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Reactivity Profiling: Covalent Modification of Single Nucleophile Peptides for Skin Sensitisation Risk Assessment

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Abbreviations: ACD, allergic contact dermatitis, LLNA, local lymph node assay, LC-MS,
liquid chromatography mass spectrometry, LC-MS/MS, liquid chromatography tandem mass
spectrometry, MRM, multiple reaction monitoring, PPD, para-phenylene diamine, DNCB,
1,4-dinitrochlorobenzene, MPT, 3-methyl-4-phenyl-1,2,5-thiadiazole-1,1-dioxide.

Abstract

The molecular basis of chemical allergy is rooted in the ability of an allergen (hapten) to modify endogenous proteins. This mechanistic understanding aided development of screening assays which generate reproducible quantitative and qualitative reactivity data. Such assays use model peptides with a limited number and type of protein nucleophiles and the data does not reflect the specificity, variety and complexity of hapten interactions with multiple nucleophiles. Building on these developments we extended the standardised approach to maximise the type and the amount of information that can be derived from an *in chemico* assay. We used a panel of six single nucleophile peptides and individually optimised the incubation conditions to favour chemical modification. Employing LC-MS/MS technique we simultaneously obtained multiple quantitative and qualitative measurements (% peptide depletion, adduct(s) formation, and peptide dimerisation for Cys containing peptide). Using these methods we obtained reactivity data for 36 chemicals of known skin sensitising potency. By optimising incubation conditions we ensured detection of all reactive chemicals. We explored the LC-MS/MS approach to generate kinetic data for 10 chemicals allowing further characterisation of reactivity and a potentially more robust quantitative reactivity descriptor. Our ultimate aim is to integrate this dataset with available physicochemical data and outputs from other predictive assays, all addressing different key steps in the induction of sensitisation, to help us make decisions about the safe use of chemicals without using animal tests. The epidermal protein target sites, modification of which may be immunogenic and lead to induction of skin sensitisation are currently unknown. Increasing the understanding of this process may help further refine *in chemico* reactivity assays as well as aid the interpretation of the reactivity data.

Keywords: skin sensitisation, covalent binding, model peptide, mass spectrometry, *in chemico*, hapten

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1. Introduction

The consumer product industry currently uses data from animal tests such as guinea pig maximisation test (Magnusson, B. *et al.* 1970) and the local lymph node assay (LLNA) (Basketter, D. A. *et al.* 2001) to determine the skin sensitising hazard and potency of a chemical and to safeguard against inducing chemical allergy in humans. Recent changes in the EU legislation will forbid marketing of cosmetic product ingredients which have been tested on animals (7th amendment to EU Cosmetic Directive (EU 2003). Major efforts are now focused on the use of *in vitro/in chemico/in silico* methods in risk assessment. Novel, multicomponent, integrated testing strategies have been suggested as a viable alternative to current animal tests (Jowsey, I. R. *et al.* 2006). It is envisaged that data from a battery of assays (addressing key steps in induction of skin sensitisation) will be combined in a weight of evidence approach, the output of which will be used in subsequent risk assessment (Maxwell, G. *et al.* 2008). An example of a multicomponent integrated strategy was published recently (Natsch, A. *et al.* 2008a).

The requirement for a chemical to covalently modify skin protein(s) has long been established as a key event in the induction of skin sensitisation (Landsteiner, K. *et al.* 1935). The exact nature of relevant covalent modifications *in vivo* is still not empirically determined for any chemical allergen (hapten) (Divkovic, M. *et al.* 2005). A limited number of studies have demonstrated that the intrinsic reactivity of sensitising chemicals with isolated proteins varies widely in terms of specificity, mechanisms and rate of reaction (eg. Meschkat, E. *et al.* 2001; Alvarez-Sanchez, R. *et al.* 2004; Aleksic, M. *et al.* 2007; Aleksic, M. *et al.* 2008). The key observation remains that the binding of chemicals is restricted to certain sites on proteins which are particularly conducive to reactivity, i.e. not all present and accessible nucleophilic residues are modified. However, due to experimental and analytical complexity of chemical

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3 interactions with proteins, simpler peptide sequences are more often utilised as adequate
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5 models for reactivity studies (Gerberick, F. *et al.* 2008).
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8 Several groups have investigated the potential of *in chemico* peptide reactivity assays
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10 to generate useful data for determination of sensitisation hazard and potency of chemicals
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12 (Kato, H. *et al.* 2003; Gerberick, G. F. *et al.* 2004; Schultz, T. W. *et al.* 2005; Aptula, A. O. *et*
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14 *al.* 2006; Gerberick, G. F. *et al.* 2007). These approaches aim to answer the key question
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16 (whether the chemical binds protein nucleophile(s)) by measuring either the disappearance of
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18 the model nucleophile (usually a peptide) or appearance of a peptide-chemical
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20 conjugate(s)/adduct(s). It is equally feasible to measure the disappearance of the test
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22 chemicals but the practical implications and often a large excess of chemical make this
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24 approach less useful. Developed with medium to high throughput capability, standardised
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26 peptide reactivity assays may be sufficient for screening purposes. However, for integration
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28 with other data types to enable a thorough risk assessment without any animal data, it is
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30 probable that the reactivity of chemicals towards protein nucleophiles must be characterised
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32 in more detail. More recent work by Natsch and colleagues (Natsch, A. *et al.* 2007; Natsch, A.
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34 *et al.* 2008b) aims to increase the amount of information that can be obtained from reactivity
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36 studies. Using peptide nucleophiles, these assays can determine both quantitative
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38 (disappearance of target peptide) and qualitative (observe the formation of covalent adducts
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40 to peptide) aspects of chemical reactivity.
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48 The published experiments show that reproducible, quantitative and qualitative
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50 reactivity data can be generated using short peptide sequences and standardised procedures.
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52 However, the screening purposes of assays published to date limits the number and type of
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54 nucleophiles investigated. The data therefore do not reflect the specificity, variety and
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56 complexity of reactions that could occur with protein nucleophiles *in vivo*. We consider that
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58 this information could provide added value for future skin sensitisation risk assessment of
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chemicals. Furthermore, such a dataset may be applicable to endpoints other than skin sensitisation although the causative relationship between covalent binding and other toxicity endpoints may not have been fully established (Evans, D. C. *et al.* 2004).

Therefore, our aim was to build on published assay developments and maximise the amount and type of information that can be derived about the reactivity of chemicals from *in chemico* approaches. We have developed a testing strategy which allows the in depth analysis of reactivity with a variety of nucleophiles in anticipation that a comprehensive reactivity data set can be used in novel risk assessment strategies integrated with data from other lines of evidence for determination of the relative sensitising potency of chemicals.

Here we show how data obtained in reactivity profiling studies can be used to (a) determine whether or not a chemical is reactive towards multiple protein nucleophiles with a high degree of confidence by observing both the disappearance of target peptide and formation of adduct(s); (b) characterise and quantify the observed reactivity (by determining the mechanism(s) of reaction(s) and reaction rate, respectively) and (c) utilise this data set to distinguish between chemicals based on their reactivity profile for use in future risk assessments.

2. Materials and Methods

2.1. Materials

Peptides were purchased from Cambridge Research Biochemicals (Cleveland, UK) as >95% pure with generic peptide sequence AcFAAXAA (where X represents a nucleophilic residue (C, K, R, H or Y). Peptides AcFAAAAA, FAAAAA and AcFAGAGA were also purchased to be used as the negative control, N-terminal nucleophile and internal standard, respectively.

Water, methanol and acetonitrile for mobile phase preparation were LC-MS grade from Riedel de Haen (Seelze, Germany). Acetonitrile and acetone were HPLC grade from Rathburns (Walkerburn, UK). All chemicals were purchased from Sigma-Aldrich (Poole, UK) except N-dodecyl gallate and 1-bromododecane (Alfa Aesar (Heysham, UK)), 2,4-pentanedione, 2-hydroxyethyl acrylate and formic acid (Fluka (Seelze, Germany)), 3-methyl-4-phenyl-1,2,5-thiadazole-1,1-dioxide (MPT) (Tocris Bioscience (Avonmouth, UK)), 3,7-dimethyl-7-hydroxyoctanal (hydroxycitronellal), trans-2-decenal, 2-hydroxypropylmethacrylate (Fisher Scientific, (Loughborough, UK)) and cyclamen aldehyde (Penta Manufacturing Co (New Jersey, USA)). All chemicals purchased were >96% pure except for ammonium hydroxide (28%), formaldehyde (36.5%), glutaraldehyde (50%), glyoxal (40%), phenylacetaldehyde (90.5%) and 2,4-heptadienal (88.9%). The factors that influenced the selection of chemical sensitisers for this study included availability, proposed mechanism of reaction (covering all common mechanistic applicability domains), as well as availability of sensitisation potency data (EC3 values from the LLNA).

