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Research article

# Expanding the applicability of the amino acid derivative reactivity assay: Determining a weight for preparation of test chemical solutions that yield a predictive capacity identical to the conventional method using molar concentration and demonstrating the capacity to detect sensitizers in liquid mixtures

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ABSTRACT

*Introduction:* The amino acid derivative reactivity assay (ADRA) is a novel *in chemico* alternative to animal testing for assessment of skin sensitization potential. The conventional ADRA protocol stipulates that test chemical solutions should be prepared to a specific molar concentration, allowing only for use of test chemicals with known molecular weights. Since many potential test substances are prepared by weight concentration or contain multiple unknown chemicals, this study was conducted to verify if it is possible to accurately assess the sensitization potential of test chemical solutions prepared at a specific weight concentration.

*Methods:* (1) Test chemical solutions for 82 chemicals were prepared at four different weight concentrations. Results were evaluated for agreement with *in vivo* results. (2) A liquid mixture comprising ten different non-sensitizers was prepared at 1 mg/mL. Ten different sensitizers of varying sensitization potencies were added individually to this mixture. The resulting pseudobinary mixtures were tested to confirm that the sensitizers could be detected.

*Results:* (1) The accuracies for test chemical solutions prepared at 0.5 and 0.2 mg/mL were 87.8% and 86.6%, respectively, which were roughly equivalent to the accuracy of 86.6% achieved with a solution prepared at the conventional molar concentration of 1 mM. In contrast, the accuracies for solutions prepared at 0.1 and 0.05 mg/mL were 82.9% and 74.4%, respectively, both of which were lower than that obtained with the conventional method. (2) Sensitizers added to the liquid mixture at 0.5 mg/mL were all correctly detected.

*Discussion:* Preparing test chemical solutions at a weight concentration of 0.5 mg/mL decreased false negatives and increased false positives while improving prediction accuracy, which suggests that the sensitization potential of mixtures can also be assessed with this method.

## 1. Introduction

In recent years, a number of alternative methods to the use of laboratory animals in testing for the sensitization potential of chemical substances have been developed, six of which have been adopted and issued as OECD test guidelines.

Of these, alternative methods for testing skin sensitization potential that address elements of the adverse outcome pathway (AOP) as endpoints include the Direct Peptide Reactivity Assay (OECD TG442C, 2015), which addresses the Molecular Initiating Event of covalent binding with proteins (haptenization); the ARE-Nrf2 Luciferase KeratinoSens™ and ARE-Nrf2 Luciferase LuSens Tests (OECD TG442D,

*Abbreviations:* ADRA, amino acid derivative reactivity assay; NAC, *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine; NAL, α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine; DPRA, direct peptide reactivity assay

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2018a), which address the Key Event of keratinocyte activation; the Human Cell Line Activation Test, U937 Cell Line Activation Test, and IL-8 Luc Assay (OECD TG442E, 2018b), which address the Key Event of dendritic cell activation; and the murine Local Lymph Node Assay (OECD429, 2010), which addresses the Key Event of activation and proliferation of antigen-specific T-cells.

The sensitization mechanism comprising the Molecular Initiating Event and other Key Events mentioned above is a complex one, that should be assessed using a combination of test methods under an Integrated Approach to Testing and Assessment (IATA) (OECD, Series on Testing & Assessment No. 255, 2016). In fact, Urbisch et al. reported in 2016 that predictions for the skin sensitization potential of test chemicals made using an IATA comprising DPRA, KeratinoSens, and h-CLAT were even more accurate than those made using LLNA when compared with human data.

In developing ADRA, we addressed the Molecular Initiating Event of covalent binding with proteins by synthesizing two nucleophilic reagents from heptapeptides. We introduced naphthalene rings to the N-termini of both cysteine and lysine, thereby synthesizing N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-N-(2-(1-naphthyl)acetyl)-L-lysine (NAL) (Fujita et al., 2014).

ADRA affords two significant improvements over DPRA: First, since it can be performed using test chemical solutions at just 1% of the concentration required by DPRA, there is virtually no precipitation of the test chemical in the test chemical solution. Second, since UV is measured at a wavelength of 281 nm, there is virtually no co-elution of the test chemical and the nucleophilic reagents during HPLC analysis. Moreover, the addition of EDTA to the NAC solution provides increased stability and prevents oxidative dimerization of cysteine derivatives (Fujita et al., 2019).

As described above, ADRA ameliorates a number of limitations that affected DPRA, but there are some issues that still require improvement. Both DPRA and ADRA require that test chemical solutions and the peptide or NAC and NAL reaction solutions be prepared with their molar concentrations at a specific ratio to each other. Thus, test chemical solutions must be prepared to a specific molar concentration.

Nevertheless, the reaction solutions used in ADRA are prepared so that the molar concentration of NAC or NAL to that of the test chemical solution is at a ratio of 1:50, and this significant excess of test chemical relative to the nucleophilic reagent means we do not anticipate that small variations in this specific ratio would have a considerable impact on reactivity.

Also, since it was necessary for the test chemical solutions used in DPRA to be prepared at the very high molar concentration of 100 mM, preparing test chemical solutions according to weight concentration often resulted in molar concentrations significantly higher than 100 mM, which we think increases the potential that the test chemical would precipitate in the reaction solution or that the peaks of the peptides would co-elute with the peaks of the test chemical during HPLC analysis. Since, however, the test chemical solutions used in ADRA are prepared at a molar concentration of 1 mM, which is just 1% of that used in DPRA, the potential for the test chemical precipitating in the reaction solution even at higher than specified concentrations remains low. Moreover, since HPLC analysis is performed at the relatively long detection wavelength of 281 nm, the potential for the peaks of NAC or NAL to co-elute with the peaks of the test chemical also remains low.

Based on the above, we sought to develop an alternative to animal testing that would be useful in assessing the sensitization potential of chemicals of unknown molecular weight by determining an optimal weight concentration for test chemical solutions. In addition, we prepared ten pseudobinary mixtures comprising 10 different non-sensitizers and one sensitizer each to verify whether or not this method was capable of assessing the sensitization potential of liquid mixtures.

**Table 1**
Test chemicals used in this study.

| № | Test substance | CAS no. | Scource[a] | Solvent[b] |
|---|---|---|---|---|
| Extreme/strong | | | | |
| 1 | Diphenylcyclopropenone | 886-38-4 | FUJIFILM Wako | Acetonitrile |
| 2 | Oxazolone | 15646-46-5 | FUJIFILM Wako | Acetonitrile |
| 3 | Benzoyl peroxide | 94-36-0 | TCI | Acetonitrile |
| 4 | Kathon CG | 56965-84-9 | Sigma-Aldrich | $H_2O$ |
| 5 | Bandrowski's base | 20048-27-5 | Alfa Aesar | Acetonitrile |
| 6 | 5-Chloro-2-methyl-4-isothiazolin-3-one | 26172-55-4 | Santa Cruz | $H_2O$ |
| 7 | p-Benzoquinone | 106-51-4 | FUJIFILM Wako | Acetonitrile |
| 8 | Tetrachlorosalicylanilide | 1154-59-2 | AccuStandard | Acetonitrile |
| 9 | 2,4-Dinitrochlorobenzene | 97-00-7 | FUJIFILM Wako | Acetonitrile |
| 10 | Glutaraldehyde | 111-30-8 | FUJIFILM Wako | $H_2O$ |
| 11 | Fluorescein isothiocyanate | 3326-32-7 | Dojindo | Acetone |
| 12 | Phthalic anhydride | 85-44-9 | FUJIFILM Wako | Acetonitrile |
| 13 | Lauryl gallate | 1166-52-5 | FUJIFILM Wako | Acetonitrile |
| 14 | Propyl gallate | 121-79-9 | FUJIFILM Wako | Acetonitrile |
| 15 | CD3 | 25646-71-3 | FF | $H_2O$ |
| 16 | Trimellitic anhydride | 552-30-7 | FUJIFILM Wako | Acetonitrile |
| 17 | Formaldehyde | 50-00-0 | FUJIFILM Wako | $H_2O$ |
| 18 | Metol | 55-55-0 | FUJIFILM Wako | $H_2O$ |
| Moderate | | | | |
| 19 | 2-Hydroxyethyl acrylate | 818-61-1 | FUJIFILM Wako | $H_2O$ |
| 20 | Glyoxal | 107-22-2 | FUJIFILM Wako | $H_2O$ |
| 21 | Vinyl pyridine | 1337-81-1 | FUJIFILM Wako | $H_2O$ |
| 22 | 2-Mercaptobenzothiazole | 149-30-4 | FUJIFILM Wako | Acetonitrile |
| 23 | Nonanoyl chloride | 764-85-2 | TCI | Acetonitrile |
| 24 | 2-Methyl-2H-isothiazol-3-one | 2682-20-4 | Sigma-Aldrich | $H_2O$ |
| 25 | 1,2-Benzisothiazoline-3-one | 2634-33-5 | TCI | Acetonitrile |
| 26 | Methyl-2-nonynoate | 111-80-8 | TCI | Acetonitrile |
| 27 | Cinnamaldehyde | 14371-10-9 | FUJIFILM Wako | Acetonitrile |
| 28 | Phenylacetaldehyde | 122-78-1 | Alfa Aesar | Acetonitrile |
| 29 | Benzylideneacetone | 122-57-6 | FUJIFILM Wako | Acetonitrile |
| 30 | 2,4-Heptadienal | 5910-85-0 | FUJIFILM Wako | Acetonitrile |
| 31 | Squaric acid | 2892-51-5 | FUJIFILM Wako | $H_2O$ |
| 32 | Trans-2-hexenal | 6728-26-3 | FUJIFILM Wako | Acetonitrile |
| 33 | Resorcinol | 108-46-3 | FUJIFILM Wako | $H_2O$ |
| 34 | Diethyl maleate | 141-05-9 | FUJIFILM Wako | $H_2O$ |
| 35 | 2-phenylpropionaldehyde | 93-53-8 | Sigma-Aldrich | Acetonitrile |
| 36 | Perillaldehyde | 2111-75-3 | FUJIFILM Wako | Acetonitrile |
| 37 | Palmitoyl Chloride | 112-67-4 | FUJIFILM Wako | Acetone |
| 38 | 1-(4-Methoxyphenyl)-1-penten-3-one | 104-27-8 | AccuStandard | Acetonitrile |
| Weak | | | | |
| 39 | α-Hexylcinnnamaldehyde | 101-86-0 | FUJIFILM Wako | Acetonitrile |
| 40 | α-Amylcinnamaldehyde | 122-40-7 | FUJIFILM Wako | Acetonitrile |
| 41 | 2,3-Butanedione | 431-03-8 | FUJIFILM Wako | $H_2O$ |
| 42 | Farnesal | 19317-11-4 | Frinton | Acetonitrile |
| 43 | Oxalic acid | 144-62-7 | FUJIFILM Wako | $H_2O$ |
| 44 | Benzyl benzoate | 120-51-4 | FUJIFILM Wako | Acetonitrile |
| 45 | 4-Allylanisole | 140-67-0 | TCI | Acetonitrile |
| 46 | Lilial | 80-54-6 | FUJIFILM Wako | Acetonitrile |
| 47 | Cyclamen aldehyde | 103-95-7 | Sigma-Aldrich | Acetonitrile |
| 48 | Imidazolidinyl urea | 39236-46-9 | Sigma-Aldrich | $H_2O$ |
| 49 | 5-Methyl-2,3-hexanedione | 13706-86-0 | TCI | Acetonitrile |
| 50 | 2,2,6,6-Tetramethyl-3,5-heptanedione | 1118-71-4 | TCI | Acetonitrile |
| 51 | Ethyleneglycol dimethacrylate | 97-90-5 | FUJIFILM Wako | Acetonitrile |
| 52 | Ethyl acrylate | 140-88-5 | FUJIFILM Wako | $H_2O$ |
| 53 | Hydroxycitronellal | 107-75-5 | FUJIFILM Wako | Acetonitrile |
| Non-sensitizer | | | | |
| 54 | Glycerol | 56-81-5 | FUJIFILM Wako | $H_2O$ |
| 55 | Hexane | 110-54-3 | FUJIFILM Wako | Acetonitrile |
| 56 | Diethyl phthalate | 84-66-2 | FUJIFILM Wako | Acetonitrile |
| 57 | Octanoic acid | 124-07-2 | FUJIFILM Wako | Acetonitrile |
| 58 | 2-Hydroxypropyl methacrylate | 923-26-2 | FUJIFILM Wako | $H_2O$ |
| 59 | 1-Butanol | 71-36-3 | FUJIFILM Wako | $H_2O$ |
| 60 | 4-Hydroxybenzoic acid | 99-96-7 | FUJIFILM Wako | Acetonitrile |
| 61 | 6-Methyl coumatrin | 92-48-8 | Sigma-Aldrich | Acetonitrile |
| 62 | Methyl salicylate | 119-36-8 | FUJIFILM Wako | Acetonitrile |
| 63 | Chlorobenzene | 108-90-7 | FUJIFILM Wako | Acetonitrile |
| 64 | Lactic acid | 50-21-5 | Sigma-Aldrich | $H_2O$ |

(*continued on next page*)

