



Utility and limitations of a peptide reactivity assay to predict fragrance allergens *in vitro*

A. Natsch ^{a,*}, H. Gfeller ^a, M. Rothaupt ^a, G. Ellis ^b

^a Givaudan Schweiz AG, Ueberlandstrasse 138, CH-8600 Duebendorf, Switzerland

^b Givaudan Schweiz AG, Rue de la Parfumerie, Genève, Switzerland

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Abstract

A key step in the skin sensitization process is the formation of a covalent adduct between the skin sensitizer and endogenous proteins and/or peptides in the skin. A published peptide depletion assay was used to relate the *in vitro* reactivity of fragrance molecules to LLNA data. Using the classical assay, 22 of 28 tested moderate to strong sensitizers were positive. The prediction of weak sensitizers proved to be more difficult with only 50% of weak sensitizers giving a positive response, but for some compounds this could also be due to false-positive results from the LLNA. LC–MS analysis yielded the expected mass of the peptide adducts in several cases, whereas in other cases putative oxidation reactions led to adducts of unexpected molecular weight. Several moderately sensitizing aldehydes were correctly predicted by the depletion assay, but no adducts were found and the depletion appears to be due to an oxidation of the parent peptide catalyzed by the test compound. Finally, alternative test peptides derived from a physiological reactive protein with enhanced sensitivity for weak Michael acceptors were found, further increasing the sensitivity of the assay.

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1. Introduction

The risk of contact sensitization is a critical issue in the development of novel ingredients for cosmetic products. A key step in the skin sensitization process is the formation of a covalent adduct between the skin sensitizer and endogenous proteins and/or peptides in the skin. The modified peptides then trigger a specific T-cell mediated immune response. One possibility to predict skin sensitization based on *in vitro* data is therefore the evaluation of the reactivity of a test compound towards peptides and proteins (reviewed by Divkovic et al., 2005). Gerberick et al. (2004a) found promising results with a peptide depletion assay, in which a peptide is incubated with an excess of a test chemical, and the unmodified peptide is detected with HPLC, assuming that loss of the peptide signal is due to

a covalent modification by the test chemical. Glutathione (GSH) and synthetic heptapeptides with a reactive Lys, His or Cys residue were tested, and the Cys-peptide gave the highest accuracy and highest sensitivity: Among 26 sensitizers, 21 gave depletion of the Cys-peptide; among 11 non-sensitizers only 1 compound depleted the peptide.

The risk for contact sensitization is currently estimated with the local lymph node assay in mice (LLNA), in which the cellular proliferation in the draining lymph nodes is measured after repeated topical application of the test compound onto the ears. Results are expressed as EC3 values indicating the concentration which induces a threefold enhanced cellular proliferation.

Here we used the peptide depletion assay proposed by Gerberick et al. (2004a) to relate peptide reactivity to our database on LLNA data for fragrance molecules and we tested several optimizations of the assay: (a) Measuring formed adducts by LC–MS analysis, (b) measuring dose-response curves and (c) using alternative peptide sequences

* Corresponding author. Tel.: +41 44 824 21 05; fax: +41 44 824 29 26.
E-mail address: andreas.natsch@givaudan.com (A. Natsch).

derived from proteins which are known to be reactive towards electrophiles instead of the synthetic sequence proposed before.

2. Materials and methods

All fragrance chemicals are commercial qualities obtained from Givaudan Schweiz, Genève, Switzerland. All other test chemicals were purchased from Fluka, Switzerland. The synthetic peptides were obtained from GenScript Inc. (Piscataway, NJ, USA). The sequences of the tested peptides are summarized in Table 1. Three novel peptide sequences were derived from proteins with specific Cys residues with a very high reactivity according to Dennehy et al. (2006). Among the 125 Cys residues identified by Dennehy et al. reacting with both target electrophiles, we selected peptides around Cys residues with very high frequency of adduct formation. Two further peptides were derived from the region with known reactive Cys residues in the human protein Keap 1 (Eggler et al., 2005; Wakabayashi et al., 2004). Among the reactive residues in Keap1, those were selected which yielded a peptide containing an aromatic amino acid for ease of HPLC detection.