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2.2. Methods

2.2.1. Measurement of Peptide Depletion

Peptide stock solutions were prepared at 2.5 mM in nitrogen purged ultrapure water aiding dissolution with a minimum volume of acetonitrile or buffer where necessary. Calibration standards were prepared for each peptide between 0.125 and 2.5 mM in ultrapure water. Phosphate and carbonate buffers (pH 7.4 and 10, respectively) were prepared at 50 mM. Test chemicals were solubilised in acetonitrile, except n-dodecyl gallate which was solubilised in acetone:acetonitrile mixture (10:90, v/v). Samples were prepared in triplicate in a 96 well plate by adding 50µl of 2.0mM peptide solution, 100µl of test chemical solution, 90µl of buffer and 10µl of 2.5mM AcFAGAGA (as internal standard) and incubated at 37°C for 24 hours. Concurrent control samples were prepared replacing the test chemical with solvent and incubated under the same conditions. Calibration standards were prepared as the control samples, replacing the 2.0 mM peptide solution with 50µl of peptide calibration standard (0.125 – 2.5mM). Samples were analysed using an Agilent (Cheshire, UK) 1100 series HPLC and Waters (Milford, MA) Quattro Micro API tandem quadrupole MS using multiple reaction monitoring (MRM) in electrospray positive mode. Samples were enclosed in the autosampler at 4°C and 2µL aliquots were injected onto a Gemini C18, 100 x 2mm, 3µ column (Phenomenex, Cheshire, UK). Separation was achieved using a 0.2mL/min flow rate with a gradient of mobile phase A (0.05% formic acid in water) and B (0.05% formic acid in acetonitrile) changing from 7 to 90% B over a total of 18 minutes. Minor alterations to the gradient were used where necessary, for example in instances where the test chemical co-eluted with either the peptide or the internal standard and resulting suppression of ionisation was affecting the accuracy of % depletion measurement. When altering the gradient was not sufficient to eliminate co-elution one of the two alternative columns were used (Phenomenex Luna 3µ

PFP(2), 150 x 2mm or Sielc (Prospect Heights, IL) Primesep 100, 5 μ , 150 x 2.1mm) with suitable mobile phases and comparable gradients.

The depletion of each parent (unchanged) peptide was measured by comparing the average concentration of peptide in the control samples against the concentration of peptide remaining in the incubation sample as determined from the internal standard calibration curve.

2.2.2. Adduct Analysis

Following measurement of peptide depletion the samples were re-analysed using identical instrument combination and mobile phases for adduct(s) detection. Sample aliquots (4 μ L) were injected onto a Gemini C₁₈, 150 x 2mm, 3 μ column (Phenomenex, Cheshire, UK) at a 0.2 mL/min flow rate (80% A : 20% B). The gradient was changed initially to 50% B, then 90% B before decreasing to 20% B over a total of 22 minutes. A precursor ion scan was performed using a common N-terminal fragment (AcFA, 260 Da) which is shared among six peptides. For peptide FAAAAA the adduct formation analysis was performed using the above conditions with the MS instrument operating in scan mode. Concurrent signals corresponding to fragment ions resulting from the loss of C-terminal Ala residues were used for confirmation. The new signals were often indicative of the nature of adduct(s), however if the reaction mechanism(s) were not apparent LC-MS^E analyses were carried out using Waters Acquity UPLC and QToF Premier (Waters). The 6 μ L sample aliquots were injected at a 0.1ml/min flow rate and the peptides were separated by a binary UPLC gradient (as for % depletion) on a Waters Acquity UPLC HSS T3, 1.8 μ m, 1 x 100mm column. The QToF Premier was set to collect both exact mass precursor and product ion information from a single injection by acquiring at both high and low collision energies. All data were acquired and processed using MassLynx ® (Waters) and the instrument calibrated with sodium

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formate solution (range m/z 50-1500) and monitored by the intermittent injection of the lock mass leucine-enkephalin.

2.2.3. Simultaneous Measurement of % Depletion and Adduct Analysis

The combined method is suitable for the six peptides that contain the common N-terminal sequence AcFA. Samples, control samples and calibration standards were prepared as described previously and analysed using the LC conditions as for adduct analysis. The MS instrument was set to perform a precursor ion scan throughout the duration of the run but switched to simultaneously perform MRM experiments at the respective retention times of the peptide and the internal standard allowing accurate quantitation.

2.2.4. Reaction Kinetics

For 10 chemicals in this dataset we attempted to measure the rates of reaction in cases where the reactivity of the chemical was confirmed by both measurement of peptide depletion and observation of adduct(s). Fresh control samples and calibration standards were prepared and analysed (with the autosampler held at 37°C) using the same methods, instrument and conditions as for the measurement of % depletion, with the exception of the starting peptide concentration (0.5mM, with the 1:100 peptide:chemical concentration ratio maintained). An incubation sample was then prepared and analysed immediately and repeatedly over approximately 24 hours, comparing the amount of unchanged peptide to the amount determined in the control sample containing no test chemical. For fast reactions, the gradient was altered to give the shortest possible run time to ensure that enough data points could be captured to allow determination of the reaction kinetics.

2.2.5. Data Analysis

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3 Peptide depletion and adduct data were processed using Microsoft® Office Excel 2003
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5 (Microsoft Corporation, Redmond, WA). For individual peptide depletion analyses box plots
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7 were generated using MATLAB® v. 7.6 (R2008a) (The MathWorks, Inc., Natick, MA, US).
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10 Spotfire DecisionSite® (TIBCO Software Inc, Somerville, MA) was used to generate two-
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12 way clustering heatmap. For the kinetic data analysis, graphs and line fits were produced
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14 using MATLAB®. Chemical structures and reactions were visualised using ChemOffice®
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17 2006 v. 10.0 (CambridgeSoft Corp., Cambridge, MA, US).
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3. Results

Initial analyses using 2,4-dinitro-1-chlorobenzene (DNCB), 2,4-pentanedione and hydroxyethyl acrylate have shown that maximum reactivity can be achieved by optimising the reaction conditions for each peptide (maximising the concentration ratio, increasing the temperature and adjusting the pH closest to the pKa of the target amino acid side chain) (data not shown). Taking into account the findings from these initial experiments as well as practical considerations, in the final protocol peptides were incubated with up to a 100 molar equivalent of the chemical (or the closest achievable concentration ratio, depending on solubility), temperature was kept at 37°C (any further temperature increase results in significant evaporation), and pH was maintained at either pH 7.4 (for Cys, His, Ala and N-term peptides) or pH 10 (for Lys, Arg and Tyr peptides).

3.1. Quantification of Reactivity – Measurement of Peptide Depletion

In total, % depletion of parent peptides was measured for 36 chemicals of known sensitising potency (Table 1) categorised into 5 potency classes: non-sensitiser, weak, moderate, strong, and extreme sensitisers based on their LLNA EC3 (Gerberick, G. F. *et al.* 2005).

Table 1

To understand the relationship between the peptide depletion and the potency indicator EC3, Spearman’s rank correlation coefficient (Spearman's ρ) was calculated for each peptide across 27 sensitising chemicals (excluding 9 non-sensitisers) (Table 2). The results suggest that

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3 LLNA EC3 strongly correlates with Lys, N-term and Cys peptide depletion values but not
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5 with His, Arg or Tyr peptide depletion. Interestingly, Cys and N-term peptide depletion data
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7 show strong correlation with Lys, but they do not strongly correlate with each other ($p = 0.03$
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9 > 0.01) thus indicating that different information is obtained using individual nucleophiles.
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15 Table 2

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20 Visually represented, individual peptide data shows a variety of patterns and ranges of values
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22 across the 5 sensitising potency categories.
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27 Figure 1

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32 In general, the scale of Lys peptide depletion for a chemical approximately indicates
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34 the strength of sensitisation potency for that chemical (Figure 1(b)). However, as ranges of
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36 depletion values are spread across several potency groups, depletion information for this
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38 peptide alone cannot distinguish between sensitising potency categories. Similar patterns are
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40 observed for N-term peptide depletion (Figure 1(f)). In contrast, the Cys peptide data
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42 demonstrates substantial reactivity for some non-sensitising chemicals (Figure 1(a)). The
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44 ranges of % depletion data overlap horizontally for the Arg peptide (Figure 1(d)) and for the
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46 His and Tyr peptides (with the exception of extreme sensitisers, Figure 1(c) and (e),
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48 respectively). The complementary nature of % depletion data from multiple peptides is
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50 evident from examples of Cys and His peptides (Figure 1(a) and (c), respectively). If a
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52 chemical of an unknown sensitising potency depleted Cys by $<50\%$ the data imply a high
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54 probability that the test chemical is a weak or non-sensitiser. Also, if the test chemical
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56 depleted His peptide $>40\%$ the data imply a high probability that the test chemical is an
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extreme sensitiser. In contrast, if the Cys peptide depletion was >50% and His peptide depletion was <40 % a confident prediction of sensitising potency could not be made.

The % depletion data for all six peptides was integrated with the rationale that the quantitative reactivity profile across six peptides could potentially better characterise the sensitisation potency of the chemical. The analysis was performed using hierarchical clustering (Han, J. 2006) using Euclidian distance metric to measure similarities between individual chemicals based on % depletion values of six peptides, and Ward method (Ward, J. H. 1963) to assess similarities between clusters of chemicals (Figure 2).

