

Refinement of the Peroxidase Peptide Reactivity Assay and Prediction Model for Assessing Skin Sensitization Potential

Cindy A. Ryan,^{*,1} John A. Troutman,^{*} Petra S. Kern,[†] Mike Quijano,[‡] Roy L.M. Dobson,[‡] Hong Jian Dai,[‡] Thomas M. Burt,[‡] and G. Frank Gerberick[§]

^{*}Global Product Stewardship, Mason Business Center, The Procter & Gamble Company, Mason, Ohio 45040

[†]Central Product Safety, Brussels Innovation Center, Procter & Gamble Eurocor, B-1853 Strombeek-Bever,

Belgium [‡]Corporate Functions Analytical, Mason Business Center, The Procter & Gamble Company,

Cincinnati, Ohio 45040 and [§]GF3 Consultancy LLC, West Chester, Ohio 45069

¹To whom correspondence should be addressed at Mason Business Center, The Procter & Gamble Company, 8700 Mason-Montgomery Road, Mason, OH 45040. E-mail: ryan.ca@pg.com.

ABSTRACT

A peptide reactivity assay with an activation component was developed for use in screening chemicals for skin sensitization potential. A horseradish peroxidase-hydrogen peroxide (HRP/P) oxidation system was incorporated into the assay for characterizing reactivity of hapten and pre-/prohapten sensitizers. The assay, named the Peroxidase Peptide Reactivity Assay (PPRA) had a predictive accuracy of 83% (relative to the local lymph node assay) with the original protocol and prediction model. However, apparent false positives attributed to cysteine depletion at relatively high chemical concentrations and, for some chemicals expected to react with the –NH₂ group of lysine, little to no depletion of the lysine peptide were observed. To improve the PPRA, cysteine peptide reactions with and without HRP/P were modified by increasing the number of test concentrations and refining their range. In addition, removal of DL-dithiothreitol from the reaction without HRP/P increased cysteine depletion and improved detection of reactive aldehydes and thiazolines without compromising the assay's ability to detect prohaptens. Modification of the lysine reaction mixture by changing the buffer from 0.1 M ammonium acetate buffer (pH 10.2) to 0.1 M phosphate buffer (pH 7.4) and increasing the level of organic solvent from 1% to 25% resulted in increased lysine depletion for known lysine reactive chemicals. Refinement of the prediction model improved the sensitivity, specificity, and accuracy for hazard identification. These changes resulted in significant improvement of the PPRA making it a reliable method for predicting the skin sensitization potential of all chemicals, including pre-/prohaptens and directly reactive haptens.

Key words: allergens; alternatives; skin sensitization; peptide reactivity; in chemico.

Determining a chemical's potential to induce allergic contact sensitization is an important factor for both occupational and consumer health risk assessments. Historically *in vivo* models such as the maximization and Buehler tests in guinea pigs and more recently the murine local lymph node assay (LLNA) have been used for identification of skin sensitizers. Enforcement of the 7th amendment of the Cosmetic Directive (EU, 2009; Council

Directive 76/768/EEC of 1976-07-27; now Cosmetics Regulation: REGULATION (EC) No 1223/2009) in 2013 for the skin sensitization endpoint resulted in a ban of *in vivo* testing of chemicals intended for use in cosmetics as well as the marketing of finished products that have either been tested on animals or that contain ingredients that have been tested on animals following the ban. Since then, the cosmetic industry has had to rely solely

on nonanimal alternatives for assessing the skin sensitization risk of new ingredients. In addition, nonanimal tests are also required by some regulatory bodies in other sectors and regions. To meet this need, the development of nonanimal-based models has been a major focus of research for more than a decade.

In 2012, the Organisation for Economic Co-operation and Development (OECD) published the Adverse Outcome Pathways (AOP) for skin sensitization (OECD, 2012a,b). The framework permits mapping of existing and novel tests to the biological events of the AOP. In the AOP for skin sensitization, the first key event, is the molecular initiating event (MIE) of covalent binding of a chemical (or metabolite or abiotic transformation product of the chemical) to nucleophilic residues of skin proteins. The ability of a chemical to react with skin proteins has long been thought to play a key role in the development of skin sensitization (Dupuis and Benezra, 1982; Gerberick et al., 2008; Landsteiner and Jacobs, 1936; Lepoittevin et al., 1998).

Skin sensitizers (or their metabolites or abiotic transformation products) are generally electrophilic in nature and can react with nucleophilic sites on proteins. This feature has been exploited to develop *in chemico* approaches for modeling the skin sensitization MIE. Although other nucleophilic amino acids have been investigated, peptides containing lysine and/or cysteine have been used most often (Ahlfors et al., 2003; Aleksic et al., 2009; Alvarez-Sanchez et al., 2003; Gerberick et al., 2004; Natsch and Gfeller, 2008).

Using model hepta-peptides containing cysteine or lysine, the Direct Peptide Reactivity Assay (DPRA) measures peptide depletion at a single peptide and test chemical concentration following 24 h incubation (Gerberick et al., 2004; OECD, 2019). In the original assessment of the DPRA, a prediction accuracy of 89% was demonstrated with a set of 82 chemicals (Gerberick et al., 2007). In the formal validation coordinated by the European Centre for the Validation of Alternative Methods (ECVAM), the overall accuracy (cumulative over 3 labs testing 22 chemicals which included 1 prohaptens, and 2 prehaptens) was 82% (76% sensitivity and 92% specificity) (EURL ECVAM, 2013).

Some sensitizers are nonelectrophilic and require either biotic (prohaptens) or abiotic (prehaptens) activation to become protein reactive (Gerberick et al., 2009; Lepoittevin, 2006; Smith and Hotchkiss, 2001). An analysis of the 231 chemicals identified as skin sensitizers in 2 published LLNA databases found that 60 chemicals, approximately 25%, are classified as pre- or prohaptens (Kern et al., 2010). Although the DPRA is not designed to detect the sensitization potential of pre- or prohaptens, many prehaptens are correctly detected as sensitizers (Patlewicz et al., 2016; Urbisch et al., 2016). However, the extent to which the assay can detect pre- or prohaptens is not known. This, along with the lack of a metabolic step to activate and identify prohaptens sensitizers, are limitations of the DPRA.

To address these limitations, a “next-generation” version of the assay was developed which incorporates a horseradish peroxidase/hydrogen peroxide (HRP/P) metabolic system for activation of prohaptens, test chemical concentration-response and mass spectrometry for determination of peptide detection (Gerberick et al., 2009; Troutman et al., 2011). Following incorporation of lysine into the assay and the development of a prediction model, the Peroxidase Peptide Reactivity Assay (PPRA) achieved a predictive accuracy of 83% (relative to the LLNA) for 26 haptens, 19 pre-/prohaptens, and 25 nonsensitizers (Troutman et al., 2011).

However, apparent false positives were attributed to cysteine depletion at relatively high test chemical concentrations and, for some chemicals expected to react with the $-NH_2$ group

of lysine, little/no depletion of the lysine peptide was observed. These data, along with a retrospective analysis of a large PPRA dataset identified opportunities to improve the assay. This paper describes the optimization of reaction conditions, improvements in the concentration-response characterization and refinement of the prediction model.

MATERIALS AND METHODS

Test chemicals and reagents. The test chemicals, with accompanying purity, molecular weight, CAS numbers, supplier, and solvent used are presented in [Supplementary Table 1](#). In total, 24 nonsensitizers and 51 sensitizers (classified according to LLNA data) were tested for this work.

Potassium Phosphate, Monobasic (catalog number PX1565-1, ACS Reagent Grade) was purchased from EM Science (Gibbstown, New Jersey). Ammonium Acetate (catalog number 238074), Ammonium Hydroxide (catalog number 320145), Potassium Phosphate, Dibasic (catalog number P-2222), Deferoxamine Mesylate Salt (catalog number D9533), Horse Radish Peroxidase (HRP: catalog number P6140), Hydrogen Peroxide (P; catalog number 216763), and DL-Dithiothreitol (DTT; catalog number D5545-16) were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). The cysteine peptide (CYS; Ac-RFAACAA-COOH) and lysine peptide (LYS; Ac-RFAAKAA-COOH) were prepared and purified by RS Synthesis (Louisville, Kentucky). N^2 -acetyl-leucine enkephalin internal standard (IS) (Ac-Tyr-Gly-Gly-Phe-Leu-COOH) (Ac-LE-IS) was purchased from RS Synthesis.

Potassium phosphate buffer (0.1 M, pH 7.4) was prepared by mixing 810 ml of 0.1 M Potassium Phosphate Dibasic with 190 ml of 0.1 M Potassium Phosphate Monobasic and adjusting the pH to 7.4 ± 0.05 with either the monobasic (to acidify) or dibasic (basify) solution; 100-mM ammonium acetate buffer was prepared, and the pH adjusted to 10.2 by addition of ammonium hydroxide.

Test chemical preparation. For the work presented here, test chemicals were prepared as 500-mM stock solutions in an appropriate solvent. Suitable solvents included acetonitrile, methanol, ethanol, isopropanol, water, dimethyl sulfoxide, and acetone (Note: In cases where a 500-mM stock concentration cannot be achieved due to solubility, it is recommended that the highest soluble concentration obtainable be used. However, if a positive result is not obtained, then the data would be considered inconclusive.). The solvent used for each chemical is provided in [Supplementary Table 1](#). For the cysteine peptide reactions, either 5 serial 1:9 or 8 serial 1:1 dilutions of the stock solutions were made in the selected solvent unless specified otherwise. For the lysine peptide reactions, 8 serial 1:2 dilutions of the stock solutions are made in the selected solvent.

Direct lysine- and cysteine-peptide incubation conditions. Direct reactivity of the test chemicals to the lysine-based synthetic peptide was determined in reactions containing either 0.1 M potassium phosphate buffer (pH 7.4) or 0.1 M ammonium acetate buffer (pH 10.2). Unless otherwise indicated, the final concentration of lysine peptide was 5 μ M in a final reaction volume of 0.3 ml. Direct reactivity of the test chemicals to cysteine-based synthetic peptide was determined in reaction mixtures containing 20 μ M cysteine peptide and 10 μ M deferoxamine in 0.1 M potassium phosphate buffer, pH 7.4 (Gerberick et al., 2009) with a final reaction volume of 0.3 ml. Incubations with lysine or cysteine (in the absence of HRP/P) peptide and no test chemical, served

as zero-depletion reference controls for comparisons with direct reactivity determinations. Sample reactions were initiated by adding 3 μ l of the cysteine chemical dilution to the cysteine reaction mixtures or 75 μ l of the lysine chemical dilution to the lysine reaction mixture. The final organic solvent content in reactions with cysteine peptide was $\leq 1\%$ and in lysine peptide reactions was 25% unless otherwise indicated. Samples were incubated at room temperature ($25 \pm 5^\circ\text{C}$), protected from light, for 24 h.

