Not Classified	NA
Dispersing agent	2.5	Not Classified	NA
Antifoaming agent	3	Not Classified	NA
Preservative	0.05	Category 1	SCL - 0.05%
Solvent	54.95	Not Classified	NA

In vivo test result for example formulation 2:

Positive skin sensitiser

Skin sensitisation classification derived from CLP threshold calculation:

There are two ingredients present in this formulation of toxicological relevance regarding skin sensitisation. These are active ingredient A (present at 20% in the formulation) and the preservative material (present at 0.05%).

Both materials are classified as category 1 skin sensitisers, however the active ingredient has a generic classification limit (GCL) of 1%, whilst the preservative has a specific concentration limit (SCL) of 0.05%.

As both ingredients are present at or above their concentration classification limits, in accordance with the harmonised classification of individual ingredients and the sensitisation classification indicated in their individual safety data sheets; the example formulation two is considered to be a skin sensitiser and warrant a H317 category 1 classification.

Formulation example 3

Coformulant material type	%	CLP skin sensitisation classification	GCL/SCL
Active Ingredient A	12	Category 1B	GCL -1%
Active Ingredient B	14	not classified	NA
Adjuvant	11	not classified	NA
Preservative	0.04	Category 1	SCL - 0.05%
Solvent	10.95	not classified	NA
Thickener	0.12	not classified	NA
Wetting agent	2.22	not classified	NA
Solvent	49.67	not classified	NA

In vivo test result for example formulation 3:

Non-sensitiser

Skin sensitisation classification derived from CLP threshold calculation:

There are two ingredients present in this formulation of toxicological relevance regarding skin sensitisation. These are active ingredient A (present at 12% in the formulation) and the preservative material (present at 0.04%).

Active ingredient A is classified as a category 1B skin sensitiser with a GCL of 1%, whilst the preservative material has a SCL of 0.05%.

8.2 Appendix 2. Chapter Two performance criteria results for the evaluation of the threshold calculation

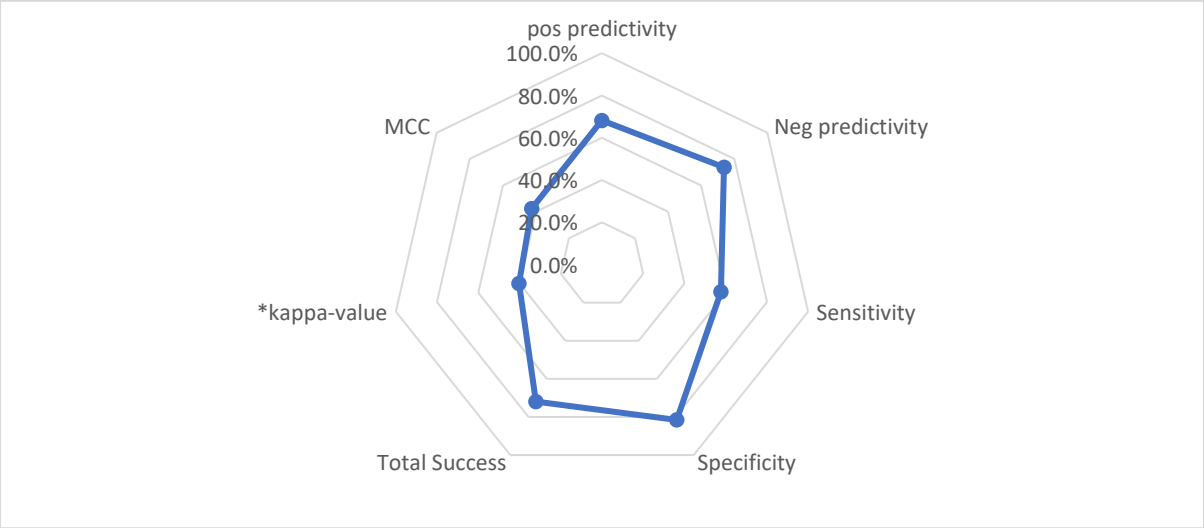

Figure 8.1 Performance criteria adopted to evaluate the prediction of skin sensitisation potential by the threshold calculation method conducted on 64 agrochemical formulations.

Positive predictivity	0%
Negative predictivity	54%
Sensitivity	0%
Specificity	100%
Total Success/Accuracy	54%
kappa-value	0.00
MCC	0.00

Table 8.1 Statistical parameters used for evaluation of the threshold calculation predictions of the FS agrochemical formulation type test set results against the *in vivo* experimental skin sensitisation results.

Positive predictivity	46%
Negative predictivity	100%
Sensitivity	100%
Specificity	60%
Total Success/Accuracy	70%
kappa-value	0.43
MCC	0.45

Table 8.2 Statistical parameters used for evaluation of the threshold calculation predictions of the SC agrochemical formulation type test set results against the *in vivo* experimental skin sensitisation results.

Positive predictivity	100%
Negative predictivity	25%
Sensitivity	67%
Specificity	100%
Total Success/Accuracy	70%
kappa-value	0.29
MCC	0.25

Table 8.3 Statistical parameters used for evaluation of the threshold calculation predictions of the EC agrochemical formulation type test set results against the in vivo experimental skin sensitisation results.

20 SC formulations	*Pred. sensitiser*	*Pred. non sensitiser*	*In vivo* dataset distribution	Value (%)
Obs. sensitiser	5 (100%)	0 (0%)	sensitiser	25%
Obs. non sensitiser	6 (40%)	9 (60%)	non sensitiser	75%
13 FS formulations	*Pred. sensitiser*	*Pred. non sensitiser*		
Obs. sensitiser	0 (0%)	6 (100%)	sensitiser	46%
Obs. non sensitiser	0 (0%)	7 (100%)	non sensitiser	54%
10 EC formulations	*Pred. sensitiser*	*Pred. non sensitiser*		
Obs. sensitiser	6 (67%)	3 (33%)	sensitiser	90%
Obs. non sensitiser	0 (0%)	1 (100%)	non sensitiser	10%
7 WG formulations	*Pred. sensitiser*	*Pred. non sensitiser*		
Obs. sensitiser	2 (100%)	0	sensitiser	29%
Obs. non sensitiser	0	5 (100%)	non sensitiser	71%

Table 8.4 Threshold calculation predictions for the SC, FS EC and WG formulation types against the *in vivo* study results.