Figure 2

The dendrogram on the left of the Figure 2 shows the progressive grouping of 36 chemicals. From a vertical line drawn by us, the chemicals are visually categorised into three clusters. Clusters 1 and 3 could be regarded as groups of weak sensitisers and strong sensitisers, respectively with some outliers. The 14 chemicals in cluster 1 are either non-sensitisers or weak sensitisers except for one strong sensitiser (p-phenylenediamine (PPD), which may require metabolic activation, so its reactivity is possibly underestimated). In contrast, the 18 chemicals in cluster 3 mostly are extreme, strong and moderate sensitisers. The exceptions are 2-acetylcyclohexanone and benzaldehyde, both of which are non-sensitisers in LLNA, and ethyl acrylate, which is a moderate sensitiser in the LLNA. Clinical reports of allergy to benzaldehyde are rare, but positive guinea pig tests indicate that evaporation and rapid conversion to benzoic acid may be responsible for lack of sensitisation in the LLNA (Klecak, G. *et al.*, 1977; Andersen, A., 2006). Ethyl acrylate is also volatile and the EC3 is possibly underestimated. Interestingly, cluster 2 contains a mixture of 4 chemicals spread over weak, moderate and strong sensitising potency categories. Nevertheless, the

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3 results obtained from this integrative analysis suggest that chemical depletion profiles do
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5 indicate some potential in predicting chemical sensitisation potency, though they are far from
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7 perfect in separating chemicals into distinct potency categories based on the LLNA EC3
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9 values. Applied to the peptides, the clustering analyses again revealed that peptides form two
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11 distinct groups based on the similarity of the % depletion data.
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15 Overall, the % depletion data show variability in some cases (up to ~10%). This
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17 presents a challenge in determining a cut-off point for % depletion even between non-
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19 sensitisers and sensitisers using either individual peptide depletion data or a combination.
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24 3.2. Confirmation of Reactivity – Adduct Analysis

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29 For majority of chemicals the % depletion data could be confirmed in the first instance by
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31 either observation of the formed adduct(s) or absence of new signals in case of negligible
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33 depletion using the precursor ion scan method. In cases where the observed signals could not
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35 be explained further, accurate mass and tandem MS analyses were conducted (detailed
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37 dataset available in Supplementary data).
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41 Adduct analyses revealed additional information about reactivity not apparent from the %
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43 depletion data. The added value in detecting adducts for confirming reactivity is evident
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45 especially in cases where % depletion is <10% as adducts were occasionally observed in
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47 instances where peptide depletion was negligible. For example, phenyl benzoate depleted Tyr
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49 peptide by -0.5%, but acylation adducts were observed. Similarly, α -hexylcinnamic aldehyde
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51 depleted Cys peptide by 7.6% and Lys peptide by 6.3%, however, Schiff base and Michael
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53 adducts were seen only with Lys peptide. However, even if the Cys peptide is 100% depleted,
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55 observation of adducts is not certain. For example, aldehydes of varying sensitising potencies
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57 (cyclamen aldehyde, hydroxycitronellal, benzaldehyde and phenylacetaldehyde) depleted Cys
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peptide between 30% and 100%, but the loss of Cys peptide was solely due to chemical induced peptide dimerisation. In cases where both adduct(s) and Cys peptide dimer were observed no reliable quantitative information could be derived about the relative contribution of these reactions to overall Cys peptide % depletion due to differences in ionisation of these species as well as lack of standards. For peptides other than Cys % depletion data >15% always resulted in observation of adduct(s). The majority of adducts observed resulted from reactions involving a single, often easily predicted mechanism. However, patterns of observed reactivity across seven peptides varied with some chemicals only reacting with a single and others with multiple peptides. Additionally, several examples of complex reactivity, such as generation of multiple adducts, hydrolysis of test chemical and/or adducts, multiple reaction mechanisms and cross-linking were observed.

Quantitative and qualitative data were reproduced simultaneously by combining the methods for % depletion measurement and adduct observation for three peptide-chemical combinations (Cys-glycerol, His-benzaldehyde and His-4-nitrobenzyl bromide). Neither % depletion or adduct information are significantly different from the data obtained in separate methods, except for % depletion values for His-benzaldehyde. Although the observed difference is significant in this case ($p < 0.05$, see Tables 1 and S1 (supplementary data) for actual values) the data from simultaneous analysis is within the expected variability for this measurement.

3.3. Further Quantification of Reactivity – Rate of Reaction Measurement

The time course of peptide concentration measurements in the kinetic version of the assay allows the reaction rate constant to be determined, provided the reaction mechanism is known. However, comparison of the chemical reactivity is not straightforward between

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3 reactions of different orders. We describe the simple and pragmatic approach we have applied
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5 at this stage, which uses the framework of the following mechanism to analyse all datasets:
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10 The high initial concentration ratio of chemical to peptide (100:1) renders this pseudo
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12 first order with respect to the peptide alone, so the peptide concentration p at time t would be
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14 given by Equation 1:
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$$p(t) = p_0 \exp(-kt) \quad \text{Equation 1}$$

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19 The rate constant k is typically estimated as minus the gradient of the best fit line of
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21 $\log p$ against t . If the mechanism is not in fact pseudo first order, the graph of $\log p$ versus t
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23 will have a varying gradient. The comparable measure of reactivity for all chemicals is taken
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25 as the initial gradient, because at time zero the concentration of peptide is known from
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27 preparation, and consistent for all cases.
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36 From the datasets produced so far, the most common deviation from pseudo first
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38 order behaviour is not a gradual curve that would suggest a higher order, but rather a point at
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40 which the reaction rate significantly decreases from its starting level or completely vanishes.
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42 This was demonstrated by about half of the datasets. In order to exclude the later time points
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44 and find the initial gradient, a threshold of 0.06 mM above the final concentration level was
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46 used (typical standard deviation of peptide concentration measured in repeated blanks was
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48 0.03 mM). Provided there were three or more data points from the start of the experiment
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50 above this threshold, a best fit straight line was fitted to $\log p$ versus time for those points,
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52 and the central value and confidence interval limits of the reaction rate were determined from
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54 the line fit.
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Figure 3 shows the results of this approach for 3,4-dihydrocoumarin and the N-term peptide, a case where the measured concentration was consistent with the pseudo first order trend of Equation 1 for the duration of the experiment.

Figure 3

In contrast, benzyl bromide and His peptide was a case where the S_N2 reaction suddenly stopped while there was still a significant amount of peptide remaining. This may result from loss of benzyl bromide by hydrolysis, and inhibition of the reaction with peptide due to hydrogen bromide generated from hydrolysis and the S_N2 reaction, once the acid has exhausted the buffer. To investigate this, the experiment was performed again with a buffer at 100 mM instead of 50 mM. The rate constant estimated with the original 50 mM buffer was 0.122 (0.033) hour^{-1} , and with the 100 mM buffer was 0.106 (0.025) hour^{-1} , which is consistent (the values in parentheses are twice the standard error). With the 100 mM buffer, though, this initial rate continued for more than 10 hours compared to about 3 hours with the 50 mM buffer. The final pH in the 100 mM buffer experiment was 3, but the reason for the reaction to stop in this case is probably still a combination of decreasing pH and hydrolysis of benzyl bromide. Furthermore, because of competition between the S_N2 reaction and hydrolysis for the benzyl bromide, the rate found represents a combined effect, which is a limitation of the simple mechanism assumed, and more experiments would be required to isolate the S_N2 rate (Figure 4). Note that other reactions observed to cease will do so for different reasons relating to their exact mechanisms.

Figure 4

There are several datasets for which the above procedure does not yield a result, and they divide into two categories: “slow” reactions where peptide depletion is minimal throughout the experiment and the rate derived is not significantly different from zero; and “fast” reactions, where the rapid initial rate allows collection of <3 data points before reaction is completed and so a best fit line cannot be generated reliably. For fast reactions, the limiting step is the length of the analytical run. This could be overcome in principle by development of an (automated) sampling procedure where the reaction is stopped by removal of the test chemical (rather than addition of another reagent). Such procedures would yield a higher throughput and increased accuracy.

With either slow or fast reactions, it is not possible to obtain a central value and confidence interval for the reaction rate constant, and instead the appropriate result is a confidence interval expressed as an upper or lower bound, respectively. For example, for slow reactions, the peptide concentration may be considered to be constant within measurement variation, and we could estimate an upper bound by assuming a decrease from the mean value (μ) over all data points at time zero to be the mean minus two standard deviations (σ) at the final time point t_{Final} . This is illustrated in Figure 5 for cinnamaldehyde and the His peptide, and yields:

$$k_{\text{UB}} = \frac{\log(\mu) - \log(\mu - 2\sigma)}{t_{\text{Final}}} \approx \frac{2\sigma}{\mu t_{\text{Final}}} \quad \text{Equation 2}$$

Figure 5

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Table 3 lists the results for the reaction rate constants calculated using the methods described above.

Table 3

4. Discussion

There has been a surge in interest in assessing skin sensitisation hazard and potency without the use of animals in recent years. Covalent modification of skin proteins is understood to be the key requirement in the induction of skin sensitisation. Chemical reactivity towards protein nucleophiles is therefore considered to be one of the key measurements. Recently published *in chemico* methods, particularly works by Gerberick and colleagues and Natsch et al (Gerberick, G. F. *et al.* 2004; Gerberick, G. F. *et al.* 2007; Natsch, A., *et al.* 2007; Natsch, A. and Gfeller, H. 2008b) have demonstrated reproducibility in generation of mainly quantitative reactivity data using standardised protocols as well as use of this data in prediction of sensitising potency with some success. It is probable however that enabling a thorough risk assessment in the future may require a more detailed characterisation of chemical reactivity. The LC-MS/MS methods we report here are based on the same principles as the published assays, but extended to include a number of additional nucleophilic target residues as well as to derive a more detailed qualitative and quantitative reactivity dataset.