Cysteine peptide incubation with HRP/P. Reaction mixtures with HRP/P were prepared only with the cysteine peptide. The majority of pre-/prohaptens are reactive with cysteine and the activation of exclusively lysine-reactive prohaptens with HRP/P was not demonstrated (Troutman et al., 2011). Therefore, it was determined that testing with cysteine with and without HRP/P and lysine without HRP/P (direct incubation) is sufficient to identify most pre-/prohaptens.

Enzyme-mediated reactivity determinations were performed in sample reactions with cysteine that were prepared as described above, but with addition of HRP and hydrogen peroxide (+HRP/P) at a final concentration of 3 U/ml and 100 μ M, respectively. Incubations with cysteine peptide and no test chemical, in the presence of HRP/P, served as zero-depletion reference controls for comparisons with enzyme-mediated test chemical reactivity determinations. Sample reactions were initiated by adding 3 μ l of a test chemical stock solution to the +HRP/P cysteine reaction mixture. The final organic solvent content in reactions with cysteine peptide +HRP/P was $\leq 1\%$. Samples were incubated at room temperature ($25 \pm 5^\circ\text{C}$), protected from light, for 24 h.

Sample processing and analysis by HPLC/MS/MS. All sample transfers and additions were conducted using a Microlab Nimbus automated liquid handling system (Hamilton Company, Reno, NV) or manually using a 96-channel pipettor (Rainin Liquidator 96, Mettler Toledo, Columbus, OH). Following the incubation period, the reaction mixtures were centrifuged at $1340 \times g$ for 3 min at RT and 0.3 ml of IS solution (3 μ g/ml N^{α} -acetyl-leucine enkephalin in 95/5 acetonitrile/water) was added to each mixture. Samples were again centrifuged at $1340 \times g$ for 3 min followed by the transfer of 50 μ l aliquots from each tube to 2 ml 96-deep well plates and 575 μ l of 2/98 acetonitrile/water was added to each well. For the original method (Troutman et al., 2011), 20 μ l of a 16 mM DTT solution (prepared in water) was added to both cysteine reaction mixtures (without and with +HRP/P) to convert collaterally formed cysteine dimer back to its monomeric form (particularly abundant in the +HRP/P incubations). In the refined method, DTT was added only to the cysteine +HRP/P reaction mixture. The cysteine samples containing DTT in sealed 96-well plates were heated in a 40°C oven for 30 min and then allowed to cool to room temperature. Injection samples were prepared by transferring 160 μ l of each sample to separate 2 ml 96-deep well plates and diluting with 640 μ l of 2/98 acetonitrile/water. All samples were mixed well and placed into a chilled (10°C) autosampler for analysis.

Analysis was primarily designed to selectively monitor 2 analytes: the cysteine peptide (monomer) and N^{α} -acetyl-leucine enkephalin IS, and in a separate method, the lysine peptide and IS. The HPLC/MS/MS methodology employed a CTC HTS-PAL autosampler (Leap Technologies, Carrboro, NC) for sample introduction. Chromatographic separation was carried out using a 10ADvp pumping system (Shimadzu, Columbia, MD). A 10 μ l aliquot of each prepared sample was injected onto a Phenomenex

Table 1. Selected Reaction Monitoring Transitions

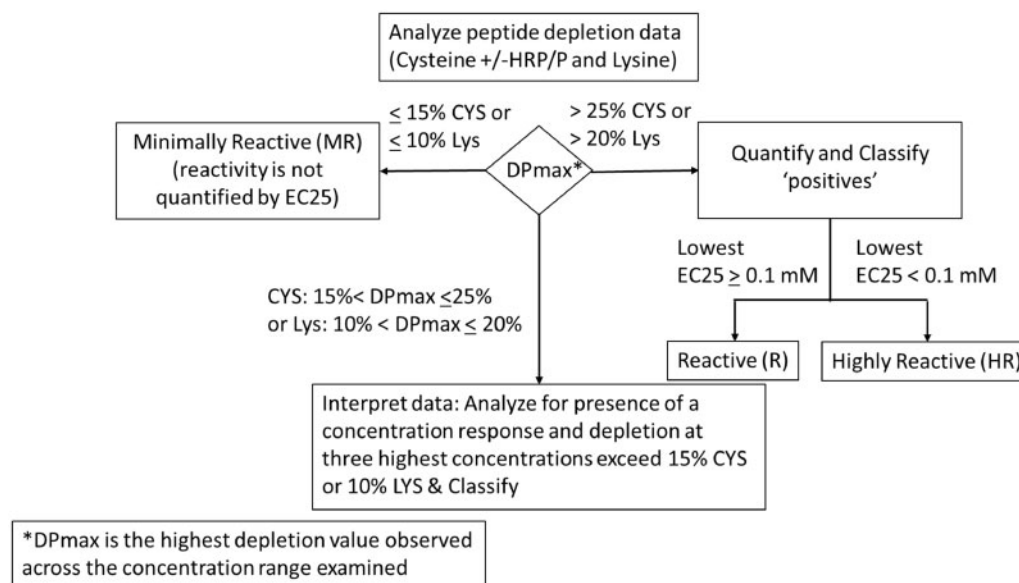
Compound Name	Precursor Ion (m/z)	Product Ion (m/z)
Cysteine ($\text{M}+\text{H}$) ⁺	751	120
Lysine ($\text{M}+2\text{H}$) ⁺⁺	389	129
Ac-LE-IS ($\text{M}+\text{H}$) ⁺	598	120

(Torrance, CA) Kinetex EVO C18 (2.1×30 mm, $2.6 \mu\text{m}$) analytical column held at room temperature. The mobile phase, at a flow rate of 0.35 ml/min, consisted of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). Gradient separation was achieved by holding at initial conditions (95% A; 5% B) for 1 min, then a 3.0 min linear gradient to 100% B for 1 min then step down to initial conditions for a total analysis time of 6 min per sample.

The entire chromatographic effluent was passed into a Sciex API 4000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada), equipped with TurboIon-Spray interface and operated in positive electrospray ionization mode. Analytes were detected using SRM (Selected Reaction Monitoring) MS/MS schemes, optimized for each peptide. The SRMs (transitions shown in Table 1) were assembled into a Multiple Reaction Monitoring protocol. Note that the cysteine disulfide peptide (ie, cysteine dimer) ($\text{M}'+2\text{H}$)⁺⁺ was also monitored. The cysteine dimer, which is chromatographically resolved from the cysteine monomer, was not quantified, but the intensity data obtained was useful for assay diagnostic purposes (eg, verify effectiveness of the DTT treatment for the +HRP/P samples and to assess the extent of collateral cysteine dimer formation in the –HRP/P samples).

For typical batch analyses, the cysteine samples were analyzed first to minimize the potential for subsequent dimer formation, followed by the lysine samples. For batches involving multiple test compounds, the direct and enzyme-mediated reactivity sample sets were analyzed in alternating fashion and each of the test chemical sample sets were bracketed by a pair of the respective zero-depletion control samples, and a third control sample inserted in the middle of each test sample concentration series. This allowed for a more-precise comparison of reactivity between direct and enzyme-mediated conditions for each test compound. Also, by using the mean signal ratios measured for the 3 closest control samples as the zero-depletion reference value, any drift in assay performance over the course of long batch runs (up to 3 days), was automatically factored out, resulting in optimal accuracy in depletion measurements.

Data analysis for peptide depletion measurements. The acceptability and integrity of the sample incubation and HPLC/MS/MS batch run was verified by analysis of zero depletion controls (+/–HRP/P) and the aggregate IS data for each test chemical sample set. SRM peak areas and area ratios (cysteine or lysine peptide-to-IS) were determined for each sample, using Sciex Analyst quantitation software. Peptide depletion in samples with test chemical was calculated by comparing the peak area or peak area ratio in samples containing test chemical to the mean of the peak area ratios calculated from the 3 closest corresponding zero-depletion control samples. In sample reactions that were evaluated in replicates, peptide depletion values for individual replicates were determined and then used to calculate mean ($N=2$) or mean and standard deviation ($N=3$) for both the direct (–HRP/P) and enzyme-mediated (+HRP/P) reactivity



1. Analyze depletion data for hazard prediction (yes/no)
2. Quantify reactivity: EC25 estimation
3. Classify chemicals: Minimally Reactive (MR), Reactive (R), Highly Reactive (HR)

For hazard prediction chemicals classified as 'MR' are considered as non-sensitizers and 'R' and 'HR' classified chemicals are considered as sensitizers.

Figure 1. Prediction model scheme for cysteine peptide reactivity following 24 h incubation of 20- μ M cysteine peptide with test chemical (0.039–5 mM) in the absence of horseradish peroxidase/hydrogen peroxide (HRP/P) (Direct) and presence of HRP/P and 5- μ M lysine peptide (Direct) with test chemical (0.011–25 mM).

determinations. During preliminary method development work, leucine enkephalin was used as the IS (LE IS). However, loss of signal for the LE IS was observed for certain chemicals (data not shown). These results indicated an apparent reactivity between the test chemical and the IS, which led to the replacement of the LE IS with N^α-acetyl-leucine enkephalin, which provided requisite stability.

Prediction model for hazard and reactivity classification. The refined prediction model is presented in Figure 1. This prediction model was determined based on the optimizations of the method described in the "Results" section. For the purpose of hazard prediction, the maximum depletion value observed across the entire concentration range (DPmax) was determined for both cysteine direct (–HRP/P) and enzyme-mediated (+HRP/P) peptide reactions and lysine direct reactions. A test chemical was classified as positive if a DPmax value greater than 25% was obtained for either the cysteine direct (–HRP/P) or enzyme-mediated (+HRP/P) reactions or if the DPmax value for lysine was greater than 20%. A test chemical was classified as "Minimally Reactive (MR)" if DPmax values for both cysteine direct (–HRP/P) and enzyme-mediated (+HRP/P) reactions were less than ≤ 15% and the DPmax for lysine was less than ≤ 10%. The method for setting the positive and negative depletion cut-off values is described in the "Results" section. If 15% < DPmax ≤ 25% for either cysteine direct (–HRP/P) or enzyme-mediated (+HRP/P) or the DPmax for lysine was > 10% but ≤ 20% the following criteria was applied to classify a test chemical as

positive: (1) a concentration-response must be apparent (ie, concentration-response plot must show a general trend of increasing depletion values with an increase in test chemical concentration, no statistical analysis is done), and (2) at least 3 terminal depletion values in the cysteine (–HRP/P) reactions or in the cysteine (+HRP/P) reactions must be > 15% depletion or at least 3 terminal depletion values in the lysine reaction must be > 10% depletion. If either of these criteria was not satisfied, the chemical was considered to be MR. If both criteria were met, the chemical was classified as Reactive (R).

