Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information for

Development of a Chromophore-Solid Phase Peptide Reaction Assay (C-SPRA) for Assessing Skin Sensitization *In Vitro*

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Table of Contents

Experimental Procedures

Test chemicals and materials

The test chemicals included *p*-benzoquinone (BQ), fluorescein-5-isothiocyanate (FITC), 2-mecarptobenzotiazole (MBT), benzylidene acetone (BA), 5-methyl-2-phenyl-2-hexanal (MPH), undec-10-enal (UE), α-hexyl cinnamic aldehyde (HCA), α-amy cinnamic aldehyde (ACA), benzyl benzoate (BB), benzyl cinnamate (BC), R(+)-limonene, benzylbutylphthalate (BBP), dibutyl phthalate (DP), and isopropanol (IPA). All test chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Special grade reagents were used for this study. The test chemicals were dissolved in HPLC grade N,N-dimethyl formamide (DMF). All chemicals were used without further purification.

Synthesis of model peptides

The Cys- and Lys-peptide resins were synthesized on TentaGel S NH_2 resin (HiPep Laboratories, Kyoto, Japan) by manual synthesis with Fmoc chemistry,[S1] and Fmoc-peptides were synthesized by the [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HBTU)–1-hydroxy benzotriazole monohydrate (HOBt) method. Initially, Fmoc-β-Ala-OH was coupled to the resin, and then the DPRA peptide sequences (Ac-RFAAKAA-βA-PEG resin and Ac-RFAAKAA-βA-PEG resin) were synthesized. The side-chain-protecting groups used were *t*-butyloxycarbonyl (Boc) for Lys, trityl (Trt) for Cys, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. The side-chain-protecting groups on the peptidyl resins were removed by incubating the peptide-resin for 2 h in deprotection solution [trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (20:1:1, v/v)]. The resins were washed 5 times, each with a deprotection solution and chloroform, and then the resins were dried completely in a desiccator. The peptide loading on the resin, peptide purity and amino acid content of each peptide-resin were evaluated by amino acid analysis. Amino acid analysis was performed using an Inertsil ODS-2 column (4.6 × 200 mm; GL Sciences, Tokyo, Japan) after samples were hydrolyzed in 6 M HCl at 110°C for 96 h in a sealed tube and then labeled with phenyl isothiocyanate. All Fmoc amino acids were purchased from HiPep Laboratories. HBTU and HOBt were purchased from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). In parallel, we synthesized the same sequences in the same conditions on the cleavable resin [TentaGel SRAM resin (HiPep Laboratories)]. Then the respective purities were checked by HPLC (GL-7400 HPLC system (GL Sciences, Tokyo, Japan) using an Inertsil ODS-3 column (4.6 × 150 mm; GL Science) with a linear acetonitrile/0.1% trifluoroacetic acid (TFA) gradient, at a flow rate of 1.0 mL/min, after the cleavage [TFA/H_2O/triisopropylsilane (20:1:1, v/v) for 2 h] (Figure S2). The peptides were analyzed using MALDI-TOF MS on an Autoflex III (Bruker Daltonics, Billerica, MA, USA) mass spectrometer with 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix.

