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Extension of the Dermal Sensitisation Threshold (DST) approach to incorporate chemicals classified as reactive

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ABSTRACT

The evaluation of chemicals for their skin sensitising potential is an essential step in ensuring the safety of ingredients in consumer products. Similar to the Threshold of Toxicological Concern, the Dermal Sensitisation Threshold (DST) has been demonstrated to provide effective risk assessments for skin sensitisation in cases where human exposure is low. The DST was originally developed based on a Local Lymph Node Assay (LLNA) dataset and applied to chemicals that were not considered to be directly reactive to skin proteins, and unlikely to initiate the first mechanistic steps leading to the induction of sensitisation. Here we have extended the DST concept to protein reactive chemicals. A probabilistic assessment of the original DST dataset was conducted and a threshold of 64 μg/cm^2 was derived. In our accompanying publication, a set of structural chemistry based rules was developed to proactively identify highly reactive and potentially highly potent materials which should be excluded from the DST approach. The DST and rule set were benchmarked against a test set of chemicals with LLNA/human data. It is concluded that by combining the reactive DST with knowledge of chemistry a threshold can be established below which there is no appreciable risk of sensitisation for protein-reactive chemicals.

1. Introduction

The concept of the Threshold of Toxicological Concern (TTC) was first introduced some 27 years ago by Rulis (1986). Rulis used historical data from experimental carcinogenicity studies on chemicals to build a probabilistic analysis of the Virtually Safe Dose (VSD). From this analysis he was able to determine a dose below which there was a low probability of appreciable risk to human health, even if a chemical were not tested. The concept was later adopted by the US Food and Drug Administration as the basis for their Threshold of Regulation (US Food and Drug Administration, 1995).

Since that time much work has been done to expand and refine the TTC concept, including development of TTC values for systemic toxicity from oral exposure including the utilisation of structural based filters such as the Cramer decision tree (Cramer et al., 1978; Munro et al., 1996) and the Cohort of Concern for carcinogens (Kroes et al., 2004). The TTC concept has gained acceptance for a number of risk assessment/management applications including flavours used in foods (JECFA, 1997; Munro et al., 1999; Renwick, 2004), genotoxic impurities in pharmaceuticals (EMEA, 2006; Muller et al., 2006) and contaminants in foods (EFSA, 2012). The TTC has also gained acceptance for its use in contaminants and ingredients in cosmetics and consumer products (SCCS/SCHER/SCENIHR, 2012). Proposals have more recently been made to expand the TTC concept to inhalation of chemicals covering both systemic and local lung effects (Carthew et al., 2009), and also for skin sensitisation (Safford, 2008; Safford et al., 2011).

Skin sensitisation resulting in allergic contact dermatitis (ACD) is an important issue for both occupational/environmental health and consumer product development. The manifestation of ACD is one of the most common immunotoxic responses, resulting in the need to accurately identify skin sensitisation hazard and to conduct safety assessments to protect human health (Kimber et al., 2001). Historically, the skin sensitisation potential and potency of ingredients has been determined using assays in animals (e.g., Guinea Pig Maximisation Test, Murine Local Lymph Node Assay [LLNA]). Ingredients shown to have no skin sensitisation potential in these models are generally considered to be non-sensitising and carry little risk to humans. Where an ingredient is shown to have skin sensitisation potential in animal assays, the risk to consumers from its inclusion in consumer products can

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be established from the relative potency observed in the assay, confirmed in a Human Repeat Insult Patch Test (HRIPT), by conducting a Quantitative Risk Assessment (QRA) as described by Api et al. (2008). The outcome of the QRA can be used as the basis for risk management measures which, given the nature of the intended product use, involves setting a maximum level at which the ingredient can be incorporated. Such a process has been used by the fragrance industry in setting use level standards for skin allergens (IFRA, 2011), and by consumer goods manufacturers for determining safe levels of use for sensitising ingredients such as preservatives.

The use of in vivo assays in making such assessments raises ethical issues on the use of animals, as well as being costly and time consuming. In addition, the EU Cosmetic Regulation now places a marketing ban on cosmetic products containing any ingredient that has been tested in animals after the regulation's effective date (European Parliament and the Council of the European Union, 2009).

Much research has been and continues to be conducted, in understanding the biological mechanisms involved in skin sensitisation with the aim of developing techniques based on in vitro, in chemico and in silico assays to determine the skin sensitisation potential and potency of chemicals (Goebel et al., 2012). In the future it is hoped that such methodologies will replace animal testing and provide information on which risk assessments for skin sensitisation can be based.