Peptide reactivity classification, expressed in terms of EC25, is an estimate of the test chemical concentration that depletes the peptide by 25%, under the experimental conditions described above. EC25 values were determined by fitting a 3-parameter log-logistic model to peptide depletion data using RExcel (www.statconn.com). Prior to analysis, all negative depletion values were set manually to zero. EC25 values for cysteine and lysine peptides were reported as "NC" (not calculated) for reactions where the chemical was assessed to be MR. To report the EC25 value based on the 3-parameter log-logistic model, the following criteria were satisfied: (1) the EC25 upper limit based on a 95% confidence interval (CI) must be within 2-fold of the EC25 (ie, EC25 Ratio [ie, upper limit/EC25] was ≤ 2), and (2) the DPmax value was 100% ± 20%. If both of these criteria were not satisfied, the EC25 value was estimated by linear interpolation.

The lowest EC25 value for chemicals that were identified as positive towards cysteine (+/–HRP/P) or lysine was used to classify chemical reactivity. Chemicals with the lowest

EC25 $\geq$ 0.1 mM were classified as “reactive” (R) and chemicals with the lowest EC25 < 0.1 mM were classified as “highly reactive (HR).” Both “R” and “HR” classified chemicals were considered as “sensitizers,” whereas “MR” chemicals were classified as “nonsensitizers.”

RESULTS

Optimization of Cysteine Peptide Reactivity Concentration-response. The reactions with cysteine +/-HRP/P were modified by refining the test chemical concentration range and increasing the number of concentrations tested from 5 (0.003, 0.03, 0.3, 3, and 30 mM) to 8 (0.04, 0.08, 0.16, 0.31, 0.63, 1.3, 2.5, and 5 mM). For this work, the cysteine -HRP/P reaction mixtures were DTT-treated postincubation. Cysteine peptide depletion with the modified concentration range was determined for a total of 15 chemicals which included 3 pre-/prohaptens, 3 direct haptens and 9 nonsensitizers considered to be false positive based on the previous cut-off values of 10% and 40% for cysteine without HRP and with HRP, respectively (Troutman et al., 2011) (Table 2). Refinement of the concentration range, specifically lowering the highest concentration tested from 30 to 5 mM, eliminated the “false positive” depletion values for 6 of the 9 nonsensitizers and reduced solubility issues. Lowering the highest concentration had no impact on the detection of the sensitizing chemicals. The weak pre-/prohaptens aniline was classified as negative in both concentration ranges when the previous cut-off values of 10% and 40% were used. When the peptide depletion data were plotted using the RExcel 3-parameter log-logistic model, the upper and lower confidence bounds based on a 95% CI, the 8-concentrations response had less uncertainty (ie, lower CI) compared with the 5-concentrations response (Figure 2).

Optimization of the Reaction Conditions With Lysine Peptide. A lysine peptide reaction was incorporated in the PPRA using a 0.1-M ammonium acetate buffer (pH 10.2) and a final organic solvent content in the reaction mixture of $\leq$ 1% (Troutman et al., 2011). To optimize the lysine reaction conditions for improved peptide depletion 2 parameters were investigated, the percent of organic solvent in the reaction mixture and the choice of buffer. A preliminary experiment was conducted with a known lysine reactive chemical, oxazolone, to assess the impact of changing the buffer and increasing the organic solvent content from 1% to 25% on lysine depletion (Figure 3). A 2- to nearly 6-fold increase in lysine peptide depletion was observed in reactions containing potassium phosphate buffer compared with those containing ammonium acetate buffer at all concentrations and both percentages of organic solvent content tested. In addition, a more modest increase in lysine depletion was observed in reactions containing 25% compared with 1% organic solvent content. Based on this preliminary experiment, both reaction condition modifications were investigated further.

A set of 24 chemicals was selected and lysine peptide depletion was determined in both potassium phosphate (pH 7.4) and ammonium acetate (pH 10.2) buffers with 25% organic solvent content at 4 concentrations (1, 3, 10, and 30 mM). This set of 24 included 18 chemicals previously shown to be lysine reactive in ammonium acetate buffer (Lalko et al., 2013; Troutman et al., 2011), 1 additional lysine reactive chemical and 5 chemicals that were not lysine reactive. A comparison of the maximum percent depletion values showed that for the majority (15/24) of the chemicals, equal or higher lysine reactivity was obtained in phosphate buffer (pH 7.4) compared with ammonium acetate buffer (pH 10.2) (Table 3). Lower, but still positive reactivity in phosphate buffer, based on the original empirical

threshold positive depletion value for lysine of $>$ 15% (Troutman et al., 2011) or the current cut-off value of 20%, was observed for 3,4-dihydrocoumarin and 2,3-butanedione. Minimal reactivity in phosphate buffer was found for 4 chemicals which were reactive in ammonium acetate; citral, methyl acrylate, lauryl gallate, and propyl gallate. 2-Methyl-2H-isothiazol-3-one, which had not been previously assessed for lysine reactivity was found to be more reactive in phosphate buffer. The remaining 6 chemicals, which included the nonsensitizer 6-methylcoumarin, were nonreactive with lysine in either buffer.

To determine whether 25% final organic solvent in the reaction mixture was optimal for use with the potassium phosphate buffer, 8 lysine reactive chemicals were tested at 8 concentrations (0.011, 0.034, 0.10, 0.31, 0.93, 2.8, 8.3, and 25 mM) with 1%, 3%, 10%, and 25% final organic solvent levels (Figure 4). Oxazolone and phthalic anhydride, both lipophilic, were not testable at 1% organic solvent level as they came out of solution when added to the lysine peptide solution. An organic solvent concentration of 25% provided enhanced peptide depletion for half of the chemicals and the final level of organic solvent had no impact on depletion for the other 4 chemicals. In addition, it was observed that increasing the number of concentrations tested from 4 to 8 and expanding the concentration range improved the characterization of the concentration response as was seen with the cysteine peptide reactions (data not shown).

Optimization of Cysteine Peptide Reactivity Without HRP/P. Evaluation of an expanded set of chemicals tested in the PPRA revealed false negative results for 12 aldehydes and 4 thiazolines which were classified as sensitizing in the LLNA and/or the DPRA (data not shown). A subset of 8 aldehydes and 2 thiazolines was selected to evaluate protocol modifications aimed at improving detection of these chemical classes. Following the 24 h incubation period and sample processing, DTT, a strong reducing agent, was added to the PPRA cysteine reaction mixtures, both with and without (+/-)HRP/P to convert cysteine dimers back to monomers prior to HPLC/MS/MS analysis. DTT postincubation treatment is absolutely needed for the cysteine +HRP/P samples as residual cysteine is nearly quantitatively oxidized to its dimer form under these reaction conditions and was originally included in the cysteine -HRP/P sample preparation for consistency (Gerberick et al., 2009). It was postulated that use of DTT in cysteine -HRP/P reaction product mixtures led to lower apparent cysteine peptide depletions for some compound classes (ie, aldehydes and thiazolinones) due to liberation of adducted monomers in a similar way that cysteine dimers are reduced back to their monomeric form. To explore this possibility, 10 selected chemicals that produced positive cysteine depletion in the DPRA were tested in the PPRA with the cysteine peptide -HRP/P in the presence and absence of DTT postincubation treatment. The results were compared with the cysteine depletion observed in the DPRA (Table 4). In the absence of DTT postincubation treatment, with the exception of citral, the DPmax of cysteine peptide increased approximately 3- to 27-fold compared with the cysteine -HRP/P reactions with DTT postincubation treatment. Moreover, 9 of the 10 chemicals without postincubation treatment with DTT had DPmax values that exceeded the positive cut-off value of 25%, compared with only 2 of the 10 in cysteine -HRP/P reactions with DTT.

Refinement of the Prediction Model for Hazard Identification and Reactivity Classification. Based on observations made with a PPRA data set from 70 chemicals generated under the previous assay conditions, threshold depletion values that were considered to

Table 2. Comparison of % Depletion Cysteine +/- HRP/P With 2 Test Concentration Ranges