**Table 1** (*continued*)

| № | Test substance | CAS no. | Scource[a] | Solvent[b] |
|---|---|---|---|---|
| 65 | 1-Bromobutane | 109-65-9 | FUJIFILM Wako | Acetonitrile |
| 66 | 2-Acethylcyclohexanone | 874-23-7 | FUJIFILM Wako | Acetonitrile |
| 67 | 4'-Methoxyacetophenone | 100-06-1 | FUJIFILM Wako | Acetonitrile |
| 68 | Ethyl benzoylacetate | 94-02-0 | FUJIFILM Wako | Acetonitrile |
| 69 | Ethyl vanillin | 121-32-4 | TCI | Acetonitrile |
| 70 | Isopropanol | 67-63-0 | FUJIFILM Wako | $H_2O$ |
| 71 | Propylene glycol | 57-55-6 | FUJIFILM Wako | $H_2O$ |
| 72 | Sulfanilamide | 63-74-1 | FUJIFILM Wako | Acetonitrile |
| 73 | Isopropyl myristate | 110-27-0 | FUJIFILM Wako | Acetonitrile |
| 74 | Benzaldehyde | 100-52-7 | Sigma-Aldrich | Acetonitrile |
| 75 | Methylparaben | 99-76-3 | FUJIFILM Wako | Acetonitrile |
| 76 | Nonanoic acid | 112-05-0 | TCI | Acetonitrile |
| 77 | Propyl paraben | 94-13-3 | FUJIFILM Wako | Acetonitrile |
| 78 | Salicylic acid | 69-72-7 | FUJIFILM Wako | Acetonitrile |
| 79 | Sulphanilic acid | 121-57-3 | FUJIFILM Wako | $H_2O$ |
| 80 | Vanillin | 121-33-5 | FUJIFILM Wako | Acetonitrile |
| 81 | Coumarin | 91-64-5 | FUJIFILM Wako | Acetonitrile |
| 82 | Vinylidene dichloride | 75-35-4 | AccuStandard | Acetonitrile |

[a] AccuStandard, AccuStandard, Inc., New Haven, CT, USA; Alfa Aesar, Alfa Aesar, Ward Hill, MA, USA; Dojindo, Dojindo Molecular Technologies, Inc., Kumamoto, Japan;FF, Synthetic Organic Chemistry Laboratories of FUJIFILM Corporation; Frinton, Frinton Laboratories, Inc., Hainesport, NJ, USA; Santa Cruz, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Sigma-Aldrich, Sigma-Aldrich Corporation, St Louis, MO, USA; TCI, Tokyo Chemical Industry Co Ltd., Tokyo, Japan; FUJIFILM Wako, FUJIFILM Wako Pure Chemical Industries Ltd., Osaka, Japan.

[b] Solvent of 0.05, 0.1, 0.2, and 0.5 mg/mL test chemical solutions.

## 2. Materials and methods

### 2.1. Test chemicals

The 82 test chemicals and their CAS numbers, suppliers, and the solvents used to prepare their test chemical solutions are summarized in Table 1. These chemicals were used to prepare test chemical solutions per different weight/volume concentrations, which were tested to determine an optimal weight concentration. Additionally, the following ten non-sensitizers were selected from these 82 test chemicals, for use in studying the assessment of mixtures: diethyl phthalate, 4-hydroxybenzoic acid, methyl salicylate, chlorobenzene, sulfanilamide, isopropyl myristate, methylparaben, propyl paraben, salicylic acid, and coumarin.

### 2.2. NAC and NAL reactivity assay

Dodium dihydrogen phosphate, disodium hydrogen phosphate, and 0.1N sodium hydroxide solution were purchased from FUJIFILM Wako Pure Chemical Corporation, ethylenediamine-N,N,N′,N′-tetraacetic acid disodium salt dehydrate (EDTA) was purchased from Dojindo Molecular Technoligiems, and both NAC and NAL were synthesized in our laboratory for use in preparing NAC and NAL stock solutions. Reagents of an equivalent grade to the NAC and NAL used in our testing are available commercially in an ADRA kit for skin sensitization testing from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Acetonitrile, acetone, and dimethyl sulfoxide (DMSO) were purchased from FUJIFILM Wako Pure Chemical Corporation for use in preparing test chemical solutions. NAC and NAL stock solutions were prepared to 6.667 μM in 100 mM sodium phosphate buffer at pH 8.0 for NAC and pH 10.2 for NAL. Test chemical solutions for each of the 82 chemicals were prepared to weight concentrations of 0.5, 0.2, 0.1, and 0.05 mg/mL in water, acetonitrile, acetone, or 5% DMSO in acetonitrile. For evaluation of mixtures, ten sensitizers were each prepared to weight concentrations of 0.5, 0.2, 0.1, and 0.05 mg/mL in an acetonitrile solution mixed with 1.0 mg/mL each of the ten non-sensitizers named in Section 2.1 “Test chemicals”.

Test samples were prepared in triplicate by adding 50 μL of the test chemical solution to 150 μL of the NAC or NAL stock solution in a 96-well microplate. Additionally, control samples without test chemicals were also prepared. The 96-well microplate was sealed by using a Resistant Embossed Seal (SHIMADZU GLC Ltd., Tokyo, Japan), shaken gently, and then incubated in the dark for 24 h at 25°C. Following incubation, 50 μL of 2.5% trifluoroacetic acid (TFA) in water was added to all samples. In addition, standard solutions for defining the calibration curve were prepared for NAC and NAL at concentrations ranging from 5.0 to 0.156 μM, after which the 96-well microplate was sealed again.

### 2.3. HPLC analysis of NAC and NAL

Acetonitrile and TFA for HPLC mobile phase preparation were purchased from FUJIFILM Wako Pure Chemical Corporation. The mobile phase (A) and (B) were prepared to 0.1%TFA in water and 0.1%TFA in acetonitrile respectively.

After incubation, NAC and NAL in all samples and standards were quantified using a LC-20A HPLC system (Prominence, Shimadzu Scientific Instruments, Kyoto, Japan) on a CAPCELL CORE C18 column (2.7 μm, 3.0 × 150 mm, Shiseido Co., Ltd., Tokyo, Japan). The flow rate was 0.3 mL/min, and the temperatures of the column oven and auto-sampler were maintained at 40°C and 4°C, respectively. 10 μL of each sample was injected, with a linear gradient from 30% B to 55% B for 9.5 min for NAC and from 20% B to 45% B for 9.5 min for NAL, followed by a rapid increase to 100% B for 0.5 min and holding 100% B for 3.5 min, then back to the initial conditions (30% B for NAC and 20% B for NAL) for a total analysis time of 20 min per sample. Finally, NAC and NAL were detected by UV absorbance at 281 nm.

### 2.4. Calculation of predictive capacity

Reactivity of the test chemical with NAC and NAL was calculated as the percent depletion based on the decrease in the NAC and NAL concentrations in the samples relative to the average concentration measured in the control. A prediction of either sensitizer or non-sensitizer was determined by mean percent depletion of NAC and NAL. The threshold value of mean percent depletion for prediction was 4.9, which is identical with the value used in ADRA with EDTA. (Fujita et al., 2019) If an average score for NAC and NAL depletion in a test chemical falls within the borderline range described below, additional testing should be performed to confirm the validity of the prediction. If the result of the second test does not agree with the first test, a third test should be performed and prediction of whether the test chemical is a sensitizer should be based on the majority of the three test results.

## 3. Results

The ADRA test method specifies to prepare test chemical solutions at a molar concentration of 1 mM. In this study, 82 test chemicals used in development of the DPRA and ADRA test methods were evaluated to verify whether test chemical solutions prepared by weight concentrations (w/v) could be used to accurately predict sensitization potential and thereby establish a means for testing chemicals of unknown molecular weight (Fujita et al., 2014; Gerberick et al., 2007; Yamamoto et al., 2015). We prepared test chemical solutions for 82 different test chemicals at four different concentrations each: 0.05, 0.1, 0.2, and 0.5 mg/mL. We then tested these chemical solutions using ADRA and summarized results for the following parameters:

1. Comparison of reactivity with NAC and NAL at each concentration of the test chemical solutions
2. Comparison of the prediction results and predictive capacity at each of the four concentrations of the test chemical solutions

**Table 2**
Reactivity of the 82 test chemicals with NAC and NAL at weight concentrations of 0.05, 0.1, 0.2, and 0.5 mg/mL as well as 1 mM, with results expressed as percent depletion of unreacted NAC and NAL.