Use of the Dermal Sensitisation Threshold, or DST, presents a potential way of reducing unnecessary animal testing without increasing the risk to consumers. The DST applies the same principles as those used to develop the TTC to define a level of skin exposure where there is no appreciable risk of skin sensitisation to an untested chemical (Safford, 2008). The method involves the fitting of probability distributions to skin sensitisation potency data for chemicals which have previously been tested. Using probability estimates from such distributions along with an estimate of the proportion of sensitisers in the world of chemicals, a probability that an untested chemical will have a potency of greater than a given value can be determined. In Safford (2008), it was suggested that a 95% probability of an untested chemical having a lower potency than the DST might be considered acceptable since this figure has been previously used in setting TTC levels.

The DST has subsequently been refined making use of the knowledge that an initial and necessary step in the induction of skin sensitisation is the binding of the chemical to skin proteins to form a hapten (Gerberick et al., 2008; Roberts and Aptula, 2008). The chemicals themselves need to be sufficiently reactive to proteins, or capable of being oxidised or metabolised to a reactive product (Aptula and Roberts, 2006; Lepoittevin, 2006). By examining their structure, chemicals can be placed into one of five reactive domains as previously described (Aptula and Roberts, 2006), or identified as non-reactive. With this method it was possible to split the DST database of sensitisers into reactive and non-reactive domains in much the same way that the Cramer classification (Cramer et al., 1978) was used to differentiate the systemic toxicity potency of chemicals in the TTC database. Thus it was possible to define a DST of 900 $\mu g/cm^2$ for chemicals identified as non-reactive (Safford et al., 2011).

In the latter publication, no proposal was made to develop a DST for chemicals falling into the reactive domain, and the recommendation for such reactive chemicals was a case-by-case risk assessment. Subsequently, it has been recognised that the development of a DST for reactive chemicals may be of use for ingredients where consumer exposure is very low. Such ingredients include fragrances which may be incorporated in products at very low levels. In this publication we describe the development of a DST for reactive chemicals using the same probabilistic principles as used in the development of the TTC and DST for non-reactive chemicals. Further, an approach to identify high potency sensitisers and indicate cases where the DST should not be applied (Roberts et al., 2015) is utilised to provide a robust process.

2. Materials and methods

2.1. Local Lymph Node Assay (LLNA) data and calculating the potency distribution

In order to establish a DST for reactive chemicals, a dataset previously used to develop a DST for non-reactive chemicals was utilised. The database has been fully described by Safford et al. (2011).

The dataset is comprised of LLNA data from a number of published sources and includes 363 chemicals for which EC3 values were available (consisting of 271 skin sensitisers and 92 non-sensitisers). The chemicals were classified into their appropriate reaction mechanistic chemistry domains using the rules defined by Aptula and Roberts (2006) and further discussed by Roberts et al. (2007, 2014). These rules place chemicals into domains which identify the most likely organic reaction mechanism leading to protein binding and include Michael acceptors, S_N2 electrophiles, S_NAr electrophiles, Schiff base formers, acylating agents, special cases, or into non-reactive/non-pro-reactive domains. Special cases are those chemicals which can be classified in one of the 5 reaction domains, although in some cases only provisionally, requiring further comment, or chemicals which do not fit any of the above domains (e.g., S_N1 electrophiles). Chemicals which require metabolism to become reactive (pro-reactive chemicals) are classified into the domain of the predicted metabolite (e.g., cinnamic alcohol is a pro-reactive Michael acceptor, therefore was classified as a Michael acceptor). A chemical is classified as non-reactive and non-pro-reactive if none of the rules for reactive domains apply. Finally, it should be noted that inorganic chemicals are considered to fall outside of the applicability domain of the DST approach.

A potency distribution was established for those reactive chemicals which were sensitisers. As in previous DST analyses, the distribution was constructed using negative log(10) EC3% values, and a Gamma distribution was fitted to the resultant histogram using EasyFitXL (MathWave Technologies).

2.2. Identification of High Potency Category Chemicals (HPCs)

In setting a DST for reactive chemicals, a value is taken from the above distribution which corresponds to a given probability that a chemical is less potent. Since this is a probabilistic approach, it is recognised that there is a probability that chemicals may be more potent than the chosen DST value. In rare cases chemicals may have extreme potency (the most potent chemical in the DST database is Benzo[a]pyrene with an EC3 value of 0.0009%). Such high potency chemicals may potentially present an unacceptable risk unless they can be identified and flagged as outside the application of the DST approach. In order to address this, additional work has been conducted to examine further the chemical structures of the highly potent chemicals in the DST database and develop structure based rules to identify particularly potent sensitisers. The rule set is described, in detail, in a concurrent publication (Roberts et al., 2015).

A material flagged by the rules is considered to be within the High Potency Category Chemicals (HPCs) classification for which the DST should not be applied. Using the HPC rule set, reactive sensitisers in the DST database were further classified into HPC and non-HPC chemicals.