Chemical	CAS No.	Historical Concentration Range (mM) ^a					Modified Concentration Range (mM)								
Nonsensitizers ^b		0.003	0.03	0.3	3.0	30	0.04	0.08	0.16	0.31	0.63	1.3	2.5	5	
1-Bromobutane	109-65-9	6.3 (6.8)	10.0 (4.0)	12.8 (1.2)	18.9 (19.0)	46.7 (79.8)	1.3 (5.7)	1.2 (0.0)	2.2 (0.2)	0.0 (4.4)	7.3 (6.8)	9.1 (8.7)	15.4 (12.7)	21.9 (26.5)	
Coumarin	91-64-5	3.9 (6.6)	6.1 (6.6)	0.0 (0.1)	4.6 (10.1)	17.9 (15.1)	0 (0)	0 (0)	0 (0)	0.7 (0)	2.0 (0)	0.2 (0)	0.1 (0)	1.8 (0)	
4-Hydroxybenzoic acid	99-96-7	33.4 (4.6)	44.2 (4.3)	44.8 (1.4)	47.2 (3.4)	54.1 (3.7)	63.1 (6.2)	63.7 (7.0)	61.9 (1.2)	60.9 (0.8)	61.0 (4.2)	61.0 (0.5)	63.5 (5.0)	63.4 (5.0)	
Hexane	110-54-3	0.7 (0.0)	0.0 (22.8)	5.0 (0.0)	5.0 (0.0)	2.98 (0.0)	0 (0)	2.5 (3.9)	2.6 (5.1)	0.2 (3.4)	0.5 (0.7)	0 (2.8)	0 (2.0)	0 (3.1)	
Isopropanol	67-63-0	9.7 (0.0)	0.0 (1.3)	0.0 (6.3)	0.0 (13.8)	0.0 (22.7)	0 (0)	3.2 (0)	1.7 (0)	0 (0)	1.0 (2.3)	3.8 (0.9)	0 (0)	1.5 (0.6)	
Nonanoic acid	112-05-0	9.1 (4.4)	0.9 (11.9)	0.0 (17.5)	0.0 (21.1)	18.5 (0.0)	0 (0)	3.6 (0)	0 (0)	0 (0.2)	0 (0)	0 (0)	1.4 (0)	0 (0)	
Propyl paraben	94-13-3	21.0 (4.3)	38.7 (10.0)	39.9 (0.4)	43.0 (6.6)	41.0 (3.0)	37.1 (0.9)	35.8 (0)	41.2 (0)	39.8 (0.2)	42.3 (0)	41.2 (0)	40.5 (0)	41.7 (0)	
Salicylic acid	69-72-7	0.1 (7.2)	2.6 (0.0)	0.0 (4.0)	6.2 (8.9)	23.4 (19.0)	4.6 (0)	4.1 (0)	3.7 (0)	3.1 (0)	5.1 (0)	2.8 (0)	4.5 (0)	10.6 (0.9)	
Tartaric acid	87-69-4	1.0 (0.0)	2.3 (1.0)	0.0 (0.0)	4.4 (2.6)	41.7 (5.1)	2.4 (0.3)	2.3 (2.6)	1.8 (1.7)	0 (0)	4.1 (1.1)	0.9 (2.7)	1.6 (2.8)	2.4 (0)	
Sensitizers															
Aniline ^c	62-53-3	0.0 (0.0)	11.0 (0.0)	7.5 (21.0)	21.5 (7.0)	31.9 (9.9)	0 (0)	9.2 (0)	0 (0)	6.7 (0)	5.6 (0)	19.0 (0)	21.7 (0)	25.6 (0)	
Eugenol ^c	97-53-0	10.9 (3.5)	41.0 (5.1)	51.3 (6.3)	48.8 (65.1)	99.4 (67.5)	76.6 (2.3)	81.2 (6.9)	81.2 (11.1)	81.1 (18.5)	77.3 (31.3)	73.0 (56.7)	69.1 (67.7)	66.9 (64.2)	
1-Naphthol ^c	90-15-3	0.0 (0.0)	15.2 (0.0)	60.2 (9.1)	98.9 (27.6)	98.1 (0.0)	25.0 (6.0)	28.9 (0)	12.4 (4.5)	94.9 (0)	98.2 (4.3)	98.8 (6.7)	98.2 (2.7)	98.0 (5.8)	
2,3-Butanedione	431-03-8	0.0 (0.8)	0.0 (3.8)	3.7 (10.1)	3.5 (29.3)	23.6 (41.8)	0 (6.8)	2.2 (6.4)	0 (5.4)	5.9 (8.5)	6.6 (16.4)	1.5 (12.2)	1.3 (27.2)	13.9 (33.3)	
Cinnamic aldehyde	104-55-2	0.0 (0.0)	0.0 (5.3)	2.6 (12.0)	36.7 (53.8)	47.4 (68.2)	0 (0)	0 (2.8)	2.1 (5.0)	0 (16.3)	0 (22.2)	0.7 (36.3)	22.3 (44.5)	22.9 (54.1)	
Diethyl maleate	141-05-9	0.0 (8.0)	0.0 (14.6)	6.9 (39.4)	8.6 (88.3)	38.7 (92.5)	0.2 (7.0)	0.3 (19.5)	5.6 (21.0)	0 (43.0)	0 (62.8)	0 (85.3)	0 (92.5)	2.4 (95.8)	

Without horseradish peroxidase/hydrogen peroxide depletion values are in parenthesis. Negative depletion values are reported as zero. Depletion values which classify the chemical as sensitizing are in bold font.

^aData previously published in Troutman et al. (2011).

^bChemicals classified as nonsensitizers based on local lymph node assay data identified as false positive based on the previous cut-off values of 40% and 10% for cysteine with HRP and without HRP, respectively (Troutman et al., 2011).

^cPre-/prohaptens.

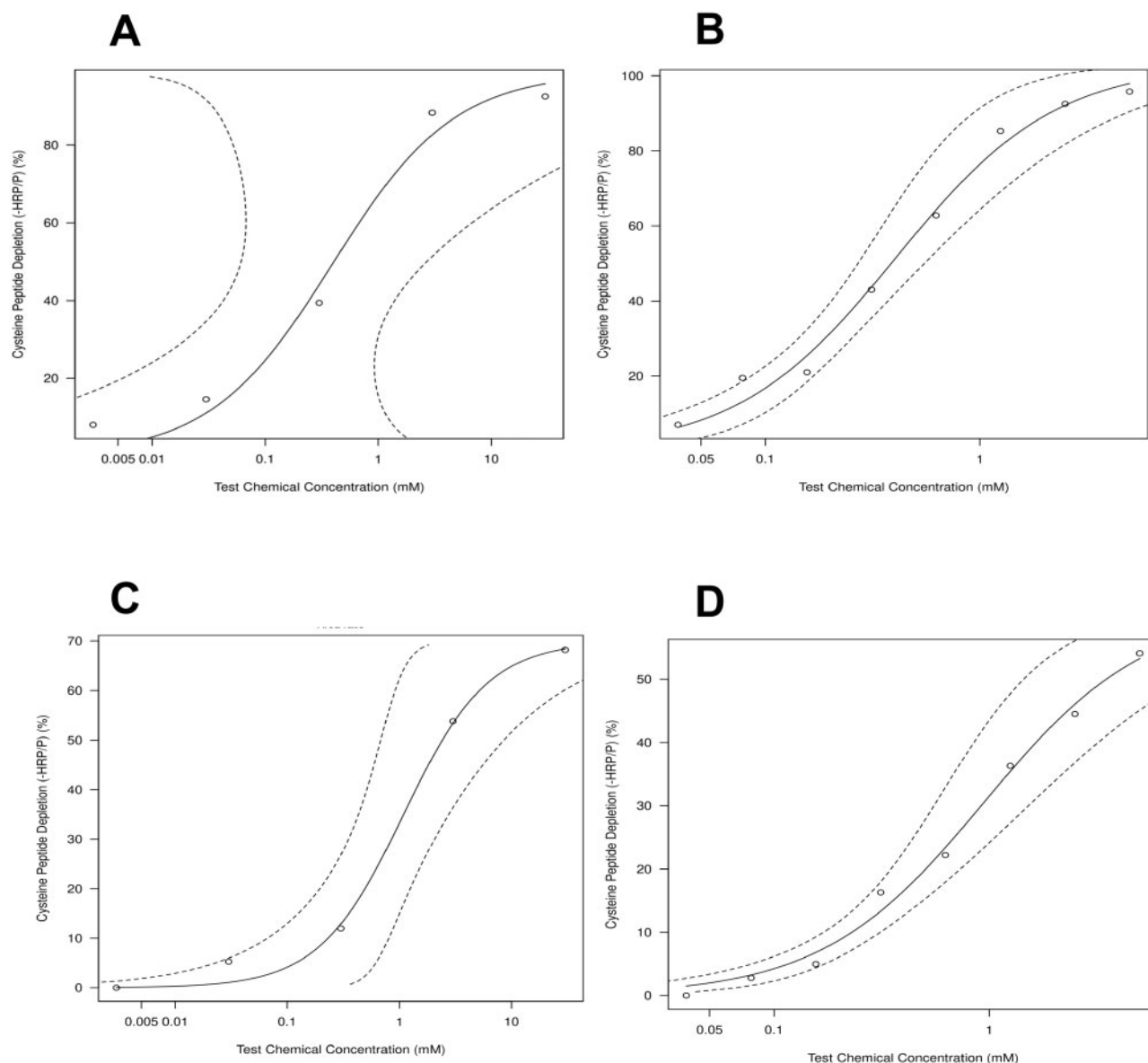


Figure 2. Three-parameter log-logistic plots with the upper and lower confidence bounds based on a 95% confidence interval comparing 5 and 8 concentration datasets of cysteine peptide depletion (direct) (–HRP/P) for 2 representative chemicals; 95% confidence limits shown as a dashed line. A, Dimethyl maleate at 5 concentrations; (B) dimethyl maleate at 8 concentrations; (C) cinnamaldehyde at 5 concentrations; (D) cinnamaldehyde at 8 concentrations.

be positive were identified empirically as depletion > 10% or > 40% for cysteine in the absence and presence of HRP/P, respectively, or > 15% for lysine without HRP/P (Troutman *et al.*, 2011). However, the adjustments made to the cysteine concentration range, and the changes made to optimize the lysine and cysteine –HRP/P reaction conditions resulted in a more sensitive assay. These method improvements required a re-examination of the prediction model to determine if there were areas for refinement.

To assess intra- and interexperiment variability in measurement of cysteine peptide depletion, 3 independent experiments were conducted and within each experiment, depletion was assessed in triplicate samples for 3 nonsensitizers, 7 prohaptens, and 5 prehaptens. One chemical concentration of 5 mM was tested in the cysteine without HRP/P and with HRP/P reaction mixtures. The intraexperiment variability for replicate samples within a study had standard deviations which ranged

from 0.1 to 6.8 for cysteine without HRP/P and 0.1 to 11.9 for cysteine with HRP/P. The interassay variability for repeated testing of the same chemical in independent runs had standard deviations which ranged from 2.0 to 8.7 for cysteine without HRP/P and 1.2 to 21.8 for cysteine with HRP/P (Supplementary File 1).

To simplify the prediction model without compromising the ability to distinguish nonsensitizers from skin sensitizing chemicals, a single threshold positive value for the cysteine peptide was set empirically to 20% depletion for both reactions, +/–HRP/P, based on observations made on review of PPRA datasets for 124 chemicals (data not shown). Peptide depletion for a majority of nonsensitizers was still generally less than 15% for lysine, so the previously defined cutoff value was retained. To establish depletion cut-off values for clear negative (nonreactive) and positive (reactive) reactions for the purpose of hazard prediction, the variability for chemicals that were classified as “nonreactive” based on a 20% depletion cut-off for both cysteine