8.3 Appendix 3. DPRA test methodology

Experimental controls

Positive controls

Cinnamic aldehyde was used as the positive control in the HPLC analysis at a concentration of 100 mM in acetonitrile for the cysteine peptide runs. Peak splitting was observed on the chromatograms for a cinnamic aldehyde lysine peptide combination. The compound 2,3-butanedione (CAS 431-03-8), at a concentration of 100 mM acetonitrile, was used as the positive control in HPLC analysis for the lysine peptide runs only. The AI 2,3-butanedione is listed among the recommended OECD Test Guideline positive prediction substances to demonstrate DPRA proficiency and has similar depletion ranges to cinnamic aldehyde (Cys: 60-100%, Lys: 10-45%) (OECD, 2019). During proficiency testing of 2,3-butanedione at the Gentronix laboratory, chromatography reliably showed a single peak only i.e., no splitting. Therefore 2,3-butanedione was considered a suitable alternative positive control chemical.

Solvent controls

Three types of reference controls (i.e., samples containing only the peptide dissolved in the appropriate solvent) were included in the HPLC run sequence:

1. Reference control A: This reference control was made with acetonitrile and was used to verify the accuracy of the calibration curve for peptide quantification.
2. Reference control B: This reference control was made with acetonitrile. Its replicates were injected at the beginning and at the end of the HPLC analysis run to verify the stability of the peptides over the HPLC analysis time.
3. Reference control C: These reference controls were prepared in each solvent used for formulation of the ten AIs or agrochemical formulations (all acetonitrile:DMSO (1:1)) and were included in every HPLC analysis run. They were used to verify that the solvent did not impact the percent peptide depletion. The appropriate reference control C was used to calculate percent peptide depletion of each test item.

A co-elution control was also used. This control was prepared without the addition of the peptides in order to determine if the AI test material was absorbed at 220 nm and had a similar retention time as the cysteine or lysine peptides, as this would have interfered with the data analysis.

Test substance incubation with cysteine and lysine peptides

The formulated test substance solutions were incubated with the cysteine or lysine peptides for approximately 22 – 26 hours at room temperature and away from light. The proportions of test material and peptide solution incubated together followed OECD test guideline procedure for the DPRA test and are indicated in table 7 presented in the appendix.

As indicated in table 6 a further test item solubility assessment was conducted on the ten AIs in the cysteine peptide buffer (sodium phosphate buffer (pH 7.5)) and the lysine peptide buffer (ammonium acetate buffer (pH 10.2)) following 22 – 26 hours incubation. Where the formation of precipitate was observed following incubation with the peptides, the samples were centrifuged (at low speed (100-400 xg)) prior to HPLC analysis (ECVAM, 2012). Consequently, the final AI concentrations in the corresponding peptide buffers were an estimation and could not be determined with precision for samples where precipitation was observed.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM Test Item*	**1:50 Ratio, Lysine Peptide** 0.5 mM Peptide, 25 mM Test Item*
750 µL cysteine peptide solution (Or pH 7.5 phosphate buffer for co-elution controls) + 200 µL acetonitrile + 50 µL test item solution Or 50 µL of suitable solvent for reference controls A, B and C or positive control solution	750 µL lysine peptide solution (Or pH 10.2 ammonium acetate buffer for co-elution controls) + 250 µL test item solution Or 250 µL solvent for reference controls

Table 8.5 DPRA Test sample preparation for HPLC analysis

**These final test item concentrations only apply if the test item was dissolved in solvent at 100 mM. Test item concentration will be less if this recommended maximum test concentration was not achieved.*

Acceptance Criteria Description	Result	Pass/Fail
Calibration curve standards	All included in curve	Pass
Linear regression R2 > 0.990	0.999	Pass
Reference Control A in acetonitrile 0.45-0.55 mM	0.521 mM	Pass
Reference Control B in acetonitrile CV <0.15	0.083	Pass
Reference Control C in acetonitrile CV <0.15 0.45-0.55 mM	0.043 0.479 mM	Pass Pass
Positive control in acetonitrile 60.8%< mean depletion of cysteine < 96.6%	69.55%	Pass

CV= Coefficient Variation, QC

Therefore, all the system suitability tests and calibration results for this experiment PASS.

Acceptance Criteria Description	Result	Pass/Fail
Control C in DMSO:Acetonitirle (1:1) CV <0.15 0.45-0.55 mM	 0.242 0.073 mM	 Fail Fail

Table 8.6 DPRA reference controls acceptance criteria

Control C in the DMSO and acetonitrile mixture show a significant depletion of the cysteine peptide and according to the OECD 442C test guideline would give non-conclusive results with respect to the formulations.

8.4 Appendix 4. KeratinoSens™ test methodology

Test item administration

At the final density of 1 X 10^6 cells/mL, the THP-1 cells were treated with 500 µL of the eight test material concentrations. The test item and THP-1 were incubated for 24 hours (± 1 hour) in 5% CO_2 at 37^{O}C. Stock solutions of each test material in their corresponding solvents (DMSO or saline) were prepared and then diluted in the same solvent in order to formulate the test solutions. Test material solutions dissolved in DMSO were diluted 1:500, whilst those dissolved in saline/media were diluted 1:100 in a 24-well plate to give the final concentrations shown in Tables 6.8 and 6.9.

Upon addition of the AI test material to the 24 well plate, any observed changes (e.g., precipitate formation or colour change) were recorded.

Controls

The vehicle control material chosen for use alongside each test material was the same solvent used to formulate the corresponding test material solution. A medium control was used for dicamba. Solvent treated cultures in which the solvent volume was equivalent to the volume used in the test material treated cultures were used as the negative control. The positive control used was DNCB. The DNCB was tested at a final concentration of 4.0 µg/mL in the 24 well plate.

Cell harvesting

Following the 24-hour incubation with test material, THP-1 cells were washed in flow cytometry staining buffer (FACS buffer, manufacturer and place, UK) and resuspended in blocking solution for approximately 15 minutes on ice and protected from light. The resuspension in blocking solution was performed to increase the specificity of antibody labelling on the target cells. After blocking, the cells were centrifuged, cell pellets were resuspended and split into three aliquots which were transferred to a 96-well microplate for antibody staining.

Cell staining with Fluorescein Isothiocyanate (FITC) labelled antibodies

Following antibody solution preparation, cells were centrifuged for five minutes in the 96-well microplate at approximately 250g at 4°C and 50 µL of antibody was added to each cell pellet in the 96-well microplate i.e., one cell pellet per sample for anti-CD86 antibody, one cell pellet per sample for anti-CD54 antibody and one cell pellet per sample for Mouse IgG_1 antibody. Cells were then incubated for approximately 30 minutes.