2.3. Benchmarking of the DST value

In order to check the robustness of the DST value determined in the above steps, a further set of chemicals, not included in the DST database, was established and classified using the HPC rules (Roberts et al., 2015). One of the sources of data used to establish the original DST data set was the ICCVAM (The Interagency Coordinating Committee on the Validation of Alternative Methods) database of sensitisers. The values were taken from this database in 2008. Since then further chemicals have been added along with EC3 values. The NICEATM (The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods) LLNA Database was downloaded from http://ntp.niehs.nih.gov/?objectid=40AFDDF1-D2B6-1850-EE321D717F291020 in June 2014. The database contained 1060 entries. However, many of these are duplicate entries (the same chemical tested more than once), inorganic chemicals, chemicals that are already in the DST database and chemicals with a dose–response pattern that does not enable a reliable EC3 to be determined. After removing these entries 72 additional chemicals for which EC3 values are available were identified for the benchmarking exercise.

2.4. Benchmarking against known fragrance allergens

The DST has been derived using a dataset of chemicals which include a diverse range of chemical types. In order to put this in perspective with respect to its use for fragrance ingredients, benchmarking was carried out against the potencies of a set of known fragrance allergens. A dataset of 55 chemically defined fragrance allergens was identified for which potency data based on both LLNA and HRIPT studies were available, and for which Weight of Evidence No Expected Sensitisation Induction Levels (WoE NESILs) have been derived based on methodology described by Api et al. (2008). Of these, 24 were not included in the DST dataset.

The LLNA EC3s and HRIPT NOELs (No Observed Effect Levels) of these fragrance ingredients were compared with the reactive DST value, and those which are more potent have been identified.

3. Results

3.1. Mechanistic chemistry domain classification

Based on mechanistic chemistry domain classification, all 363 chemicals were divided into two groups – those which were assigned to a reactive domain (Michael acceptors, Schiff bases, S_N2, S_NAr, acylating agents, special cases), including pro-reactive chemicals, were classified as reactive and those which were not assigned to one of these domains were classified as non-reactive. Table 1 shows the numbers of skin sensitisers and non-sensitisers in these two domains (reactive and non-reactive).

3.2. Calculating a DST for chemicals within the reactive domain

A histogram showing the distribution of negative log(10) EC3% values for the 233 chemicals assigned to a reactive domain which were sensitisers is shown in Fig. 1. In the figure a Gamma distribution has been fitted to the data, consistent with the development of the DST for non-reactive materials. The distribution – Gamma (2.7582; 0.56732; −2) is shown in Fig. 1.

As in the establishment of a DST for chemicals falling into the non-reactive domain (Safford et al., 2011), a probabilistic approach was taken to determine a DST value for chemicals falling into the reactive domains as follows:

- The proportion of skin sensitisers in the world of chemicals has previously been estimated as 20% based on an examination of the ELINCS (European LIst of Notified Chemical Substances) database (Safford, 2008). This figure was also found to be valid by Keller et al. (2009) who examined Annex I of Directive 67/548 EEC (3366 chemicals), the European Flavour and Fragrance Association database (EFFA, 2008 – 1487 chemicals) and the IFRA/RIFM dataset of fragrance ingredients.
- As a predictive tool for skin sensitisation, mechanistic chemistry domain classification has been shown to have a sensitivity of 86%, and a specificity of 64% (Safford et al., 2011).

Using these figures it was calculated that 86% of the 20% sensitisers will be correctly identified as sensitisers based on reactivity (i.e., 17.2% of all chemicals), and 36% of the 80% non-sensitisers will be incorrectly identified as sensitisers (i.e., 28.8% of all chemicals). The probability that a chemical classified as reactive is a sensitiser was calculated to be 0.374 (=17.2/(17.2 + 28.8)).

For the purposes of setting a DST for reactive chemicals, a value was derived such that 95% of all chemicals in the DST database are either non-sensitisers or have a potency which is less than this value (i.e., only 5% of chemicals in the database have an EC3 value lower than the DST). In order to obtain this, the percentile that needs to be taken from the distribution shown in Fig. 1 was calculated as follows:

- Probability of a chemical classified as reactive being a skin sensitiser = 0.374.
- Required probability that an untested chemical will have an EC3 value < DST = 0.05.
- Probability of a sensitising reactive chemical having an EC3 < DST = 0.05/0.374 = 0.134.
- Probability of a sensitising reactive chemical having an EC3 > DST = 1–0.134 = 0.866 (86%).

Table 1
Numbers of chemicals in the Dermal Sensitization Threshold (DST) database falling into the reactive and non-reactive domains.