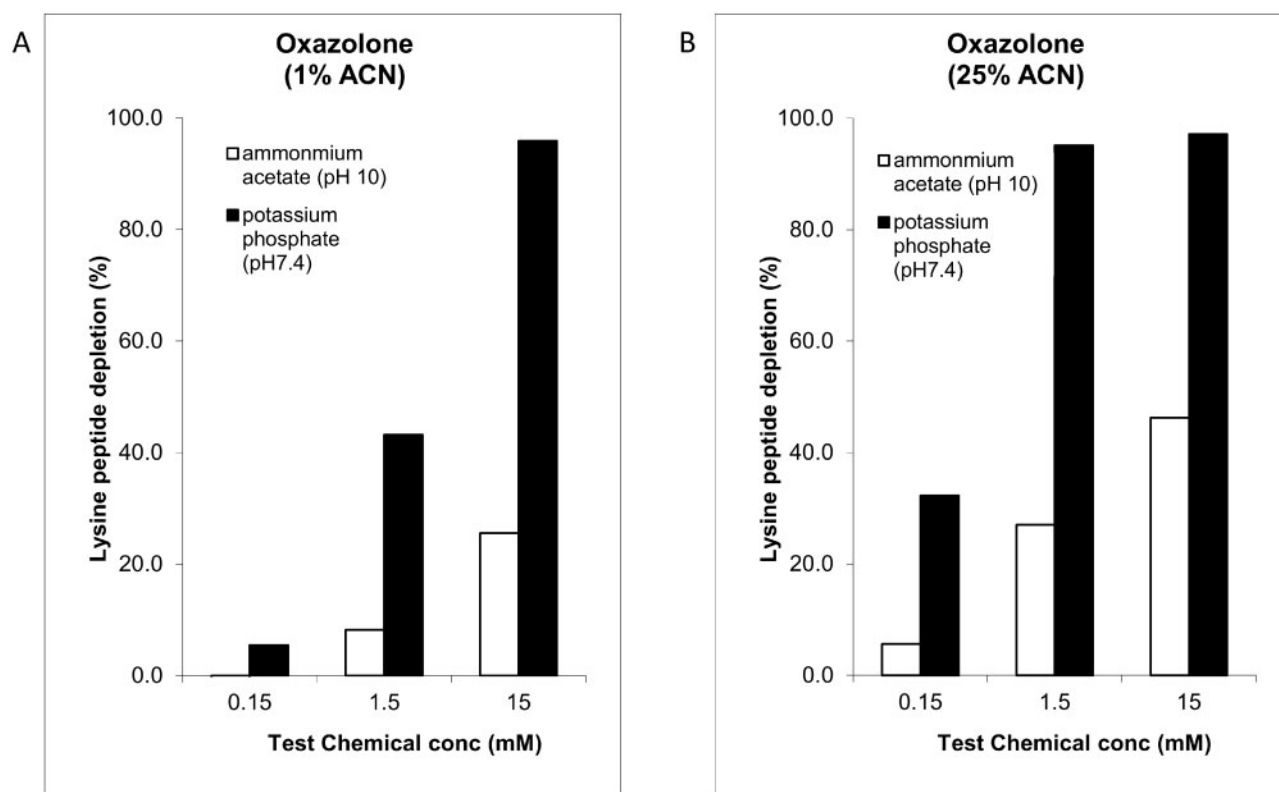


Figure 3. Lysine reactivity (no horseradish peroxidase/hydrogen peroxide) with oxazolone in reactions in potassium phosphate buffer (pH 7.4) and ammonium acetate buffer (pH 10.2) with either (A) 1% final organic solvent concentration or (B) 25% final organic solvent content (acetonitrile; ACN).

Table 3. Maximum Lysine Depletion Values for 24 Chemicals Conditions in 25% Organic Solvent in 0.1-M Potassium Phosphate Buffer (pH 7.4) and 0.1-M Ammonium Acetate Buffer (pH 10.2)

Test Chemical	Maximum Depletion, DPmax % (Conc)	
	Potassium phosphate buffer (pH7.4)	Ammonium acetate buffer (pH10.2)
Glutaraldehyde ^a	100	88.8
Oxazolone ^a	99.3	66.8
Phenyl isocyanate ^a	99.1	51.2
Phthalic anhydride ^a	99.0	35.0
Maleic anhydride ^a	95.3	29.6
Hexamethylene diisocyanate ^a	94.6	37.7
Toluene 2,6-diisocyanate ^a	100	39.7
Hexahydrophthalic anhydride ^a	97.0	19.5
Trimellitic anhydride ^a	82.3	18.3
Hydroxycitronellal	99.0	68.5
Diethyl acetaldehyde ^a	92.3	92.9
2,3-Butanedione ^a	56.4	76.6
3,4-Dihydrocoumarin ^a	51.2	71.3
2-Methyl-2H-isothiazol-3-one	34.4	7.4
Propyl gallate ^a	0	75.0
Methyl acrylate ^a	3.0 (10 mM)	82.2
Lauryl gallate ^a	1.1	46.1
Citral ^a	12.4	43.9
2-Phenylpropionaldehyde ^a	4.5 (10 mM)	12.1
Cyclamen aldehyde	8.7 (10 mM)	0
2-Mercaptobenzothiazole	3.2	1.4
Ethylenediamine (free base)	0	0
2-Methylundecanal ^a	4.3 (10 mM)	0
6-Methylcoumarin (NS)	0	0

DPmax was observed at the 30 mM test concentration unless indicated otherwise. Negative depletion values are reported as zero.

^aPreviously reported to be lysine reactive in ammonium acetate buffer in either [Lalko et al. \(2013\)](#) or [Troutman et al. \(2011\)](#).

Abbreviation: NS, nonsensitizer.

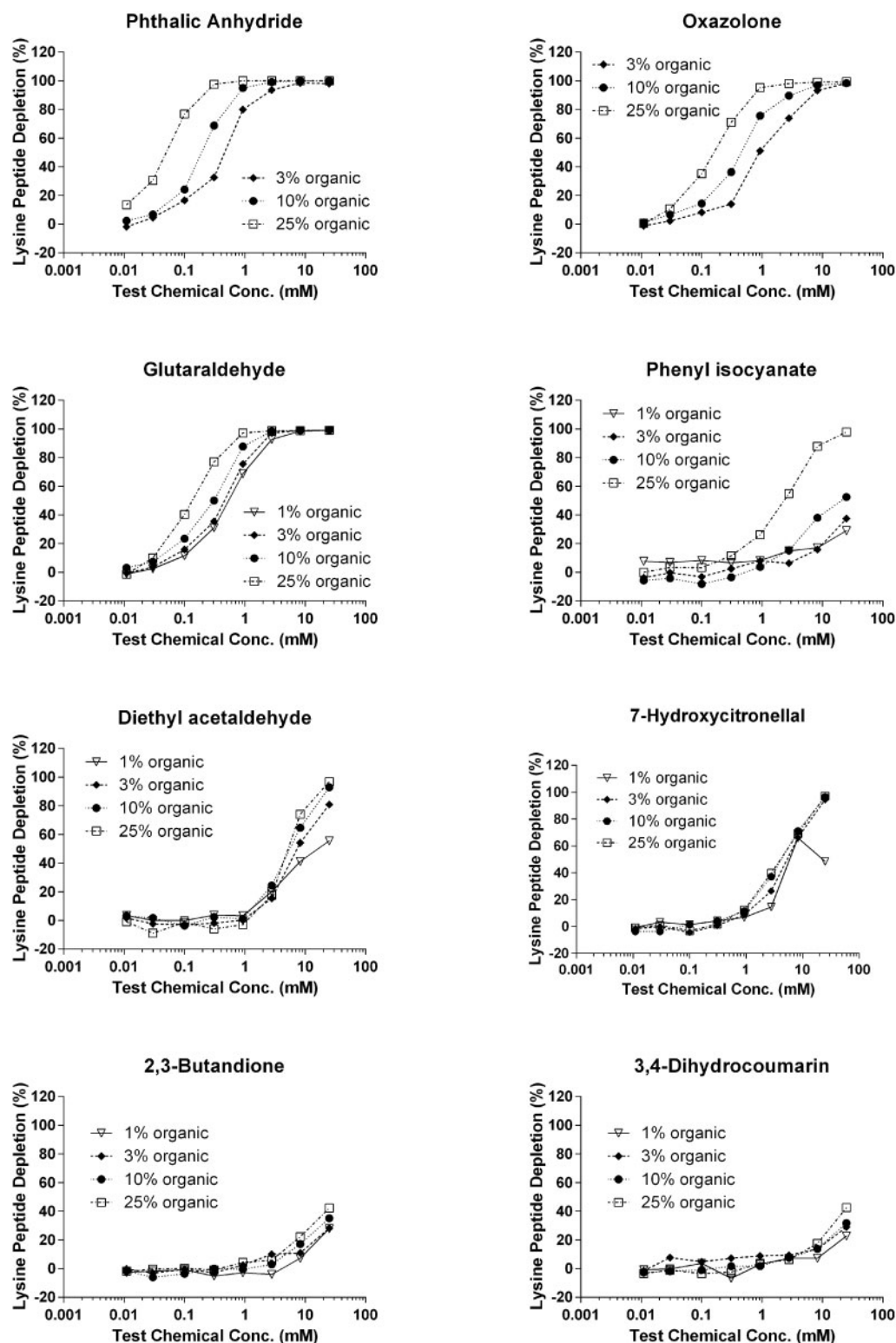


Figure 4. Concentration-response profiles for a subset of lysine reactive chemical in reactions containing lysine peptide only (no horseradish peroxidase/hydrogen peroxide) and 1% (except phthalic anhydride and oxazolone), 3%, 10%, and 25% organic content in 0.1-M potassium phosphate (pH 7.4) buffer.

reactions, was examined (Supplementary File 1). Of the 3 non-sensitizers, all met the “nonreactive” criteria for both cysteine reactions (+/-HRP/P). Seven prohaptens met the criteria for the -HRP/P cysteine reaction and 2 met the criteria for the +HRP/P reaction. None of the 5 prehaptens tested met the “nonreactive”

criteria for either of the cysteine reactions. The median SD for the reactions with less than 20% depletion was calculated to be 2.45%. Upper and lower limits were set at 2 standard deviations below and above the 20% and 15% threshold cut-offs for cysteine and lysine, respectively (Supplementary File 1). For the

Table 4. Percent Cysteine (CYS) Depletion Observed in the Direct Peptide Reactivity Assay (DPRA) and the Maximum Depletion Observed Across the Concentration Range in the Peroxidase Peptide Reactivity Assay (PPRA) for the CYS –HRP/P Reaction Mixture With and Without DL-dithiothreitol (DTT)

Chemical	DPRA % CYS Depletion ^a	PPRA Maximum % CYS Depletion	
		CYS –HRP/P with DTT	CYS –HRP/P without DTT
Aldehydes			
Cinnamic aldehyde	70.6	21.8	78.4
Citral	85.7	8.8	7.2
Lillial	14.0	13.2	90.9
Isocyclocitral	15.9	14.7	97.9
Laevo Perilla aldehyde	31.9	15.0	40.1
2-Methylundecanal	100	28.3	82.7
2,4-Dimethyl-3-cyclohexen-1-carboxaldehyde	100	4.8	59.1
α -Methyl cinnamic aldehyde	10.4	8.8	58.3
Thiol derivatives			
1,2-Benzisothiazolin-3-one	97.7	22.5	67.6
2-Mercaptobenzothiazole	97.9	25.2	100

^aAs reported in Natsch et al. (2013) or Hoffmann et al. (2018).