After antibody staining, cells were washed with FACS buffer and cell pellets were resuspended in 400 µL of FACS buffer in a flow cytometry sample tube. Before each flow cytometry acquisition, PI solution at a final concentration of 0.625 µg/mL was added to each sample.

All steps required for cell staining with antibodies were performed on ice/refrigerated and protected from light as directed (ECVAM, 2014b).

Relative fluorescence intensity calculation

The Relative Fluorescence Intensity (RFI) was used as an indicator of CD86 and CD54 expression. Based on the Mean Fluorescence Intensity (MFI), the RFI of CD86 and CD54 were calculated according to the following equation:

$$RFI = \frac{\text{MFI of test item treated cells - MFI of test item treated isotype cells}}{\text{MFI of solvent treated cells - MFI of solvent treated isotype cells}} \times 100$$

The cell viability was recorded for each concentration of every test item using the isotype control cells.

Calculation of the EC150 (for CD86) and EC200 (for CD54)

Where test substances are predicted as positive in the h-CLAT assay, effective concentration (EC) values leading to the 150-fold and 200 fold inductions (in CD86 and CD54 respectively) can be determined. The EC values are calculated using the test data in the following equations:

$$EC150 \text{ (for CD86)} = B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

EC200 (for CD54) = B_{dose} + [(200 – B_{RFI}) / (A_{RFI} – B_{RFI}) x (A_{dose} – B_{dose})]

Where

A_{dose} is the lowest concentration in µg/mL leading to a RFI >150 (CD86) or 200 (CD54)

B_{dose} is the highest concentration in µg/mL leading to a RFI <150 (CD86) or 200 (CD54)

A_{RFI} is the RFI at the lowest concentration with a RFI of >150 or 200 accordingly

B_{RFI} is the RFI at the highest concentration with a RFI of <150 or 200 accordingly

		Concentrations on the Microplate (µM)											
		Highest conc. →	→	→	→	→	→	→	→	→	→	→	Lowest conc.
Test Item ID	**Concentration in Solvent (mM)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
acetamiprid	200.0	2000	1000	500	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98
acibenzolar-s-methyl	25.00	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24	0.12
benzovindiflupyr	25.00	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24	0.12
chlorantraniliprole	25.00	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24	0.12
chlorothalonil	30.00	300	150	75	37.50	18.75	9.38	4.69	2.34	1.17	0.59	0.29	0.15
cyantraniliprole	50.00	500	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24
dicamba	200.0	2000	1000	500	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98
mesotrione	200.0	2000	1000	500	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98
pinoxaden	100.0	1000	500	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49
AI1	25.00	250	125	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24	0.12

Table 8.7 KeratinoSens™ active ingredient 12 microplate dosing concentrations.

		Concentrations on the Microplate (µM)											
		Highest conc. →	→	→	→	→	→	→	→	→	→	→	Lowest conc.
Test Item ID	**Concentration in Solvent (mM)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
SYN1	312.50	312.50	250	200	160	128	102.40	81.92	65.54	52.43	41.94	33.55	26.84
SYN2	1024	1024	819.20	655.36	524.29	419.43	335.54	268.44	214.75	171.80	137.44	109.95	87.96
SYN3	1000	1000	800	640	512	409.60	327.68	262.14	209.72	167.77	134.22	107.37	85.90
SYN4	44	44	29.33	19.56	13.04	8.691	5.794	3.863	2.575	1.717	1.145	0.763	0.509
SYN5	26.60	26.60	17.73	11.82	7.881	5.254	3.503	2.335	1.557	1.038	0.692	0.461	0.308
SYN6	2.15	2.15	1.72	1.38	1.10	0.88	0.71	0.56	0.45	0.36	0.29	0.23	0.19
SYN7	21.64	21.64	17.31	13.85	11.08	8.86	7.09	5.67	4.54	3.63	2.90	2.32	1.86
SYN8	33.08	33.08	22.05	14.70	9.80	6.53	4.36	2.90	1.94	1.29	0.86	0.57	0.38
SYN9	397.80	397.80	265.20	176.80	117.87	78.58	52.39	34.92	23.28	15.52	10.35	6.90	4.60
SYN10	114	114	91.20	72.96	58.37	46.69	37.36	29.88	23.91	19.13	15.30	12.24	9.79

Table 8.8 KeratinoSens™ agrochemical formulations 12 microplate dosing concentrations

Active ingredient	Highest soluble concentration in solvent (mg/mL)	Selected solvent
acetamiprid	400.0	DMSO
acibenzolar-s-methyl	60.00	DMSO
benzovindiflupyr	500.0	DMSO
chlorantraniliprole	250.0	DMSO
chlorothalonil	10.00	DMSO
cyantraniliprole	100.0	DMSO
dicamba	100.0	Saline
mesotrione	300.0	DMSO
pinoxaden	150.0	DMSO
AI1	150.0	DMSO

Table 8.9 AI pre-test solubility assessment for the KeratinoSens™ assay.

Test formulation	Highest soluble test concentration (µg/mL)	Selected solvent
SYN 1	5000	Saline
SYN 2	78.13	Saline
SYN 3	250	Saline
SYN 4	875	Saline
SYN 5	39.06	Media
SYN 6	3.91	Saline
SYN 7	5000	Saline
SYN 8	10	DMSO
SYN 9	500	Saline
SYN 10	156.30	Saline

Table 8.10 Agrochemical formulation pre-test solubility assessment for the KeratinoSens™ assay

Active ingredient	Final Concentration in Cell Culture Medium (µg/mL)
acetamiprid	800.0
acibenzolar-s-methyl	30.00
benzovindiflupyr	15.63
chlorantraniliprole	62.50
chlorothalonil	20.00
cyantraniliprole	100.0
dicamba	1000
mesotrione	600.0
pinoxaden	150.0
AI1	75.00

Table 8.11 AI formulated test concentrations for the cell culture medium for the KeratinoSens™ assay

Control chemicals

Dimethyl sulfoxide was selected as the negative control material. Vehicle controls consisted of DMSO solvent-treated cultures in which the volume of DMSO (1%) was equivalent to that in the test item-treated cultures.

Cinnamic aldehyde (CAS number 14371-10-9, >99% purity) was used as the positive control material for the KeratinoSens™ assay. It was tested at final concentrations of 64, 32, 16, 8 and 4 µM.