The peptide reactivity assay in HPLC vials was conducted as described by Gerberick et al. (2004a) with the following modifications: Concentration of the test peptide was 0.25 mM instead of 0.5 mM and the concentration of the phosphate buffer was 20 mM instead of 100 mM. With these modifications less peptide precipitation was observed. The samples were incubated for 24 h at 30°C with shaking at 140 rpm prior to analysis by HPLC with a DAD detector. Five microliter of the samples was injected onto a Waters polarity column, 2.1 mm ID × 150 mm, 5-Micron. The mobile phase consisted of ammonium formate (20 mM) in 5% formic acid (pH 5) (A) and methanol (B). A gradient with 85% (A) for 1 min and then from 85% (A) to 100% (B) over 17 min at a flow rate of 0.15 mL/min was used for separation. Chromatograms were extracted at 214 nm. Peptide depletion was expressed as the concentration of the peptide compared to control samples with the peptide in solvent only. To measure dose-response curves, reactivity assays were performed under equal conditions in microtiter plates in a final volume of 100 µL. The unreacted cysteine in the parent peptide was then determined by addition of 50 µL of a solution of monobromobimane (0.75 mM in 100 mM NaCO₃, pH 8.8) and

reading of the fluorescence at 385/480 nm. GSH depletion assays were performed under equal incubation conditions as for the other peptides, but unreacted GSH was detected with the enzymatic test described by Tietze (1969). To detect adducts of the peptides with the test chemicals, LC–MS analysis was performed on a Finnigan LCQ classic Mass spectrometer (Thermo Finnigan, San Jose, CA, USA) operated in the ESI(+) mode. The ion source was operated at 5 kV, the sheath gas flow rate was set to 40 arb and the aux gas flow rate to 10 arb. The temperature of the capillary was kept at 220 °C. Mass spectra were recorded from 200 to 2000 amu. For the peptide Cor1–C420 a ZORBAX Eclipse XDB-C18 column, 2.1 mm ID, 150 mm, 5-Micron (Agilent Technologies) was used. The mobile phase consisted of H₂O (A) and CH₃CN (B) each containing 1% HOAc (v/v). The solvent flow was 300 µL/min and the following gradient was used: 0–0.5 min, 80% A; 0.5–6 min, from 80% A to 98% B; 6–10 min, 98% B. For the peptide Cys-react a VYDAC C18 column modified for proteins and peptides, 2.1 mm ID, 250 mm, 5-Micron, (VYDAC, Hesperida, CA, USA) and the same mobile phase was used. The solvent flow was 250 µL/min with the following gradient: 0–0.5 min, 95% A; 0.5–10 min, from 95% A to 95% B; 10–12 min, 95% B. LLNA data on fragrance materials were determined under standard conditions as defined in the OECD guideline 429. Further, LLNA data are either from the literature and are summarized in Gerberick et al. (2004a,b) or from the Research institute on fragrance materials RIFM.

3. Results

3.1. Screening for peptide depletion

Table 2 lists the peptide depletion results for all test chemicals with the original Cys-peptide (CysReact) and with GSH along with their sensitization potential as predicted from the LLNA. Twenty-one of 28 moderate and strong sensitizers yielded a peptide depletion of >50% and one moderate sensitizer gave >20% depletion. Moderate allergens which gave no peptide depletion included dihydroeugenol, 3,4-dihydrocoumarin and benzylsalicylate. These chemicals probably need a metabolic activation in order to become sensitizers. The moderate sensitizers bisabolene and longifolene were not reactive *per se*, but peptide depletion was noted in the case of bisabolene after oxidation under pure O₂ for 7 days. Among the weak aller-