| № | Test substance | Mw | Depletion of NAC | | | | | | | | | | Depletion of NAL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.05 mg/ml | | 0.1 mg/ml | | 0.2 mg/ml | | 0.5 mg/ml | | 1 mM[1] | | 0.05 mg/ml | | 0.1 mg/ml | | 0.2 mg/ml | | 0.5 mg/ml | | 1 mM[1] | |
| | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **Extreme/strong** | | | | | | | | | | | | | | | | | | | | | | |
| 1 | Diphenylcyclopropenone | 206.24 | 7.2 | 0.8 | 17.1 | 0.3 | 29.1 | 0.5 | 54.4 | 0.4 | 28.7 | 1.0 | 0.0 | 0.1 | 2.5 | 0.2 | 2.9 | 0.7 | 3.5 | 0.4 | 6.3 | 1.2 |
| 2 | Oxazolone | 217.22 | 35.5 | 0.9 | 59.9 | 1.1 | 83.5 | 0.5 | 89.6 | 0.2 | 85.3 | 0.3 | 59.7 | 0.4 | 69.7 | 0.1 | 67.8 | 0.5 | 74.3 | 0.4 | 80.1 | 0.8 |
| 3 | Benzoyl peroxide | 242.23 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 50.4 | 0.2 | 54.9 | 0.3 | 60.0 | 0.2 | 76.2 | 0.2 | 50.6 | 2.7 |
| 4 | Kathon CG | 115.15 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 99.6 | 0.7 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | -0.4 | 0.5 |
| 5 | Bandrowski's base | 318.38 | 84.0 | 2.1 | 97.3 | 0.7 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 0.0 | 0.0 | 2.2 | 0.1 | 3.4 | 0.5 | 14.7 | 0.8 | 5.7 | 0.4 |
| 6 | 5-Chloro-2-methyl-4-isothiazolin-3-one | 149.6 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 0.0 | 0.0 | 1.9 | 0.9 | 13.7 Co | 0.8 | 0.0 Co | | 17.7 | 1.1 |
| 7 | p-Benzoquinone | 108.09 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 96.5 | 0.2 | 60.5 | 0.2 | 73.2 | 0.3 | 82.3 | 0.1 | 88.1 | 0.6 | 83.5 | 1.1 |
| 8 | Tetrachlorosalicylanilide | 351.01 | 29.6 | 0.9 | 34.0 | 1.0 | 36.5 | 1.6 | 68.3 | 1.3 | 43.4 | 4.2 | 2.0 | 0.2 | 1.9 | 0.1 | 1.0 | 0.2 | 9.6 | 0.3 | 2.1 | 0.3 |
| 9 | 2,4-Dinitrochlorobenzene | 202.55 | 41.4 | 0.1 | 66.8 | 0.2 | 90.3 | 0.3 | 100.0 | 0.0 | 89.3 | 2.0 | 0.0 | 0.0 | 1.5 | 0.4 | 4.6 | 0.7 | 6.3 | 0.5 | 6.1 | 1.3 |
| 10 | Glutaraldehyde | 100.12 | 2.9 | 0.9 | 0.1 | 0.1 | 1.6 | 0.9 | 25.1 | 1.3 | 0.8 | 0.3 | 39.5 | 0.2 | 35.6 | 0.6 | 58.4 | 0.2 | 85.2 | 0.2 | 53.1 | 2.9 |
| 11 | Fluorescein isothiocyanate | 389.38 | 45.0 | 0.3 | 61.0 | 0.3 | 79.3 Co | 0.4 | 84.5 Co | 1.2 | 73.6 | 0.8 | 49.4 | 1.2 | 71.2 | 0.4 | 88.2 | 0.3 | 97.9 | 0.1 | 98.1 | 0.6 |
| 12 | Phthalic anhydride | 148.12 | 0.0 | 0.0 | 0.5 | 0.6 | 0.6 | 1.0 | 9.6 | 6.2 | 0.3 | 0.3 | 80.3 | 1.8 | 85.8 | 2.1 | 89.1 | 3.2 | 95.6 | 0.1 | 96.9 | 1.1 |
| 13 | Lauryl gallate | 338.44 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 40.3 | 3.1 | 67.3 | 0.1 | 85.8 | 0.3 | 95.0 | 0.0 | 19.0 | 0.4 |
| 14 | Propyl gallate | 212.2 | 97.6 | 0.2 | 98.5 | 0.2 | 100.0 | 0.0 | 100.0 | 0.0 | 97.1 | 0.1 | 25.7 | 0.4 | 44.6 | 0.3 | 64.6 | 0.9 | 62.3 | 0.8 | 56.4 | 1.4 |
| 15 | CD3 | 271.38 | 92.1 | 0.7 | 93.9 | 0.2 | 94.7 | 1.7 | 97.8 | 0.1 | 76.6 | 1.3 | 4.7 | 0.2 | 7.7 | 0.4 | 13.2 | 6.7 | 12.9 | 0.2 | 16.5 | 0.5 |
| 16 | Trimellitic anhydride | 192.13 | 0.1 | 0.2 | 0.0 | 0.0 | 2.5 | 0.0 | 4.5 | 0.8 | 1.9 | 0.4 | 70.0 | 0.5 | 87.2 | 4.3 | 95.8 | 0.3 | 97.5 | 0.3 | 97.0 | 1.2 |
| 17 | Formaldehyde | 30.03 | 25.8 | 0.9 | 51.6 | 2.2 | 67.3 | 2.2 | 85.8 | 0.6 | 25.7 | 1.1 | 0.1 | 0.1 | 0.7 | 0.6 | 0.6 | 0.2 | 0.0 | 0.0 | 1.6 | 1.9 |
| 18 | Metol | 221.23 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 8.5 | 0.2 | 12.2 | 0.2 | 17.4 | 0.4 | 22.2 | 0.3 | 22.8 | 1.1 |
| **Moderate** | | | | | | | | | | | | | | | | | | | | | | |
| 19 | 2-Hydroxyethyl acrylate | 116.12 | 96.7 | 0.3 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 3.0 | 0.3 | 7.2 | 0.3 | 12.6 | 0.5 | 23.4 | 0.5 | 16.3 | 4.7 |
| 20 | Glyoxal | 58.04 | 11.6 | 0.5 | 17.6 | 1.0 | 18.7 | 1.4 | 33.9 | 0.5 | 12.8 | 0.5 | 0.1 | 0.1 | 1.4 | 0.9 | 2.2 | 0.1 | 1.9 | 0.2 | 0.8 | 0.5 |
| 21 | Vinyl pyridine | 105.14 | 13.2 | 0.3 | 25.5 | 3.8 | 43.7 | 1.2 | 81.5 | 0.3 | 18.1 | 1.0 | 0.7 | 0.3 | 2.4 | 0.7 | 7.7 | 5.8 | 0.0 | 0.0 | 7.7 | 1.8 |
| 22 | 2-Mercaptobenzothiazole | 167.25 | 32.7 | 0.7 | 51.2 | 0.2 | 84.2 | 2.0 | 100.0 | 0.0 | 56.6 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 6.9 | 0.8 | 0.3 | 0.8 |
| 23 | Nonanoyl chloride | 176.68 | 0.0 | 0.0 | 1.6 | 0.6 | 11.1 | 2.1 | 11.3 | 1.6 | 7.4 | 0.4 | 12.6 | 1.6 | 28.0 | 3.0 | 45.4 | 1.6 | 64.1 | 1.2 | 39.4 | 1.8 |
| 24 | 2-Methyl-2H-isothiazol-3-one | 115.15 | 97.8 | 0.1 | 90.2 | 0.0 | 90.0 | 0.1 | 81.8 | 0.1 | 94.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 2.6 | 4.6 | 0.0 Co | | 7.0 | 2.2 |
| 25 | 1,2-Benzisothiazoline-3-one | 151.19 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 100.0 | 0.0 | 0.7 | 0.2 | 0.6 | 0.1 | 2.4 | 0.8 | 7.9 | 0.8 | 0.4 | 0.7 |
| 26 | Methyl-2-nonynoate | 168.23 | 2.8 | 0.4 | 6.2 | 0.3 | 18.2 | 1.9 | 35.7 | 1.1 | 15.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.3 | 7.3 | 0.5 | 1.4 | 0.2 |
| 27 | Cinnamaldehyde | 132.16 | 6.9 | 0.4 | 28.3 | 0.2 | 43.0 | 1.6 | 74.8 | 0.4 | 37.0 | 0.9 | 0.0 | 0.0 | 1.5 | 0.7 | 2.6 | 0.5 | 12.3 | 0.2 | 13.1 | 1.3 |
| 28 | Phenylacetaldehyde | 120.15 | 9.3 | 0.7 | 13.4 | 0.1 | 20.6 | 0.5 | 44.8 | 0.5 | 22.4 | 2.5 | 28.7 | 0.6 | 77.0 | 0.8 | 94.5 Co | 0.4 | 83.2 Co | 0.3 | 98.3 | 1.5 |
| 29 | Benzylideneacetone | 146.19 | 7.9 | 0.7 | 12.7 | 0.5 | 19.8 | 0.4 | 39.1 | 0.7 | 44.9 | 0.2 | 2.9 | 1.0 | 2.0 | 0.3 | 1.7 | 0.2 | 2.0 | 0.0 | 7.2 | 1.0 |
| 30 | 2,4-Heptadienal | 110.15 | 20.0 | 0.3 | 44.5 | 0.5 | 45.7 Co | 0.6 | 53.5 Co | 0.0 | 35.8 | 0.2 | 3.6 | 1.0 | 13.3 | 0.1 | 17.1 | 0.4 | 37.9 | 0.4 | 50.1 | 1.3 |
| 31 | Squaric acid | 114.06 | 2.3 | 0.9 | 4.8 | 0.7 | 4.3 | 0.7 | 2.9 | 0.8 | 0.8 | 0.3 | 0.6 | 0.6 | 0.8 | 0.0 | 0.0 | 0.0 | 0.3 | 0.3 | 0.5 | 0.2 |
| 32 | Trans-2-hexenal | 96.14 | 52.0 | 0.2 | 72.3 | 0.2 | 86.7 | 0.1 | 97.1 | 0.0 | 80.4 | 0.4 | 2.0 | 0.3 | 4.1 | 0.6 | 8.5 | 0.1 | 21.6 | 0.1 | 12.3 | 1.1 |
| 33 | Resorcinol | 110.11 | 2.3 | 0.4 | 6.2 | 1.0 | 11.1 | 0.9 | 13.4 | 0.3 | 4.0 | 0.3 | 3.1 | 5.0 | 0.1 | 0.1 | 0.8 | 0.2 | 0.3 | 0.3 | 2.2 | 0.7 |
| 34 | Diethyl maleate | 172.18 | 33.1 | 0.3 | 25.4 | 1.4 | 77.8 | 0.3 | 96.3 | 0.1 | 31.8 | 0.5 | 1.0 | 0.7 | 0.6 | 1.0 | 1.2 | 0.4 | 3.0 | 0.4 | 7.7 | 1.9 |
| 35 | 2-phenylpropionaldehyde | 134.18 | 3.9 | 0.9 | 12.4 | 1.2 | 16.6 | 0.9 | 29.1 | 0.3 | 8.0 | 0.3 | 4.2 | 0.4 | 6.2 | 0.3 | 7.3 | 0.3 | 17.1 | 0.3 | 4.6 | 1.3 |
| 36 | Perillaldehyde | 150.22 | 11.7 | 0.6 | 29.0 | 0.5 | 51.2 | 0.6 | 85.7 | 0.0 | 29.1 | 1.7 | 0.7 | 0.1 | 4.6 | 0.3 | 7.1 | 0.3 | 13.5 | 0.4 | 18.5 | 1.0 |
| 37 | Palmitoyl Chloride | 274.87 | 6.9 | 0.6 | 8.6 | 0.7 | 7.7 | 0.3 | 19.7 | 0.9 | 5.8 | 0.6 | 78.1 | 2.0 | 98.0 | 0.3 | 93.9 | 3.5 | 100.0 | 0.0 | 95.9 | 2.5 |
| 38 | 1-(4-Methoxyphenyl)-1-penten-3-one | 190.24 | 0.9 | 0.4 | 2.6 | 0.6 | 3.0 | 1.1 | 11.3 | 0.3 | 1.6 | 0.7 | 1.4 | 0.2 | 0.8 | 0.2 | 0.0 | 0.0 | 6.8 | 0.5 | 4.2 | 1.6 |
| **Weak** | | | | | | | | | | | | | | | | | | | | | | |
| 39 | α-Hexylcinnnamaldehyde | 216.32 | 0.0 | 0.0 | 2.7 | 0.4 | 2.9 | 0.5 | 4.1 | 0.4 | 0.0 | 0.0 | 0.4 | 0.6 | 2.2 | 0.3 | 1.9 | 0.8 | 4.4 | 1.0 | 1.1 | 0.9 |
| 40 | α-Amylcinnamaldehyde | 202.29 | 0.0 | 0.0 | 2.2 | 0.8 | 2.1 | 0.4 | 3.1 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 2.3 | 0.2 | 1.3 | 0.1 | 3.8 | 0.6 | 1.6 | 0.4 |