	Sensitisers	Non-sensitisers	Total
Reactive	233	33	266
Non-reactive	38	59	97
Total	271	92	363

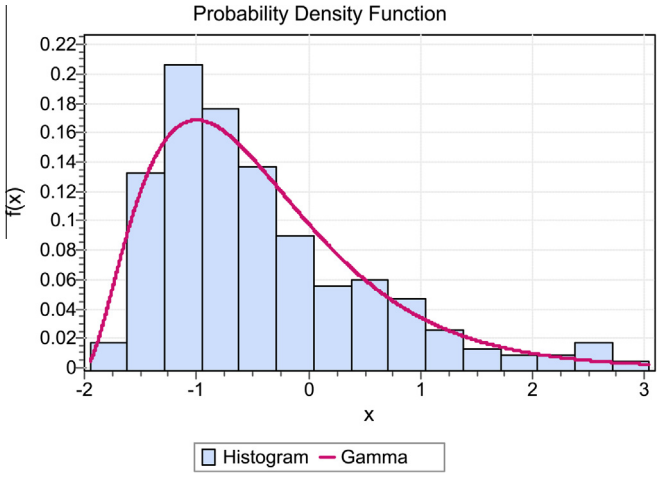

Fig. 1. Histogram showing the distribution of negative log(10) EC3% values for the 233 sensitisers in the Dermal Sensitisation Threshold (DST) database falling into the reactive domain, and associated Gamma distribution.

Thus in order to define a DST based on the 95th percentile the 86.6th percentile from the distribution in Fig. 1 was taken. This value is 0.59124 (negative log(10) EC3%), which is equivalent to 64 μg/cm^2.

3.3. Identification of HPC materials

The DST value of 64 μg/cm^2 is calculated to be the 95th percentile of EC3 values in reactive chemicals. Thus it is expected that 5% of reactive chemicals will be more potent than this value. In the DST database, 33 chemicals are more potent than 64 μg/cm^2. Looking at these chemicals it is clear that some are highly potent sensitisers. As an example, Table 2 shows the EC3 values for the ten most potent sensitisers in the DST database.

Such high potency sensitisers would present an unacceptable risk of sensitisation if a DST of 64 μg/cm^2 were to be used on its own. In order to address this, chemical structures of chemicals in the DST database were examined using the HPC approach described by Roberts et al. (2015) and classified into HPC and non-HPC classes.

Table 3 shows numbers of chemicals in the DST database defined as HPC, non-HPC and non-reactive and their sensitisation potential in the LLNA.

Of the 266 chemicals in the DST database falling into the reactive domain, 56 were classified as HPC and 209 as non-HPC. One chemical, potassium dichromate, is inorganic, and falls outside of the applicability domain of the approach. The sensitiser, 2,2-azobis-phenol originally placed in the non-reactive domain, was classified as HPC. One reactive chemical classified as HPC (methyl hexadecanesulfonate) was a non-sensitiser in the LLNA (this LLNA result is considered anomalous, methyl hexadecanesulfonate being strongly positive in a guinea pig test (Roberts and Basketter, 1997). Of the 209 non-HPC chemicals, 177 were sensitisers.

Only one non-HPC chemical (hexyl salicylate) had an EC3 value of less than 64 μg/cm^2 (45 μg/cm^2). This LLNA result is considered to be anomalous, and hexyl salicylate has been shown in an HRIPT to be at worst a weak sensitiser in humans. In fact, the NESIL for hexyl salicylate is 35,400 μg/cm^2, which is 500 times higher (less potent) than the reactive DST.

3.4. Validation of the DST value

Of the 72 additional chemicals with LLNA test results from the NICEATM LLNA Database, 18 were classified as HPC, and 54 as non-HPC. Of the 54 non-HPC chemicals only 1 was more potent than the proposed DST value of 64 μg/cm^2. This was ethyl-2-(hydroxymethyl)-1,3-propanediol triacrylate, with an EC3 of 32 μg/cm^2.

It should be emphasised that the EC3 values taken from the database were taken at face value and were not scrutinised.

Table 2
Ten most potent sensitisers in the Dermal Sensitisation Threshold (DST) database along with Local Lymph Node Assay EC3 values.

Chemical name	EC3 (μg/cm^2)
Benzo[a]pyrene	0.225
4′-Hydroxy chalcone	0.5
Oxazolone	0.75
Diphenylcyclo-propenone	0.75
Chlorothalonil	1.0
1-Chloromethylpyrene	2.5
7,12-Dimethylbenz[α]anthracene	2.5
5-Chloro-2-methyl-4-isothiazolin-3-one	2.5
p-Benzoquinone	2.5
1-Methyl-3-nitro-1-nitrosoguanidine	7.5

Table 3
Numbers of chemicals in the Dermal Sensitisation Threshold (DST) database defined as High Potency Category Chemicals (HPC), non-HPC and non-reactive and shown to be sensitisers or non-sensitisers, along with respective percentages of the totals.