Procedure using Cys-peptide resin

Cys-peptide resin (0.5 mg as dry resin) was placed in a LibraTube (HiPep Laboratories), washed 6 times with DMF. 1) The swelled resin was incubated with 1 mL of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Dojindo Laboratories, Kumamoto, Japan) solution [in pH 7.5 phosphate buffer (20 mM)-DMF (2:1)] for 30 min at room temperature (20°C – 25°C) (moles of peptide : moles of DTNB = 1:80 – 1:100), and then washed 6 times with DMF for removal of TNBs and the unreacted DTNB. 2) The Cys-peptide resin was incubated with 1 mL of 10 mM dithiothreitol (DTT) (FUJIFILM Wako Pure Chemical Corporation) solution [in pH 7.5 phosphate buffer (20 mM)-DMF (2:1)] (the release solution) for 5 min at room temperature, and then the optical density ($OD_{control}$) of the resin filtrate at 412 nm was measured using a UV spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). 3) After washing 6 times with DMF, the Cys-peptide resin was incubated with test compound solution (in DMF) at room temperature for 24 h (moles of peptide : moles of 100 mM test chemicals = 1:800 – 1:1000). 4) After washing 6 times with DMF, the Cys-peptide resin was incubated with 1 mL of 10 mM DTNB solution [in pH 7.5 phosphate buffer (20 mM)-DMF (2:1)] for 30 min at room temperature and then washed 6 times with DMF for removal of TNBs and the unreacted DTNB. 5) The Cys-peptide resin was incubated with 1 mL of the release solution for DTNB for 5 min at room temperature and then the OD_{test} of the resin filtrate at 412 nm was measured using a UV spectrophotometer.

Procedure using Lys-peptide resin

Lys-peptide resin (0.5 mg as dry resin) was placed in a LibraTube, washed 6 times with DMF. 1) The swelled resin was incubated with 1 mL of 10 mM picric acid (PA) (FUJIFILM Wako Pure Chemical Corporation) solution [in water-DMF (1:1)] for 30 min at room temperature (moles of peptide : moles of PA = 1:80 – 1:100), and then washed 6 times with DMF for removal of the unreacted picric acid. 2) The Lys-peptide resin was incubated with 1 mL of 2% sodium hydroxide (NaOH) aqueous solution (the release solution) for 5 min at room temperature, and then the optical density ($OD_{control}$) of the resin filtrate at 380 nm was measured using a UV spectrophotometer. 3) After washing 6 times with DMF, the Lys-peptide resin was incubated with test compound solution (in DMF) at room temperature for 24 h (moles of peptide : moles of 100 mM test chemicals = 1:800 – 1:1000). 4) After washing 6 times with DMF, the Lys-peptide resin was incubated with 1 mL of 10 mM PA solution [in water-DMF (1:1)] for 30 min at room temperature, washed 6 times with DMF for removal of unreacted PA. 5) The Lys-peptide resin was incubated with 1 mL of the release solution for PA for 5 min at room temperature and then the OD_{test} of the resin filtrate at 380 nm was then measured using a UV spectrophotometer.

Calculation of reactivity of test chemicals with peptide resins
The peptide reactivity was expressed as percent depletion based on the decrease in the amount of unreacted peptide after the reaction with chemical relative to the amount of peptide initially measured [Eq. (1)].

$$\text{Peptide depletion ratio (\%)} = [1 - (OD_{test} / OD_{control})] \times 100 \quad (1)$$

where, $OD_{control}$ is the OD obtained from the initially measured resin, and OD_{test} is the OD obtained after reaction with a chemical.

References

S1 W. C. Chan and P. D. White, *Fmoc solid phase peptide synthesis: A practical approach*, Oxford University Press, New York, 2000.