| 41 | 2,3-Butanedione | 86.09 | 12.1 | 0.8 | 33.5 | 0.6 | 87.3 | 1.4 | 100.0 | 0.0 | 41.2 | 0.9 | 7.9 | 0.1 | 8.5 | 0.2 | 13.3 | 2.4 | 29.5 | 0.3 | 25.5 | 1.2 |
| 42 | Farnesal | 220.35 | 6.1 | 0.2 | 18.8 | 0.8 | 25.6 | 0.9 | 21.3 | 0.2 | 17.2 | 0.6 | 0.8 | 0.2 | 6.8 | 7.7 | 6.6 | 0.9 | 8.1 | 0.5 | 8.8 | 1.4 |
| 43 | Oxalic acid | 90.03 | 0.6 | 1.0 | 0.3 | 0.4 | 0.0 | 0.0 | 0.6 | 0.2 | 0.0 | 0.0 | 0.5 | 0.2 | 0.2 | 0.2 | 1.4 | 0.4 | 1.0 | 0.1 | 3.6 | 1.0 |
| 44 | Benzyl benzoate | 212.24 | 0.6 | 0.5 | 0.5 | 0.8 | 0.1 | 0.2 | 3.5 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 0.4 | 0.1 | 1.0 | 0.5 | 3.9 | 0.2 | 2.8 | 1.1 |
| 45 | 4-Allylanisole | 148.2 | 8.3 | 1.5 | 19.5 | 0.9 | 35.5 | 0.2 | 61.2 | 0.4 | 27.0 | 0.4 | 0.1 | 0.2 | 2.8 | 0.1 | 7.2 | 0.4 | 2.9 | 0.3 | 3.9 | 2.6 |
| 46 | Lilial | 204.31 | 5.1 | 1.1 | 7.4 | 0.8 | 9.4 | 0.2 | 17.1 | 0.2 | 7.0 | 0.8 | 0.7 | 0.7 | 1.1 | 0.2 | 1.6 | 0.6 | 4.5 | 0.1 | 3.9 | 0.9 |
| 47 | Cyclamen aldehyde | 190.28 | 1.7 | 0.6 | 1.1 | 0.5 | 2.8 | 0.5 | 13.7 | 0.2 | 3.1 | 0.4 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 3.5 | 0.1 | 1.7 | 0.6 |
| 48 | Imidazolidinyl urea | 388.29 | 4.6 | 0.2 | 11.4 | 1.1 | 19.4 | 0.7 | 36.7 | 1.8 | 35.4 | 3.1 | 0.3 | 0.3 | 0.1 | 0.2 | 1.1 | 0.7 | 2.8 | 0.2 | 2.0 | 0.2 |
| 49 | 5-Methyl-2,3-hexanedione | 128.17 | 3.4 | 0.5 | 3.0 | 1.0 | 4.1 | 0.3 | 11.8 | 1.1 | 6.4 | 0.2 | 4.4 | 0.3 | 14.8 | 0.2 | 33.5 | 0.4 | 73.7 | 0.8 | 34.8 | 2.8 |
| 50 | 2,2,6,6-Tetramethyl-3,5-heptanedione | 184.28 | 0.8 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 4.6 | 0.7 | 0.5 | 0.5 | 0.1 | 0.2 | 0.0 | 0.1 | 0.2 | 0.2 | 3.2 | 0.2 | 1.6 | 0.8 |
| 51 | Ethyleneglycol dimethacrylate | 198.22 | 1.6 | 0.2 | 2.8 | 1.4 | 7.5 | 0.2 | 19.5 | 0.2 | 5.9 | 0.5 | 0.2 | 0.2 | 0.5 | 0.4 | 0.8 | 0.5 | 5.4 | 0.2 | 2.6 | 1.6 |
| 52 | Ethyl acrylate | 100.12 | 71.8 | 0.2 | 96.5 | 0.2 | 100.0 | 0.0 | 100.0 | 0.0 | 87.8 | 0.1 | 3.7 | 0.4 | 5.9 | 0.3 | 12.1 | 0.6 | 30.1 | 0.5 | 13.7 | 0.4 |
| 53 | Hydroxycitronellal | 172.26 | 1.9 | 0.7 | 9.1 | 0.6 | 16.4 | 0.3 | 29.5 | 0.7 | 17.5 | 0.3 | 0.0 | 0.0 | 0.4 | 0.3 | 1.0 | 0.7 | 5.2 | 0.1 | 1.5 | 0.7 |
| **Non-sensitizer** | | | | | | | | | | | | | | | | | | | | | | |
| 54 | Glycerol | 92.09 | 0.4 | 0.7 | 0.2 | 0.3 | 0.3 | 0.4 | 0.7 | 0.2 | 0.3 | 0.3 | 0.7 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.1 | 0.7 | 1.2 |
| 55 | Hexane | 86.18 | 1.6 | 0.4 | 2.7 | 0.6 | 0.2 | 0.3 | 3.6 | 0.3 | 0.2 | 0.3 | 2.3 | 0.3 | 0.3 | 0.2 | 0.1 | 0.0 | 3.8 | 0.1 | 0.9 | 0.9 |
| 56 | Diethyl phthalate | 222.24 | 2.0 | 0.2 | 1.8 | 1.1 | 0.6 | 0.4 | 4.0 | 0.7 | 0.0 | 0.0 | 2.6 | 0.3 | 0.4 | 0.2 | 0.6 | 0.3 | 4.2 | 0.4 | 1.7 | 1.4 |
| 57 | Octanoic acid | 144.21 | 1.9 | 0.6 | 1.4 | 0.3 | 0.9 | 0.8 | 3.6 | 0.9 | 0.0 | 0.0 | 2.2 | 0.5 | 0.0 | 0.0 | 0.4 | 0.3 | 3.7 | 0.3 | 1.2 | 0.5 |
| 58 | 2-Hydroxypropyl methacrylate | 144.17 | 4.0 | 0.6 | 5.9 | 0.6 | 9.5 | 1.0 | 22.0 | 0.8 | 2.4 | 0.5 | 0.4 | 0.3 | 0.6 | 0.3 | 1.2 | 0.1 | 3.1 | 0.2 | 2.2 | 0.6 |
| 59 | 1-Butanol | 74.12 | 1.0 | 0.9 | 0.7 | 0.4 | 0.6 | 0.6 | 1.2 | 0.7 | 0.1 | 0.2 | 0.5 | 0.3 | 0.4 | 0.4 | 1.2 | 0.4 | 0.3 | 0.5 | 0.8 | 0.5 |
| 60 | 4-Hydroxybenzoic acid | 138.12 | 2.0 | 0.4 | 4.0 | 0.6 | 1.7 | 0.3 | 3.6 | 0.6 | 0.0 | 0.0 | 2.0 | 0.3 | 0.0 | 0.0 | 0.2 | 0.3 | 2.9 | 0.1 | -1.4 | 1.0 |
| 61 | 6-Methyl coumatrin | 160.17 | 2.0 | 0.6 | 1.5 | 0.9 | 1.1 | 0.6 | 3.1 | 0.5 | 0.0 | 0.0 | 2.2 | 0.1 | 0.0 | 0.0 | 0.1 | 0.2 | 2.9 | 0.1 | 1.0 | 0.7 |
| 62 | Methyl salicylate | 152.15 | 1.6 | 0.5 | 1.8 | 0.2 | 0.2 | 0.2 | 3.1 | 0.6 | 0.0 | 0.0 | 1.9 | 0.1 | 0.1 | 0.1 | 0.4 | 0.3 | 3.0 | 0.1 | 0.4 | 0.6 |
| 63 | Chlorobenzene | 112.56 | 1.5 | 0.4 | 0.7 | 0.2 | 0.6 | 0.6 | 2.2 | 0.4 | 0.0 | 0.0 | 2.2 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 3.0 | 0.1 | 0.7 | 0.7 |
| 64 | Lactic acid | 90.08 | 0.2 | 0.2 | 0.2 | 0.4 | 3.4 | 4.9 | 0.3 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.3 | 0.5 | 0.2 | 0.0 | 0.1 | 2.8 | 0.6 |
| 65 | 1-Bromobutane | 137.02 | 1.5 | 0.1 | 1.6 | 0.4 | 0.3 | 0.5 | 2.6 | 0.3 | 0.1 | 0.1 | 2.2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 1.9 | 1.7 | 2.4 | 1.6 |
| 66 | 2-Acethylcyclohexanone | 140.18 | 1.7 | 0.3 | 0.9 | 0.1 | 5.3 | 0.3 | 11.9 | 0.4 | 1.3 | 0.6 | 3.1 | 0.0 | 1.2 | 0.8 | 0.1 | 0.1 | 2.8 | 1.5 | 0.3 | 0.6 |
| 67 | 4'-Methoxyacetophenone | 150.17 | 0.1 | 0.1 | 2.2 | 0.3 | 2.8 | 0.2 | 5.7 Co | 0.6 | 3.3 | 0.4 | 0.0 | 0.1 | 2.8 | 1.3 | 1.4 | 0.3 | 3.1 | 0.0 | 3.3 | 0.6 |
| 68 | Ethyl benzoylacetate | 192.21 | 2.9 | 0.4 | 4.7 | 0.5 | 5.4 | 0.5 | 10.2 | 0.3 | 4.4 | 0.3 | 0.4 | 0.3 | 1.0 | 0.3 | 1.6 | 0.1 | 4.5 | 0.2 | 5.3 | 1.3 |
| 69 | Ethyl vanillin | 166.17 | 0.9 | 0.2 | 2.5 | 1.2 | 3.2 | 1.0 | 5.4 | 0.4 | 1.4 | 0.3 | 0.0 | 0.0 | 3.1 | 0.6 | 0.8 | 0.1 | 2.6 | 0.1 | 2.1 | 0.8 |
| 70 | Isopropanol | 60.1 | 0.0 | 0.0 | 0.5 | 0.9 | 0.6 | 1.0 | 0.7 | 0.7 | 0.1 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 1.5 | 0.4 | 1.1 | 0.2 | 2.8 | 0.6 |
| 71 | Propylene glycol | 76.09 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.4 | 0.0 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 0.0 | 0.0 | 0.7 | 0.3 | 1.0 | 0.2 | 2.2 | 0.7 |
| 72 | Sulfanilamide | 172.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.5 | 2.7 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 2.9 | 0.4 | 0.9 | 0.1 | 3.2 | 0.4 | 1.4 | 0.7 |
| 73 | Isopropyl myristate | 270.45 | 0.0 | 0.0 | 0.1 | 0.2 | 0.6 | 0.6 | 2.4 | 0.3 | 0.0 | 0.0 | 0.6 | 0.3 | 2.6 | 0.5 | 0.7 | 0.3 | 2.8 | 0.2 | 1.8 | 1.4 |
| 74 | Benzaldehyde | 106.12 | 9.9 | 0.9 | 20.1 | 0.2 | 51.5 | 2.7 | 100.0 | 0.0 | 25.3 | 1.0 | 2.2 | 0.0 | 4.1 | 0.2 | 10.6 | 0.5 | 13.7 | 0.1 | 2.9 | 1.0 |
| 75 | Methylparaben | 152.15 | 0.1 | 0.1 | 0.4 | 0.7 | 0.3 | 0.5 | 2.5 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 2.5 | 0.8 | 0.2 | 0.1 | 2.1 | 0.1 | 1.3 | 0.5 |
| 76 | Nonanoic acid | 158.24 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 1.7 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 3.4 | 0.5 | 0.3 | 0.2 | 2.3 | 0.1 | 1.3 | 0.7 |
| 77 | Propyl paraben | 180.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.8 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 2.0 | 0.3 | 0.0 | 0.0 | 2.0 | 0.1 | 1.9 | 3.1 |
| 78 | Salicylic acid | 138.12 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.9 | 0.5 | 0.0 | 0.0 | 0.1 | 0.1 | 2.3 | 0.3 | 0.2 | 0.2 | 3.1 | 0.3 | 0.4 | 0.6 |
| 79 | Sulphanilic acid | 173.19 | 0.4 | 0.6 | 0.2 | 0.2 | 0.2 | 0.3 | 0.8 | 0.8 | 0.5 | 0.4 | 0.5 | 0.1 | 0.4 | 0.3 | 1.3 | 0.3 | 0.0 | 0.0 | 0.4 | 0.2 |
| 80 | Vanillin | 152.15 | 0.9 | 0.7 | 3.1 | 0.3 | 5.5 | 1.0 | 4.6 | 0.5 | 1.5 | 0.3 | 0.5 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 3.0 | 0.3 | 1.9 | 1.5 |
| 81 | Coumarin | 146.14 | 0.2 | 0.3 | 0.5 | 0.3 | 0.0 | 0.0 | 1.2 | 0.5 | 0.5 | 0.5 | 1.3 | 0.7 | 0.2 | 0.3 | 0.0 | 0.0 | 1.5 | 0.1 | 3.9 | 2.4 |
| 82 | Vinylidene dichloride | 96.94 | 0.2 | 0.4 | 0.2 | 0.1 | 0.0 | 0.0 | 0.9 | 0.4 | 0.9 | 0.4 | 0.7 | 0.1 | 0.4 | 0.3 | 0.0 | 0.0 | 2.4 | 0.2 | -0.3 | 0.7 |