		Sensitisers		Non-sensitisers		Total	
		No.	%	No.	%	No.	%
Reactive	HPC	55	20.4	1	1.1	56	15.5
	Non-HPC	177	65.6	32	34.8	209	57.7
Non-reactive		38	14.1	59	64.1	97	26.8
Total		270		92		362	

Abbreviation: HPC, High Potency Category Chemicals.

Many of the EC3 values for the 72 chemicals were submitted by industry, and no reports were available for review of the data.

3.5. Benchmarking against known fragrance allergens

The 55 fragrance allergens used in the benchmarking exercise, along with their LLNA EC3s, HRIPT NOEL and LOEL (Lowest Observed Effect Level, where determined) and WoE (Weight of Evidence) NESIL are listed in Table 4.

It can be seen that, in general, the potencies of these fragrance materials are lower than for the materials in the DST dataset. So, based on LLNA EC3 values, none of the fragrance ingredients fall into the potent category (classification according to Gerberick et al., 2001), and only 1 falls into the strong category. This is illustrated in Fig. 2 in which a comparison is made between the percentage of materials in the DST dataset falling into each category and those in the fragrance dataset.

All 55 fragrance allergens were classified as non-HPC. Based on the LLNA result, only one material has an EC3 value which is less than the proposed DST value of 64 μg/cm^2. This is hexyl salicylate, with an EC3 of 45 μg/cm^2. As has been previously noted, in a HRIPT with hexyl salicylate, a dose of 35,433 μg/cm^2 failed to induce sensitisation, and it is likely that the actual potency of this material in humans is well below the proposed DST.

Based on results from HRIPT studies, two materials have NOELs which are lower than the reactive DST value. These are trans-2-hexenal and methyl 2-nonynoate (methyl octine carbonate), each with a NOEL of 24 μg/cm^2. However, the LLNA EC3 values for the two materials are considerably higher at 1012 and 625 μg/cm^2 (see Table 4).

4. Discussion

A DST for reactive chemicals has been defined using the same probabilistic principles used to determine TTC values (Rulis, 1986; Munro et al., 1996), and subsequently used to determine an overall DST (Safford, 2008) and a DST for non-reactive materials (Safford et al., 2011). The analysis conducted was based on LLNA EC3 values for 271 chemicals, 233 of which were classified as reactive according to the principles given by Aptula and Roberts (2006). The DST was taken from the distribution of the EC3 values, taking into account the proportion of skin sensitisers in the world of chemicals, and the predictivity and sensitivity of reactivity classification as a tool for prediction of sensitisers.

Based on all of these factors, a DST for reactive chemicals has been determined to be 64 μg/cm^2. This is based on a 95% probability that materials defined as reactive will either be non-sensitisers or will have a sensitisation potency which is less than this value.

In determining this value, it has been assumed that the incidence of skin sensitisers in the world of chemicals is 20%. This was based on an analysis of the ELINCS database, listing chemicals registered between 1981 and 2005 (Safford, 2008) in which 409 chemicals were classified R43 (may cause sensitisation by skin

Table 4
Fragrance allergens used in the benchmarking exercise including potency data in the Local Lymph Node Assay and Human Repeated Insult Patch Test data.