The ability to generate both quantitative (% depletion) and qualitative (adduct(s)) data in truly simultaneous fashion utilising a single analytical run will increase the assay throughput. Bespoke analytical procedures to overcome ionisation suppression or co-elution were occasionally necessary for some peptide-chemical combinations. This could be problematic if a standardised protocol was required. The assay however uses a negative control peptide and an internal standard peptide which improves the quantification. Additionally, flexibility of this approach allows us to study reactions of protein nucleophiles with more than one chemical at a time and reaction of one chemical with more than one protein nucleophile at a time. It is important to note that the incubation conditions were optimised to the individual

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3 peptides to maximise the reactivity *in chemico* rather than a representing the physiological
4 conditions.
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8 The % depletion data reported here is comparable to the published data with Lys, Cys
9 and His peptides used by Gerberick and colleagues (Gerberick, G. F. *et al.* 2004; Gerberick,
10 G. F., *et al.* 2007). Although the actual % depletion values here are higher in some cases, this
11 is expected because higher concentration of the reactants, compared to the ones used in the
12 protocol by Gerberick et al (Gerberick, G. F., *et al.* 2007). Maximising the reaction rate in
13 this way increases confidence in detection of weakly reactive chemicals. The Cor1 peptide %
14 depletion data (Natsch, A. *et al.* 2007; Natsch, A. and Gfeller, H. 2008b) is not directly
15 comparable as the Cor1 peptide sequence contains three nucleophilic residues (AcNKKCDLF,
16 derived from Dennehy, M. K. *et al.* 2006), which mutually influence the overall reactivity
17 and make data interpretation significantly more complex. For example, Natsch and
18 colleagues reported difficulties in observing Schiff base adducts with Cor1 peptide (Natsch,
19 A. and Gfeller, H. 2008b), whereas significant % depletion confirmed by observation of
20 Schiff base adduct as well as their intermediates was seen for a number of weakly reactive
21 aldehydes with at least one of the primary amines in our peptide panel. In contrast, the
22 additional Arg residue in Cys, His and Lys peptides used by Gerberick and colleagues does
23 not appear to significantly change the reactivity of the target nucleophiles. The % depletion
24 data summarised above together with published reactivity datasets demonstrate that a strong
25 association exists between sensitising potency and reactivity with model peptides, although
26 the relationship is often not straightforward.
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53 Confirmation of reactivity (by characterisation of adduct(s) or confirmation of their
54 absence within limits of detection) was often needed due to overall data variability of
55 approximately 10% and in cases of uncharacteristic % depletion. The majority of predicted
56 reactions were confirmed employing routine adduct characterisation in this approach. In
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3 addition, a variety of reactivity patterns and several examples of complex reactivity were also
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5 observed. For example, benzyl bromide formed triple adducts with Cys and Lys and only
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7 single adducts with other nucleophilic peptides. A moderate sensitiser, 3-methyl-4-phenyl-
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9 1,2,5-thiadiazole-1,1-dioxide (MPT) (EC₅₀ 1.4), reacted via Michael addition only with Cys
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11 peptide. With all other peptides (except His) Schiff bases were formed with the major
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13 hydrolysis products 1-phenyl-1,2-propane dione which has a comparable sensitising potency
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15 (EC₅₀ 1.3). This could indicate that the diketone hydrolysis product is responsible for the
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17 sensitising potency of MPT.
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22 A number of chemicals have more than one electrophilic site and therefore a
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24 capability to react via different mechanisms. For example, trans-2-decenal, α,β -unsaturated
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26 aliphatic aldehyde with two electrophilic centres, formed Michael adducts with Cys and His
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28 peptides, whereas both Schiff base and Michael adduct were seen with Lys. Additionally,
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30 with the N-term peptide evidence exists for both adduct types as well as a cross-linked
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32 species. Complex cross-linked structures (cyclised diquinone adducts, (Person, M. D. *et al.*
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34 2003) were observed in reaction of Lys and N-term peptides with benzoquinone (and
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36 hydroquinone). However, no evidence was obtained for intermolecular cross-linking with
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38 formaldehyde, which is a well known cross-linking chemical. Cross-linking only occurs
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40 between formaldehyde modified thiols and/or primary amines which react with other amino
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42 acid side chains (Arg, Tyr, Asp, Glu, His and Trp) forming stable methylene bridges and this
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44 does not occur between formaldehyde modified primary amino groups (Metz, B. *et al.* 2004;
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46 Metz, B. *et al.* 2006). The most complex reactivity was observed with oxazolone, an extreme
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48 sensitiser reacting via three electrophilic sites with the majority of peptides (except His and
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50 Arg) generating a variety of adducts some of which are additionally subject to hydrolysis. It
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52 is currently not clear how complex reactivity may generally affect the sensitisation potency of
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54 chemicals. This is currently a major knowledge gap and much work is needed to investigate
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3 the relevance of such examples in a system closer to *in vivo* skin conditions, for example
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5 utilising cell lines, 3D skin models or even *ex vivo* human skin. It is likely that a thorough
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7 understanding of all aspects of chemical reactivity starting from *in chemico* through to *in vivo*
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9 (or *ex vivo*) may help us rationalise the toxicological effects.
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13 An unusual example is modification of C-termini of some peptides (including the
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15 negative control peptide) by 4-nitrobenzyl bromide. Carboxyl groups are not classically
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17 thought to be targeted by sensitising chemicals, however if a carboxylic anion can be
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19 generated a reaction with an electrophilic group can occur. The modification of carboxyl
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21 groups in addition to all of the other nucleophilic sites in this peptides panel could help
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23 rationalise extreme sensitising potency of 4-nitrobenzyl bromide. The ability of the assay to
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25 detect adducts and rationalise depletion is also applicable to cases where reactivity needs to
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27 be assessed for more than one chemical (in mixture or formulation) and the interaction
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29 between the components (solvents or test chemicals) could affect overall chemical reactivity.
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31 For example, benzyl bromide can react with the negative control peptide (by modifying the
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33 C-term) in presence of ammonia, resulting in ~20% depletion.
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39 Approximately one third of sensitisers are not directly reactive and require some form of
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41 activation (Smith, C. K. *et al.* 2001) either via spontaneous oxidation on air exposure or
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43 metabolic activation in the epidermis (prehaptens and prohaptens, respectively, (Lepoittevin,
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45 J. P. 2006). This necessitates the inclusion of an activation step in reactivity testing.
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47 Preliminary experiments are underway to investigate whether reactivity of sensitisers
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49 activated by “skin-like” cocktails of metabolising enzymes (Bergstrom, M. A. *et al.* 2007)
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51 can be evaluated using this panel of single nucleophile peptides.
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5. Conclusion

In conclusion, we have developed a robust and flexible procedure to obtain detailed quantitative and qualitative information on chemical reactivity with typical protein nucleophiles *in chemico*. We demonstrated the added value in observing peptide adducts for confirmation of % depletion data as well as further describing reactivity, explaining outliers and rationalising sensitising potency. The combination of (quantitative and qualitative) reactivity profiles from a panel of single nucleophile peptides shows greater ability to separate chemicals based on potency when compared to a single peptide. Additionally, we have demonstrated the feasibility of obtaining reaction rate constants providing additional means of quantifying reactivity. Considerable effort is now directed towards further method development which will allow reactions to be stopped without further reagent addition and evaluating a larger kinetic dataset. Increasing our understanding of critical epidermal protein targets *in vivo* and their immunogenicity may lead to more specific reactivity assays in the near future.

The dataset generated to date is a complex representation of reactivity of a panel of known sensitisers with single nucleophile model peptides. Significant further effort is needed to demonstrate how this detailed dataset can be fully utilised to characterise sensitising potency of chemicals. It is generally accepted that data from single assay will not be sufficient to inform the risk assessment and the major future challenge remains integration of multiple data types to obtain information about chemicals that can be used in risk assessment for skin sensitisation.

Supplementary data

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>. Table S1 in the supplementary data lists detailed information about observed adducts for 7 peptides tested

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with 36 chemicals. Figure S1 shows a comparison of the chromatographic traces obtained for His peptide and 4-nitrobenzyl bromide sample using quantitative % depletion measurement (A) and qualitative adduct analysis (C) with those obtained using a simultaneous method combining quantitative and qualitative analyses (B and D, respectively).

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Figure legends:

Figure 1. Box plots summarising the peptide depletion values for chemicals in each sensitising potency class (NS=Non-sensitiser, W=Weak, M=Moderate, S=Strong, E=Extreme, (Gerberick et al, 2005)) and for each peptide: (a) Cys, (b) Lys, (c) His, (d) Arg, (e) Tyr, and (f) N-term. The boxes show the first quartile, median and third quartile of the peptide depletion values. The whiskers extend to the furthest data points within 1.5 times the interquartile range from the first or third quartile as appropriate, and outliers beyond that level that are shown as circles (○).

Figure 2. Two-way clustering of 36 chemicals % depletion data across 6 peptides using heat map visualisation. Each row represents a chemical with the 6 columns of peptide depletion values (excludes the negative control peptide depletion data). Scales of the depletion values are colour coded by blue (high depletion), black (moderate depletion), and yellow (low depletion). Chemicals are visually categorised into three clusters, whereas peptides formed two clusters, based on similarity of the data.

Figure 3. Time course of N-term peptide concentration during reaction with 3,4-dihydrocoumarin (●), and fit to the data assuming a mechanism that is pseudo first order with respect to the peptide (line).

Figure 4. Natural logarithm of His peptide concentration during reaction with benzyl bromide, expressed as a percentage of the average concentration of the blank samples. Circles and dashed line: data and fit from experiment with buffer concentration 50 mM; Triangles and solid line: data and fit from experiment with buffer concentration 100 mM. The fits are to

the data points that correspond to the initial rates in the respective experiments, which are consistent between different buffer concentrations. The fine dotted lines show the rate that would be inferred from the final depletion value from experiments with 50mM and 100 mM buffer.