Figures and Legends

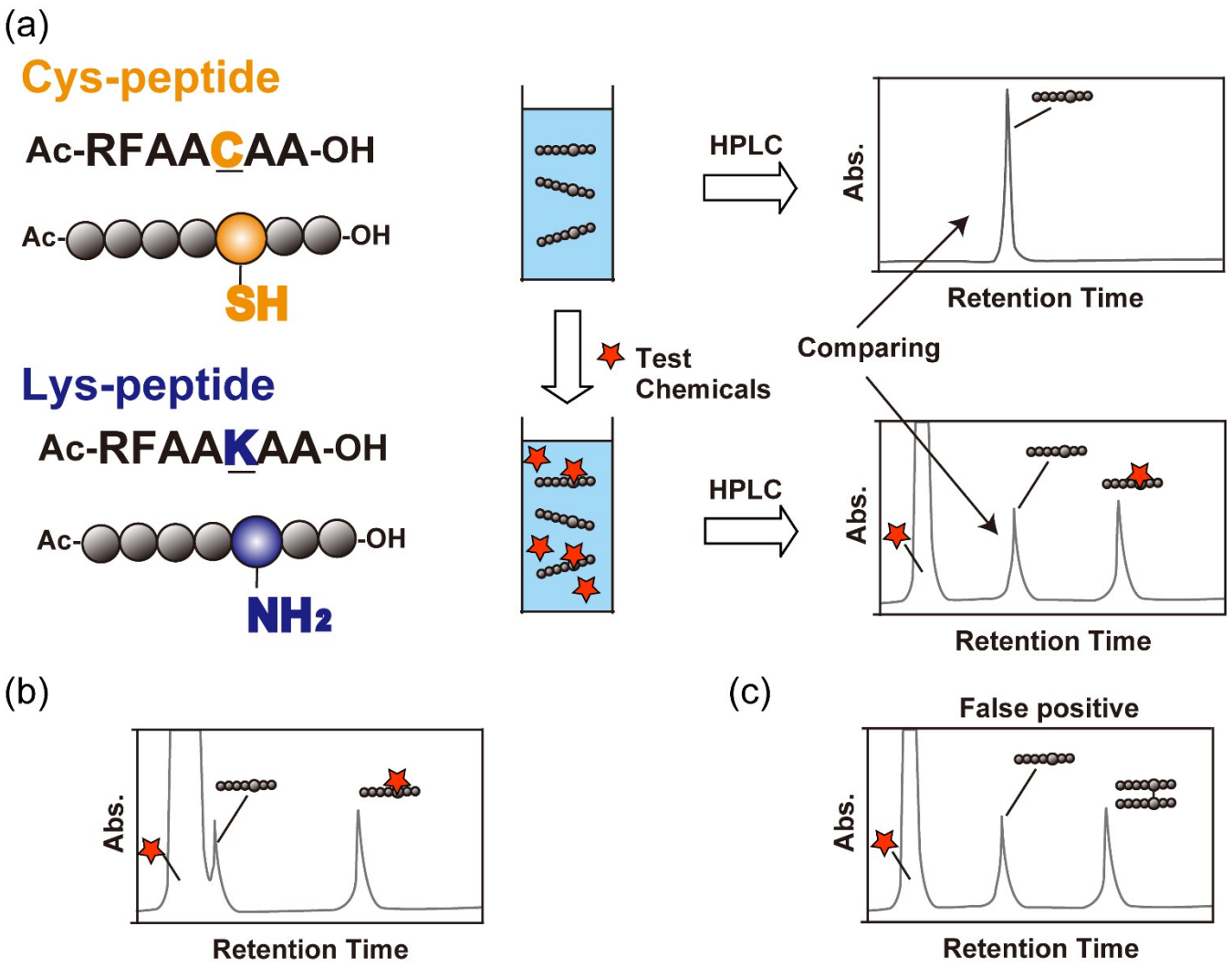

Fig. S1 (a) Scheme of DPRA described in OECD Test Guideline (TG) 442C. The reactivity of the chemical substances to Cys- or Lys- containing peptides has been measured by monitoring the depletion of peptide using HPLC analysis. According to the OECD TG 442C, the test substances were dissolved in an appropriate solvent to prepare a 100 mM solution. Cys- and Lys- peptides are reacted for 24 h with the test chemical at 1:10 and 1:50 ratio respectively. Amounts of unreacted peptides in the samples with/without a test chemical are compared by reverse-phase HPLC with UV detection at 220 nm. (b) A representative drawback of DPRA. The drawback ii), retention times of the unreacted test chemical and peptide may overlap (co-elution). (c) A representative drawback of DPRA. The drawback iii), the free thiol of a Cys-containing peptide can easily dimerize upon oxidation, potentially creating false positives.