Active ingredient	Concentration in Solvent (mM)	Concentration on the microplate (µM)	Test item observations upon addition to the microplate
acetamiprid	200.0	2000	None
acibenzolar-s-methyl	25.0	250.0	Precipitation at highest four concentrations tested
benzovindiflupyr	25.0	250.0	None
chlorantraniliprole	25.0	250.0	None
chlorothalonil	30.0	300.0	Precipitation at highest concentration tested
cyantraniliprole	50.0	500.0	Precipitation at highest two concentrations tested
dicamba	200.0	2000	None
mesotrione	200.0	2000	Slight colour change at highest concentration tested
pinoxaden	100.0	1000	None
AI1	25.0	250.0	Precipitation at highest three concentrations tested

Table 8.12 Active ingredient maximum test concentrations used for the KeratinoSens™ assay and microplate observations

Test formulation	Concentration in Solvent (mM)	Concentration on the microplate (µM)	Test item observations upon addition to the microplate
SYN 1	200.0	2000	Small amount of precipitation in top concentration
SYN 2	102.4	1024	Small amount of precipitation in top concentration
SYN 3	70.64	706.4	None
SYN 4	70.71	707.1	None
SYN 5	85.15	851.5*	None
SYN 6	110.3	1103	None
SYN 7	138.5	1385	None
SYN 8	13.23	132.3	None
SYN 9	79.56	795.6	None
SYN 10	22.80	228.0	None

Table 8.13 Agrochemical formulation maximum test concentrations used for the KeratinoSens™ assay and microplate observations

*1:2 dilution in DMSO needed

The ten agrochemical AIs and formulations were dissolved in DMSO and any precipitation observed, upon addition of the test material formulations to the KeratinoSens™ exposure medium, was recorded and is reported in Tables 3.10 and 3.11. Serial dilutions were made from the initial stock solutions for each of the test materials. Twelve concentrations using a 2-fold dilution scheme with DMSO as the solvent were administered. The twelve concentrations used for each AI and formulation are listed in Tables 8.13 and 8.14.

Culture of the KeratinoSensTM human keratinocyte cell line

The KeratinoSens™ cell line is an immortalised and genetically modified human adherent HaCat keratinocyte cell line (Schoop et al., 1999). A vial of the human keratinocyte cell line was obtained under licence from the assay developer Givaudan (Givaudan SA, Switzerland). This was used to create

an in-house master stock of the KeratinoSens™ human keratinocyte cell line in the Gentronix laboratory and working stocks were created. All cell stocks were stored in liquid nitrogen. Cells were maintained in D-MEM media, supplemented with 9.1% heat inactivated foetal calf serum and G418 (Gibco, Life Technologies, UK), for a maximum of 25 passages. On the day prior to testing, cells were seeded into 96 well microplates at a concentration of 10,000 cells per well.

Test material administration

The KeratinoSens^{TM} cells were placed in 96-well pates and grown for 24 hours at 37^{o}C. The cell medium was removed, the cells were treated with 50 µL of the test material or positive control solution and solvent control cultures were treated with the same volume of solvent. Four concurrent 96-well microplates were prepared for each KeratinoSens™ test, three white, flat-bottomed 96-well microplates (for luminescence measurement) and one clear, flat bottomed 96-well microplate (for the MTT cytotoxicity assessment).

The treated plates were incubated for 48 hours at 37^{o}C in a humidified atmosphere of 5% CO_2. At the end of this treatment, the cells were washed, and the luciferase production measured by flash luminescence.

Endpoint measurements

Microscopic observation to evaluate the presence or absence of precipitate - transparent plate

After the 48-hour incubation period, the presence or absence of precipitate/emulsion was determined in each well by microscopic inspection.

Luminescence flash signal to evaluate induction signal (white plates)

After approximately 48 hours incubation with the test item, positive control or solvent, cells were lysed in passive lysis buffer (Promega Corp, UK). Passive lysis buffer was added to each well and the cells were incubated for 20 minutes, after which 50 µL of luciferase substrate (Promega Corp) was

added to each well. One second after this addition, the luciferase signal was integrated for two seconds.

Absorbance signal to evaluate the cytotoxicity (transparent plate)

Cytotoxicity was measured in parallel using a MTT reduction test. Following a 48-hour incubation with the test item, positive control or solvent control, cells were incubated with MTT for 4 hours under the same humidified atmosphere and temperature conditions (5% CO^2 and 37°C). After this incubation period, the cells were then dissolved in sodium dodecyl sulphate (SDS) and the absorption at approximately 600nM was determined for each well using a plate reader (Tecan Infinite F500).

Active ingredient	Observations following 48-hour exposure period with test item
acetamiprid	None
acibenzolar-s-methyl	None
benzovindiflupyr	Precipitation at highest 3 concentrations tested
chlorantraniliprole	Precipitation at highest 2 concentrations tested
chlorothalonil	Precipitation at highest 3 concentrations tested
cyantraniliprole	Precipitation at highest 5 doses tested
dicamba	None
mesotrione	None
pinoxaden	None
AI1	None

Table 8.14 Observations following the test item exposure period for the KeratinoSens™ assay

8.5 Appendix 5. KeratinoSens™ raw data results

Active Ingredient	I_{max} values for Replicates (Rep) 1 - 5					
	Rep 1 I_{max}	Rep 2 I_{max}	Rep 3 I_{max}	Rep 4 I_{max}	Rep 5 I_{max}	Mean I_{max}
acetamiprid	NA	NA	1.40	1.21	1.47	1.36
acibenzolar-s-methyl	NA	NA	1.06	0.98	1.00	1.01
benzovindiflupyr	12.12	3.20	NA	NA	NA	7.66
chlorantraniliprole	1.19	MTT	1.10	0.94	1.28	1.13
chlorothalonil	17.85	7.49	NA	NA	NA	12.67
cyantraniliprole	NA	NA	1.17	1.05	1.06	1.10
dicamba	1.14	MTT	1.19	1.17	1.31	1.20
mesotrione	NA	NA	1.69	2.42	1.74	1.95
pinoxaden	26.00	21.34	NA	NA	NA	23.66
AI1	NA	NA	2.68	3.25	2.68	2.87

Table 8.15 Imax values from the KeratinoSens™ assay for the ten active ingredients

Key: NA = Not Applicable – test item was not tested in this experimental run, MTT = Cytotoxicity data from the MTT assay was unacceptable for this experimental run.