Abbreviation: HRP/P, horseradish peroxidase/hydrogen peroxide.

cysteine reactions, less than 15.1% depletion was a clear negative response and greater than 24.9% depletion was a clear positive. These values were rounded to 15% and 25% for use in the prediction model. For lysine reactions, less than 10.0% depletion was a clear negative response and greater than 20.0% depletion was a clear positive. The use of lower/upper limits allows chemicals that exhibit marginal depletion to be further analyzed based on a set of criteria (described in the “Materials and Methods” section) to classify chemicals as positive or negative.

A subset of 67 of the 70 chemicals reported in Troutman et al. (2011), was tested using the refined PPRA protocol, including 18 chemicals identified as pre- and/or prohaptens. The maximum peptide depletion values and hazard prediction obtained with the original protocol and prediction model were compared with those obtained with the refined protocol with both the original prediction model (Troutman et al., 2011) and the new prediction model (Table 5). The performance of the original test method and prediction model, the refined test method with the original prediction model, and the refined method with the refined prediction model was evaluated using Cooper statistics (Figure 5). Compared with the original test method and prediction model, use of the refined test method with the original prediction provided only a modest increase in the accuracy from 82% to 84%, the positive predictivity from 81% to 82% and negative predictivity from 84% to 89%. Compared with the original test method and prediction model, use of the refined test method and prediction model resulted in a more substantial improvement, increasing the overall accuracy to 88%, the positive predictivity to 85%, and the negative predictivity to 95%.

Previously the estimated test chemical concentration that depletes peptide by 15% (EC15) was explored as a possible means for rank ordering skin sensitization potency (Troutman et al., 2011). Because the percent depletion cut-off value for defining a positive response with the cysteine peptide was changed to $\geq 25\%$ for cysteine and 20% for lysine, the estimated test chemical concentration that depleted peptide by 25% (EC25) was calculated. The lowest EC25 value observed was used to classify chemicals as either “Reactive” (EC25 > 0.1 mM) or “HR” (EC25 < 0.1 mM) (Table 6). Observations less than the first quartile minus 1.5 * IQR or greater than the third quartile plus 1.5 *

IQR (where IQR = interquartile range) are declared outliers. Seven of the 48 chemicals were identified as outliers. Median EC25 values for each sensitization category, excluding outliers, were 0.625 mM (nonsensitizers), 0.075 mM (weak), 0.156 mM (moderate), and 0.061 mM (strong/extreme). Figure 6 presents these data graphically along with the distribution of the lowest EC25 values for each sensitization category. The average EC25 values, with outliers retained, were 1.31 mM (nonsensitizers), 0.54 mM (weak), 0.37 mM (moderate), and 0.24 mM (strong/extreme).

DISCUSSION

Around 25% of skin sensitizing chemicals are classified as either pro- and/or prehaptens (Casati et al., 2016; Kern et al., 2010) so reliable hazard identification methods are critical for these chemicals. Examination of OECD Test guideline assays (eg, DPRA, KeratinoSens, h-CLAT) found that a combination of these can identify the sensitization potential of prehaptens and most prohaptens (Patlewicz et al., 2016; Urbisch et al., 2016). However, prohaptens are outside of the applicability domain for methods in OECD Test Guideline 442C, so opportunities exist for improved reactivity assays. The PPRA determines chemical reactivity towards 2 synthetic peptides containing either lysine or cysteine. Reactions with the cysteine peptide are performed without and with a horseradish peroxidase-hydrogen peroxide oxidation system (+/–HRP/P) for identifying directly reactive haptens and pre-/prohaptens, respectively. Following the addition of the lysine peptide to the assay (Troutman et al., 2011), further protocol modifications were made to improve the assay.

In a previous evaluation of the PPRA, 9 of 25 chemicals classified as nonsensitizers (based on LLNA data) were misclassified as sensitizing (Troutman et al., 2011). These false positives were attributed to peptide depletion values which exceeded the threshold cutoffs at a single and often high concentration. Depletion at the highest test chemical concentration was likely the result of mass spectrometer ion suppression of the peptide analyte (20 μ M) in the presence of an unnaturally high chemical concentration of 30 mM which (at least partially) cochromatographed with the peptide. Reducing the highest test chemical

Table 5. Comparison of Maximum Peptide Depletion Values and Hazard Predictions Obtained With the Original (as Published in Troutman et al., 2011) and Refined Peroxidase Peptide Reactivity Assay Protocols

Chemical	CAS No.	Cysteine (–HRP/P; Direct)		Cysteine (+HRP/P)	Lysine (–HRP/P; Direct)		Prediction			
		DPmax original	DPmax refined		DPmax original	DPmax refined	DPmax ^a original	DPmax refined	HAZ id Original ^b	HAZ id Refined ^b
Nonsensitizers										
1-Bromobutane	109-65-9	79.8	34.8	46.7	35.3	4.4	1.8	S	S	S
1-Butanol	71-36-3	4.2	14.7	11.7	17.4	6.5	2.9	NS	S	NS
3-Phenoxy propiononitrile	3055-86-5	4.6	6.7	5.9	8.3	1.9	6.6	NS	NS	NS
4-Hydroxybenzoic acid	99-96-7	4.6	0.0	54.1	66.2	0.0	0.2	S	S	S
4-Methoxyaceto-phenone	100-06-1	5.5	0.0	17.2	12.5	2.2	7.0	NS	NS	NS
6-Methylcoumarin	92-48-8	7.3	0.0	4.9	12.4	10.9	0.0	NS	NS	NS
Benzaldehyde	100-52-7	1.9	99.6	21.3	66.8	13.3	29.3	NS	S	S
Chlorobenzene	108-90-7	4.1	3.6	10.2	12.0	8.4	5.1	NS	NS	NS
Coumarin	91-64-5	15.1	5.7	17.9	16.6	27.7	0.0	S	NS	NS
Diethyl phthalate	84-66-2	2.2	1.1	2.1	0.0	5.7	2.7	NS	NS	NS
Glycerol	56-81-5	2.7	13.5	5.4	8.5	5.6	10.5	NS	S	NS
Hexane	110-54-3	22.8	0.0	5.0	12.0	6.4	1.0	S	NS	NS
Isopropanol	67-63-0	22.7	2.0	9.7	9.2	0.0	1.6	S	NS	NS
Lactic acid	50-21-5	4.6	5.2	19.4	0.0	2.8	3.6	NS	NS	NS
Methyl paraben	99-76-3	7.4	2.8	36.3	38.7	4.1	3.6	NS	NS	S
Methyl salicylate	119-36-8	9.0	0.0	35.0	31.7	10.0	6.7	NS	NS	S
Nonanoic acid	112-05-0	21.1	19.3	18.5	3.4	4.6	7.3	S	S	NS
Octanenitrile	124-12-9	7.0	9.3	10.1	12.0	6.4	6.5	NS	NS	NS
Octanoic acid	124-07-2	5.7	0.0	16.3	18.6	10.3	0.0	NS	NS	NS
Propyl paraben	94-13-3	10.0	9.0	43.0	47.2	4.3	6.2	S	S	S
Propylene glycol	57-55-6	2.6	0.0	14.4	15.2	5.4	8.2	NS	NS	NS
Salicylic acid	69-72-7	19.0	29.9	23.4	15.6	9.7	4.4	S	S	S
Streptomycin sulfate	3810-74-0	4.4	9.2	1.0	5.8	10.5	5.4	NS	NS	NS
Sulphanilamide	63-74-1	3.4	11.0	9.4	5.4	5.7	14.1	NS	S	NS
Tartaric acid	87-69-4	5.1	10.0	41.7	10.6	11.4	0.0	S	NS	NS
Weak sensitizers—direct haptens										
2-Methylundecanal	110-41-8	6.5	76.3	2.8	19.8	22.0	0.2	NS	S	S
2,3-Butanedione	431-03-8	41.8	99.8	23.6	51.4	32.4	47.7	S	S	S
7-Hydroxycitronellal	107-75-5	2.3	99.5	1.9	15.7	17.9	78.5	S	S	S
α-Amyl cinnamaldehyde	122-40-7	11.0	6.0	14.6	31.0	8.1	3.0	S	NS	S
Citral	5392-40-5	25.3	100.0	37.8	33.1	31.4	10.9	S	S	S
Diethyl acetaldehyde	97-96-1	30.8	100.0	35.5	93.0	20.6	98.2	S	S	S
Ethyl acrylate	140-88-5	78.2	99.6	59.9	76.9	29.6	4.3	S	S	S
Farnesal	19317-11-4	11.2	21.0	20.8	37.0	31.8	11.1	S	S	S
Hexyl cinnamaldehyde	101-86-0	6.5	5.4	7.9	21.7	0.0	1.6	NS	NS	NS
Methyl acrylate	96-33-3	84.9	99.9	70.0	82.9	60.3	7.2	S	S	S
Moderate sensitizers—direct haptens										
2-Hydroxyethyl acrylate	818-61-1	92.3	99.9	91.1	86.9	78.0	7.0	S	S	S
3,4-Dihydrocoumarin	119-84-6	26.1	41.0	0.0	7.4	55.6	46.5	S	S	S
Benzylidene acetone	122-57-6	85.2	99.7	46.4	44.1	27.6	9.4	S	S	S
Cinnamic aldehyde	104-55-2	68.2	92.1	47.4	35.0	4.0	9.3	S	S	S
Diethyl maleate	141-05-9	92.5	99.6	38.7	42.0	38.1	4.7	S	S	S
Glyoxal	107-22-2	93.7	92.9	86.2	50.8	67.0	97.1	S	S	S
Phenylacet-aldehyde	122-78-1	5.8	42.1	21.6	50.2	3.9	14.7	NS	S	S
2-Phenylpropionaldehyde	93-53-8	11.2	28.1	27.1	35.6	0.4	12.1	S	S	S
Strong/extreme—direct haptens										
p-Benzoquinone	106-51-4	100.0	99.9	100.00	99.8	92.8	98.6	S	S	S
Fluorescein-5-isothiocyanate	3326-32-7	33.8	51.6	44.1	21.5	37.7	54.8	S	S	S
B-Propiolactone	57-57-8	91.2	97.7	91.4	43.4	49.8	40.2	S	S	S
Glutaraldehyde	111-30-8	88.0	98.8	59.7	49.2	63.4	99.0	S	S	S
Hexahydrophthalic anhydride	85-42-7	0.0	19.5	29.5	32.3	29.4	98.4	S	S	S
Oxazolone	15646-46-5	13.9	95.2	34.3	27.0	49.1	99.1	S	S	S
Weak sensitizers—pre-/prohaptens										
Aniline	62-53-3	9.9	10.2	31.9	62.6	9.9	4.3	S	S	S
Cinnamyl alcohol	104-54-1	13.8	68.6	23.8	72.9	11.6	3.6	S	S	S
Eugenol	97-53-0	67.5	89.7	99.4	79.5	6.3	3.8	S	S	S
Methyl eugenol	93-15-2	10.0	78.0	60.4	62.9	1.1	3.4	S	S	S
Pentachlorophenol	87-86-5	0.0	13.3	42.9	57.4	13.5	0.0	S	S	S