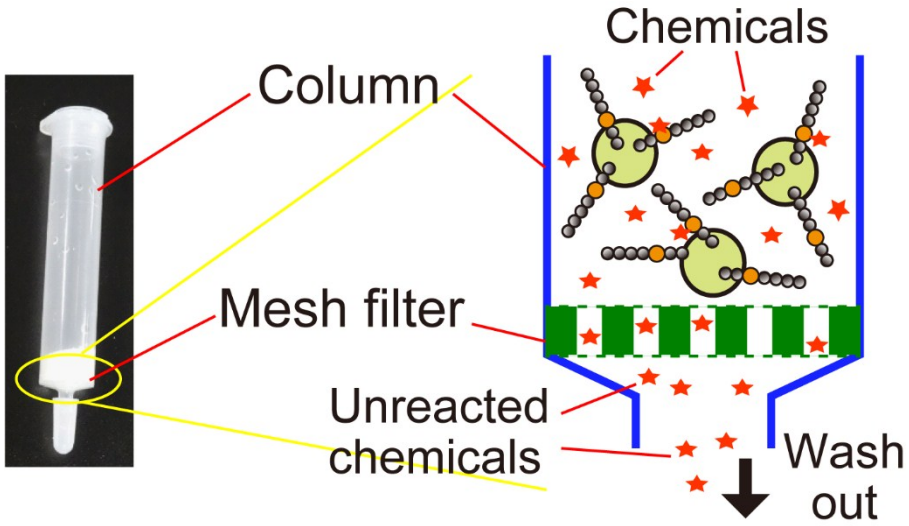

Fig. S2 Outline of the column used in C-SPRA.

