Patel et al.:

Comparison of *In Chemico* Skin Sensitization Methods and Development of *In Chemico* Skin Photosensitization Assays

Supplementary Data

Tab. S1: Stability of the model Cys- and Lys-heptapeptides
A) Some batches of acetonitrile have a negative impact on Cys-peptide stability. Hence, stability of Cysteine peptide was assessed at 0 h and after 24 h and 48 h of incubation. Triplicate samples were analyzed by HPLC-UV, retention time (min, minutes) and measured peak areas (AU, Approximate Units) are shown in the table. Concentration of each sample was calculated from peak areas using standard calibration curve equation number 1 (Supplementary Fig. S2D). Note that the calibration curve samples and peptide stability samples were made at the same time and calibration curve samples were analyzed after 24 ± 2 h at 25 ± 2.5°C. Calculated Standard Deviation (SD) and Relative Coefficient of Variance (RCV) values for nine reference controls are shown below. As per OECD guidelines, acetonitrile batches with < 15% RCV were used for further experiments. B) Additionally, stability of Lys-peptide was analyzed for 24 h, as indicated. Acetonitrile batches with < 11.6% RCV were used for further analysis.

S1A

Cys-peptide stability			
Replicates	**Area (AU)**	**Conc. (mM)**	**RT (min)**
1) 0 h	1780053	0.514	9.581
2) 0 h	1789939	0.517	9.575
3) 0 h	1801172	0.520	9.566
1) 24 h	1725287	0.498	9.568
2) 24 h	1729875	0.499	9.558
3) 24 h	1760887	0.508	9.565
1) 48 h	1707299	0.493	9.540
2) 48 h	1708723	0.493	9.556
3) 48 h	1723970	0.497	9.561
AVG	1747467	0.504	
SD	36052.30	0.010	
RCV	2.06	2.07	

S1B

Lys-peptide stability			
Replicates	**Area (AU)**	**Conc. (mM)**	**RT (min)**
1) 0 h	1516869	0.497	7.199
2) 0 h	1480658	0.485	7.153
3) 0 h	1517486	0.497	7.122
1) 24 h	1520534	0.498	7.078
2) 24 h	1518453	0.498	7.075
3) 24 h	1526394	0.500	7.069
AVG	1513399	0.496	
SD	16407.55	0.005	
RCV	1.08	1.08	

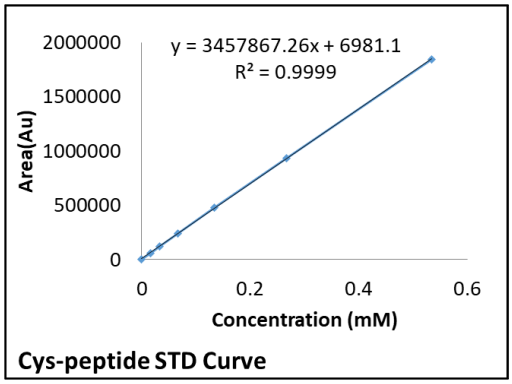
doi:10.14573/1811011s

Tab. S2: Generation of standard calibration curves for model peptides
A) Standards were prepared in a solution of 20% acetonitrile: phosphate buffer (Cys-peptide) and/or ammonium acetate buffer (Lys-peptide). Standards of the stock solutions covering the range from 0.534 mM - 0.0167 mM were prepared by serial dilution. 10 ml of dilution buffer was prepared by mixing 8 ml of buffer (pH 7.5 phosphate buffer for Cys-peptide, pH 10.2 ammonium acetate buffer for Lys-peptide) with 2 ml of acetonitrile. The detailed preparation scheme of the standards is described below. B,C) 5 µl of each standard was injected and analyzed by HPLC, using a fixed wavelength detector at 220 nm. Peak areas were obtained by appropriate integration and plotted against Cys-peptide (S2B) and Lys-peptide (S2C) concentrations to generate standard calibration curves. D) Summary of Linear Equation and r^2 values obtained from standard curve analysis of four replicates, performed on four independent days, are shown in the table. Representative standard calibration curve shown in supplementary figure S2B and figure S2C correspond to equation number 1 for Cys- and Lys-peptides.