Table 1
Peptides used in this study

Name	Sequence	Sequence origin
CysReact	Ac-RFAACAA	Cys-peptide described by Gerberick et al. (2004)
Keap-257	Ac-KYDCEQR	Reactive Cys257 in human Keap 1
Keap-297	Ac-DSRCKDY	Reactive Cys297 in human Keap 1
RP17-34	Ac-KRVCEEF	Cys34 in Human ribosomal protein S17, amino acid 7 changed from I to F for ease of detection in HPLC
Cor1–C420	Ac-NKKCDLF	Cys420 of human Coronin 1 C; amino acid 7 changed from I to F for ease of detection in HPLC
Cofil1-138	Ac-QANCYEE	Cys138 in Cofilin-1

Table 2
Peptide depletion of fragrance chemicals with GSH and CysReact

Chemical or trivial name	CAS Nr.	EC3 values LLNA	Sensitisation class	GSH Depletion (%)	CysReact Depletion (%)
Oxazolone	15646-46-5	0.003	Strong	98.4 ± 0.1 ^a	95.4 ± 1.7 ^b
1-Chloro-2,4-dinitrobenzene	97-00-7	0.04	Strong	93.5 ± 7.2	100 ± 0
Lauryl gallate	1166-52-5	0.3	Strong	24.4 ± 9.9	94.4 ± 0.9
Formaldehyde	50-00-0	0.7	Strong	90.5 ± 2.3	66.9 ± 1.5
Methyl 2-octynoate	111-80-8	<0.5	Strong	n.d.	100 ± 0
7-(3-Methylbutyl)-2H-1,5-benzodioxepin-3(4H)-one	362467-67-2	1.4	Moderate	45.4 ± 5.7	100 ± 0 ^c
5,6,7-trimethyl-(2E)-2,5-Octadien-4-one	357650-26-1	1.6	Moderate	82.2 ± 0.3	100 ± 0
Isoeugenol	97-54-1	1.8	Moderate	55.1 ± 3.3	100 ± 0
Longifolene	475-20-7	1.8	Moderate	n.d.	2.1 ± 5.6
Oxidised Longifolene	475-20-7	n.d.	Moderate	n.d.	0.5 ± 10.1
Diethyl maleate	141-05-9	2.1	Moderate	99 ± 0.2	98.2 ± 1.6
1-Spiro[4.5] dec-7-en-7-yl-4-penten-1-one	224031-70-3	2.2	Moderate	32.5 ± 4.1	98.8 ± 2.13
trans-2-Decenal	3913-81-3	2.5	Moderate	36 ± 1.4	100 ± 0
(Z)-ethyl 2-acetyldec-2-enoate		2.6	Moderate	30.9 ± 3	77.5 ± 13.2
trans-Anethole	104-46-1	2.7	Moderate	2.1 ± 3	21.7 ± 9
Benzyl salicylate	118-58-1	2.9	Moderate	−6.7 ± 3.6	−9.5 ± 6.7
Galbanone	56973-85-4	3	Moderate	61.4 ± 1.9	75.5 ± 4.73
Phenylacetaldehyde	122-78-1	3	Moderate	20.4 ± 2.3	65.8 ± 2.73
Cinnamic aldehyde	104-55-2	3.1	Moderate	21.3 ± 3.3	85.9 ± 12.5
α-Methyl cinnamic aldehyde	101-39-3	4.5	Moderate	12.6 ± 2.3	7.5 ± 4.7
Citrathal R (reaction product of citral)		4.9	Moderate	18.1 ± 9.1	100 ± 0
Citral	5392-40-5	~5	Moderate	21.2 ± 3	94.8 ± 5.3
trans-2-Hexenal	6728-26-3	5.5	Moderate	61.9 ± 2.2	100 ± 0
3,4-Dihydrocoumarin	119-84-6	5.6	Moderate	−7.1 ± 1.3	4 ± 9.4
Damascone beta	23726-91-2	6.7	Moderate	81.5 ± 0.4	100 ± 0