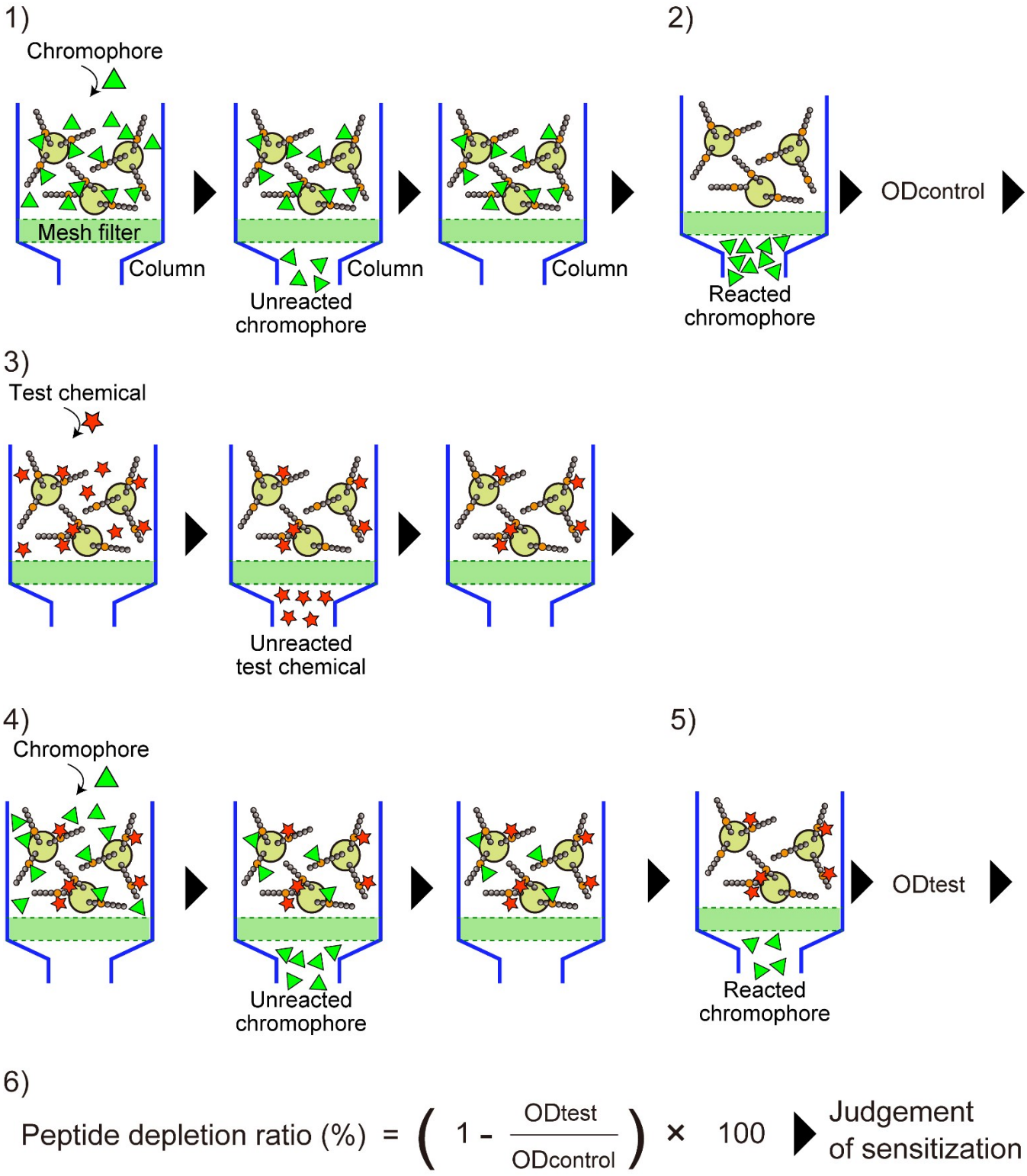

Fig. S3 Schematics of filtration of unreacted compound using a column in C-SPRA and of assessing skin sensitization using C-SPRA. The procedures are as follows; 1) The chromophore (DTNB for Cys-peptide resins or PA for Lys-peptide resins) was added to the peptide resins and then the unreacted chromophores were removed out by washing with the solvent. 2) the release solution (DTT solution for Cys or 2% NaOH solution for Lys) was added for removal and collection of reacted chromophores and $OD_{control}$ was obtained by UV measurement. 3) A test chemical was added. After the reaction process (24 h) the resins were washed. 4) The chromophore was added to the resins and then the unreacted chromophores were removed out. 5) The release solution was added for removal and collection of reacted chromophores and OD_{tesr} was obtained by UV measurement. 6) Peptide depletion ratio was obtained using $OD_{control}$ and OD_{test}.