Co : Co-elution

**Table 3**
LLNA categories, ADRA results at each of five concentrations, and DRPA results.

| № | Test substance | EC3 value[e] | LLNA category[e] | Human data[f] | ADRA | | | | | | | | | | DPRA[h] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0.05 mg/mL | | 0.1 mg/mL | | 0.2 mg/mL | | 0.5 mg/mL | | 1 mM[g] | | |
| 1 | Diphenylcyclo propenone | 0.0003 | Extreme | S | NS[b] | (3.6)[d] | S | (9.799) | S | (15.97) | S | (28.92) | S | (17.5) | S |
| 2 | Oxazolone | 0.003 | Extreme | S | S[c] | (47.6) | S | (64.8) | S | (75.65) | S | (81.97) | S | (82.7) | S |
| 3 | Benzoyl peroxide | 0.004 | Extreme | S | S | (75.2) | S | (77.4) | S | (79.98) | S | (88.08) | S | (75.3) | S |
| 4 | Kathon CG | 0.008 | Extreme | S | S | (50.0) | S | (50.0) | S | (50) | S | (49.8) | S | (49.8) | S |
| 5 | Bandrowski's base | 0.008 | Extreme | – | S | (42.0) | S | (49.7) | S | (51.7) | S | (57.35) | S | (52.9) | S |
| 6 | 5-Chloro-2-methyl-4-isothiazolin-3-one | 0.009 | Extreme | – | S | (50.0) | S | (50.9) | S | (56.85) | S | (50) | S | (58.8) | S |
| 7 | p-Benzoquinone | 0.0099 | Extreme | S | S | (80.2) | S | (86.6) | S | (91.16) | S | (94.07) | S | (90.0) | S |
| 8 | Tetrachlorosalicylanilide | 0.04 | Extreme | S | S | (15.8) | S | (17.9) | S | (18.74) | S | (38.92) | S | (22.7) | S |
| 9 | 2,4-Dinitrochlorobenzene | 0.05 | Extreme | S | S | (20.7) | S | (34.2) | S | (47.46) | S | (53.16) | S | (47.7) | S |
| 10 | Glutaraldehyde | 0.1 | Strong | S | S | (21.2) | S | (17.8) | S | (30) | S | (55.16) | S | (26.9) | S |
| 11 | Fluorescein isothiocyanate | 0.14 | Strong | – | S | (47.2) | S | (66.1) | S | (83.73) | S | (91.18) | S | (85.8) | S |
| 12 | Phthalic anhydride | 0.16 | Strong | NS | S | (40.2) | S | (43.1) | S | (44.85) | S | (52.62) | S | (48.6) | S |
| 13 | Lauryl gallate | 0.3 | Strong | S | S | (70.1) | S | (83.7) | S | (92.89) | S | (97.51) | S | (59.5) | S |
| 14 | Propyl gallate | 0.32 | Strong | S | S | (61.7) | S | (71.6) | S | (82.32) | S | (81.13) | S | (76.7) | S |
| 15 | CD3 | 0.6 | Strong | – | S | (48.4) | S | (50.8) | S | (53.93) | S | (55.35) | S | (46.6) | S |
| 16 | Trimellitic anhydride | 0.6 | Strong | – | S | (35.1) | S | (43.6) | S | (49.14) | S | (50.96) | S | (49.4) | S |
| 17 | Formaldehyde | 0.61 | Strong | S | S | (12.9) | S | (26.1) | S | (33.92) | S | (42.89) | S | (13.6) | S |
| 18 | Metol | 0.8 | Strong | S | S | (54.3) | S | (56.1) | S | (58.69) | S | (61.1) | S | (61.4) | S |
| 19 | 2-Hydroxyethyl acrylate | 1.4 | Moderate | S | S | (49.8) | S | (53.6) | S | (56.3) | S | (61.7) | S | (58.1) | S |
| 20 | Glyoxal | 1.4 | Moderate | S | S | (5.9) | S | (9.5) | S | (10.4) | S | (17.9) | S | (6.8) | S |
| 21 | Vinyl pyridine | 1.6 | Moderate | – | S | (7.0) | S | (13.9) | S | (25.7) | S | (40.8) | S | (12.9) | S |
| 22 | 2-Mercaptobenzothiazole | 1.7 | Moderate | S | S | (16.3) | S | (25.6) | S | (42.2) | S | (53.4) | S | (28.4) | S |
| 23 | Nonanoyl chloride | 1.8 | Moderate | – | S | (6.3) | S | (14.8) | S | (28.3) | S | (37.7) | S | (23.4) | NS |
| 24 | 2-Methyl-2H-isothiazol-3-one | 1.9 | Moderate | S | S | (48.9) | S | (45.1) | S | (46.3) | S | (40.9) | S | (50.5) | S |
| 25 | 1,2-Benzisothiazoline-3-one | 2.3 | Moderate | S | S | (50.3) | S | (50.3) | S | (51.2) | S | (53.9) | S | (50.2) | S |
| 26 | Methyl-2-nonynoate | 2.5 | Moderate | S | NS | (1.4) | NS | (3.1) | S | (9.6) | S | (21.5) | S | (8.2) | S |
| 27 | Cinnamaldehyde | 3 | Moderate | – | NS | (3.5) | S | (14.9) | S | (22.8) | S | (43.6) | S | (25.0) | S |
| 28 | Phenylacetaldehyde | 3 | Moderate | S | S | (19.0) | S | (45.2) | S | (57.5) | S | (64.0) | S | (60.3) | S |
| 29 | Benzylideneacetone | 3.7 | Moderate | S | S | (5.4) | S | (7.3) | S | (10.8) | S | (20.5) | S | (26.1) | S |
| 30 | 2,4-Heptadienal | 4 | Moderate | – | S | (11.8) | S | (28.9) | S | (31.4) | S | (45.7) | S | (42.9) | S |
| 31 | Squaric acid | 4.3 | Moderate | S | NS | (1.5) | NS | (2.8) | NS | (2.1) | NS | (1.6) | NS | (0.6) | S |
| 32 | Trans-2-hexenal | 5.5 | Moderate | S | S | (27.0) | S | (38.2) | S | (47.6) | S | (59.3) | S | (46.3) | S |
| 33 | Resorcinol | 5.5 | Moderate | S | NS | (2.7) | NS | (3.2) | S | (5.9) | S | (6.9) | NS | (3.1) | NS |
| 34 | Diethyl maleate | 5.8 | Moderate | S | S | (17.1) | S | (13.0) | S | (39.5) | S | (49.7) | S | (19.8) | S |
| 35 | 2-phenylpropionaldehyde | 6.3 | Moderate | S | NS | (4.1) | S | (9.3) | S | (11.9) | S | (23.1) | S | (6.3) | S |
| 36 | Perillaldehyde | 8.1 | Moderate | S | S | (6.2) | S | (16.8) | S | (29.1) | S | (49.6) | S | (23.8) | S |
| 37 | Palmitoyl Chloride | 8.8 | Moderate | – | S | (42.5) | S | (53.3) | S | (50.8) | S | (59.8) | S | (50.8) | S |
| 38 | 1-(4-Methoxyphenyl)-1-penten-3-one | 9.3 | Moderate | – | NS | (1.2) | NS | (1.7) | NS | (1.5) | S | (9.0) | NS | (2.9) | S |
| 39 | α-Hexylcinnnamaldehyde | 11 | Weak | S/NS | NS | (0.2) | NS | (2.5) | NS | (2.4) | NS | (4.3) | NS | (0.6) | NS |
| 40 | α-Amylcinnamaldehyde | 11 | Weak | S/NS | NS | (0.0) | NS | (2.3) | NS | (1.7) | NS | (3.4) | NS | (0.8) | NS |
| 41 | 2,3-Butanedione | 11 | Weak | – | S | (10.0) | S | (21.0) | S | (50.3) | S | (64.8) | S | (33.4) | S |
| 42 | Farnesal | 12 | Weak | – | NS | (3.5) | S | (12.8) | S | (16.1) | S | (14.7) | S | (13.0) | S |
| 43 | Oxalic acid | 15 | Weak | – | NS | (0.5) | NS | (0.3) | NS | (0.7) | NS | (0.8) | NS | (1.8) | NS |
| 44 | Benzyl benzoate | 17 | Weak | NS | NS | (0.3) | NS | (0.5) | NS | (0.6) | NS | (3.7) | NS | (1.4) | NS |
| 45 | 4-Allylanisole | 18 | Weak | – | NS | (4.2) | S | (11.1) | S | (21.3) | S | (32.0) | S | (15.4) | S |
| 46 | Lilial | 19 | Weak | S | NS | (2.9) | NS | (4.2) | S | (5.5) | S | (10.8) | S | (5.4) | S |
| 47 | Cyclamen aldehyde | 22 | Weak | – | NS | (0.9) | NS | (0.6) | NS | (1.4) | S | (8.6) | NS | (2.4) | S |
| 48 | Imidazolidinyl urea | 24 | Weak | S | NS | (2.4) | S | (5.7) | S | (10.2) | S | (19.7) | S | (18.7) | S |
| 49 | 5-Methyl-2,3-hexanedione | 26 | Weak | S | NS | (3.9) | S | (8.9) | S | (18.8) | S | (42.7) | S | (20.6) | S |
| 50 | 2,2,6,6-Tetramethyl-3,5-heptanedione | 27 | Weak | – | NS | (0.4) | NS | (0.0) | NS | (0.1) | NS | (3.9) | NS | (1.1) | NS |
| 51 | Ethyleneglycol dimethacrylate | 28 | Weak | S | NS | (0.9) | NS | (1.6) | NS | (4.1) | S | (12.5) | NS | (4.3) | S |
| 52 | Ethyl acrylate | 28 | Weak | S | S | (37.8) | S | (51.2) | S | (56.0) | S | (65.1) | S | (50.8) | S |
| 53 | Hydroxycitronellal | 33 | Weak | S | NS | (1.0) | NS | (4.7) | S | (8.7) | S | (17.4) | S | (9.5) | S |
| 54 | Glycerol | NC[a] | NS[b] | NS | NS | (0.5) | NS | (0.1) | NS | (0.2) | NS | (0.6) | NS | (0.5) | NS |
| 55 | Hexane | NC | NS | NS | NS | (2.0) | NS | (1.5) | NS | (0.2) | NS | (3.7) | NS | (0.5) | NS |
| 56 | Diethyl phthalate | NC | NS | NS | NS | (2.3) | NS | (1.1) | NS | (0.6) | NS | (4.1) | NS | (0.9) | NS |
| 57 | Octanoic acid | NC | NS | NS | NS | (2.1) | NS | (0.7) | NS | (0.7) | NS | (3.6) | NS | (0.6) | NS |
| 58 | 2-Hydroxypropyl methacrylate | NC | NS | – | NS | (2.2) | NS | (3.2) | S | (5.364) | S | (12.56) | NS | (2.3) | S |
| 59 | 1-Butanol | NC | NS | NS | NS | (0.8) | NS | (0.5) | NS | (0.9) | NS | (0.8) | NS | (0.5) | NS |
| 60 | 4-Hydroxybenzoic acid | NC | NS | NS | NS | (2.0) | NS | (2.0) | NS | (0.972) | NS | (3.267) | NS | (-0.7) | NS |
| 61 | 6-Methyl coumatrin | NC | NS | – | NS | (2.1) | NS | (0.7) | NS | (0.626) | NS | (3.033) | NS | (0.5) | NS |
| 62 | Methyl salicylate | NC | NS | NS | NS | (1.8) | NS | (0.9) | NS | (0.263) | NS | (3.058) | NS | (0.2) | NS |
| 63 | Chlorobenzene | NC | NS | – | NS | (1.9) | NS | (0.4) | NS | (0.301) | NS | (2.57) | NS | (0.4) | NS |
| 64 | Lactic acid | NC | NS | NS | NS | (0.1) | NS | (0.5) | NS | (1.975) | NS | (0.185) | NS | (1.4) | NS |
| 65 | 1-Bromobutane | NC | NS | – | NS | (1.8) | NS | (0.9) | NS | (0.199) | NS | (2.258) | NS | (1.2) | S |
| 66 | 2-Acethylcyclohexanone | NC | NS | – | NS | (2.4) | NS | (1.0) | NS | (2.66) | S | (7.358) | NS | (0.8) | S |
| 67 | 4'-Methoxyacetophenone | NC | NS | NS | NS | (0.1) | NS | (2.5) | NS | (2.082) | NS | (4.375) | NS | (3.3) | NS |
| 68 | Ethyl benzoylacetate | NC | NS | – | NS | (1.6) | NS | (2.8) | NS | (3.465) | S | (7.393) | NS | (4.9) | NS |
| 69 | Ethyl vanillin | NC | NS | – | NS | (0.4) | NS | (2.8) | NS | (1.992) | NS | (4.007) | NS | (1.7) | NS |
| 70 | Isopropanol | NC | NS | NS | NS | (0.0) | NS | (0.3) | NS | (1.04) | NS | (0.888) | NS | (1.5) | NS |
| 71 | Propylene glycol | NC | NS | NS | NS | (0.1) | NS | (0.0) | NS | (0.63) | NS | (0.528) | NS | (1.1) | NS |

(*continued on next page*)

**Table 3** (*continued*)

| № | Test substance | EC3 value[e] | LLNA category[e] | Human data[f] | ADRA | | | | | | | | | | DPRA[h] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0.05 mg/mL | | 0.1 mg/mL | | 0.2 mg/mL | | 0.5 mg/mL | | 1 mM[g] | | |
| 72 | Sulfanilamide | NC | NS | NS | NS | (0.0) | NS | (1.4) | NS | (0.812) | NS | (2.955) | NS | (0.7) | NS |
| 73 | Isopropyl myristate | NC | NS | NS | NS | (0.3) | NS | (1.4) | NS | (0.639) | NS | (2.585) | NS | (0.9) | NS |
| 74 | Benzaldehyde | NC | NS | S | S | (6.0) | S | (12.1) | S | (31.0) | S | (56.8) | S | (14.1) | NS |
| 75 | Methylparaben | NC | NS | – | NS | (0.1) | NS | (1.5) | NS | (0.3) | NS | (2.3) | NS | (0.7) | NS |
| 76 | Nonanoic acid | 21 (False +) | NS | – | NS | (0.1) | NS | (1.7) | NS | (0.2) | NS | (2.0) | NS | (0.6) | NS |
| 77 | Propyl paraben | NC | NS | NS | NS | (0.1) | NS | (1.0) | NS | (0.0) | NS | (1.9) | NS | (1.0) | NS |
| 78 | Salicylic acid | NC | NS | NS | NS | (0.1) | NS | (1.2) | NS | (0.1) | NS | (2.5) | NS | (0.2) | – |
| 79 | Sulphanilic acid | NC | NS | – | NS | (0.4) | NS | (0.3) | NS | (0.748) | NS | (0.387) | NS | (0.5) | NS |
| 80 | Vanillin | NC | NS | – | NS | (0.7) | NS | (1.6) | NS | (2.734) | NS | (3.777) | NS | (1.7) | NS |
| 81 | Coumarin | NC | NS | S | NS | (0.8) | NS | (0.3) | NS | (0) | NS | (1.307) | NS | (2.2) | NS |
| 82 | Vinylidene dichloride | NC | NS | – | NS | (0.5) | NS | (0.3) | NS | (0.009) | NS | (1.64) | NS | (0.3) | NS |

"-": No data.
[a] Not calculated.
[b] Non-sensitizer.
[c] Sensitizer.
[d] Mean depletion of NAC and NAL shown in parentheses.
[e] Fujita et al. (2019).
[f] Urbisch et al. (2015).
[g] Result judged from depletion of NAC and NAL (Fujita et al., 2019).
[h] Result judged from depletion of Cys peptide (cysteine peptide) and Lys peptide (lysine peptide) (Gerberick et al., 2007).

### 3.1. Reactivity with NAC and NAL at each of four concentrations

Table 2 shows NAC and NAL depletion values obtained from test chemical solutions prepared at four different weight concentrations for 82 test chemicals. Depletion of NAC and NAL by chemical solutions prepared at 0.5, 0.2, 0.1, and 0.05 mg/mL was color-coded to represent increases and decreases in comparison to depletion by the chemical solution prepared at 1 mM. Dark blue indicates increased more than 20%, light blue indicates increased by more than 10%, light red indicates decreased by more than 10%, and dark red indicates decreased by more than 20%. Depletion of NAC was weight concentration-dependent for sensitizers, with a greater number of red frames for higher weight concentrations, indicating greater depletion than the 1 mM solution, and higher number of blue frames at lower concentrations, indicating less depletion than the 1 mM solution. In contrast, several non-sensitizers exhibited greater depletion than the 1 mM solution at high weight concentrations, but only one chemical exhibited lower depletion. NAL depletion results were similar to those of NAC but smaller in effect size. The number of chemicals that exhibited high NAL depletion was lower than that of NAC, but the number of chemicals that exhibited less depletion was as the same as for NAC.

### 3.2. Prediction results and predictive capacity at four different concentrations

Table 3 shows prediction results obtained from test chemical solutions prepared at four different weight concentrations of each of the 82 test chemicals evaluated using ADRA.

Nine sensitizers (methyl-2-nonynoate, cinnamaldehyde, 2-phenylpropionaldehyde, farnesal, 4-allylanisol, lilial, imidazolidinylurea, 5-methyl-2,3-hexanedione, and hydroxycitronellal) were correctly identified as sensitizers at concentrations of 1 mM in solution but were incorrectly identified as non-sensitizing at 0.1 or 0.05 mg/mL. Three non-sensitizers (2-hydroxypropyl methacrylate, 2-acethylcyclohexanone and ethyl benzoylacetate) were incorrectly identified as sensitizers at 0.5 or 0.2 mg/mL. In contrast, sensitizers (resorcinol,1-(4-Methoxyphenyl)-1-penten-3-one, cyclamen aldehyde, and ethyleneglycol dimethacrylate) were incorrectly identified as non-sensitizers at a concentration of 1 mM in solution. However, when prepared at 0.5 or 0.2 mg/mL, each was correctly identified as a sensitizer.