Active Ingredient	$EC_{1.5}$ values (µM) for Replicates (Rep) 1 - 5					
	Rep 1 $EC_{1.5}$	Rep 2 $EC_{1.5}$	Rep 3 $EC_{1.5}$	Rep 4 $EC_{1.5}$	Rep 5 $EC_{1.5}$	Mean $EC_{1.5}$
acetamiprid	NA	NA	n.i.	n.i.	n.i.	n.i.
acibenzolar-s-methyl	NA	NA	n.i.	n.i.	n.i.	n.i.
benzovindiflupyr	8.60	4.69	NA	NA	NA	6.65
chlorantraniliprole	n.i.	MTT	n.i.	n.i.	n.i.	n.i.
chlorothalonil	<0.15	<0.15	NA	NA	NA	<0.15
cyantraniliprole	NA	NA	n.i.	n.i.	n.i.	n.i.
dicamba	n.i.	MTT	n.i.	n.i.	n.i.	n.i.
mesotrione	NA	NA	781.10	651.30	1324	918.80
pinoxaden	28.16	29.03	NA	NA	NA	28.60
AI1	NA	NA	23.54	41.83	21.01	28.79

Table 8.16 EC1.5 values from the KeratinoSens™ assay for the ten active ingredients

Key: n.i. = no induction above threshold, NA = Not Applicable – test item was not tested in this experimental run, MTT = Cytotoxicity data from the MTT assay was unacceptable for this experimental run.

Active Ingredient	IC_{50} values (µM) for Replicates (Rep) 1 - 5					
	Rep 1 IC_{50}	Rep 2 IC_{50}	Rep 3 IC_{50}	Rep 4 IC_{50}	Rep 5 IC_{50}	Mean IC_{50}
acetamiprid	NA	NA	>2000	>2000	>2000	>2000
acibenzolar-s-methyl	NA	NA	>250	>250	>250	>250
benzovindiflupyr	24.63	20.85	NA	NA	NA	22.74
chlorantraniliprole	>250	MTT	>250	>250	>250	>250
chlorothalonil	1.12	0.83	NA	NA	NA	0.97
cyantraniliprole	NA	NA	459.70	>500	>500	>500
dicamba	>2000	MTT	>2000	>2000	>2000	>2000
mesotrione	NA	NA	>2000	>2000	>2000	>2000
pinoxaden	110	104.90	NA	NA	NA	107.50
AI1	NA	NA	193.20	175.20	188.10	185.50

Table 8.17 IC50 values from the KeratinoSens™ assay for the ten active ingredients

Key: NA = Not Applicable – test item was not tested in this experimental run, MTT = Cytotoxicity data from the MTT assay was unacceptable for this experimental run.

Positive control induction values							Positive control acceptance criteria	
Cinnamic aldehyde	4 µM	8 µM	16 µM	32 µM	64 µM	$EC_{1.5}$	$EC_{1.5}$	I_{max} 64 µM
rep 1	1.06	1.18	1.38	1.97	3.18	19.33	TRUE	TRUE
rep 2	1.30	1.33	1.81	2.81	4.69	10.87	TRUE	TRUE
rep 3	1.21	1.44	1.65	2.22	4.95	10.13	TRUE	TRUE
rep 4	1.11	1.31	1.53	2.54	5.22	15.02	TRUE	TRUE
rep 5	1.26	1.47	1.89	2.75	9.82	8.66	TRUE	FALSE
Mean	1.19	1.35	1.65	2.46	5.57	12.80		

Table 8.18 Numerical results for the positive control cinnamic aldehyde in the KeratinoSens™ assay

Historical mean	22.01 µM
STDEV	10.82
Historical mean +2STDEV	43.65 µM
Historical mean -2STDEV	0.380 µM

Table 8.19 Gentronix Laboratory historic control data for cinnamic aldehyde positive control in the KeratinoSens™ assay

	% standard deviation blanks	
rep 1	14.51	accepted
rep 2	15.19	accepted
rep 3	12.27	accepted
rep 4	12.95	accepted
rep 5	12.11	accepted

Table 8.20 Solvent control variability

8.6 Appendix 6. H-CLAT assay methodology

Test item administration of the CV75 Dose Finding Assay

The THP-1 cells at a final density of 1 x 10^6 cells/mL were treated with 500 µL of the of test material for 24 hours for approximately one hour at 37°C in a 5% CO_2 incubator. Eight AI concentrations in a 2-fold dilution scheme were administered. Solvent control cultures were treated with the same volume used in nine AIs using DMSO and a medium control was used for dicamba only.

Following the 24-hour incubation with the test material, a cell viability test was conducted. The cells were harvested and stained with propidium iodide (PI) at a concentration of 0.625µg/mL. The staining was performed in the absence of light and at 4^0C. Approximately 10000 living cells (i.e., cells exhibiting low or no PI staining) were collected and analysed using flow cytometry. The percentage of living cells (PI negative) was used to determine the cell viability. The flow cytometry software indicated the cell viability (% total) of the cell sample taken, or, if necessary, it was calculated as follows:

Cell viability = (number of living cells / total number of acquired cells) X 100

At least two cell viability assays were included at each test material concentration. On every occasion independently prepared test material formulations and control solutions were used, with separate cell passages, to derive a reliable CV75. The mean of the two runs was then used to set the test material dose range for measuring the CD54 and CD86 expression.

As indicated in OECD Test Guideline 442E (OECD, 2018a), the CV75 value was calculated by log-linear interpolation using the following equation:

Log CV75 = ((75 - c) X Log(b) – (75 - a) X Log(d)) / a – c

Where a, b, c and d are represented in a dose response curve as shown in Figure 8.2.

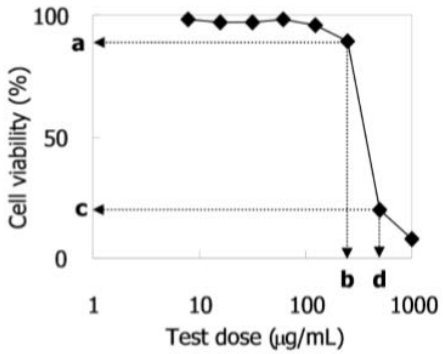

Figure 8.2 CV75 determination dose response curve (OECD, 2018a)

a – the minimum value of cell viability over 75%

c – the maximum value of cell viability below 75%

b and d - the test material concentrations leading to the value of cell viability of a and c respectively

Agrochemical formulation and Active ingredient test material formulation

Following the dose-range finding experimentation, eight final concentrations of the test materials were selected for the measurement of CD54 and CD86 expression. The highest test material concentration used for all substances was CV75 X 1.2 and a 1.2-fold dilution series was created to generate the seven remaining test concentrations. Where no cytotoxicity was observed following cell incubation with a test material, the eight dosing concentrations were prepared using a 1.2 -fold dilution series from the highest soluble concentration previously determined. The final maximum test concentration did not exceed 5000 µg/mL for test materials prepared in saline or culture medium, or 1000 µg/mL for test materials prepared in DMSO.