Dihydroeugenol	2785-87-7	6.8	Moderate	0 ± 0.8	0.8 ± 2.89
2-methyl-3-(4-(2-methylpropyl)phenyl)-propanal	6658-48-6	9	Moderate	6.1 ± 5.1	100 ± 0
Cyclal C	68039-49-6	3 to 10	Moderate	−2.1 ± 2.1	100 ± 0
Bisabolene	495-62-5	8.6	Moderate	−5.4 ± 3.2	−3.7 ± 9.5
Oxidised Bisabolene	495-62-5	n.d.	Moderate	25.8 ± 0.2	100 ± 0
6-(1-methylpropyl)quinoline	65442-31-1	11.2	Weak	3.2 ± 4.9	6.3 ± 2.4
Eugenol	97-53-0	13	Weak	n.d.	54.3 ± 0
α-Hexyl cinnamic aldehyde	101-86-0	14	Weak	32.3 ± 3.1	63.7 ± 9
3-methyl-(5Z)-5-cyclotetradecen-1-one	259854-71-2	16.4	Weak	1 ± 1.6	6 ± 3.1
Lylal	31906-04-4	17.1	Weak	8.2 ± 6.4	95.2 ± 2
1-(Cyclopropylmethyl)-4-methoxybenzene	16510-27-3	16.7	Weak	1 ± 6.9	−1.56 ± 1.25
Benzyl cinnamate	103-41-3	18.4	Weak	6.9 ± 2.3	−1.5 ± 2.7
Lilial	80-5406	19	Weak	13.6 ± 1.3	47 ± 3.7
6-Ethyl-3-methyl-6-octen-1-ol	26330-65-4	19.3	Weak	7.7 ± 1.4	−3.1 ± 7.2
Cinnamic alcohol	104-54-1	21	Weak	−1.8 ± 4.4	10.8 ± 2
Cyclamen aldehyde	103-95-7	22.3	Weak	4.1 ± 4.1	100 ± 0
Cis-6-nonenal	2277-19-2	23.1	Weak	n.d.	53.4 ± 6.6
Hydroxycitronellal	107-75-5	23	Weak	38.3 ± 0.8	77.1 ± 4.18
Cyclopropanecarboxylic acid, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester	477218-42-1	27.8	Weak	1.5 ± 2.5	10.8 ± 3.5
Methyl atrarate	4707-47-5	28.1	Weak	−3 ± 2.6	100 ± 0
Ambrettolide	63286-42-0	28.8	Weak	−0.8 ± 3.1	8.3 ± 4.47
Linalool	78-70-6	30	V.weak/none	−9.2 ± 5.1	1.7 ± 2.5
1,1,3-Trimethyl-3-phenylindane	3910-35-8	43.3	V.weak/none	4.4 ± 5.6	12.8 ± 6.7
Isopropyl myristate	110-27-0	44	V.weak/none	−2.2 ± 7.3	4.2 ± 0.9
2-methyl-butanoic acid hexyl ester	10032-15-2	54.8	V.weak/none	n.d.	8.6 ± 5.5
Geraniol	106-24-1	57	V.weak/none	−9.2 ± 0.8	−1.39 ± 1.14
Hexenol-2-trans	928-95-0	60	V.weak/none	−1 ± 4	17.6 ± 1.4
Pyridine	110-86-1	72	V.weak/none	−3.9 ± 3.9	9.9 ± 7.2
2,3-dihydro-2,3,3-trimethyl-1H-Inden-1-one	54440-17-4	>100	V.weak/none	−0.5 ± 3.6	7.6 ± 1.65
6-methoxy-2,6-dimethyl octanal		>100	V.weak/none	8.2 ± 1.4	52 ± 5.9
Glycerol	56-81-5	>100	V.weak/none	−8.9 ± 3	4.2 ± 6
Propylene glycol	57-55-6	>100	V.weak/none	−4 ± 8.5	12.8 ± 6.8
Methyl salicylate	119-36-8	>20	V.weak/none	5.6 ± 8.8	2.4 ± 0.35
Ethylene brassylate	105-95-3	>30	V.weak/none	8.6 ± 1.1	−2.2 ± 18.3
Calone	28940-11-6	>30	V.weak/none	n.d.	3.8 ± 0.7
2-Hydroxybenzoic acid	69-72-7	>40	V.weak/none	−6.8 ± 2.3	−20.8 ± 12