Table 5. (continued)

Chemical	CAS No.	Cysteine (–HRP/P; Direct)		Cysteine + HRP/P	Lysine (–HRP/P; Direct)		Prediction			
		DPmax original	DPmax refined	DPmax original	DPmax refined	DPmax ^a original	DPmax refined	HAZ id Original ^b	HAZ id Refined ^b	HAZ id Refined ^c
Moderate sensitizers—pre-/prohaptens										
1-Naphthol	90-15-3	27.6	71.2	98.9	91.0	35.7	11.7	S	S	S
2-Methoxy-4-methylphenol	93-51-6	34.2	8.3	99.0	93.1	5.7	22.2	S	S	S
3-Aminophenol	591-27-5	3.7	18.4	97.7	55.0	18.6	9.7	S	S	S
5-Amino-2-methylphenyl	2835-95-2	69.7	37.4	84.0	30.7	39.2	7.9	S	S	S
Dihydroeugenol	2785-87-7	9.7	5.0	99.0	87.0	7.4	10.7	S	S	S
Isoeugenol	97-54-1	90.5	100.0	71.0	100.0	7.7	0.8	S	S	S
Strong/extreme sensitizers—pre-/prohaptens										
1,4-Phenylenediamine	106-50-3	98.0	99.6	100	99.9	13.4	3.2	S	S	S
2-Aminophenol	95-55-6	29.9	83.0	99.1	86.6	8.3	3.4	S	S	S
2-Nitro-1,4-phenylenediamine	5307-14-2	93.7	42.1	100.0	85.9	8.3	4.7	S	S	S
3-Methylcatechol	488-17-5	98.0	99.6	100	99.8	74.9	37.3	S	S	S
Hydroquinone	123-31-9	56.7	99.5	100	41.9	100	19.4	S	S	S
Propyl gallate	121-79-9	98.4	99.8	96.1	89.9	68.1	2.2	S	S	S
Lauryl gallate	1166-52-5	71.2	2.3	88.4	93.2	7.8	4.0	S	S	S

Negative depletion values are reported as zero. Nonsensitizer/sensitizer classifications are based on local lymph node assay data.

^aLysine reactions were conducted in the original protocol with 0.1-M ammonium acetate buffer (pH10.2) (Troutman et al., 2011).

^bClassification based on the previous depletion cut-off values of > 40%, > 10%, and > 15% for cysteine with horseradish peroxidase/hydrogen peroxide (HRP/P), cysteine without HRP/P, and lysine, respectively (Troutman et al., 2011).

^cClassification based on the refined depletion values for clear positive values of > 25% and > 20% for cysteine with and without HRP/P, and lysine, respectively. Clear negative calls for depletion values are < 15% and < 10% for cysteine with and without HRP/P, and lysine, respectively. Depletion values falling between the clear positive and negative cut-offs require additional evaluation.

concentration from 30 to 5 mM and adjusting the depletion cut-off values for positive responses from 40% depletion for the +HRP/P reaction and 10% for the –HRP/P reaction to 25% for both cysteine reactions reduced the number of false positives, minimized solubility issues encountered with the higher concentration and reduced the potential for ion suppression effects.

Some chemicals which cause cysteine depletion in the DPRA were not producing similar depletion in the “direct” (–HRP/P) cysteine reaction in the PPRA. The reaction mixture for cysteine is the same for both assays except for postincubation addition of DTT to both PPRA cysteine reaction mixtures, +HRP/P and –HRP/P. Removal of DTT from the cysteine –HRP/P reaction improved detection of reactive aldehydes and thiazol derivatives, while not compromising the assay's ability to detect pre-/prohaptens. Some of the differences noted between the DPRA % cysteine depletion values and the PPRA DPmax values for cysteine –HRP/P without DTT may be due to the use of the more sensitive and selective HPLC/MS/MS detection system in the PPRA methodology.

In addition to adduct formation, side reactions can occur in the cysteine/test chemical reaction mixture which lead to apparent cysteine peptide depletion (Natsch and Emter, 2017). Oxidation driven cysteine peptide dimerization can occur in parallel to adduct formation and is often triggered by aldehydes (Natsch et al., 2007). Peptide reactivity has been characterized for most of the chemicals tested (Table 4): citral and α -methyl cinnamic aldehyde are only adduct forming; cinnamic aldehyde, laevo perilla aldehyde, 2-mercaptobenzothiazole, 1,2-benzisothiazolin-3-one, are adduct forming and oxidizing (ie, cysteine peptide depletion results from adduct formation and

dimerization); and lillial is reported to be only oxidizing (Natsch and Gfeller, 2008). Removal of DTT from the cysteine –HRP/P reaction mixture increased cysteine depletion for those chemicals that also drive cysteine dimerization. It's not known whether oxidative dimerization plays a direct role in the mechanism of skin sensitization or if it's observation in peptide reactivity assays is the result of test method design and timing. It was however shown, that reactive thiols generate a range of cysteine oxidation products, including inter- and intraprotein disulfides, which serve as a basis for redox signaling pathways (Ma, 2013). It is important to note that the PPRA (and DPRA) are intended to be screening methods and are not expected to fully characterize all reaction chemistry. The addition of dimer formation monitoring for the cysteine –HRP/P reaction in the PPRA adds valuable information regarding the nature of the observed depletion.

Although some chemicals deplete the lysine peptide to a greater degree than the cysteine peptide, it is rare to find chemicals that react exclusively with lysine. Because most reactions occur with the cysteine peptide, the reaction mixture with lysine +HRP/P was dropped from the PPRA to streamline the assay. It was observed that some lipophilic chemicals had decreased solubility in the lysine reaction mixture at 1% organic solvent content. Therefore, the organic solvent content of the lysine reaction mixture was evaluated to see if test chemical solubility, and thus peptide depletion could be improved. In addition, further simplification of the assay by using a single buffer, 0.1 M potassium phosphate pH 7.4, for both peptides was explored. The change in buffer to potassium phosphate and the increase in organic solvent content to 25% for the lysine peptide reaction resulted in increased depletion for most of the lysine-

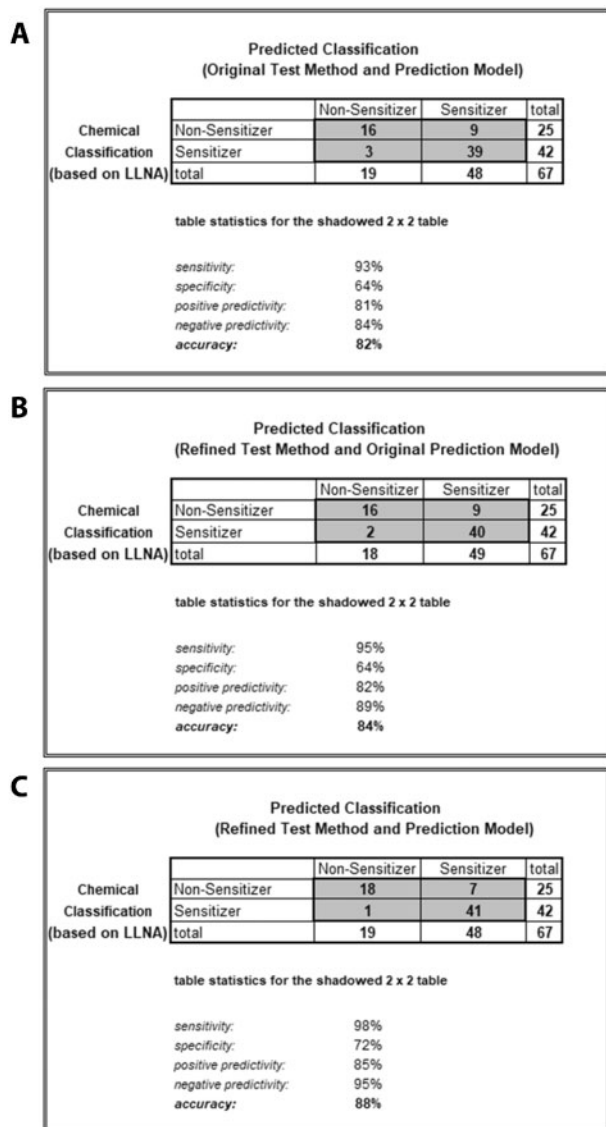


Figure 5. Comparison of Cooper Statistics for Methods and Prediction Models. A, Original test method and prediction model, (B) refined test method and original prediction model, (C) refined test method and prediction model. Abbreviation: LLNA, local lymph node assay.

reactive chemicals. Therefore, these changes were adopted into the test protocol.

Changes to the cysteine and lysine reaction mixtures were made to reduce the number of false positives and improve detection for weak sensitizers, aldehydes, and thiazol derivatives. These changes prompted the need for a re-examination of the prediction model. Adjustments made to cutoff depletion values for classifying clear negative and positive responses improved the sensitivity, specificity, and accuracy of the assay for hazard identification (Figure 5).

With the refined PPRA method and prediction model, 7 of the 25 chemicals classified as nonsensitizing (based on LLNA data) were found to be reactive. The covalent-bonding reactions between electrophilic chemicals and nucleophilic amino acids in proteins are used to group chemical structures associated with skin sensitization into protein reactivity domains (Aptula et al., 2005). No reactivity domain could be assigned to 5 structurally related chemicals (Roberts et al., 2007b): methyl and

propyl paraben are esters of 4-hydroxybenzoic acid; methyl salicylate is a salicylic acid ester; salicylic acid and 4-hydroxybenzoic acid are phenolic acids with the hydroxy group in the ortho- and para-positions, respectively (Supplementary Figure 1). The false positive predictions for 4-hydroxybenzoic acid, methyl paraben, propyl paraben, and methyl salicylate, were attributed to the peptide depletion only in the cysteine +HRP/P reaction. It is reasonable to assume that the paraben esters become hydrolyzed to 4-hydroxybenzoic acid which reacts with the cysteine peptide under +HRP/P (noting that it behaves as a prohaptens in the PPRA). Although negative in the LLNA, the parabens and methyl salicylate are classified as rare human contact allergens (Basketter et al., 2014) and 4-hydroxybenzoic acid was positive in a guinea pig maximization test (Basketter and Scholes, 1992). 1-Bromobutane, also reported to be positive in the DPRA (Natsch et al., 2013), is classified as an S_N2 electrophile. Its negative response in the LLNA has been attributed to its hydrophobicity (Basketter et al., 1992; Roberts et al., 2007a). Benzaldehyde is a Schiff base electrophile (Roberts et al., 2007b) classified as nonsensitizing by the LLNA, has induced sensitization in a Human Repeat Insult Patch Test (Natsch et al., 2012) and produced a positive response in the KeratinoSens assay (Natsch et al., 2013).