During the pre-test solubility assessment for the test formulations 1% Pluronic was also explored as a potential solvent. It was explored because it is used frequently as a vehicle in the LLNA testing of formulations. Confirmation of Pluronic as a usable solvent for this h-CLAT test would allow for the removal of the solvent variable when comparing results to the *in vivo* LLNA test. However, this type of Pluronic was extremely viscous. Vortexing and sonicating did not improve the solubility and resulted in oily globules within a cloudy media. Pluronic was immiscible with the THP-1 media, as such testing proceeded with the most appropriate solvents identified pre-test solubility.

8.7 Appendix 7. H-CLAT assay raw data results

Active Ingredient	Eight active ingredient test formulation concentrations (µg/mL)							
	dilution 1	dilution 2	dilution 3	dilution 4	dilution 5	dilution 6	dilution 7	dilution 8
acetamiprid	800.00	666.70	55.60	463.00	385.80	321.50	267.90	223.30
acibenzolar-s-methyl	30.00	25.00	20.83	17.36	14.47	12.06	10.05	8.37
benzovindiflupyr	15.63	13.03	10.85	9.05	7.54	6.28	5.23	4.36
chlorantraniliprole	62.50	52.08	43.40	36.17	30.14	25.12	20.93	17.44
chlorothalonil	150.00	125.00	104.20	86.81	72.34	60.28	50.23	41.86
cyantraniliprole	200.00	166.70	138.90	115.70	96.45	80.38	66.98	55.82
dicamba	5000.00	4167.00	3472.00	2894.00	2411.00	2009.00	1674.00	1395.00
mesotrione	600.00	500.00	416.70	347.20	289.40	241.10	200.90	167.40
pinoxaden	150.00	125.00	104.20	86.81	72.34	60.28	50.23	41.86
AI1	75.00	62.50	52.08	43.40	36.17	30.14	25.12	20.93

Table 8.21 AI h-CLAT test formulation concentrations

Test formulation	Eight Agrochemical test formulation concentrations (µg/mL)							
	dilution 1	dilution 2	dilution 3	dilution 4	dilution 5	dilution 6	dilution 7	dilution 8
SYN1	83.22	69.35	57.79	48.16	40.13	33.44	27.87	23.23
SYN2	78.13	65.11	54.26	45.21	37.68	31.40	26.17	21.80
SYN3	250	208.30	173.60	144.70	120.60	100.50	83.72	69.77
SYN4	875.00	729.20	607.60	506.40	422	351.60	293	244.20
SYN5	39.06	32.55	27.13	22.60	18.84	15.70	13.08	10.90
SYN6	2.87	2.40	2.00	1.66	1.39	1.16	0.96	0.80
SYN7	89.47	74.56	62.13	51.78	43.15	35.96	29.96	24.97
SYN8	10	8.33	6.94	5.79	4.82	4.02	3.35	2.79
SYN9	500	416.70	347.20	289.40	241.10	200.90	167.40	139.50
SYN10	147.10	122.60	102.20	85.13	70.94	59.12	49.26	41.05

Table 8.22 Agrochemical formulation h-CLAT test concentrations

Agrochemical AI h-CLAT performance evaluation data

N	*h-CLAT. positive*	*h-CLAT. negative*
in vivo. sens	1	3
in vivo. non sens	2	4

Table 8.23 Agrochemical AI h-CLAT results confusion matrix

Positive predictivity	33.3%
Negative predictivity	57.1%
Sensitivity	25.0%
Specificity	66.7%
Total Success	50.0%
**kappa-value*	-0.09

Table 8.24 Agrochemical formulation h-CLAT performance evaluation data

Active ingredient	Test item observations upon addition to the 24-well plate	Test item observations following exposure period
acetamiprid	None	None
acibenzolar-s-methyl	Precipitation at seven highest concentrations tested (30.00 – 10.05 µg/mL) in both runs 1 and 2	None
benzovindiflupyr	None	None
chlorantraniliprole	None	None
chlorothalonil	None	None
cyantraniliprole	Precipitation at highest concentration tested (200.0 µg/mL) in run 1 and again at the two highest concentration tested (200.0 and 166.7 µg/mL) in run 2	Small amounts of precipitation at highest four doses tested (200.0 – 115.7 µg/mL) in run 1 only
dicamba	None	None
mesotrione	Slight colour change at six highest concentrations tested (600.0 – 241.1 µg/mL) in both runs 1 and 2	None
pinoxaden	None	None
AI1	None	None

Table 8.25 Recorded changes following AI test substance administration to h-CLAT 24 well plate.

Agrochemical formulation h-CLAT performance evaluation data

N	*h-CLAT. positive*	*h-CLAT. negative*
in vivo. sens	2	4
in vivo. Non sens	3	1

Table 8.26 Agrochemical formulation h-CLAT results confusion matrix

Positive predictivity	40.0%
Negative predictivity	20.0%
Sensitivity	33.3%
Specificity	25.0%
Total Success	30.0%
**kappa-value*	-0.40

Table 8.27 Agrochemical formulation h-CLAT performance evaluation data

8.8 Appendix 8. Defined approach formulation and AI raw data

AI two out of three defined approach results

N	Pred. Bad	Pred. Neutral	Pred. Good
Obs. Bad	3	1	0
Obs. Neutral	0	0	0
Obs. Good	4	1	1

Table 8.28 3x3 confusion matrix for the two/three skin sensitisation defined approach on the AI test material

Statistics	**Value**
Total Success (%)	*40.0%*
**kappa-value*	*0.09*
N	*10*

Table 8.29 two/three AI Classification Success Rates

Agrochemical formulation weight of evidence and performance evaluation data

N	*Pred. Sens*	*Pred. Non Sens*
in vivo. sens	3	2
in vivo. non sens	2	1

Table 8.30 Agrochemical formulation WoE approach confusion matrix

Positive predictivity	60.0%
Negative predictivity	33.3%
Sensitivity	60.0%
Specificity	33.3%
Total Success	50.0%
**kappa-value*	-0.07

Table 8.31 Agrochemical formulation WoE performance evaluation data

8.9 Appendix 9. SENS-IS solvent material list

Controls	Material	Supplier	Batch number	Description	Concentration	Storage
vehicle/negative control	Phosphate Buffered Saline	Gibco	1905421	Clear solution	Pure	Room temperature
vehicle/negative control	Olive oil	Puget	18215V0114	Yellow solution	Pure	Room temperature, protected from light and humidity
vehicle/negative control	Dimethyl sulfoxide	Pan biotech	H170410	Viscous clear solution	Pure	Room temperature, protected from light and humidity
Irritation positive control	Sodium Lauryl Sulfate	Invitrogen	1927138	10% dilution clear solution	5% (commercial solution diluted at 50% in PBS)	Room temperature, protected from light and humidity
Sensitisation positive control	2, 4,6-trinitrobenzene sulfonic acid	Sigma	BCBV5717	Clear yellow solution	1% solution	-20°C