### 3.3. Predictive capacity of each of four different concentrations of test chemical solutions

Predictivity determined by ADRA toward LLNA was based on accuracy, sensitivity, and specificity. Accuracy and sensitivity of the 0.5 and 0.2 mg/mL test solutions were equal to or better than the 86.6% and 81.1% values obtained for accuracy and sensitivity, respectively, of the 1 mM solution, but specificity was lower. Accuracy and sensitivity of the 0.1 and 0.05 mg/mL were lower than those observed for the 1 mM solution, and specificity of 0.1 and 0.05 mg/mL solutions was not different (96.6%) from that of the 1 mM solution. Detailed comparisons are summarized in Table 4.1.

Predictivity of ADRA toward human sensitivity was based on accuracy, sensitivity, and specificity. Accuracy, sensitivity, and specificity of 0.5 and 0.2 mg/mL solutions were equal to or higher than the 90.2%, 88.2%, and 94.1% accuracy, sensitivity, and specificity values obtained using the 1 mM solution. Accuracy and sensitivity of 0.1 and 0.05 mg/mL solutions exhibited decreased accuracy and sensitivity, but similar specificity, compared to the 1 mM solution. Detailed comparisons are summarized in Table 4.2.

### 3.4. Detection of sensitizers in liquid mixtures of non-sensitizers

We prepared 0.25 mg/mL solutions of ten different non-sensitizers (diethyl phthalate, 4-hydroxybenzoic acid, methyl salicylate, chlorobenzene, sulfanilamide, isopropyl myristate, methylparaben, propyl paraben, salicylic acid, and coumarin) to verify whether sensitizers can be detected in mixtures. This mixture was not reactive with NAC and NAL, consistent with being characterized as non-sensitizing. We added NAC and NAL to the mixtures and performed HPLC analysis to verify that NAC and NAL did not coelute with the chemicals in the mixture (Fig. 1). We then prepared pseudobinary mixtures by adding one each of ten different sensitizers with varying sensitization potencies and evaluated these mixtures using ADRA (Fig. 2). These results were compared to past results in which the sensitizers were tested individually. The sensitizers and non-sensitizers used in this experiment were selected based on results of the previous weight concentration experiments so that three different levels of LLNA sensitization potency (extreme/strong, n = 3; moderate, n = 4, and weak, n = 3) were represented. Test chemical solutions of these sensitizers were prepared at 0.05, 0.1, 0.2, and 0.5 mg/mL.

**Table 4.1**
ADRA predictive capacity at each of five concentrations, and DPRA results for 82 chemicals.

| | | ADRA | | | | | | | | | | | | | | DPRA[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.05 mg/mL | | | 0.1 mg/mL | | | 0.2 mg/mL | | | 0.5 mg/mL | | | 1 mM[b] | | | 100 nM | | |
| | | S | NS | Total | S | NS | Total | S | NS | Total | S | NS | Total | S | NS | Total | S | NS | Total |
| Chemical classification[a] | S | 33 | 20 | 53 | 40 | 13 | 53 | 44 | 9 | 53 | 47 | 6 | 53 | 43 | 10 | 53 | 46 | 7 | 53 |
| | NS | 1 | 28 | 29 | 1 | 28 | 29 | 2 | 27 | 29 | 4 | 25 | 29 | 1 | 28 | 29 | 3 | 25 | 28 |
| | total | 34 | 48 | 82 | 41 | 41 | 82 | 46 | 36 | 82 | 51 | 31 | 82 | 44 | 38 | 82 | 49 | 32 | 81 |
| | | Sensitivity: | | 62.3% | Sensitivity: | | 75.5% | Sensitivity: | | 83.0% | Sensitivity: | | 88.7% | Sensitivity: | | 81.1% | Sensitivity: | | 86.8% |
| | | Specificity: | | 96.6% | Specificity: | | 96.6% | Specificity: | | 93.1% | Specificity: | | 86.2% | Specificity: | | 96.6% | Specificity: | | 89.3% |
| | | Positive predictivity: | | 97.1% | Positive predictivity: | | 97.6% | Positive predictivity: | | 95.7% | Positive predictivity: | | 92.2% | Positive predictivity: | | 97.7% | Positive predictivity: | | 93.9% |
| | | Negative predictivity: | | 58.3% | Negative predictivity: | | 68.3% | Negative predictivity: | | 75.0% | Negative predictivity: | | 80.6% | Negative predictivity: | | 73.7% | Negative predictivity: | | 78.1% |
| | | Accuracy: | | 74.4% | Accuracy: | | 82.9% | Accuracy: | | 86.6% | Accuracy: | | 87.8% | Accuracy: | | 86.6% | Accuracy: | | 87.7% |

[a] Based primarily on LLNA data S: Sensitizer, NS: Non-sensitizer.
[b] Based on depletion of NAC and NAL (Fujita et al., 2019).
[c] Based on depletion of cysteine peptide and lysine peptide (Gerberick et al., 2007).



**Table 4.2**
Comparision of predictive capacity of 51 chemicals for 0.05, 0.1, 0.2, and 0.5 mg/mL of test chemical solution and Potency Data (vs human data).

| | | ADRA | | | | | | | | | | | | | | DPRA[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.05 mg/mL | | | 0.1 mg/mL | | | 0.2 mg/mL | | | 0.5 mg/mL | | | 1 mM[b] | | | | | |
| | | S | NS | total | S | NS | Total | S | NS | Total | S | NS | Total | S | NS | Total | S | NS | Total |
| Chemical classification[a] | S | 23 | 11 | 34 | 27 | 7 | 34 | 31 | 3 | 34 | 32 | 2 | 34 | 30 | 4 | 34 | 31 | 3 | 34 |
| | NS | 1 | 16 | 17 | 1 | 16 | 17 | 1 | 16 | 17 | 1 | 16 | 17 | 1 | 16 | 17 | 1 | 16 | 17 |
| | total | 24 | 27 | 51 | 28 | 23 | 51 | 32 | 19 | 51 | 33 | 18 | 51 | 31 | 20 | 51 | 32 | 19 | 51 |
| | | Sensitivity: | | 67.6% | Sensitivity: | | 79.4% | Sensitivity: | | 91.2% | Sensitivity: | | 94.1% | Sensitivity: | | 88.2% | Sensitivity: | | 91.2% |
| | | Specificity: | | 94.1% | Specificity: | | 94.1% | Specificity: | | 94.1% | Specificity: | | 94.1% | Specificity: | | 94.1% | Specificity: | | 94.1% |
| | | Positive predictivity: | | 95.8% | Positive predictivity: | | 96.4% | Positive predictivity: | | 96.9% | Positive predictivity: | | 97.0% | Positive predictivity: | | 96.8% | Positive predictivity: | | 96.9% |
| | | Negative predictivity: | | 59.3% | Negative predictivity: | | 69.6% | Negative predictivity: | | 84.2% | Negative predictivity: | | 88.9% | Negative predictivity: | | 80.0% | Negative predictivity: | | 84.2% |
| | | Accuracy: | | 76.5% | Accuracy: | | 84.3% | Accuracy: | | 92.2% | Accuracy: | | 94.1% | Accuracy: | | 90.2% | Accuracy: | | 92.2% |

[a] Based primarily on human data
[b] Result judged from depletion of NAC and NAL (Fujita et al., 2019).
[c] Result judged from depletion of Cys peptide (cysteine peptide) and Lys peptide (lysine peptide) (Gerberick et al., 2007).

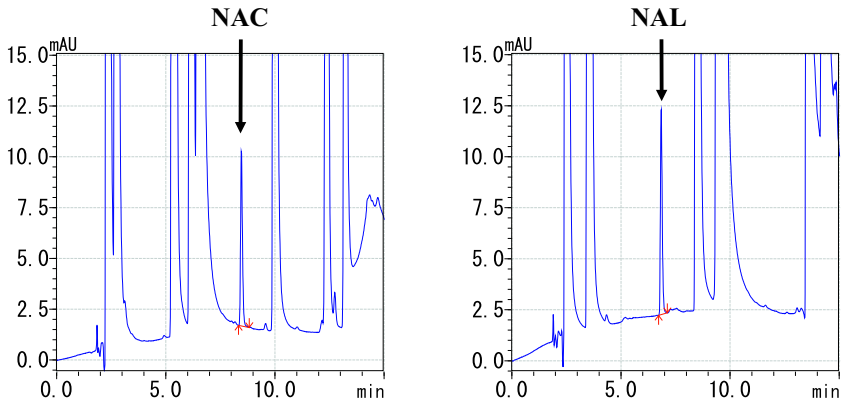


**Fig. 1.** HPLC chromatograms of NAC and NAL in a mixture comprising 10 non-sensitizers.
A mixture of 5 μM of either NAC or NAL and 0.25 mg/mL each of 10 non-sensitizers was analyzed by HPLC after incubation for 24 h at 25 °C. NAC was detected at about 8.5 min and NAL was detected at about 7 min.

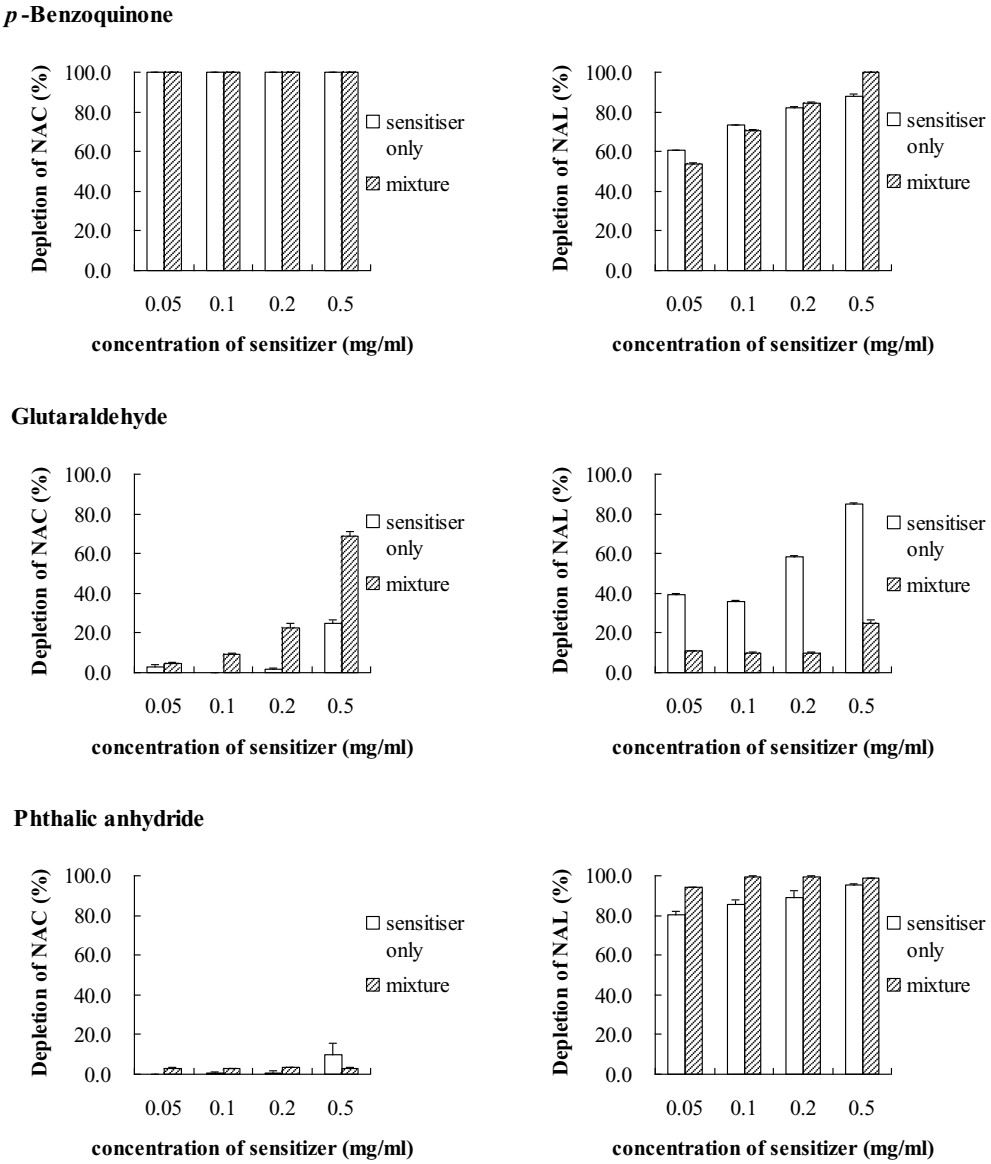


**Fig. 2.** Comparison of NAC and NAL depletion values in ADRA when sensitizers of various sensitization potencies are added to a liquid mixture.
This figure shows NAC and NAL depletion values for ten different sensitizers at weight concentrations of 0.05, 0.1, 0.2, and 0.5 mg/mL when measured individually as well as when added to a liquid mixture comprising 10 different types of non-sensitizers. Cells containing results for an individual sensitizer are not highlighted. Cells containing results for mixtures containing a sensitizer are highlighted.