Test material	LLNA EC3 (μg/cm^2)	HRIPT NOEL (μg/cm^2)	HRIPT LOEL (μg/cm^2)	WoE NESIL (μg/cm^2)
Hexyl salicylate	45	35,433		35,400
Methyl 2-octynoate (methyl heptine carbonate)	<125	118	194	110
Cinnamaldehyde	262[a]	591	775	590
Isoeugenol	498[a]	250	775	250
2-Hexylidene cyclopentanone	600	300	500	300
Benzyl salicylate	725	17,717		17,700
1-(5,5-Dimethyl-1-cyclohexene-1-yl)pent-4-en-1-one	745	2500		2500
Allyl phenoxyacetate	775	709		700
3-Propylidenephthalide	350	945	2760	920
Phenylacetaldehyde	962[a]	591	1181	590
trans-2-Hexenal	1012[a]	24	236	24
p-t-Butyl-dihydrocinnamaldehyde (bourgenol)	1075	1181	7087	1100
α-Methyl cinnamic aldehyde	1125	3543		3500
Farnesol	1200[a]	2755	6897	2700
6-Methyl-3,5-heptadien-2-one	>1250	118	1299	110
Methyl 2-nonynoate (methyl octine carbonate)	<1250 (estimate 625[b])	24	118	24
Citral	1414	1400	3876	1400
2-Methoxy-4-methylphenol	1450	118		118
Dibenzyl ether	1575	2362		2300
2-Phenylpropionaldehyde	1575	388	1938	380
Isocyclocitral	1825	7087		7000
p-Mentha-1,8-dien-7-al	2175[a]	709	2760	700
p-t-Butyl-α-methylhydro-cinnamic aldehyde (BMHCA)	2372[a]	4125	29,528	4100
α-Hexyl-cinnamaldehyde	2372[a]	23,622		23,600
p-Isobutyl-α-methyl hydrocinnamaldehdye	<2500	2362		2300
α-Amylcinnamaldehyde	2942[a]	23,622		23,600
Cinnamyl nitrile	>2500	1063	1938	1060
Menthadiene-7-methyl formate	>2025	1063	6900	1060
Methoxy dicyclopentadiene carboxaldehyde	>2500	5000		5000
Carvone	2675	2657		2650
Eugenol	2703[a]	5906		5900
α-Butylcinnamaldehyde	2775[a]			1000
Vetiver aceatate	2910[a]	2362		2300
α-Methyl-1,3-benzodioxole-5-propionaldehyde	4100	11,811	15,000	11,800
Geraniol	3525[a]	11,811		11,800
3 & 4-(4-Hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde (HMPCC)	4275[a]	4000		4000
Benzyl cinnamate	4600	4720		4700
2-Nonyn-1-al dimethyl acetal	>5000	23,622		23,000
Cinnamyl alcohol	5250[a]	3000	4724	3000
α-iso-Methylionone	5450	70,866		70,000
Hydroxycitronellal	5612[a]	5000	5906	5000
4-Methoxy-α-methyl benzenpropanal	5900	5905		5900
α-Amylcinnamyl alcohol	>6250	3543		3500
Benzaldehyde	>6250	590	2760	590
Isocyclogeraniol	>6250	3898	7752	3800
OTNE	6825	47,244		47,200
β,β,3-Trimethyl benzenepropanol	>7500	9900		9900
1-Octen-3-yl acetate	>7500	3543	6900	3500
D-Limonene	10,075[a]	10,000		10,000
DL-Citronellol	10,875	29,528		29,500
Benzyl alcohol	>12,500	5906	8858	5900
Benzyl benzoate	>12,500	59,050		59,000
Coumarin	>12,500	3543	8858	3500
Vanillin	>12,500	1181		1100
Linalool	12,650[a]	15,000		15,000

Abbreviations: LLNA, Local Lymph Node Assay; HRIPT, Human Repeat Insult Patch Test; NOEL, No Observed Effect Level; WoE NESIL, Weight of Evidence No Expected Sensitisation Induction Level.

[a] EC3 value is a vehicle weighted average from multiple Local Lymph Node Assays.

[b] Estimate based on log-linear extrapolation (Ryan et al., 2007), all doses tested down to the lowest of 1250 μg/cm^2 resulted in SI values >3.

contact) out of an estimated 2082 tested chemicals. In a more recent publication (Angers-Loustau et al., 2011), a similar analysis using the EC New Chemicals Database (NCD; which replaces the ELINCS database) showed a higher proportion of sensitisers. As of 2008, there were 5288 individual substances registered in the NCD, from which 3792 reported having been tested for skin sensitisation hazard assessment. Of these chemicals, 1047 (28%) were classified R43. This increase in the overall incidence may reflect a higher proportion of positive results in the 1710 chemicals tested from 2005 to 2008, or may reflect a difference in the methodology used to analyse the figures. However, as was noted in the Safford (2008) publication, at the time of analysis only 6% of the chemicals in the ELINCS database were registered as cosmetic and personal care product ingredients. A much larger proportion of the chemicals (25%) were intermediates (i.e., chemicals used for the synthesis of other chemicals) which might be expected to be more chemically reactive than cosmetic and personal care product ingredients, and therefore more likely to be sensitisers. Other chemicals