Table 8.32 SENS-IS solvent material list

8.10 Appendix 10. Positive and negative control results for all experimental runs

Key

Positive ■ negative □

Experimental controls for SYN1

Table 8.33 Positive and negative control results for all experimental runs

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	10	4
SENS-IS	3	8	0
REDOX	4	16	0
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	14	5
SENS-IS	5	11	5
REDOX	4	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN2

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	10	4
SENS-IS	3	8	0
REDOX	4	16	0
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	14	5
SENS-IS	5	11	5
REDOX	4	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN3

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	14	5
SENS-IS	5	11	5
REDOX	4	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	22	11	2
SENS-IS	2	7	2
REDOX	1	14	1
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN4

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	17	13	1
SENS-IS	2	8	6
REDOX	2	15	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	14	5
SENS-IS	5	11	5
REDOX	4	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	18	6	1
SENS-IS	5	5	0
REDOX	5	14	2
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN5

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	19	13	14
SENS-IS	1	10	3
REDOX	5	17	3
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	17	13	1
SENS-IS	2	8	6
REDOX	2	15	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN6

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	19	13	14
SENS-IS	1	10	3
REDOX	5	17	3
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	17	13	1
SENS-IS	2	8	6
REDOX	2	15	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #4:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN7

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	14	5
SENS-IS	5	11	5
REDOX	4	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	22	11	2
SENS-IS	2	7	2
REDOX	1	14	1
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN8

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	10	4
SENS-IS	3	8	0
REDOX	4	16	0
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	23	14	5
SENS-IS	5	11	5
REDOX	4	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN9

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	19	13	14
SENS-IS	1	10	3
REDOX	5	17	3
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	17	13	1
SENS-IS	2	8	6
REDOX	2	15	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experimental controls for SYN10

Experiment #1:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	19	13	14
SENS-IS	1	10	3
REDOX	5	17	3
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #2:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	17	13	1
SENS-IS	2	8	6
REDOX	2	15	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

Experiment #3:

	Positive control for irritation	Positive controls for sensitisation	Negative control
Number of overexpressed genes	SLS 5%	TNBS 1%	DMSO 100%
Irritation	21	10	4
SENS-IS	6	5	4
REDOX	5	16	4
Irritation Outcome	POSITIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	POSITIVE	NEGATIVE

8.11 Appendix 11. SENS-IS gene induction results of the experimental runs for the ten test formulations

Key

Positive ■ negative □

Table 8.34 SENS-IS gene induction results of the three experimental runs for each of the ten test formulations

	Experimental run 1		Experimental run 2		Experimental run 3	
SYN1	10% (in PBS)	10% (DMSO)	50% (DMSO)	10% (DMSO)	10% (DMSO)	1% (DMSO)
Irritation	**14**	**17**	**23**	**16**	**15**	**6**
SENS-IS	**7**	**6**	**4**	**3**	**5**	**3**
REDOX	**7**	**7**	**8**	**7**	**7**	**3**
Irritation Outcome	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE
Sensitisation Outcome	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE

SYN2	50% (PBS)	10% (PBS)	10% (PBS)	1% (PBS)	10% (PBS)	1% (PBS)
Irritation	**13**	**12**	**8**	**5**	**5**	**4**
SENS-IS	**10**	**10**	**4**	**4**	**1**	**5**
REDOX	**15**	**12**	**9**	**5**	**7**	**6**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE

SYN3	50% (PBS)	10% (PBS)	100%	50% (PBS)	100%	50% (PBS)
Irritation	**9**	**0**	**3**	**1**	**2**	**1**
SENS-IS	**6**	**6**	**2**	**3**	**2**	**4**
REDOX	**4**	**4**	**2**	**1**	**2**	**6**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIV	NEGATIVE

SYN4	10% (in PBS)	10% (DMSO)	100%	50% (PBS)	100%	50% (PBS)
Irritation	**7**	**4**	**14**	**1**	**13**	**5**
SENS-IS	**3**	**5**	**11**	**1**	**4**	**1**
REDOX	**2**	**3**	**11**	**5**	**7**	**3**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE

SYN5	10% (in PBS)	10% (DMSO)	100%	50% (PBS)	100%	50% (PBS)
Irritation	**7**	**4**	**14**	**13**	**9**	**11**
SENS-IS	**1**	**3**	**6**	**5**	**3**	**6**
REDOX	**6**	**5**	**5**	**6**	**5**	**6**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

	Experimental run 1		Experimental run 2		Experimental run 3	
SYN6	50% (PBS)	10% (PBS)	50% (PBS)	10% (PBS)	1% (PBS)	0.1% (PBS)
Irritation	**14**	**14**	**5**	**10**	**10**	**4**
SENS-IS	**5**	**10**	**3**	**4**	**5**	**8**
REDOX	**8**	**8**	**8**	**8**	**13**	**7**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE

	Experimental run 1		Experimental run 2			Experimental run 3	
SYN7	50% (PBS)	10% (PBS)	100%	50% (PBS)	10% (PBS)	100%	50% (PBS)
Irritation	**7**	**10**	**0**	**2**	**6**	**3**	**1**
SENS-IS	**5**	**5**	**1**	**1**	**4**	**1**	**3**
REDOX	**5**	**6**	**0**	**1**	**4**	**3**	**3**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

	Experimental run 1		Experimental run 2		Experimental run 3	
SYN8	50% (PBS)	10% (PBS)	10% (PBS)	1% (PBS)	1% (PBS)	0.1% (PBS)
Irritation	**13**	**6**	**2**	**2**	**2**	**1**
SENS-IS	**1**	**7**	**5**	**6**	**3**	**4**
REDOX	**7**	**8**	**9**	**8**	**9**	**4**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE

SYN9	10% (PBS)	10% (DMSO)	50% (PBS)	10% (PBS)	100%	50% (PBS)
Irritation	**6**	**8**	**16**	**7**	**19**	**17**
SENS-IS	**0**	**4**	**6**	**2**	**4**	**4**
REDOX	**5**	**4**	**6**	**5**	**5**	**2**
Irritation Outcome	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	POSITIVE
Sensitisation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