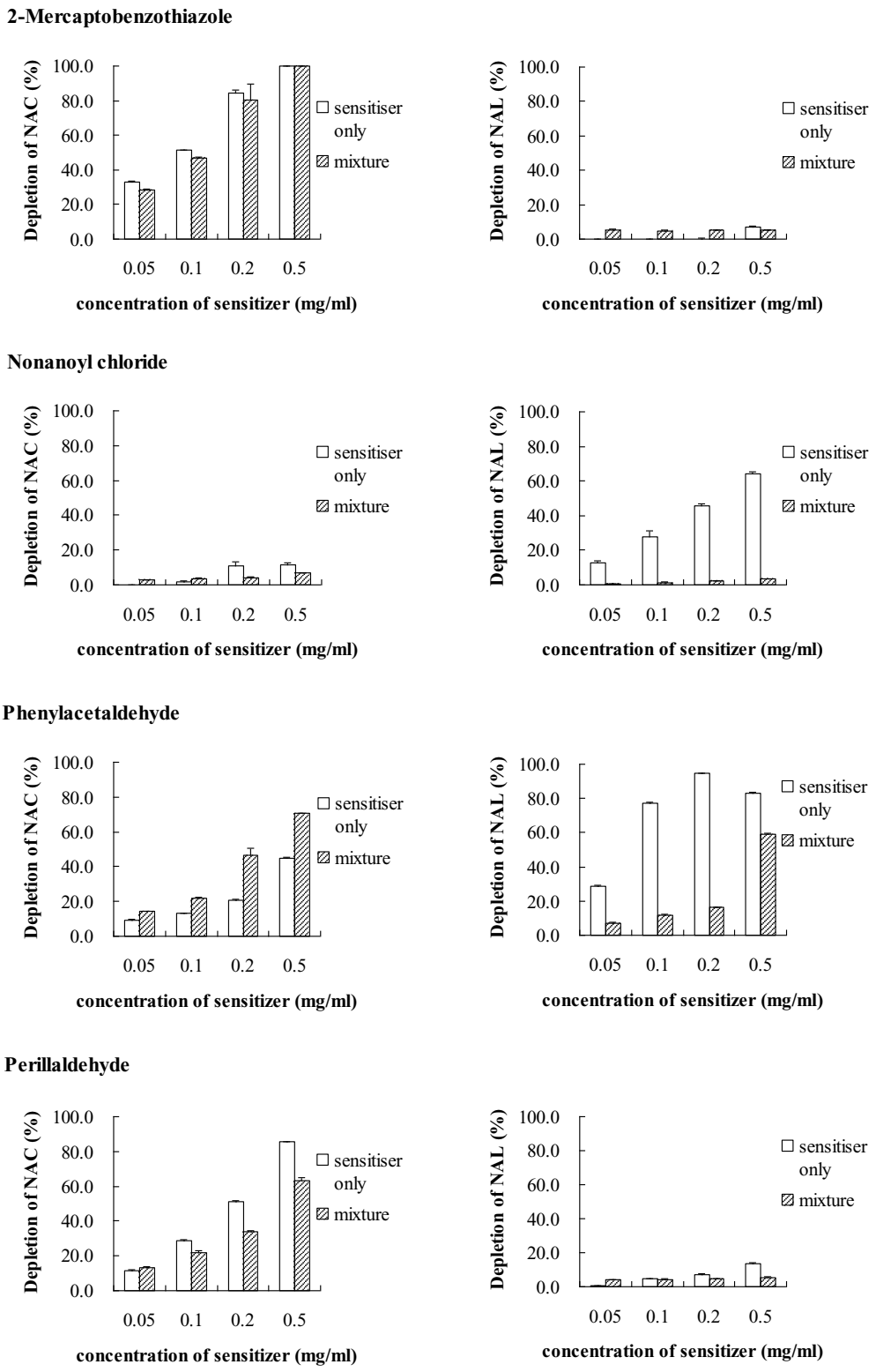


**Fig. 2.** (*continued*)

One sensitizer, perillaldehyde at 0.5 and 0.2 mg/mL, exhibited a 15% or greater decrease in NAC depletion compared to the sensitizer tested individually. Five sensitizers (nonanoyl chloride at 0.1, 0.2 and 0.5 mg/mL; phenylacetaldehyde at 0.05, 0.1, 0.2, and 0.5 mg/mL; glutaraldehyde at 0.1, 0.2, and 0.5 mg/mL; 2,3-butanedione at 0.2 and 0.5 mg/mL; and 5-methyl-2,3-hexanedione at 0.1, 0.2, and 0.5 mg/mL) exhibited 15% or more decreases in NAL depletion compared to the sensitizers tested individually.

Prediction results for sensitizers in pseudobinary mixtures are show in Table 6. Prediction results from testing sensitizers individually and in pseudobinary mixtures at 0.5 mg/mL correctly identified each of the ten test chemicals as sensitizers using both methods. However, at 0.2

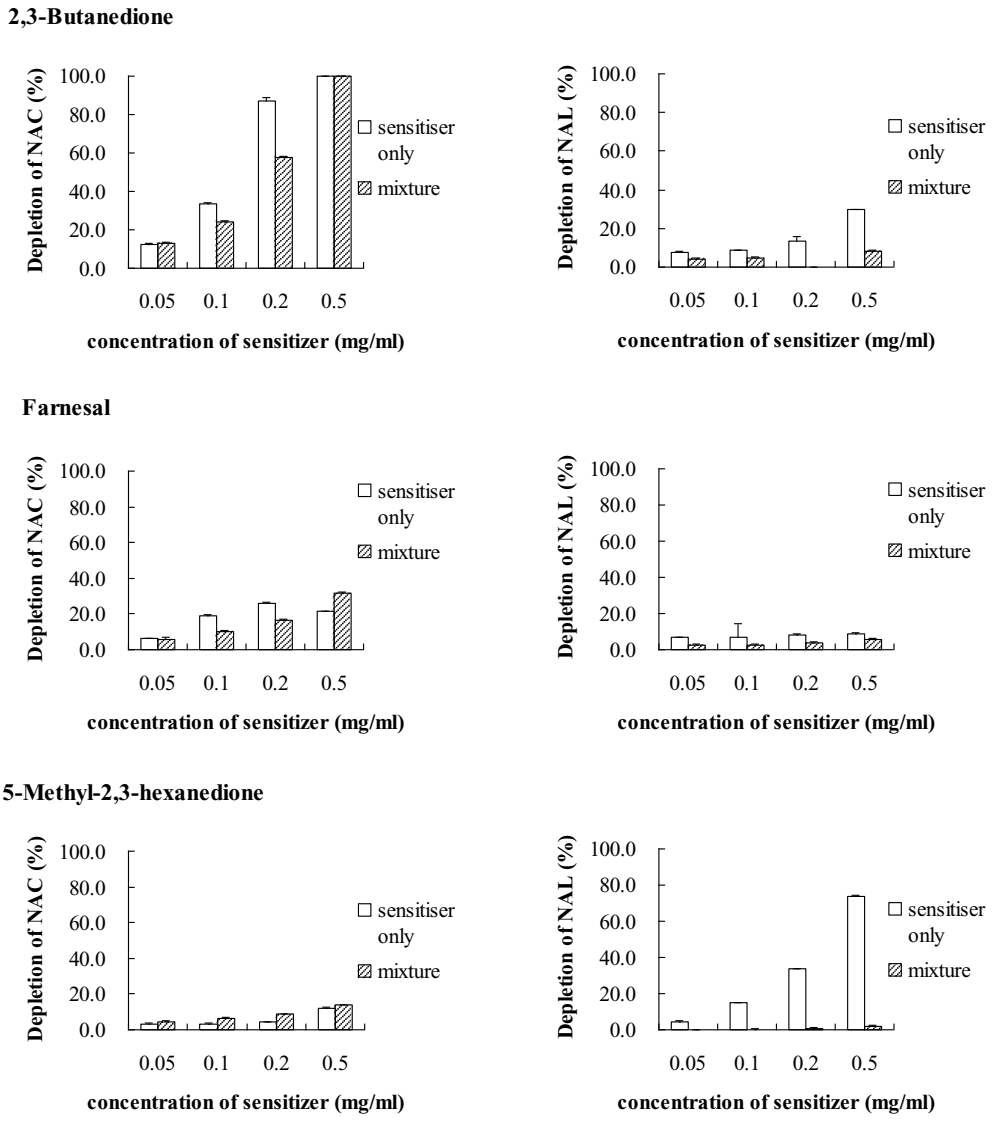


**Fig. 2.** (*continued*)

**Table 5**
LLNA sensitization potency and ADRA predictive capacity at each of five concentrations.

| LLNA category | Number of chemicals | Predictive capacity (%) (ADRA vs LLNA) | | | | |
|---|---|---|---|---|---|---|
| | | 0.05 mg/mL | 0.1 mg/mL | 0.2 mg/mL | 0.5 mg/mL | 1 mM[a] |
| Extreme/strong | 18 | 94.4 (17/18) | 100.0 (18/18) | 100.0 (18/18) | 100.0 (18/18) | 100.0 (18/18) |
| Moderate | 20 | 70.0 (14/20) | 80.0 (16/20) | 90.0 (18/20) | 95.0 (19/20) | 85.0 (17/20) |
| Weak | 15 | 13.3 (2/15) | 40.0 (6/15) | 53.3 (8/15) | 66.7 (10/15) | 53.3 (8/15) |
| Non-sensitizer | 29 | 96.6 (28/29) | 96.6 (28/29) | 93.1 (27/29) | 86.2 (25/29) | 96.6 (28/29) |

[a] Based on depletion of NAC and NAL (Fujita et al., 2019).

and 0.1 mg/mL, nonanoyl chloride and 5-methyl-2,3-hexanedione were both incorrectly predicted to be non-sensitizers. Furthermore, at 0.05 mg/mL, farnesal and 5-methyl-2,3-hexanedione were both incorrectly predicted to be non-sensitizers.

Although none of the ten non-sensitizers individually or in pseudobinary mixtures exhibited any co-elution during HPLC analysis, glutaraldehyde co-eluted with NAL at all four weight concentrations. Furthermore, since p-benzoquinone analysis at 0.5 mg/mL had appropriate peaks, but inconsistent baseline throughout all chromatograms, reference values were used for NAC and NAL depletion, as shown in Table 6.

## 4. Discussion

Both DPRA (OECD TG 442C, 2015) and ADRA (Fujita et al., 2014; Yamamoto et al., 2015) use HPLC analysis to measure unreacted levels of peptides or amino acid derivatives, which are used as nucleophilic reagents. Each of these tests require that molar concentrations of the test chemicals and the nucleophilic reagents are at a specific ratio in the reaction solution, which means that the molecular weight of the test chemicals must be known.

The ability to prepare test chemical solutions based on weight concentration would allow expansion of prediction of sensitization

**Table 6**
Prediction results for sensitizers individually and in pseudobinary mixture.

| No | Test substance | EC3 value | LLNA category | Concentration of each sensitizer | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.05 mg/mL | | | 0.1 mg/mL | | | 0.2 mg/mL | | | 0.5 mg/mL | | |
| | | | | Sensitizer + Mixture[a] | | Sensitizer only | Sensitizer + Mixture | | Sensitizer only | Sensitizer + Mixture | | Sensitizer only | Sensitizer + Mixture | | Sensitizer only |
| 7 | p-Benzoquinone | 0.0099 | Extreme | S[b] | (76.7)[d] | S | (80.2) | S | (85.3) | S | (86.6) | S | (92.2) | S | (91.2) | S | (100)[e] | S | (94.1) |
| 10 | Glutaraldehyde | 0.1 | Strong | S | (7.9)[Co] | S | (21.2) | S | (9.7)[Co] | S | (17.8) | S | (16.2)[Co] | S | (30.0) | S | (46.7)[Co] | S | (55.2) |
| 12 | Phthalic anhydride | 0.16 | Strong | S | (48.4) | S | (40.2) | S | (51.0) | S | (43.1) | S | (51.5) | S | (44.9) | S | (50.9) | S | (52.6) |
| 22 | 2-Mercaptobenzothiazole | 1.7 | Moderate | S | (16.7) | S | (16.3) | S | (26.0) | S | (25.6) | S | (42.8) | S | (42.2) | S | (52.6) | S | (53.4) |
| 23 | Nonanoyl chloride | 1.8 | Moderate | NS[c] | (1.5) | S | (6.3) | NS | (2.4) | S | (14.8) | NS | (3.2) | S | (28.3) | S | (5.0) | S | (37.7) |
| 28 | Phenylacetaldehyde | 3 | Moderate | S | (10.6) | S | (26.7) | S | (16.6) | S | (62.9) | S | (31.4) | S | (72.7) | S | (65.0) | S | (59.1) |
| 36 | Perillaldehyde | 8.1 | Moderate | S | (8.6) | S | (6.2) | S | (13.2) | S | (16.8) | S | (19.2) | S | (29.1) | S | (34.4) | S | (49.6) |
| 41 | 2,3-Butanedione | 11 | Weak | S | (8.7) | S | (10.0) | S | (14.6) | S | (21.0) | S | (28.7) | S | (50.3) | S | (54.2) | S | (64.8) |
| 42 | Farnesal | 12 | Weak | NS | (4.1) | NS | (3.5) | S | (6.3) | S | (12.8) | S | (10.0) | S | (16.1) | S | (18.3) | S | (14.7) |
| 49 | 5-Methyl-2,3-hexanedione | 26 | Weak | NS | (2.3) | NS | (3.9) | NS | (3.4) | S | (8.9) | NS | (4.7) | S | (18.8) | S | (7.8) | S | (42.7) |

[a] Mixture of 10 non-sensitizers as described in Section 2.1.
[b] Sensitizer.
[c] Non-sensitizer.
[d] Mean depletion of NAC and NAL shown in parentheses.
[e] Accurate depletion values not obtained due to inconsistent baseline across HPLC charts.
[Co] Co-elution observed in either NAC or NAL, or both.

potential to substances with unknown molecular weights, which would mean that ADRA could be used to test mixtures used in cosmetic ingredients. Therefore, we determined an optimal weight concentration for predictive capacity equivalent to the conventional ADRA test method and verified that this approach was capable of detecting sensitizers contained in liquid mixtures.