(continued on next page)

Table 2 (continued)

Chemical or trivial name	CAS Nr.	EC3 values LLNA	Sensitisation class	GSH Depletion (%)	CysReact Depletion (%)
Hedione	24851-98-7	>40	V.weak/none	3 ± 2.3	4.1 ± 2.3
Benzyl benzoate	120-51-4	>50	V.weak/none	−2.1 ± 0.1	0.2 ± 0.8
Vanillin	121-33-5	>50	V.weak/none	−2.8 ± 3.2	36.8 ± 20.1
Diethyl phthalate	84-66-2	>50	V.weak/none	−4.6 ± 1.1	0.5 ± 1.02
Methyl paraben	99-76-3	>50	V.weak/none	−14 ± 1.1	1.2 ± 1.08
Benzyl alcohol	100-51-6	>50	V.weak/none	−0.7 ± 10.8	5.5 ± 0.8
Phenyl ethyl alcohol	60-12-8	>50	V.weak/none	−0.3 ± 0.9	4 ± 1.9
β-Ionone	79-77-6	NA	V.weak/none ^d	5.7 ± 4.2	52.5 ± 1.42

^a Decrease of GSH concentration as measured with the enzymatic method of Tietze (1969).

^b Decrease of CysReact concentration as measured with HPLC.

^c The reactive species was later found to be the contaminant 4-(4-methylpentyl)-catechol.

^d Based on Guinea pig maximisation test.

gens tested, only 8 of 16 gave peptide depletion which was significantly above a 10% threshold, and among the chemicals rated as very weak/non-sensitizers by the LLNA or GPMT there were 4 false-positives among 23 chemicals tested (3 out of 22 if only LLNA data are taken into account). In agreement with the data of Gerberick et al. (2004a), GSH is less reactive and depletion for most chemicals was less pronounced.

3.2. Dose-response of peptide depletion with different peptides

To quantify reactivity, the assay with a single concentration of the test chemicals has limitations. For a series of test compounds, dose-response curves were therefore measured to better compare reactivity. The alternative peptides described in the materials and methods section were included in these assays in order to find the most reactive test peptides. Fig. 1 shows the dose-response curves for β-damascone. The lowest reactivity was noted for *N*-acetyl-Cys, and the highest reactivity for the peptide Cor1–C420, which has two Lys residues next to Cys. Table 3 lists EC50 values for a range of compounds towards the

different test peptides in comparison to the LLNA EC3 values. All the tested compounds with low EC3 values in the LLNA had a high reactivity towards the different peptides, with the exception of α-methyl cinnamic aldehyde. The peptide Cor1–C420 gave the lowest EC50 values for all the test compounds, indicating that it is the most reactive peptide and it could therefore be used in the future as a more sensitive test peptide for *in vitro* reactivity assays.

3.3. LC–MS analysis

Simply measuring peptide depletion does not give any information on the reactions taking place between the peptide and the test chemicals. Therefore, LC–MS analysis was used in a next step. Table 4 lists the observed adducts detected for several compounds. In many cases Michael-addition adducts with α,β-unsaturated ketones and aldehydes of expected molecular weight were detected. As an example the LC–MS analysis of the adduct between Cor1–C420 and Galbanone is shown in Fig. 2. Some adducts had mass shifts of +16 or +32, indicating that oxidations took place. Especially interesting is the case of isoeugenol: a range of different adducts were observed, reflecting the high reactivity of this molecule. Currently different oxidation and probably dimerization processes are the only explanation for these apparent molecular masses, but further work is needed for structure elucidation of these adducts. For many aldehydes significant peptide depletion was observed but no adducts were found. Detailed analysis showed that with the peptide CysReact the aldehydes induced precipitation of the peptide, and the resuspended precipitate was found by LC–MS to be the oxidized dimer of the peptide (MW 1499). Similarly, in reactions with Cor1–C420 a peak with MW 1816 corresponding to the oxidized dimer was found in reactions of several aldehydes (e.g. Citral, cinnamic aldehyde, 2-methyl-3-(4-(2-methylpropyl)phenyl)-propanal; Fig. 2.). Furthermore, a significant peak with MW 798.5 was observed in incubations of hydroxycitronellal with CysReact, and a peak with MW 956.3 was formed upon incubation of this aldehyde with Cor1–C420. These molecular