Figure 5. Time course of His peptide concentration during reaction with cinnamaldehyde (●). The reaction is sufficiently slow that the data points do not display any significant trend over time. We estimate an upper bound for the rate (as shown by the solid line) by assuming the peptide concentration decreases from the mean to the mean minus two standard deviations of the data points (both levels shown by dashed lines) over the duration of the experiment.

Figure S1. A comparison of the qualitative and quantitative data obtained for His peptide and 4-nitrobenzyl bromide sample using stand alone methods for quantitative and qualitative analyses to the data obtained using simultaneous analysis (combined qualitative and quantitative method). The data was obtained using optimal gradients for each analysis. Chromatographic traces of (A) quantitative % depletion measurement using stand alone; (B) quantitative % depletion data obtained using simultaneous method; (C) qualitative adduct analysis using stand alone method (D) qualitative adduct data obtained using a simultaneous method.

Tables

Table 1. Individual peptide % depletion data for 7 peptides tested with 36 chemicals (n=3).

Chemical	Potency category (%EC3)*	Cys (pH7.4)		Lys (pH 10)		His (pH 7.4)		Arg (pH 10)		Tyr (pH 10)		N-term (pH 7.4)		Ala (pH 7.4)	
		% dep	SD	% dep	SD	% dep	SD	% dep	SD	% dep	SD	% dep	SD	% dep	SD
oxazolone	extreme (0.003)	82.3	0.38	99.2	0.17	-7.63	3.61	-15.3	4.03	22.7	3.41	96.0	0.00	-3.56	8.29
benzoquinone	extreme (0.0099)	100	0.00	100	0.00	100	0.00	20.0	8.69	0.25	1.32	100	0.00	3.38	0.91
DNCB	extreme (0.06)	97.4	0.31	97.7	0.90	5.87	9.20	-1.73	4.90	93.3	11.1	39.8	6.63	6.79	3.01
4-nitrobenzyl bromide	extreme (0.05)	95.0	0.48	100	0.00	84.8**	7.54	14.6	5.86	99.9	0.14	100	0.00	24.1	0.74
glutaraldehyde	strong (0.1)	74.4	1.15	100	0.00	1.45	3.38	6.24	8.70	0.86	2.70	96.2	0.00	9.57	1.42
hydroquinone	strong (0.11)	100	0.00	98.2	0.26	31.9	0.41	-1.08	1.66	0.42	1.26	83.8	0.16	3.54	0.84
PPD	strong (0.16)	99.0	0.82	25.0	5.61	27.3	3.79	22.6	8.75	-12.6	2.75	-3.00	1.86	1.65	1.65
benzyl bromide	strong (0.2)	100	0.00	77.3	1.61	40.1	10.1	3.24	6.14	60.5	0.91	94.8	0.80	-0.08	0.27
n-dodecyl gallate	strong (0.3)	72.8	3.77	85.3	2.53	-5.29	5.19	3.02	4.79	-2.04	5.12	6.17	1.91	-8.45	4.42
formaldehyde	strong (0.61)	31.5	2.16	26.2	6.26	-11.1	5.63	34.0	3.32	1.27	2.40	100	0.00	4.92	1.20
isoeugenol	moderate (1.7)	90.4	0.14	96.1	0.71	21.6	5.14	6.16	2.91	11.7	0.44	95.6	0.23	12.1	6.61
MPT	moderate (1.4)	100	0.00	61.5	10.5	3.50	6.82	0.92	8.24	29.0	1.44	90.2	2.16	-13.2	6.93
glyoxal	moderate (1.4)	-7.23	1.50	42.7	1.41	4.04	6.80	100	0.00	-0.13	1.57	100	0.00	1.42	1.26
2-hydroxyethyl acrylate	moderate (1.4)	100	0.00	100	0.00	14.1	6.83	10.8	9.02	3.73	2.78	55.3	3.92	-3.94	6.14
trans-2-decenal	moderate (2.5)	91.7	0.38	57.9	3.77	17.9	5.32	10.4	1.95	6.95	1.62	49.3	0.88	2.51	1.31
cinnamaldehyde	moderate (3.0)	97.3	0.00	58.8	1.64	18.9	4.16	14.4	8.54	-4.19	4.18	81.3	0.62	12.6	2.74
phenylacetaldehyde	moderate (3.0)	100	0.00	100	0.00	-0.13	4.37	35.0	1.61	-0.61	7.81	92.1	0.26	-3.98	1.45
2,4-heptadienal	moderate (4.0)	90.8	0.39	90.7	2.02	14.5	1.64	74.5	0.31	3.01	0.90	90.2	0.76	6.68	7.65
3,4-dihydrocoumarin	moderate (5.6)	-5.13	2.84	38.1	3.20	2.60	1.85	-1.62	6.89	6.60	4.50	61.3	0.62	12.9	2.68
12-bromo-1-dodecanol	moderate (6.9)	51.1	11.6	13.0	8.63	9.94	2.96	11.7	1.16	1.68	1.82	5.22	8.92	-2.39	1.67
hexylcinnamaldehyde	weak (11)	7.59	1.90	6.27	3.21	-2.64	8.63	-17.8	7.62	-7.30	2.45	1.55	2.28	5.85	0.76
1-bromododecane	weak (18)	74.4	11.3	10.2	1.90	4.87	9.31	-9.60	4.43	-4.27	4.43	-14.6	2.57	7.86	1.04
phenyl benzoate	weak (20)	76.2	3.16	20.3	5.60	9.75	3.88	-9.32	4.05	-0.54	3.39	-2.02	0.81	10.3	0.39

cinnamyl alcohol	weak (21)	22.7	1.13	8.38	3.17	4.94	1.72	16.2	1.38	16.6	2.63	32.8	3.23	1.17	3.74
cyclamen aldehyde	weak (22)	33.5	2.79	7.19	5.48	3.41	0.82	11.8	3.73	5.16	1.77	81.4	0.80	5.87	1.41
ethyl acrylate	weak (28)	92.4	0.14	94.0	2.75	3.94	4.63	-4.92	4.66	-0.64	1.03	30.0	2.11	3.40	0.98
hydroxycitronelal	weak (33)	72.7	4.18	10.9	6.48	6.36	1.23	0.97	4.64	4.45	1.38	91.2	0.57	3.69	1.63
glycerol	non-sensitiser	-1.47**	2.07	1.04	2.23	-8.93	1.04	-3.17	5.23	-0.64	3.89	-3.66	1.84	3.48	1.42
1-bromobutane	non-sensitiser	11.0	4.68	0.04	1.46	3.31	2.01	-4.40	1.14	-3.74	4.81	3.61	3.11	-3.30	3.23
2-acetylcyclohexanone	non-sensitiser	74.8	1.80	49.6	1.03	3.93	1.66	53.6	8.33	20.0	1.02	35.7	5.14	11.0	1.24
6-methyl coumarin	non-sensitiser	-7.59	0.78	12.7	3.48	7.26	3.38	-4.76	1.83	3.66	2.94	9.79	4.99	8.68	1.04
salicylic acid	non-sensitiser	-8.59	2.87	-9.06	5.93	2.56	1.24	-7.02	3.04	-5.85	1.33	2.47	1.79	-21.0	9.17
lactic acid	non-sensitiser	-12.7	0.38	1.16	0.26	9.44	2.85	12.5	3.55	-4.77	3.41	-11.7	0.55	1.41	1.69
benzaldehyde	non-sensitiser	100	0.00	26.7	0.14	36.2**	0.97	36.5	5.27	-10.5	9.97	82.1	0.49	4.62	1.70
2-hydroxypropyl methacrylate	non-sensitiser	100	0.00	33.1	1.65	17.1	2.60	8.46	4.47	0.10	6.24	6.96	9.67	10.2	4.77
1-butanol	non-sensitiser	10.1	9.61	6.27	4.17	-4.95	1.66	4.43	16.5	-8.36	6.03	5.96	9.99	-2.64	5.80

*Potency category and % EC3 data reported from Gerberick et al (2005); ** - values reproduced in simultaneous analysis were: for Cys-glycerol -4.03% (SD 1.51, $p=0.16$), for His-4-nitrobenzyl bromide 82.34% (SD 1.51, $p=0.62$) and for His-benzaldehyde 14.53% (SD 3.05, $p<0.05$).

Table 2: Spearman rank correlations between EC3s and depletion values for 6 peptides, and between three peptides that show strong correlation with EC3. A correlation coefficient (Spearman's ρ) is calculated based on 27 sensitisers (9 non-sensitisers are excluded). A corresponding p value indicates whether the correlation is statistically significant or not.

Variable 1	Variable 2	Spearman's ρ	p value (Prob> ρ)
EC3	Lys Depletion (%)	-0.69	<0.0001*
EC3	N-term Depletion (%)	-0.49	0.009*

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EC3	Cys Depletion (%)	-0.45	0.017*
EC3	His Depletion (%)	-0.21	0.28
EC3	Arg Depletion (%)	-0.12	0.55
EC3	Tyr Depletion (%)	-0.25	0.21
Lys Depletion (%)	Cys Depletion (%)	0.70	0.0001*
Lys Depletion (%)	N-term Depletion (%)	0.68	0.0001*
Cys Depletion (%)	N-term Depletion (%)	0.37	0.03

* statistically significant correlation

Table 3. Rate constants determined from kinetic data as described in the text. Values in parentheses are twice the standard error.