The only false negative sensitizer, hexyl cinnamaldehyde, is also negative in the DPRA (Natsch et al., 2013) and h-CLAT, a cell-based assay (Nukada et al., 2012). Based on its chemical structure it could theoretically bind to peptide nucleophiles via Michael addition or Schiff base formation, but its reactivity might be sterically hindered (Basketter et al., 2015). Hexyl cinnamaldehyde is classified as a chemical that rarely induces skin sensitization in humans (Basketter et al., 2014). Although problematic in *in vitro* assays, it serves as a positive control chemical for the LLNA, reliably producing positive responses (Basketter et al., 2015; Dearman et al., 2001).

With the original method and prediction model, EC15 values were used to classify reactivity. A trend for higher DPmax values and lower median EC15 values were observed for moderate, strong and extreme sensitizers, including both haptens and pre-/prohaptens, compared with weak sensitizers (Troutman et al., 2011). With the change in the cutoff depletion value for clear positives, calculation of an EC25, rather than an EC15, was explored as a means of binning a chemical's reactivity as either "Reactive" (EC25 > 0.1 mM) or "HR" (EC25 < 0.1 mM) (Table 6). The median EC25 value of 0.625 mM for the 7 nonsensitizers misclassified as sensitizers, was higher than the median EC25 values for weak, moderate, and strong/extreme sensitizers. The strong/extreme sensitizers had the lowest median EC25 of 0.061 mM, but the median value for the moderate sensitizers was higher than the value for the weak sensitizers. A clearer trend for decreasing EC25 values with increasing potency was observed using the group average. However, within each potency classification the number of Reactive and Highly Reactive chemicals was similar, with 30%–50% of the chemicals being highly reactive. As such, no correlation was established between EC25 values and sensitization potency. Use of PPRA data for classification of sensitization potency remains an area for further model refinement. However, it is important to keep in mind that sensitization potency does not depend solely on protein reactivity and that other events of the AOP such as dermal penetration, keratinocyte inflammatory responses, and dendritic cell activation, are contributing factors. In addition, the reaction kinetics of the MIE may also play a role in sensitization potency. Rate constants derived from a kinetic DPRA have been shown to correlate with LLNA potency classifications for

TABLE 6. Calculated EC25 Values for Peptide-reactive Test Chemicals Using the Refined Test Method and Prediction Model

Chemical	CAS No.	EC25 (mM)			Overall Lowest EC25	Reactivity Classification
		Cysteine (–HRP/P)	Cysteine (+HRP/P)	Lysine		
Nonsensitizers misclassified as sensitizers						
1-Bromobutane	109-65-9	3.547	1.928	NC ^a	1.928	Reactive
4-Hydroxybenzoic acid	99-96-7	NC	<0.625	NC	<0.625	Reactive
Benzaldehyde	100-52-7	0.125	1.312	19.188	0.125	Reactive
Methyl paraben	99-76-3	NC	<0.039	NC	<0.039	Highly reactive
Methyl salicylate	119-36-8	NC	2.862	NC	2.862	Reactive
Propyl Paraben	94-13-3	NC	<0.039	NC	<0.039	Highly reactive
Salicylic acid	69-72-7	3.566	NC	NC	3.566	Reactive
Weak sensitizers—direct haptens						
2-Methylundecanal	110-41-8	0.12	NC	NC	0.12	Reactive
2,3-Butanedione	431-03-8	0.053	1.462	10.804	0.053	Highly reactive
7-Hydroxycitronellal	107-75-5	0.361	NC	4.12	0.361	Reactive
α-Amyl cinnamaldehyde ^b	122-40-7	NC	2.204	NC	2.204	Reactive
Citral ^b	5392-40-5	4.56	1.9	NC	1.9	Reactive
Diethyl acetaldehyde	97-96-1	0.034	0.369	1.927	0.034	Highly reactive
Ethyl acrylate	140-88-5	0.072	0.224	NC	0.072	Highly reactive
Farnesal ^b	19317-11-4	NC	2.102	NC	2.102	Reactive
Methyl acrylate	96-33-3	0.028	0.177	NC	0.028	Highly reactive
Moderate sensitizers—direct haptens						
2-Hydroxyethyl acrylate	818-61-1	0.017	0.05	NC	0.017	Highly reactive
3,4-Dihydrocoumarin ^b	119-84-6	2.714	NC	8.918	2.714	Reactive
Benzylidene acetone	122-57-6	0.088	1.405	NC	0.088	Highly reactive
Cinnamic aldehyde	104-55-2	0.289	0.283	NC	0.283	Reactive
Diethyl maleate	141-05-9	0.084	1.917	NC	0.084	Highly reactive
Glyoxal	107-22-2	0.181	1.327	1.937	0.181	Reactive
Phenylacetaldehyde	122-78-1	1.859	<0.039	NC	<0.039	Highly reactive
2-Phenylpropionaldehyde	93-53-8	4.305	0.474	NC	0.474	Reactive
Strong/extreme—direct haptens						
p-Benzoquinone	106-51-4	<0.039	<0.313	0.251	<0.039	Highly reactive
Fluorescein-5-isothiocyanate ^b	3326-32-7	1.56	>5	4.77	1.56	Reactive
B-Propiolactone	57-57-8	0.149	1.586	10.543	0.149	Reactive
Glutaraldehyde	111-30-8	0.564	0.205	0.083	0.083	Highly reactive
Hexahydrophthalic anhydride ^b	85-42-7	NC	1.739	0.38	0.38	Reactive
Oxazolone	15646-46-5	0.123	0.678	0.101	0.101	Reactive
Weak sensitizers—pre-/prohaptens						
Aniline	62-53-3	NC	<0.078	NC	<0.078	Highly reactive
Cinnamyl alcohol	104-54-1	4.486	0.075	NC	0.075	Highly reactive
Eugenol	97-53-0	0.326	<5	NC	0.326	Reactive
Methyl eugenol	93-15-2	0.205	0.753	NC	0.205	Reactive
Pentachlorophenol	87-86-5	NC	0.054	NC	0.054	Highly reactive
Moderate sensitizers—pre-/prohaptens						
1-Napthol	90-15-3	1.143	<0.313	NC	<0.313	Reactive
2-Methoxy-4-methylphenol	93-51-6	NC	<0.039	>8.333	<0.039	Highly reactive
3-Aminophenol	591-27-5	NC	<0.312	NC	<0.312	Reactive
5-Amino-2-methylphenyl	2835-95-2	0.932	0.399	NC	0.399	Reactive
Dihydroeugenol	2785-87-7	NC	<0.156	NC	<0.156	Reactive
Isoeugenol	97-54-1	0.033	<0.039	NC	0.033	Highly reactive
Strong/extreme sensitizers—pre-/prohaptens						
1,4-Phenylenediamine	106-50-3	0.011	0.007	NC	0.007	Highly reactive
2-Aminophenol	95-55-6	0.206	<0.12	NC	<0.12	Reactive
2-Nitro-1,4-phenylenediamine	5307-14-2	1.499	<0.156	NC	<0.156	Reactive
3-Methylcatechol	488-17-5	<0.039	<0.039	2.731	<0.039	Highly reactive
Hydroquinone ^b	123-31-9	0.485	<2.5	NC	0.485	Reactive
Propyl gallate	121-79-9	<0.039	<0.039	NC	<0.039	Highly reactive
Lauryl gallate	1166-52-5	NC	0.008	NC	0.008	Highly reactive

Nonsensitizer/sensitizer classifications are based on local lymph node assay data.

^aNC, not calculated. The chemical did not test positive with the peptide reaction mixture.

^bChemical's overall lowest EC25 value identified as an outlier in the data set.

Abbreviation: HRP/P, horseradish peroxidase/hydrogen peroxide.

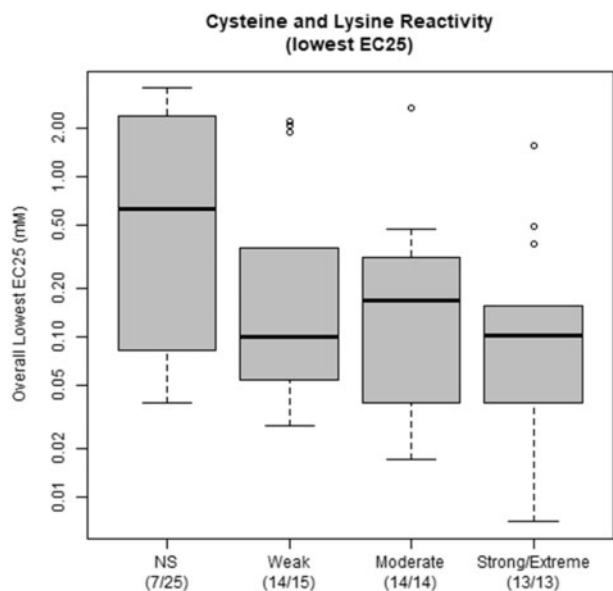


Figure 6. Box plots of the lowest EC25 values for each chemical and sensitization category. The box gives the distribution of EC25 values between the first and third quartiles. The median (second quartile) is shown as the black line within the box and the whiskers indicate the greatest and least EC25 values excluding outliers. Outliers, determined by the interquartile range, are shown as open circles.

chemicals belonging to the Michael acceptor reaction mechanistic domain (Roberts and Natsch, 2009).

In conclusion, refinements made to the PPRA method and prediction model improved assay performance for hazard identification and reliable detection for pre- and prohaptens. Given the additional analytical resources needed for the PPRA, the benefits of the PPRA as a replacement for the DPRA for use in routine screening are being explored. In cases where the test chemical coelutes with the cysteine peptide in the DPRA or has minimal reactivity in the DPRA and is predicted to be a pro-/pre-hapten, the PPRA provides an alternative method for evaluating peptide reactivity. None of the current OECD skin sensitization alternatives are intended to be used as stand-alone assays, but rather used together within a defined approach (DA) to predict sensitization potential. Most current DAs include DPRA data as the input to address the MIE in the AOP (Kleinstreuer et al., 2018). Use of PPRA data to address KE1 in the currently available DAs is underway. Conduct of an interlaboratory ring trial and development of a plan for obtaining regulatory acceptance remain as future possibilities.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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