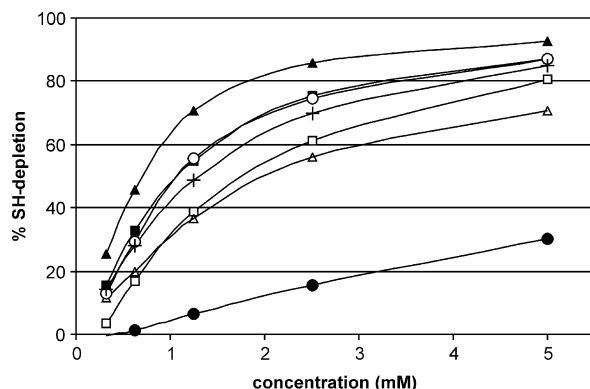


Fig. 1. Dose-response for the peptide depletion of different test peptides at 0.25 mM in presence of different doses of β-damascone. Acetyl-Cys, closed circle; Keap-297, open circle; Keap-257, closed square; CysReact, open square; Cor1–C420, closed triangle; Cofilin-138, open triangle; RP17-34, open diamond.

Table 3
Comparison of reactivity of selected compounds with different peptides

	LLNA EC3	Acetyl-Cys	Cys- React	Cor1–C420	Cofil1-138	Keap-257	Keap-297	RP17-34
α -Methyl cinnamic aldehyde	4.5	>10 ^a	>10	>10	>10	>10	>10	>10
Cinnamic aldehyde	3.1	3.06	2.74	1.39	2.53	4.04	3.00	2.20
Citral	5	>10	7.03	4.86	>10	9.81	>10	9.39
Diethylmaleate	2.1	0.48	<0.31	<0.31	<0.31	<0.31	<0.31	<0.31
5,6,7-trimethyl-(2E)-2,5-Octadien-4-one	1.6	1.55	0.40	0.26	0.53	0.33	0.35	0.31
β -ionone	n.a.	>10	8.93	6.53	11.21	>10	>10	6.65
Galbanone	3	>10	6.51	2.63	11.02	4.30	4.38	3.47
β -Damascone	6.7	>10	1.78	0.70	1.99	1.08	1.10	1.31
Eugenol	13	>10	4.27	2.26	8.82	7.25	9.51	3.89
Isoeugenol	1.8	1.42	0.63	0.57	1.25	1.01	0.85	0.58

^a Given is the EC50 value in mM indicating the concentration of test chemical giving 50% depletion of 0.25 mM of the test peptide after 24 h incubation. Peptide depletion measured as loss of the free SH group determined with monobromobimane.

Table 4
Peptide adducts observed with LC MS

Parent molecule	MW	Test peptide and MW of peptide	Observed adduct (M + H)	Comment
5,6,7-trimethyl-(2E)-2,5-Octadien-4-one	166	CysReact, 750.5	916.6	
5,6,7-trimethyl-(2E)-2,5-Octadien-4-one	166	Cor1–C420, 909	1075.4	
Galbanone	192	Cor1–C420, 909	1101.4	
Cinnamic aldehyde	132	CysReact, 750.5	883.6	
Citral	152	CysReact, 750.5	903.3, traces	
β -Damascone	192	Glutathione, 307	500	
α -methyl cinnamic aldehyde	146	CysReact, 750.5	897.4, traces	
Diethylmaleate	172	CysReact, 750.5	923.3	
Trans-2-hexenal	98	CysReact, 750.5	849.3	
Dec-2-enal	154	CysReact, 750.5	937.2	Expected mass + 32
β -Ionone	192	CysReact, 750.5	975.4	Expected mass + 32
β -Ionone	192	Cor1–C420, 909	1133.4	Expected mass + 32
Isoeugenol	164	GSH, 307	633.9 and 798	Corresponds to GSH with di-mer or tri-mer of isoeugenol
Isoeugenol	164	CysReact, 750.5	931.3/947.3/1077.5	Expected mass + 16/Expected mass + 32/750.5 + 2 × 164 – 2
Isoeugenol	164	Cor1–C420, 909	1089.4	Expected mass + 16