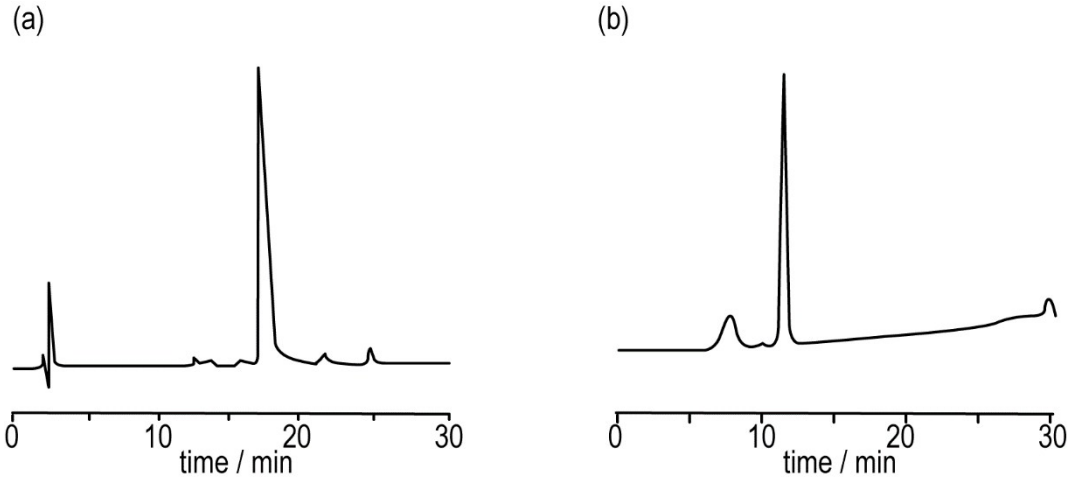

Fig. S4 HPLC for Cys (a) and Lys (b) peptides separated on an ODS column (150×4.6 mm) with MilliQ water (containing 0.1% TFA) using a gradient from 5% to 95% acetonitrile (containing 0.08% TFA) over 30 min, 1.0 mL/min; detection at 220 nm. MALDI-TOF MS showed 821.6 for Cys-peptide ($[M+H]^+$ = 822.0 (calc.)) (a) and 846.7 for Lys-peptide ($[M+H]^+$= 847.1 (calc.))(b).

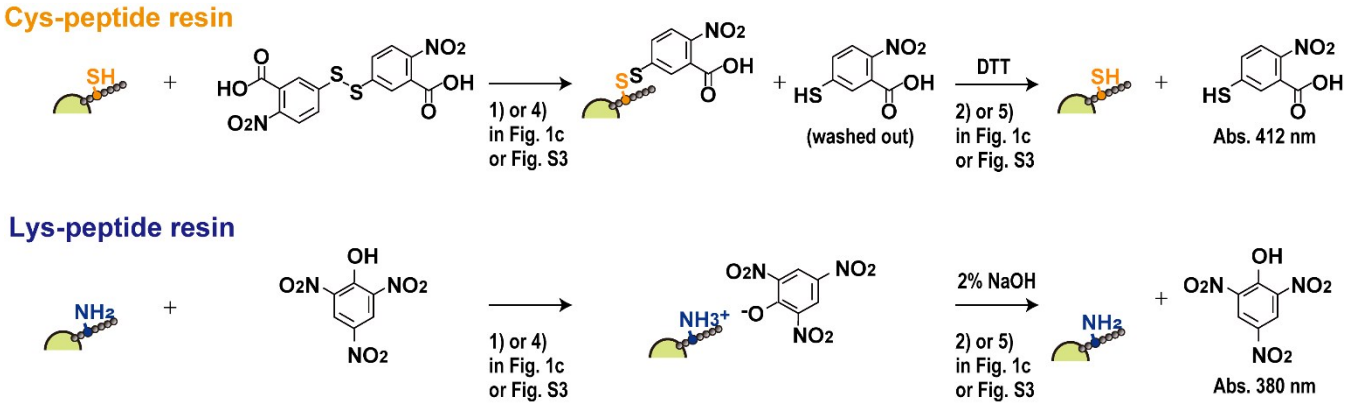

Fig. S5 Methods for UV detection using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and picric acid (PA) in C-SPRA.

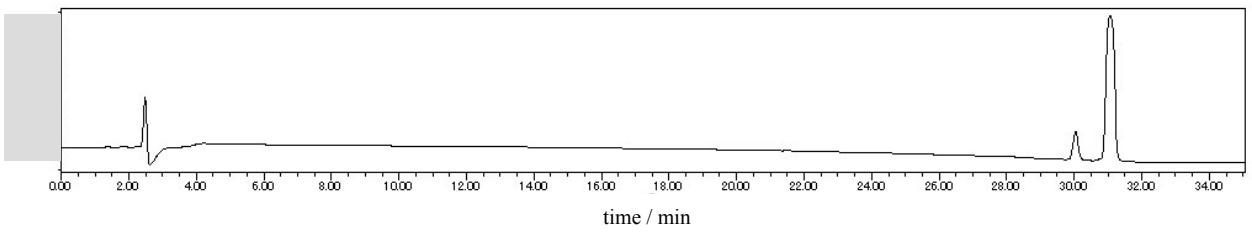

Fig. S6 HPLC for R(+)-limonene separated on an ODS column (150×4.6 mm) with MilliQ water (containing 0.1% TFA) using a gradient from 5% – 95% acetonitrile (containing 0.08% TFA) over 30 min, and 95% acetonitrile for 5 min (30 min – 35 min) , 1.0 mL/min; detection at 210 nm.