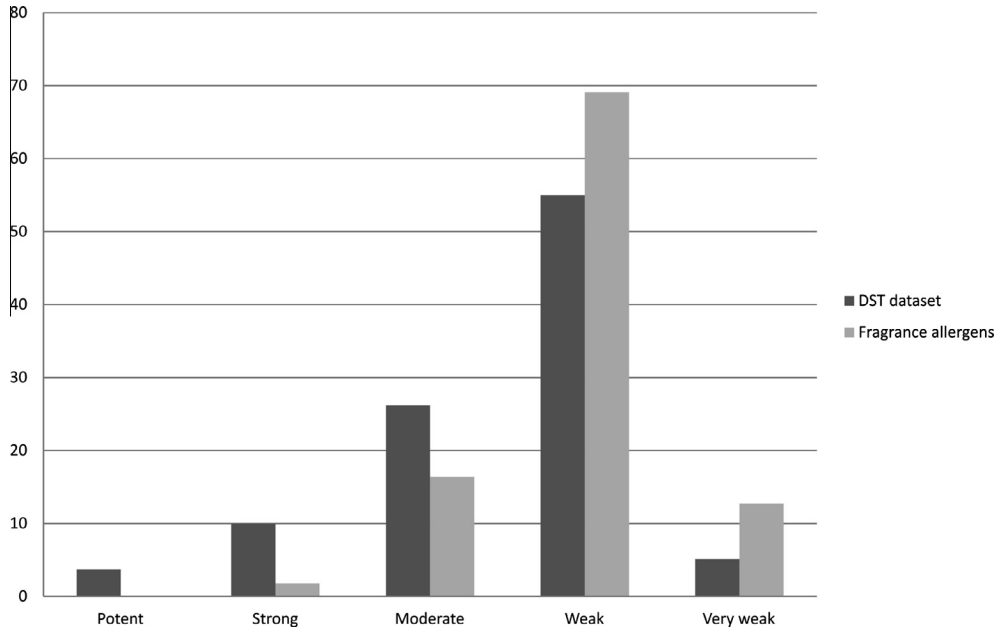

Fig. 2. Comparison of potency classifications of chemicals in the fragrance allergens data set vs. those in the Dermal Sensitisation Threshold (DST) database.

include colouring agents, photochemicals and process regulators, making up a further 27% of the chemicals. Use of a dataset that contains such a diverse range of chemicals, including many reactive intermediates, might be expected to provide a high estimate of the incidence of sensitisers in the world of chemicals. Since the current target application of the DST is cosmetic and personal care product ingredients which, in the most part, do not depend on reactivity for their consumer use, it is considered pertinent to use the 20% incidence figure in this analysis.

Examination of the chemicals used in the DST database showed that some are highly potent, and have EC3 values which are much lower than 64 μg/cm^2, in some cases an order of magnitude or more lower. These high potency sensitisers would present an unacceptable risk of sensitisation if a DST of 64 μg/cm^2 were to be used on its own. In order to address this, additional work has been conducted to examine further the chemical structures of the high potency chemicals in the DST database and to identify chemical features that may be used to identify particularly potent sensitisers (i.e., those with a EC3 value of <64 μg/cm^2). The chemical features identified can then be used to screen out materials for which the reactive DST should not apply. This process can be regarded as analogous to the Cohort of Concern approach for carcinogens as described by Kroes et al. (2004) and which is used to screen out high potency carcinogens in the TTC process.

A full description of the chemistry of the HPC classification is provided in the associated publication by Roberts et al. (2015). Use of this additional screen strengthens the DST approach by screening out chemicals highly potent sensitisers. Based on the DST approach described here, incorporating the HPC classification, and the previously published DST approach for chemicals in the non-reactive domain (Safford et al., 2011), a proposed overall DST process is shown in Fig. 3. In the overall process, there are two decision points based on chemical property information. First, to decide if the chemical belongs to a reactive mechanistic domain or not, which determines whether or not the non-reactive DST can be applied. Second, for chemicals that are classed as reactive, to decide whether or not the chemical falls into the HPC classification, which determines if the reactive DST can be applied. In many cases these chemical property based decisions can be made by application of chemical structure rules (Aptula and Roberts, 2006 for reactive mechanistic domains; Roberts et al., 2015 for DST). In cases of uncertainty, investigative chemistry can be carried out to resolve the uncertainty. The nature of this investigative chemistry required will depend on the chemical under consideration, and may involve, for example: experiments with model nucleophiles to determine whether reactions occur and if so under what conditions; identification of reaction products; kinetics with model nucleophiles to determine rate constants; competition experiments to determine reactivity relative to that of a sensitizer with known potency.

Application of this process to the 363 chemicals in the DST database resulted in only one chemical, hexyl salicylate, which had an EC3 value of less than the appropriate DST (64 μg/cm^2 for a non-HPC chemical). This LLNA result is considered to be anomalous, and the actual potency in humans is 500 times lower than the reactive DST, so this is not considered to be missed high potency chemical. However, since the HPC rules were derived from the high potency chemicals in the DST database, it perhaps not surprising that they provide such good predictions.

Of the 72 further chemicals from the NICEATM database, 14 were classified as non-reactive, and so would have a DST of 900 μg/cm^2. The most potent chemical in the group was endo-tropine-3-mesylate with an EC3 value of 1105 μg/cm^2. Of the 58 chemicals classified as reactive, 40 classified as non-HPC, and to which the DST of 64 μg/cm^2 would be applicable. Ethyl-2-(hydroxymethyl)-1,3-propanediol triacrylate was the only non-HPC chemical with an EC3 of <64 μg/cm^2 (EC3 32 μg/cm^2). For this chemical the reactive moiety is the acrylic group (Michael acceptor). Most acrylates are weaker than predicted from their reactivity as measured by kinetics (Roberts and Natsch, 2009), attributed to their tendency to polymerise rapidly. Even the monoacrylates which are well predicted have EC3 values around 250 μg/cm^2 (e.g., 2-hydroxyethyl acrylate). Therefore the HPC rules exclude acrylates. The present compound is a tri-acrylate in which each acrylic group can react independently of the others, so it may act as a cross-linking agent and this may be the cause of its higher than predicted potency (Roberts et al., 2015). The HPC rule may need modification to allow for the cross-linking effect, which in this case appears to correspond to a factor of 10 in the potency value.