Peptide	Chemical	Rate constant (hour ⁻¹)
Cys	benzyl bromide	Fast
Cys	1-bromododecane	Slow, <0.00381
Cys	DNCB	Fast
Cys	cinnamaldehyde	Fast
Cys	MPT	1.46 (0.16)
Cys	phenyl benzoate	0.0516 (0.0063)
His	benzyl bromide	0.106 (0.025)

His	DNCB	Slow, <0.00369
His	cinnamaldehyde	Slow, <0.00274
N-term	benzyl bromide	Fast
N-term	3,4-dihydrocoumarin	0.0613 (0.0067)
N-term	cinnamaldehyde	Fast
Lys	benzyl bromide	2.048 (0.033)
Lys	1-bromododecane	Slow, <0.00826
Lys	DNCB	0.255 (0.039)
Lys	3,4-dihydrocoumarin	0.104 (0.026)
Lys	MPT	0.134 (0.071)
Lys	n-dodecyl gallate	0.275 (0.049)
Lys	phenyl benzoate	0.057 (0.028)
Lys	cinnamaldehyde	0.351 (0.099)
Tyr	benzyl bromide	Fast
Tyr	DNCB	0.3630 (0.0051)
Tyr	MPT	Slow, <0.00879

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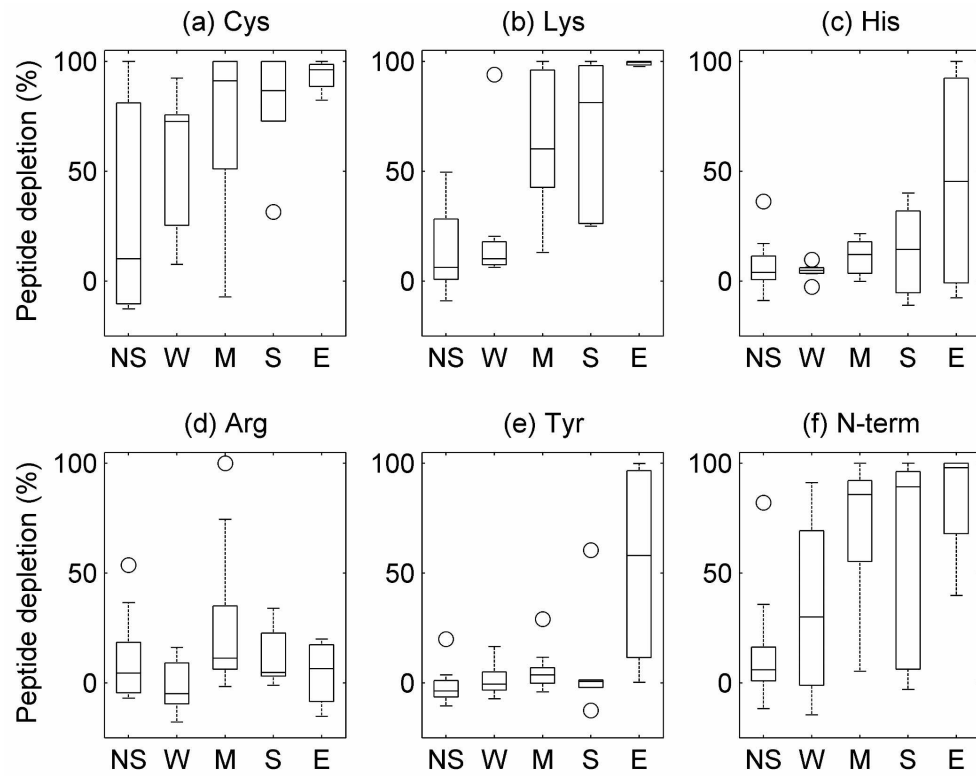