weights would correspond to the MW of the parent peptides + 48, which could be explained by the peptide oxidized at the cysteine to cysteic acid, but further work is needed to confirm this hypothesis. The same peaks were also noted after incubations of the peptides with several other aldehydes. Taken together, these data suggest that many tested aldehydes catalyze different peptide oxidation reactions by some unknown mechanism, and peptide depletion does not always indicate formation of covalent adducts.

4. Discussion

The high accuracy and sensitivity of the peptide depletion assay reported by Gerberick et al. (2004a) prompted us to use this assay with a larger dataset of known reference molecules and many fragrance chemicals of known sensitization potential in order to evaluate its use for the fragrance industry. For those molecules tested in both studies, reproducibility was high and we also found a very good positive prediction of moderate and strong allergens.

In many cases, adducts of these moderate allergens with the peptides could also be verified with LC–MS. We included more weak allergens in our study, and the correct prediction of the LLNA data for these molecules proved to be more difficult. However, some of the weak sensitizers according to the LLNA, which gave no peptide depletion, do not possess structural alerts for skin sensitization (e.g. 3-methyl-(5Z)-5-cyclotetradecen-1-one, 1-(Cyclopropylmethyl)-4-methoxybenzene, Ambrettolide). The possibility that these molecules are false-positive in the LLNA, and not false-negative in the peptide reactivity assay cannot be ruled out. LC–MS analysis revealed a limitation of the assay: Several weakly or moderately sensitizing aldehydes would be correctly classified as sensitizers by our results and three of them were also tested and rated positive in the Gerberick study, but the observed depletion appears not to be due to a peptide reactivity of the compound, which is the underlying assumption of the test. Therefore, these results may be classified as ‘false-correct’. For one aldehyde (6-methoxy-2,6-dimethyl octanal) which was non-sensitizing in the LLNA a positive result was found