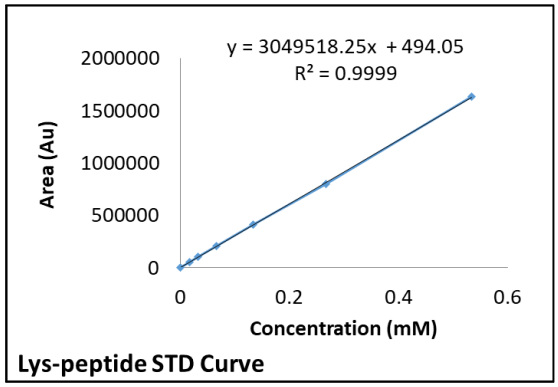
S2A Preparation of Cys- and Lys-peptide standards
STD 1 preparation (0.534 mM): 800 µl of the peptide stock solution (0.667 mM) + 200 µl acetonitrile

Standard (STD)	Standard volume	Dilution buffer volume	End concentration (mM)
STD 2	500 µl of STD 1	500 µl	0.2670
STD 3	500 µl of STD 2	500 µl	0.1335
STD 4	500 µl of STD 3	500 µl	0.0668
STD 5	500 µl of STD 4	500 µl	0.0334
STD 6	500 µl of STD 5	500 µl	0.0167
STD 7	-	1000 µl	0.0000

S2B



S2C



S2D

Sr. No.	Standard Curve Analysis	
	Cys-Peptide STD Curve	Lys-Peptide STD Curve
	Equation and r^2 value	Equation and r^2 value
1	y = 3457867.26 x + 6981.09 r^2 = 0.9999	y = 3049518.25x + 494.05 r^2 = 0.9999
2	y = 3492562.78x + 9655.15 r^2 = 0.9999	y = 3060144.74x - 2001.77 r^2 = 0.9999
3	y = 3353031.51x + 8861.19 r^2 = 0.9997	y = 3110584.85x - 3.39 r^2 = 0.9999
4	y = 3297081x + 2110.662 r^2 = 0.9999	y = 3084965x -0.161654 r^2 = 0.9999

Tab. S3: Analysis of reference controls
A-D) 750 µl Cys-peptide + 250 µl acetonitrile (S3A, S3C) and 750 µl Lys-peptide + 250 µl acetonitrile (S3B, S3D) at 0 h (reference control A) or after 24 h incubation/ at the end of run of experiment (reference control B) by HPLC-UV. Reference control A was used to verify accuracy of the calibration curve for peptide quantification, whereas reference control B was used to check the stability of peptide during the course of DPRA experiment.

S3A

Cys-peptide reference control A		
Replicates	**Area (AU)**	**RT (min)**
Rep-1	1863.8	9.711
Rep-2	1946.8	9.722
Rep-3	1965.0	9.689
AVG	1925.20	
SD	53.95	
RCV	2.80	

S3B

Lys-peptide reference control A		
Replicates	**Area (AU)**	**RT (min)**
Rep-1	1667.2	7.028
Rep-2	1660.5	6.96
Rep-3	1665.8	6.896
AVG	1664.5	
SD	3.53	
RCV	0.21	

S3C

Cys-peptide reference control B		
Replicates	**Area (AU)**	**RT (min)**
Rep-1	1915.3	9.573
Rep-2	1924.7	9.566
Rep-3	1915.0	9.566
Rep-4	2163.4	9.569
Rep-5	2175.2	9.569
Rep-6	2135.7	9.568
AVG	2038.2	
SD	132.00	
RCV	6.48	

S3D

Lys-peptide reference control B		
Replicates	**Area (AU)**	**RT (min)**
Rep-1	1730.1	6.907
Rep-2	1699.6	6.941
Rep-3	1705.7	6.938
Rep-4	1750.5	6.764
Rep-5	1724.4	6.716
Rep-6	1715.7	6.725
AVG	1721	
SD	18.36	
RCV	1.07	

Tab. S4: Retention time (RT) of proficiency compounds obtained from co-elution controls
Co-elution controls for each test chemical were prepared without peptide, to verify whether the test chemical absorbs at 220 nm and has a distinct retention time from that of the model peptide. As seen in Fig. 1, retention time for Cys-peptide is 9.4 min and for Lys-peptide it is 6.7 min. Co-elution controls were prepared using phosphate buffer, pH 7.5 (750 µl buffer + 200 µl of acetonitrile + 50 µl test chemical) and ammonium acetate buffer, pH 10.2 (750 µl buffer + 250 µl of test chemical). RTs for individual compounds did not vary between the two buffers and are shown below.