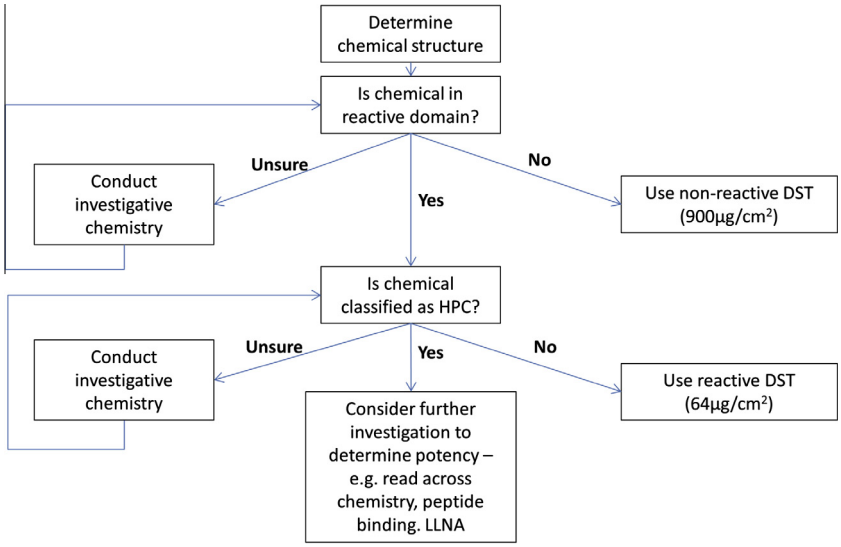

Fig. 3. Proposed overall process for the Dermal Sensitization Threshold (DST) evaluation of chemicals. *Abbreviations:* DST, Dermal Sensitisation Threshold; LLNA, Local Lymph Node Assay; HPC, High Potency Category Chemicals.

In the fragrance ingredient dataset, 8 of the 55 chemically defined fragrance allergens were classified as non-reactive. The most potent non-reactive fragrance ingredient was menthadiene-7-methyl formate with an EC3 of >2025 μg/cm^2, and a WoE NESIL of 1060 μg/cm^2. All of the reactive fragrance ingredients were classified as non-HPC, and the DST value of 64 μg/cm^2 could be applied. One material has an EC3 value which is less than this proposed DST value. This is hexyl salicylate, with an EC3 of 45 μg/cm^2. As has been previously noted, in a HRIPT with hexyl salicylate, a dose of 35,433 μg/cm^2 failed to induce sensitisation, and it is likely that the actual sensitisation potency of this material in humans is well below the proposed DST.

For two materials, trans-2-hexenal and methyl 2-nonynoate, the LLNA values of 1012 and 625 μg/cm^2 (estimate) respectively were much higher than the proposed DST. However, HRIPT studies gave NOEL values for both of 24 μg/cm^2, which is lower that the DST. This highlights the issue that potency in the LLNA may not always reflect the potency seen in the HRIPT, and not an issue for the DST which is based on LLNA data. It is worthy of note that the HRIPT LOELs for these two fragrances were 236 and 118 μg/cm^2 respectively, and that the actual NOELs may well be higher than 64 μg/cm^2.

The DST values for chemicals in the non-reactive domain (900 μg/cm^2) and those classified as non-HPC in the reactive domain (64 μg/cm^2) are intended to be used as default NESILs for chemicals where no sensitisation data exist. It is proposed that the value would then be used in the standard QRA approach to determine an Acceptable Exposure Level (AEL) by applying appropriate Sensitisation Assessment Factors (SAFs) as described by Api et al. (2008). Based on the analysis carried out in this publication, and in the previous publication on the non-reactive DST (Safford et al., 2011) the proposed process and DST values shown in Fig. 3 provide a robust way forward in conducting risk assessments for chemicals used at low levels in consumer products, and for which sensitisation data are not available.

Where chemicals are classified as HPC, it will be necessary to carry out further investigations, which could include in silico, in chemico, in vitro or in vivo assays, to assess their potency and define a NESIL as appropriate.

We have demonstrated here that the reactive DST utilised in conjunction with the HPC rules can establish a level where there is no appreciable risk of sensitisation for protein-reactive chemicals. This is particularly the case for ingredients used in fragrance, cosmetic and personal care products. The DST is expected to have applicability outside the space of these ingredients; however additional work will be needed to benchmark the DST against human data for other chemical functional/use classes.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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