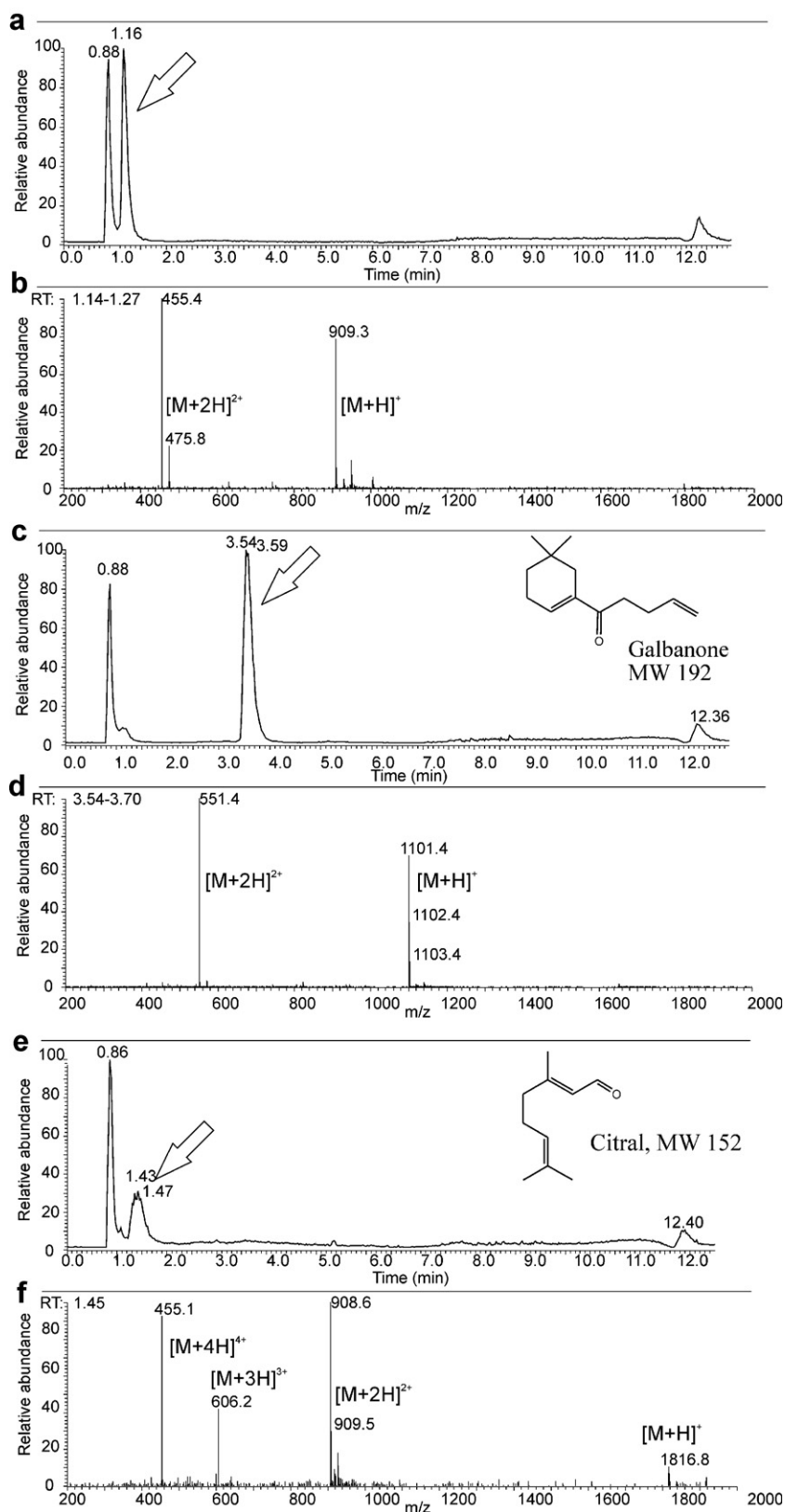


Fig. 2. LC–MS analysis of parent peptide Cor1–C420 and adducts/dimers formed after incubation with test chemicals. (a) Total ion chromatogram of the parent peptide; (b) MS-analysis of the peak at RT 1.16 min; (c) Total ion chromatogram of the peptide reacted with Galbanone; (d) MS-analysis of the peak at RT 3.6 min; (e) Total ion chromatogram of the peptide reacted with Citral and (f) MS-analysis of the peak at RT 1.45 min.

in the depletion assay probably also due to peptide oxidation (false-positives). Therefore, the assay does not yet help to classify aldehydes which have no α,β -unsaturation, and these compounds are probably better predicted with molecular modelling as proposed by Roberts et al. (2006). Another option is to use Lys-containing peptides and reaction conditions which facilitate Schiff-base formation and to screen aldehydes for their potential to form Schiff-bases. Nevertheless, especially the reactivity of α,β -unsaturated compounds and phenolic allergens can be rated with the current peptide assay. The proposed modifications with (i) more reactive peptides derived from physiologically reactive proteins, (ii) dose-response curves and (iii) LC–MS analysis will help for an estimation of the reactivity and the sensitization risk of novel members of these important structural classes early in the development process without the use of animal testing. Especially, the novel peptide Cor1–C420 derived from the proteomic reactivity study of Dennehy et al. (2006) appears interesting: in addition to its very high reactivity towards electrophiles it is perfectly soluble and no precipitates are observed. Eventual oxidation can therefore be monitored in LC–MS along with adduct formation, which will make this assay even more meaningful.

In several cases LC–MS analysis indicated formation of unexpected adducts or peptide oxidation products. Thus, isoeugenol gave several adducts which currently can only be explained by dimerization/trimerization of the test chemicals and several oxidation reactions. Elucidation of the structure of these and other adducts will be a subject

of further studies to better understand the peptide reactivity of these compounds.

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