Test Chemical	RT (min)
Cinnamaldehyde	13.97
2-4-Dinitrochlorobenzene	14.31
Formaldehyde	8.81
Oxazolone	12.77
Benzylideneacetone	14.15
Farnesal	16.09
2-3 Butanedione	11.16
1-Butanol	13.68
Lactic Acid	14.07
6-Methylcoumarin	14.03
4-Methoxyacetophenone	13.66

Tab. S5: DPRA Cysteine 1:10/Lysine 1:50 prediction model

Average (Cys- and Lys-peptide) % Depletion	Reactivity Class	Prediction
0% < Mean % depletion < 6.38%	Minimal Reactivity	Non-Sensitizer
6.38% < Mean % depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % depletion < 100%	High Reactivity	Sensitizer

Tab. S6: ADRA calibration curves

A-D) 1.25 mM each of NAC and NAL stock solutions were prepared in 100 mM phosphate buffer at pH 9.5 and pH 12, respectively. Standards were prepared in a solution of (1250:1750) acetonitrile: phosphate buffer (pH 9.5). Detailed dilution scheme (A, B) and representative standard calibration graphs (C,D) are shown below. E) Summary of Linear Equation and r^2 values obtained from analysis of standard curve analysis of four replicates are shown in the table. Note that stability of NAC and NAL for 24 h was checked before standard calibration curve analysis. Note that most of the data presented in the manuscript was validated on two different HPLC systems, Agilent technology 1100 series, Shimadzu LC2010CHT. Standard calibration curve analysis for equation no. 1-5 was performed on Shimadzu LC2010CHT, whereas analysis for equation no. 6 was done using Agilent technology 1100 series, for NAC. For NAL, standard calibration curve analysis was performed using Agilent technology 1100 series..

S6A Preparation of NAC Standards

STD 1 (0.5 mM): 400 µl of the peptide stock solution (1.25 mM) + 600 µl buffer, pH 9.5

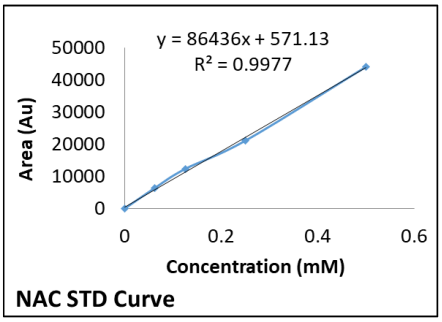
Standard (STD)	Volume of Standard	Volume of Dilution Buffer	End Concentration (mM)
STD 2	500 µl of STD 1	500 µl	0.25
STD 3	500 µl of STD 2	500 µl	0.125
STD 4	500 µl of STD 3	500 µl	0.0625
STD 5	-	1000 µl	0.00

S6B Preparation of NAL Standards

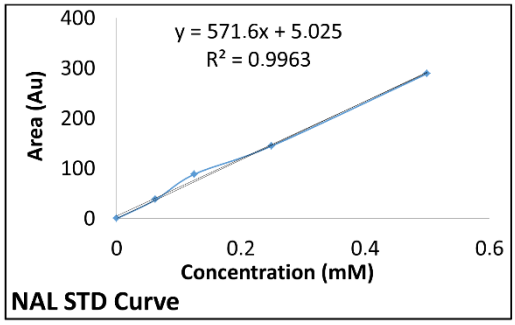
STD 1 (0.5 mM): 400 µl of the peptide stock solution (1.25 mM) + 600 µl buffer, pH 12.0

Standard (STD)	Volume of Standard	Volume of Dilution Buffer	End Concentration (mM)
STD 2	500 µl of STD 1	500 µl	0.25
STD 3	500 µl of STD 2	500 µl	0.125
STD 4	500 µl of STD 3	500 µl	0.0625
STD 5	-	1000 µl	0.000

S6C



S6D



S6E

Sr. No.	Standard Curve Analysis	
	NAC STD Curve	NAL STD Curve
	Equation and r^2 value	Equation and r^2 value
1.	y = 86435.60x + 571.13 r^2 = 0.9977	y = 571.60 x + 5.03 r^2 = 0.9963
2.	y = 218989.20x + 512.93 r^2 = 0.9998	y = 566.64x + 2.38 r^2 = 0.9997
3.	y = 226544.80x- 133.25 r^2 = 0.9999	y = 578.84x + 0.43 r^2 = 1
4.	y = 249657.20x -1894.13 r^2 = 0.9984	y =600.88x + 0.76 r^2 = 0.9999
5.	y = 231407.60x +115.08 r^2 = 0.9998	y = 760.601x + 3.93 r^2 = 0.9996
6.	y = 382.44x -1.65 r^2 = 0.9997	y = 664.12x - 0.72 r^2 = 0.9999