SYN10	10% (PBS)	10% (DMSO)	10% (DMSO)	1% (DMSO)	1% (DMSO)	0.1% (DMSO)
Irritation	**13**	**14**	**12**	**3**	**5**	**6**
SENS-IS	**7**	**2**	**1**	**3**	**4**	**2**
REDOX	**9**	**9**	**8**	**11**	**9**	**3**
Irritation Outcome	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
Sensitisation Outcome	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE

8.12 Appendix 12. Chapter Three active ingredient test material *in silico* model entry information

Active Ingredient	CAS Number	SMILES	OECD toolbox – identified metabolites with potential for covalent binding
Acetamiprid	135410-20-7	CC(=NC#N)N(C)CC1=CN=C(C=C1)Cl	Skin metabolite – Schiff base formation
Acibenzolar-s-methyl	7135158-54-2	CSC(=O)C1=C2C(=CC=C1)N=NS2	Skin metabolite – nucleophilic substitution (S_N2)
Benzovindiflupyr	1072957-71-1	O=C(Nc1cccc2c1C3C(=C(Cl)Cl)C2CC3)c4cn(nc4C(F)F)C	Skin metabolite 1 – Schiff base formation Skin metabolite 2 & 4 – acylation
Chlorantraniliprole	736994-63-1	CC1=CC(=CC(=C1NC(=O)C2=CC(=NN2C3=C(C=CC=N3)Cl)Br)C(=O)NC)Cl	None
Chlorothalonil	1897-45-6	c1(c(c(c(Cl)c(c1Cl)Cl)C#N)Cl)C#N	None
Cyantraniliprole	736994-63-1	Cc1cc(cc(c1NC(=O)c2cc(nn2c3c(cccn3)Cl)Br)C(=O)NC)C#N	Skin metabolite 1 – Schiff base formation Skin metabolites 3,6, 7 & 8 – acylation
Dicamba	1918-00-9	c1(c(c(ccc1Cl)Cl)OC)C(=O)O	Skin metabolite – Schiff base formation
Mesotrione	104206-82-8	CS(=O)(=O)C1=CC(=C(C=C1)C(=O)C2C(=O)CCCC2=O)[N+](=O)[O-]	None
Pinoxaden	243973-20-8	O=C(OC1=C(C(=O)N2N1CCOCC2)c3c(cc(cc3CC)C)CC)C(C)(C)C	None
AI1	Agreed Confidential information		

Table 8.35 Active ingredient smiles codes and metabolites identified by the OECD toolbox containing structural alerts for protein binding associated with skin sensitisation.

8.13 Appendix 13. Copy of published GARD assay agrochemical AI evaluation research

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Comparison of the predictive nature of the Genomic Allergen Rapid Detection (GARD) assay with mammalian assays in determining the skin sensitisation potential of agrochemical active ingredients

William Masinja[a,c,*], Claire Elliott[a,b], Sandeep Modi[a], Steven J. Enoch[c], Mark T.D. Cronin[c], Elizabeth F. McInnes[a], Richard A. Currie[a]

[a] Syngenta, International Research Centre, Jealott's Hill, Bracknell, Berks RG42 6EY, United Kingdom
[b] Pennan Consulting Limited, Aspect House, Waylands Avenue, Wantage, Oxon OX12 9FF, United Kingdom
[c] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, United Kingdom

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ABSTRACT

Alternatives to mammalian testing are highly desirable to predict the skin sensitisation potential of agrochemical active ingredients (AI). The GARD assay, a stimulated, dendritic cell-like, cell line measuring genomic signatures, was evaluated using twelve AIs (seven sensitisers and five non-sensitisers) and the results compared with historical results from guinea pig or local lymph node assay (LLNA) studies. Initial GARD results suggested 11/12 AIs were sensitisers and six concurred with mammalian data. Conformal predictions changed one AI to a non-sensitiser. An AI identified as non-sensitising in the GARD assay was considered a potent sensitiser in the LLNA. In total 7/12 GARD results corresponded with mammalian data. AI chemistries might not be comparable to the GARD training set in terms of applicability domains. Whilst the GARD assay can replace mammalian tests for skin sensitisation evaluation for compounds including cosmetic ingredients, further work in agrochemical chemistries is needed for this assay to be a viable replacement to animal testing. The work conducted here is, however, considered exploratory research and the methodology needs further development to be validated for agrochemicals. Mammalian and other alternative assays for regulatory safety assessments of AIs must provide confidence to assign the appropriate classification for human health protection.

1. Introduction

Allergic contact dermatitis is caused by an adverse immune response to chemical haptens (Rustemeyer et al., 2012; Kaplan et al., 2012). For compounds such as agrochemicals, the identification of skin sensitising properties is an important part of regulatory hazard assessment to ensure safety during manufacture and use. Currently there is no globally harmonised position on the use of *in vitro* alternatives for regulatory purposes. Consequently, agrochemicals are routinely tested for skin sensitisation using *in vivo* tests such as the guinea pig tests and the local lymph node assay (LLNA) (Basketter et al., 2012; Gwaltney-Brant, 2014). Recently attempts have been made to identify non animal-based methods with good predictive power for chemical hazard identification in a bid to reduce laboratory animal use (Alloul-Ramdhani et al., 2014; Doe and Botham, 2019; Reisinger et al., 2015; Ivan de Ávila et al., 2019). In accordance with Article 62 of the European Regulation (EC) No. 1107/2009, concerning the placing of plant protection products on the market; the use of *in vivo* mammalian test methods should only be used as a last resort. Where available non-animal test methods should be used and promoted (EC, 2009) and several such *in vitro* assays have been developed for skin sensitisation. The Genomic Allergen Rapid Detection (GARD) assay is one of the more recent assays with as yet unknown potential for agrochemicals and therefore it was selected for evaluation in this investigation.

The GARD assay is a cell-based, *in vitro* alternative to animal testing which assesses skin sensitisation by measuring the biomarker signature in chemical-stimulated, human MUTZ-3 cells (Johansson et al., 2011). The MUTZ-3 cell line serves as a surrogate for dendritic cells (DC) and changes in transcription in the genes can be linked to processes involved in skin sensitisation (Rovida et al., 2013; Masterson et al., 2002). The GARD assay measures transcriptional changes in 200 genes associated with sensitisation (Johansson et al., 2011). The 200 gene biomarker signature includes transcripts involved in oxidative stress, dendritic cell maturation and cytokine responses (Johansson et al.,

* Corresponding author at: Syngenta, International Research Centre, Jealott's Hill, Bracknell, Berks RG42 6EY, United Kingdom.
E-mail address: william.masinja@syngenta.com (W. Masinja).

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