The stratum corneum barrier function allows substances with a molecular weight of 500 or less to permeate to subcutaneous tissue (Bos & Meinardi, 2000). Converting this molecular weight from the conventional ADRA test chemical molar concentration of 1 mM results in a weight concentration of 0.5 mg/mL. Using this value as an upper limit, we compared reactivity with NAC and NAL of 82 chemicals prepared at the following four concentrations: 0.5, 0.2 0.1, and 0.05 mg/mL.

Chemicals prepared at 1 mM that were incorrectly identified as non-sensitizers because of depletion values slightly lower than the criteria for being classified as sensitizers tended to be correctly identified as sensitizers when prepared by weight concentration. However, false positive rates increased. We considered chemicals that did not fit this trend.

One millimolar CD3 (MW 271.38; 0.27 mg/mL) resulted in 73.6% NAC depletion. Depletion values for weight concentration preparations were 92.1% for 0.05 mg/mL, 93.9% for 0.1 mg/mL, 94.7% for 0.2 mg/mL, and 97.8% for 0.5 mg/mL. Although the 0.2, 0.1, and 0.05 mg/mL weight concentrations were lower than the 1-mM concentration, the depletion values were higher.

CD3 is a derivative of p-phenylenediamine that exhibits extremely high oxidative reactivity, and reacts with proteins and other compounds and also forms polymers, resulting in a structure with no sensitization potential (Pot, Coenraads, Goebel, & Blömeke, 2015). It is possible that polymerization of the test substance occurred while the test chemical solution or reactivity solution were being prepared or during subsequent incubation, which could reduce the depletion value obtained from testing the 1 mM concentration. In our laboratory, we have obtained NAC depletion values of 95% or higher from tests of 1 mM solutions of CD3 (Data not shown).

One millimolar lauryl gallate (MW 338.44; 0.34 mg/mL) resulted in 19% NAL depletion. The depletion values for weight concentration preparations were 40.3% for 0.05 mg/mL, 67.3% for 0.1 mg/mL, 85.8% for 0.2 mg/mL, and 95.0% for 0.5 mg/mL, all of which were higher than that observed in the 1 mM preparation.

Lauryl gallate is a pre-hapten, which exhibits sensitization potential when oxidized. Acetonitrile was used as a solvent for lauryl gallate in the present study for weight concentration preparations. The solvent used in the previous study evaluating a 1 mM test chemical solution was 5% DMSO/acetonitrile. Since lauryl gallate is highly hydrophobic, with a CLogP value of 6.8, it is unlikely to oxidize in an aqueous reaction solution. Based on this, it much more likely that lauryl gallate would undergo oxidation in acetonitrile rather than 5% DMSO/acetonitrile, likely explaining the differences in results between the 1 mM solution and the weight concentration preparations.

As shown in Table 2, although no coelution with either NAC or NAL was found during evaluation of the conventional 1 mM test chemical solutions, some of the 82 chemicals tested in this study did coelute with NAL or NAC at weight concentrations of 0.5 and 0.2 mg/mL. Fluorescein isothiocyanate and 2,4-heptadienal both coeluted with NAC. In addition, 5-chloro-2-methyl-4-isothiazolin-3-one and phenylacetaldehyde both coeluted with NAL at 0.5 and 0.2 mg/mL, while 2-methyl-2H-isothiazol-3-one coeluted with NAL at 0.5 mg/mL.

2,4-heptadienal, phenylacetaldehyde, and 2-methyl-2H-isothiazol-3-one showed a very small amount of interference at the retention time of NAL in the conventional 1 mM test chemical solution. Greater peaks at the retention time of NAL in these solutions when prepared by weight concentration was likely due to higher absolute amounts of these compounds in the these preparations.

Fluorescein isothiocyanate and 5-chloro-2-methyl-4-isothiazolin-3-one are highly reactive and break down in the reaction solution,

resulting in numerous trace peaks, which may contribute to interference with either NAC or NAL (No data shown).

All of the chemicals that coeluted with NAC or NAL were highly reactive, and even when the test chemical coeluted with either NAC of NAL, the peak area relative to the control was small, resulting in prediction as sensitizers. We anticipate that highly reactive test chemicals will break down in the reaction solution and produce multiple trace peaks, resulted in a high likelihood they will be identified as sensitizers, similar to the five test chemicals that coeluted in this study.

Relative to the 1 mM test chemical preparations, the test chemical solutions for all 82 test chemicals were more highly concentrated at 0.5 mg/mL. Sixty-two of the 82 test chemicals prepared at 0.2 mg/mL were more highly concentrated than the 1 mM preparations. Higher concentrations in these preparations is the likely reason for increased reactivity with NAC. Conversely, only 12 of the 82 test chemicals were more concentrated in the 0.1 mg/mL preparations than the corresponding 1 mM preparations, and only one of the 82 test chemicals was more concentrated in the 0.05 mg/mL preparation, which may explain decreased reactivity with NAC at these concentrations. ADRA is designed for incubation of a molar excess of test chemical relative to NAC and NAL (50:1). Based on this molar excess, we anticipated that small changes in concentrations would not cause significant changes in reactivity. However, we found that reactivity of many of the test chemicals was concentration-dependent in weight concentration preparations. Since the concentration of the test chemicals in the reaction solutions were between 0.0125 and 0.125 mg/mL, and the concentrations of NAC and NAL were roughly 1.5 μg/mL, these extremely low concentrations may have resulted in a low number of collisions between molecules, thereby causing significant changes in reactivity at different test chemical concentrations. The molar ratio of nucleophilic reagents to that of the test chemicals used in ADRA is roughly equivalent to that used in DPRA, but the concentrations of nucleophilic reagents and test chemicals used in ADRA are just 1% of those used in DPRA, which may be why, relative to DPRA, ADRA exhibited fewer collisions between molecules and an overall reduction in reactivity.

Therefore, when the reaction rate of NAC/NAL and test chemicals decreases, variations in depletion may increase. Therefore, the range of dispersion of depletion of 82 chemicals prepared by weight concentration and the average SD value (bracket) were calculated. These values were 0.0 to 2.1 (0.4), 0.0 to 3.8 (0.5), 0.0 to 4.9 (0.6), 0.0 to 6.2 (0.5), and 0.0 to 4.2 (0.5) in 0.1, 0.2, 0.5 mg/mL, and 1 mM solutions, respectively, for NAC. These values were 0.0 to 5.0 (0.4), 0.0 to 7.7 (0.5), 0.0 to 6.7 (0.6), 0.0 to 1.7 (0.3), and 0.0 to 4.7 (1.1) in 0.1, 0.2, 0.5 mg/mL, and 1 mM solutions, respectively, for NAL. Based on these results, the decline in reactivity of chemical substances had no significant effect on the variation in depletion.

Based on these results, test chemical solutions prepared at 0.2 mg/mL yielded similar values for accuracy with similar levels of sensitivity and specificity as those prepared at 1 mM.

The 82 chemicals tested during this study had an average molecular weight of 164.0, and conversion from a weight concentration of 0.2 mg/mL resulted in a molar concentration of 1.2 mM. Thus, it might be reasonable for test chemical solutions prepared at 0.2 mg/mL to exhibit similar levels of accuracy, sensitivity, and specificity as those prepared at 1 mM.

When weight concentrations of chemicals were higher than 0.2 mg/mL, the ratio of test chemical to NAC/NAL was higher on average than the ratio of test chemical to NAC/NAL in the molar concentration preparations, resulting in increased reaction rate and a higher number of false positives. In contrast, at weight concentrations lower than 0.2 mg/mL of test chemicals, the ratios of test chemicals to NAC/NAL were lower on average than those in the molar concentration preparations, and reaction rates decreased. Therefore, although sensitivity decreased, specificity and false negatives increased.

Although the ADRA test method specifies that testing should be performed on test chemical solutions prepared at a molar concentration of 1 mM, we found that testing at a more highly concentrated weight concentration of 0.5 mg/mL resulted in fewer false negatives, but more false positives. However, in the context of predicting sensitization potential for safety hazard assessment, it is preferable to eliminate false negatives. Table 5 shows the predictive capacity of ADRA relative to LLNA for the 82 chemicals tested in this study at four weight concentrations as well as at the conventional molar concentration of 1 mM, grouped by LLNA sensitization potencies of extreme/strong, moderate, weak, and non-sensitizer. Across each of the five concentrations shown in Table 5, predictive capacity decreased as LLNA sensitization potency decreased. However, test chemical solutions prepared at weight concentrations of 0.5 mg/mL exhibited higher accuracy, especially for predicting moderate and weak sensitizers, than the other four concentrations. Accuracy for non-sensitizers at 0.5 mg/mL was only 86.2%, which was the lowest value obtained from any of the concentration levels. Thus, of the four weight concentrations used in this study, 0.5 mg/mL test chemical solutions exhibited the highest accuracy and sensitivity, and we recommend use of test chemical solutions prepared at weight concentrations of 0.5 mg/mL when ADRA is performed to predict sensitization potential of test substances with unknown molecular weights.

Evaluation of the ability of ADRA to detect sensitizers contained in liquid mixtures comprising 10 types of non-sensitizers resulted in one sensitizer, perillaldehyde, that exhibited a 15% or more decrease in NAC depletion compared to individual preparations, and five sensitizers (nonanoyl chloride, phenylacetaldehyde, glutaraldehyde, 2,3-butanedione, and 5-methyl-2,3-hexanedione) exhibited 15% or more decreases in NAL depletion:,

As there was no correlation with dose, lower NAC depletion by perillaldehyde in liquid mixtures was likely a result of normal variability. It is possible that significantly lower NAL depletion in liquid mixtures containing glutaraldehyde, nonanoyl chloride, phenylacetaldehyde, and 5-methyl-2,3-hexanedione was a result of partial reaction of the amino groups contained in sulfanilamide, which is a non-sensitizer, with the sensitizers in the pseudobinary mixtures. Furthermore, glutaraldehyde coelution with NAL may have interfered with accurate depletion value calculation. Since *p*-benzoquinone at 0.5 mg/mL had normal peaks but an inconsistent baseline throughout all HPLC chromatograms, it could not be quantified accurately, and reference values were used for NAC and NAL depletion (Data not shown). This may have been due to highly reactive quinone structures of *p*-benzoquinone which may have reacted with non-sensitizers in the pseudobinary mixture, resulting in a peak pattern with an inconsistent baseline.

Prediction results at each weight concentration for test chemicals that are sensitizers per LLNA differed from the results obtained when testing sensitizers individually for two test chemicals—0.05, 0.1, and 0.2 mg/mL concentrations of nonanoyl chloride and 0.1 and 0.2 mg/mL concentrations of 5-methyl-2,3-hexandione—which yielded negative results. No NAC depletion was observed for either of these test chemicals, but they yielded positive results when tested individually. Despite this, negative results were obtained in the pseudobinary mixtures as no reaction was observed. The likely cause was that sulfanilamide reacted with the test chemicals, which reduced reactivity with NAL.

Based on our results, we were able to obtain positive results for each of the ten sensitizers evaluated when their test chemical solutions were prepared at a weight concentration of 0.5 mg/mL and included in a pseudobinary mixture. Moreover, test chemicals with an LLNA sensitization potency of extreme/strong all yielded positive results in this study even at the minimum weight concentration of 0.05 mg/mL. However, evaluation at 0.5 mg/mL tended to increase depletion values above those observed with 1 mM preparations, resulting in more false positives.

In this study, we demonstrated that test chemicals with unknown molecular weights can be tested with the same predictive capacity as

conventional DPRA or ADRA test preparations when test chemical solutions are prepared at 0.5 mg/mL. Moreover, we also demonstrated that sensitizers of all levels of sensitization potency, from strong to weak, can be accurately identified as sensitizers when test chemical solutions prepared at 0.5 mg/mL are included in a pseudobinary liquid mixture at a final concentration of 0.0125 mg/mL, thereby indicating ADRA's usefulness in a top down approach for assessing the sensitization potential even of mixtures of unknown molecular weight. However, coelution of test substances with NAC or NAL during HPLC analysis was problematic for some liquid mixtures, demonstrating that some types of liquid mixtures cannot be used for evaluation of sensitization potential using this method. This suggests the necessity for future studies to establish analytical techniques that are not affected by interference between solution components and test substances.

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