Table **S**1 Reactivity of test chemicals to cysteine- and lysine-peptide resins determined by percent depletion.

Test Chemicals	log Kow	LLNA Potency category	Mechanism	DPRA[a,b]		C-SPRA			
				Mean depletion ratio (%)	Results[c]	Cys depletion ratio (%)	Lys depletion ratio (%)	Mean depletion ratio (%)	Results[d]
p-Benzoquinone (BQ)	0.25	Extreme	Michael acceptor	95.0[a]	P	94.2 ± 0.6	92.9 ± 1.1	93.6	P
Fluorescein-5-isothiocyanate (FITC)	4.69	Strong	Acyl-transfer	80.6[a]	P	74.6 ± 10.1	98.4 ± 0.3	86.5	P
2-Mercaptobenzothiazole (MBT)	2.86	Moderate	Acyl-transfer	48.8[a]	P	24.8 ± 1.0	7.3 ± 3.8	16.1	P
Benzylidene acetone (BA)	2.04	Moderate	Michael acceptor	48.1[a]	P	28.1 ± 1.6	22.1 ± 4.2	25.1	P
5-Methyl-2-phenyl-2-hexenal (MPH)	3.77	Moderate	Michael acceptor /Schiff base	-	-	57.3 ± 12.1	42.0 ± 9.9	49.6	P
Undec-10-enal (UE)	4.12	Moderate	Schiff base	0.00[b]	N[e]	18.3 ± 3.8	19.1 ± 1.7	18.7	P
α-Hexyl cinnamic aldehyde (HCA)	4.82	Weak	Michael acceptor /Schiff base	0.00[a]	N[e]	2.3 ± 1.6	14.9 ± 0.3	8.61	P
α-Amyl cinnamic aldehyde (ACA)	4.33	Weak	Michael acceptor /Schiff base	2.25[a]	N[e]	4.8 ± 1.1	17.3 ± 2.7	11.1	P
Benzyl benzoate (BB)	3.54	Weak	SN2	1.60[a]	N[e]	4.2 ± 3.6	14.1 ± 2.5	9.15	P
Benzyl cinnamate (BC)	4.06	Weak	Michael acceptor /SN2	0.65[b]	N[e]	3.4 ± 1.2	17.3 ± 3.9	10.4	P
R(+)-Limonene	4.83	Weak	Non-binding[e]	8.56[b]	P	14.7 ± 3.5	8.3 ± 1.5	11.5	P
Butylbenzylphthalate (BBP)	4.84	Non-sensitizer	Non-binding	0.00[b]	N	4.6 ± 1.6	7.1 ± 1.9	5.83	N
Dibutyl phthalate (DP)	4.61	Non-sensitizer	Non-binding	0.00[b]	N	3.0 ± 1.3	2.8 ± 0.7	2.89	N
Isopropanol (IPA)	0.28	Non-sensitizer	Non-binding	0.25[a]	N	6.5 ± 5.3	3.4 ± 1.4	4.94	N
None	-	-	-	-	-	0.0 ± 0.0	0.9 ± 0.6	0.45	-

[a] Data from Natsch et al., 2013. [b] Data from Otsubo et al., 2017. [c] Threshold of 6.38% average peptide depletion was used to discriminate between 'P' (positive) and 'N' (negative). [d] Threshold of 8 % mean peptide depletion was used to discriminate between 'P' (positive) and 'N' (negative). [e] Because they are sensitizers, they showed "false negatives" in DPRA. [e]Pure limonene should not react. The sample contained oxidized limonene, which reacts with the peptides.