Figure 1

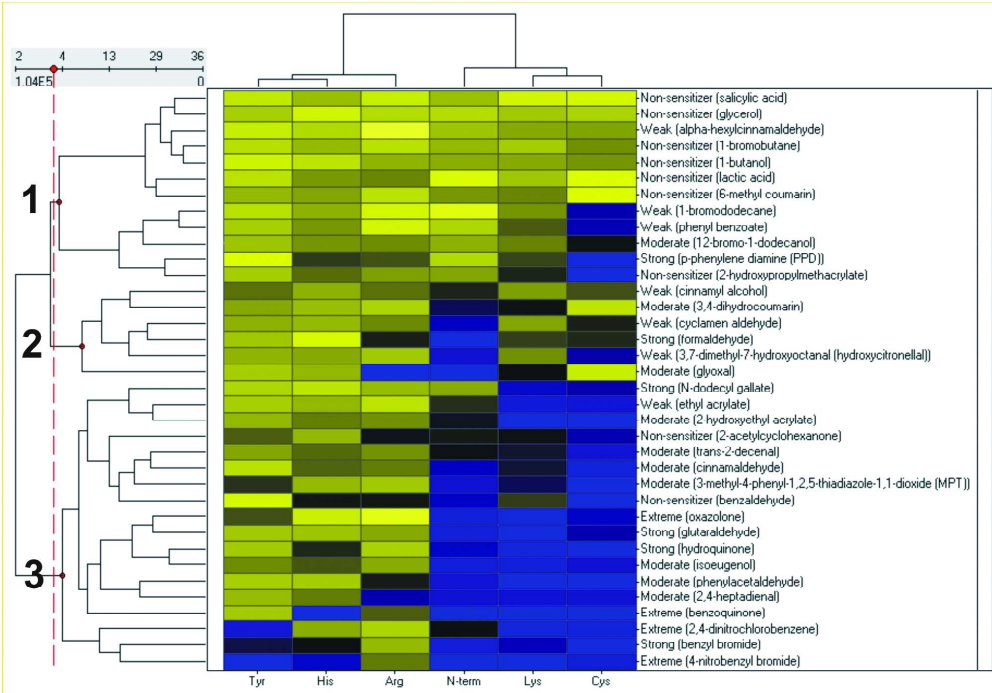


Figure 2

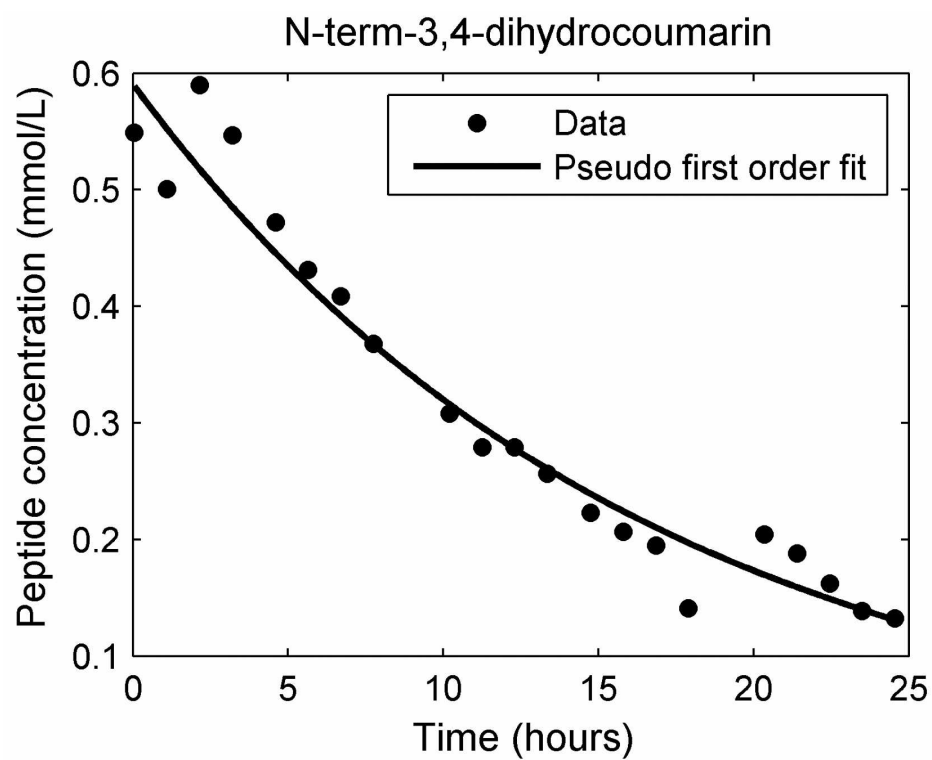


Figure 3

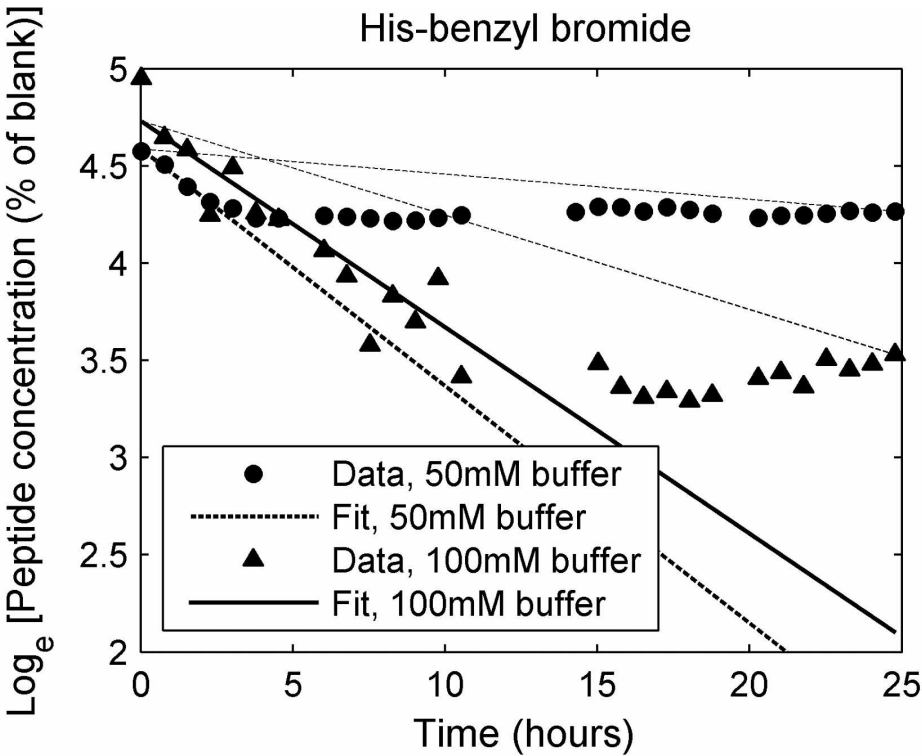


Figure 4

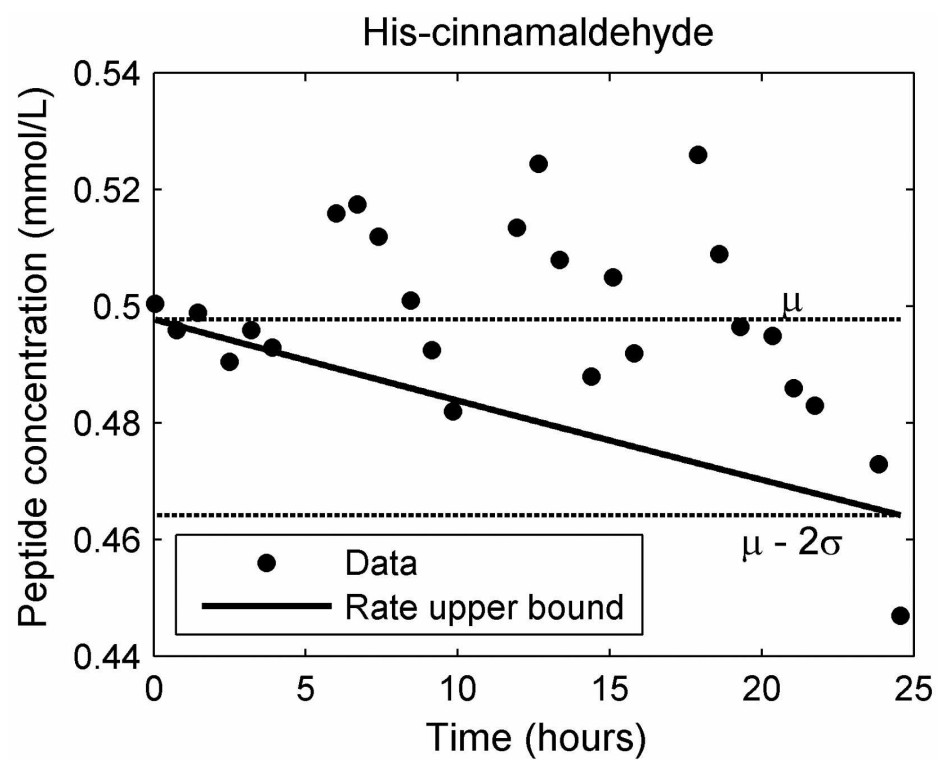
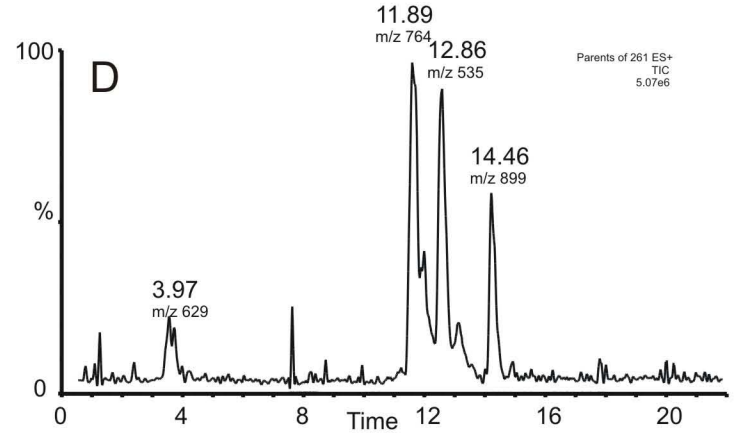
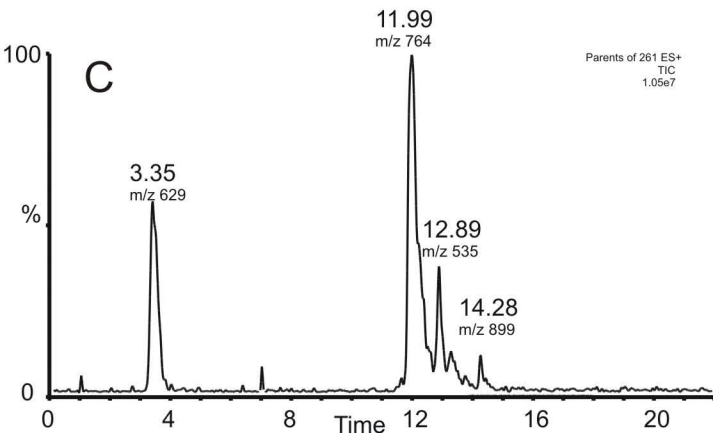
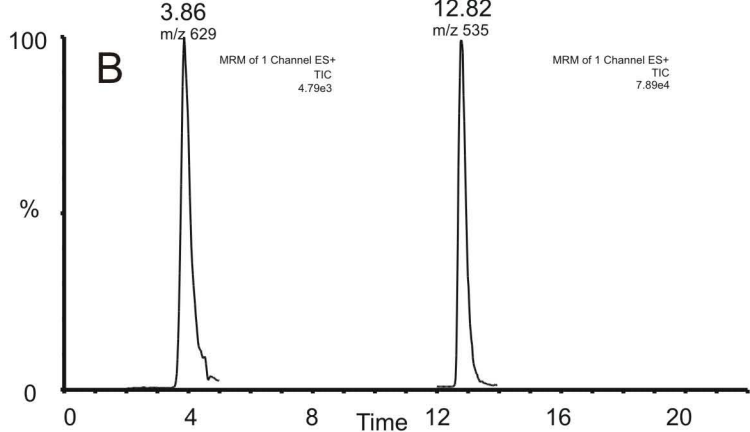
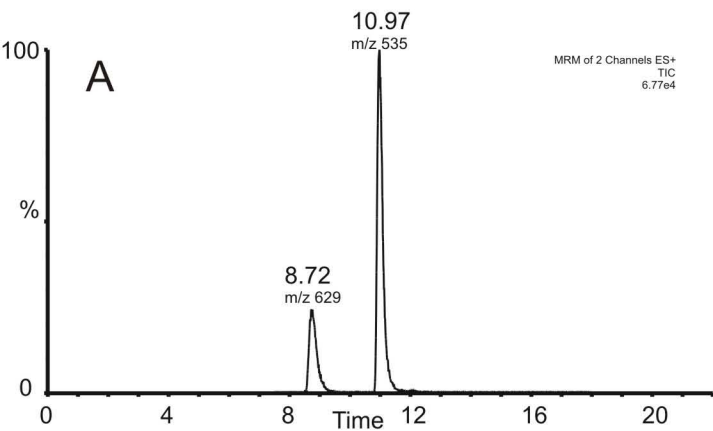


Figure 5



Supplementary data.

Table S1. Detailed information on adducts observed for 7 peptides tested with 36 chemicals

*Cys_d – Cys peptide dimerisation

Chemical	Cys (AcFAACAA, MW 594)			
	Cys _d *	MS signals observed (m/z)	Δ mass	Description of mechanism(s)
oxazolone	No	812 756	217 161	Michael addition followed by hydrolysis
benzoquinone	No	701	106	Michael addition
DNCB	Yes	761	166	S _N Ar
4-nitrobenzyl bromide	No	730 865	135 270	S _N 2 reaction Double S _N 2 adduct, either on thiol or on thiol and carboxyl group (C-term)
glutaraldehyde	No	677 759	82 164	Schiff base-like reaction, double adduct, possibly due to polymerisation of glutaraldehyde or interaction with acetonitrile
hydroquinone	No	719 735	124 140	Oxidation of hydroquinone to benzoquinone, then Michael addition followed by formation of sulfoxide or sulphone
PPD	Yes	701 717 733	106 122 138	Michael addition Formation of sulfoxide and sulphone
benzyl bromide	Yes	685 775 865	90 180 270	S _N 2 (single, double and triple adducts)
n-dodecyl gallate	Yes	915	320	Schiff base – like reaction
formaldehyde	No	607	12	Schiff base-like reaction
isoeugenol	No	775	180	Oxidation of isoeugenol followed by Michael addition
MPT	No	801	207	Michael addition
glyoxal	No	-	-	-
2-hydroxyethyl acrylate	No	710	116	Michael addition
trans-2-decenal	Yes	749 781	154 186	Michael addition followed by formation of sulphone
cinnamaldehyde	Yes	709 725	114 132	Schiff base – like reaction Michael addition
phenylacetaldehyde	Yes	-	-	Cys peptide dimerisation
2,4-heptadienal	No	687 (703 and 719) 704 (721 and 737)	92 (108 and 124) 110 (126 and 142)	Schiff base-like reaction (followed by formation of sulfoxide and sulphone) Michael addition (followed by formation of sulfoxide and sulphone)
3,4-dihydrocoumarin	Yes	-	-	Cys peptide dimerisation
12-bromo-1-dodecanol	No	779	184	S _N 2 reaction
hexylcinnamaldehyde	No	-	-	-
1-bromododecane	Yes	763	169	S _N 2 reaction
phenyl benzoate	Yes	699	104	Acylation with a loss of phenyl
cinnamyl alcohol	No	727	132	Michael addition
cyclamen aldehyde	Yes	-	-	Cys peptide dimerisation
ethyl acrylate	No	695	100	Michael addition
hydroxycitronelal	Yes	-	-	-
glycerol	No	-	-	-
1-bromobutane	No	651	56	S _N 2 reaction
2-acetylcyclohexanone	No	749 689	154 94	Schiff base-like reaction and formation of sulphone Unknown

6-methyl coumarin	Yes	754	160	Michael addition OR Acylation
salicylic acid	No	-	-	-
lactic acid	No	-	-	-
benzaldehyde	Yes	-	-	Cys peptide dimerisation
2-hydroxypropyl methacrylate	No	739 681 725	143 86 130	Michael addition followed by hydrolysis; unknown
1-butanol	No	-	-	-
Chemical	Lys (AcFAAKAA, MW 619)			
	MS signals observed (m/z)	Δ mass	Description of mechanism(s)	
oxazolone	791	171	Acylation, loss of ethanol and imine formation	
benzoquinone	multiple	-	Cyclised diquinone cross-linked species	
DNCB	786	166	S _N Ar reaction	
4-nitrobenzyl bromide	755 890	135 270	Single and double S _N 2 adducts	
glutaraldehyde	1304 + multiple fragment signals	-	Cross-linked species	
hydroquinone	multiple	-	Cyclised diquinone cross-linked species	
PPD	725 661	106 40	Michael addition of quinine diimine (product of PPD oxidation) Unknown	
benzyl bromide	710 800 890	90 180 270	Single, Double and Triple S _N 2 adduct	
n-dodecyl gallate	940	320	Schiff base formation	
formaldehyde	632	12	Schiff base formation	
isoeugenol	800 946	180 326	Oxidation of isoeugenol followed by Michael addition Double Michael addition	
MPT	878	258	Double Schiff base adduct from an MPT hydrolysis product	
glyoxal	660	40	Schiff base formation	
2-hydroxyethyl acrylate	735 691 851 807 763	115 71 231 187 143	Michael addition followed by hydrolysis Double Michael addition followed by hydrolysis of one adduct and hydrolysis of the second adduct	
trans-2-decenal	910	290	Double adduct (Schiff base and Michael adduct)	
cinnamaldehyde	734 752	114 132	Schiff base formation Michael addition	
phenylacetaldehyde	722	102	Schiff base formation	
2,4-heptadienal	712	92	Schiff base formation	
3,4-dihydrocoumarin	768	148	Michael addition OR Acylation	
12-bromo-1-dodecanol	804	184	S _N 2 reaction	
hexylcinnamaldehyde	818 838	198 217	Schiff base formation Michael addition	
phenyl benzoate	724	104	Acylation	
cyclamen aldehyde	790	170	Schiff base formation	
ethyl acrylate	720 764	100 144	Michael addition Double Michael addition followed by hydrolysis of both adducts	
hydroxycitronelal	772	152	Schiff base formation	
1-bromobutane	676	56	S _N 2 reaction	
2-acetylcyclohexanone	742 748	122 128	Schiff base formation Unknown	
6-methyl coumarin	780	160	Michael addition OR Acylation	
benzaldehyde	706	86	Schiff base formation	
2-hydroxypropyl methacrylate	706	86	Michael addition followed by hydrolysis	
	His (AcFAAHAA, MW 629)			

Chemical	MS signals observed (m/z)	Δ mass	Description of mechanism(s)
benzoquinone	737	108	Michael addition
DNCB	795	166	S _N Ar reaction
4-nitrobenzyl bromide	764 899	135 270	S _N 2 reaction Double S _N 2 adduct (one on His and one on the C-term carboxyl group)
hydroquinone	737	108	Oxidation of hydroquinone to benzoquinone followed by Michael addition
PPD	multiple	-	Evidence of cross-link?
benzyl bromide	719	90	S _N 2 reaction
isoeugenol	809	180	Oxidation of isoeugenol followed by Michael addition
2-hydroxyethyl acrylate	745 700 816 772	116 72 187 143	Michael addition followed by hydrolysis Double Michael addition followed by hydrolysis of one adduct and hydrolysis of both adducts
trans-2-decenal	783	154	Michael addition
cinnamaldehyde	761	132	Michael addition
2,4-heptadienal	739 771	110 142	Michael addition Unknown
ethyl acrylate	729	100	Michael adduct
hydroxycitronelal	755	126	Unknown
benzaldehyde	717 806	88 176	Single and Double Schiff base adducts
Arg (AcFAARAA, MW 647)			
Chemical	MS signals observed (m/z)	Δ mass	Description of mechanism(s)
4-nitrobenzyl bromide	783	135	S _N 2 reaction
glutaraldehyde	730	82	Schiff base formation
PPD	755	106	Michael addition of quinine diimine (oxidation product of PPD) weak signal
benzyl bromide	738	90	S _N 2 reaction
formaldehyde	660 690 720	12 42 72	Single Schiff base adduct Double Schiff base adduct (intermediate) Triple Schiff base adduct (intermediate)
MPT	752 778	105 131	Addition of MPT hydrolysis product Schiff base formation with MPT hydrolysis product
glyoxal	688 728 670	40 80 22	Schiff base formation Double Schiff base adduct Ring like structure formation
cinnamaldehyde	780 896	132 248	Michael addition Double adduct (Michael addition and Schiff base formation)
phenylacetaldehyde	764 900	116 252	unknown unknown
2,4-heptadienal	712	64	Unknown
2-acetylcyclohexanone	752 742	104 94	Formation of a ring-like structure Unknown
benzaldehyde	736	88	Schiff base formation
Tyr (AcFAAYAA, MW 654)			
Chemical	MS signals observed (m/z)	Δ mass	Description of mechanism(s)
oxazolone	872 816 758	217 162 104	Acylation (three electrophilic sites – three adduct possible) followed by hydrolysis of an adduct further adduct hydrolysis
DNCB	821	166	S _N Ar reaction
4-nitrobenzyl bromide	790	135	S _N 2 reaction
benzyl bromide	745	90	S _N 2 reaction
MPT	759	105	Acylation with and MPT hydrolysis product
2,4-heptadienal	747	92	Schiff base like reaction

12-bromo-1-dodecanol	839	184	S _N 2 reaction
phenyl benzoate	759	104	Acylation with loss of phenyl group
1-bromobutane	711	56	S _N 2 reaction
2-acetylcyclohexanone	783	128	Unknown
Chemical	N-term (FAAAAA, MW 520)		
	MS signals observed (m/z)	Δ mass	Description of mechanism(s)
oxazolone	692	171	Imine formation with loss of ethanol
benzoquinone	841 multiple	321 -	Triple Michael adduct Cyclised diquinone cross-linked species
DNCB	687	166	S _N Ar reaction
4-nitrobenzyl bromide	656 791	135 270	Single S _N 2 adduct Double S _N 2 adduct (at the N-term amine and C-term carboxyl group)
glutaraldehyde	multiple signals	-	Multiple glutaraldehyde adducts resulting from polymerisation and/or interaction with acetonitrile
hydroquinone	737 multiple	216 -	Double Michael adduct Cyclised diquinone cross-linked species
benzyl bromide	611	90	S _N 2 reaction
formaldehyde	533	12	Schiff base formation
isoeugenol	701	180	Oxidation followed by Michael addition
MPT	753	232	Schiff base formation with a hydrolysis product of MPT
glyoxal	561	40	Schiff base formation
2-hydroxyethyl acrylate	637 593	116 72	Michael addition followed by hydrolysis
trans-2-decenal	657 675 multiple	136 154 -	Schiff base formation Michael addition Evidence of a cross-linked species
cinnamaldehyde	635	114	Schiff base formation
phenylacetaldehyde	623	102	Schiff base formation
2,4-heptadienal	613	92	Schiff base formation
3,4-dihydrocoumarin	669	148	Michael addition OR Acylation
12-bromo-1-dodecanol	705	184	S _N 2 reaction
hexylcinnamaldehyde	719	198	Schiff base formation
cinnamyl alcohol	653 635	132 114	Michael addition Evidence of cinnamaldehyde Schiff base adduct (?possible conversion of alcohol to aldehyde or contamination?)
cyclamen aldehyde	693	172	Schiff base formation
ethyl acrylate	621	100	Michael addition
hydroxycitronelal	675	154	Schiff base formation
2-acetylcyclohexanone	643	122	Schiff base formation
6-methyl coumarin	681	160	Michael addition OR Acylation
benzaldehyde	609	88	Schiff base formation
Chemical	Ala (AcFAAAAA, MW 562)		
	MS signals observed (m/z)	Δ mass	Description of mechanism(s)
4-nitrobenzyl bromide	698	135	Single S _N 2 adduct at the carboxyl terminus