

# Application News

## No. L531

### High Performance Liquid Chromatography

## Analysis of DPRA (Direct Peptide Reactivity Assay) for Skin Sensitization Testing Using Prominence™-i

Animal bioassay involving the use of guinea pigs or mice was once the mainstream method of skin sensitization testing. However, a total ban on animal testing of cosmetics enacted in the EU in 2013 made it necessary to establish alternative testing methods which do not use animals. DPRA (direct peptide reactivity assay) is one of these alternative methods used to evaluate the potency of skin sensitization by examining the reactivity between a test chemical and peptides. This method uses two types of peptides, one containing cysteine and another containing lysine, which are mixed and reacted with the test chemical for 24 hours before using HPLC to determine the percent peptide depletion. The skin sensitization of the test chemical is evaluated from the determined percent depletion value of each peptide.

In this article, we performed an analysis based on "TG 442C OECD GUIDELINE FOR THE TESTING OF CHEMICALS *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)"<sup>1)</sup>. The Prominence-i integrated high performance liquid chromatograph (PDA model) was used as the analytical instrument. The rack in the autosampler of this instrument was used to perform 24-hour incubation of the test chemical and peptides. The Shim-pack™ HR-ODS (100 mm L. x 2.1 mm i.d., 3 µm) was used for the column.

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### Test Chemicals

Three test chemicals were selected for use from the proficiency<sup>2)</sup> substances listed in Annex 2 of the above guideline. Testing was performed on the sensitizers of formaldehyde (strong), benzylideneacetone (moderate), and the non-sensitizer of lactic acid to determine the percent depletion values of the cysteine peptide and the lysine peptide.

### Sample Solution Preparation

A sample of 100 mmol/L of each test chemical was prepared using acetonitrile. Both the cysteine peptide<sup>3)</sup> solution and lysine peptide<sup>3)</sup> solution were adjusted to a 0.667 mmol/L concentration in the buffer immediately prior to analysis. Each solution was mixed as shown in Tables 1 and 2. The concentrations of these solutions were, with respect to a 0.5 mmol/L peptide concentration, 5 mmol/L for the cysteine peptide solution (1:10 concentration ratio), and 25 mmol/L for the lysine peptide solution (1:50 concentration ratio). These solutions were put into glass vials and incubated for 24 hours in the rack of the autosampler set to 25 °C.

Reference controls A to C were prepared to confirm no increases or decreases in peptides due to operational factors, such as incubation or analysis. Reference control A was measured at the start of consecutive analysis to verify the suitability of the HPLC system. Reference control B was measured before and after measuring the test solution to verify the stability of reference controls over the analysis time. Reference control C was measured together with the test solution to verify that the sample solvent did not affect the percent peptide depletion. The co-elution controls are samples for confirming no elution of impurities at the peptide elution time. Cinnamic aldehyde was used as the positive control. This compound has a known percent peptide depletion value and is used to verify that the sequence of operations and analysis are performed correctly.

### Analysis

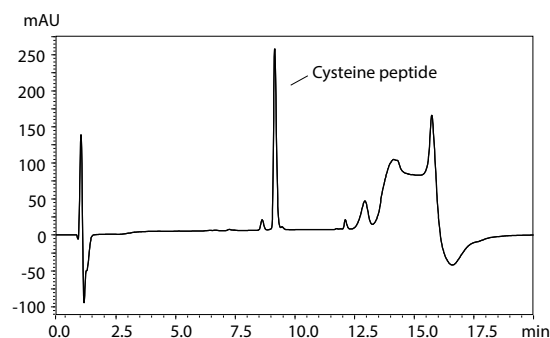
Following incubation, each solution was analyzed under the conditions in Table 3. Calibration curves were created from the peptide standard solutions and then analysis was performed in order starting from reference control A. Each solution was analyzed in triplicate to check that measurement is achieved without variation. Figs. 1 and 2 show the chromatograms of reference control A for both cysteine peptide and lysine peptide.

**Table 1 Preparation of Sample Solutions for Cysteine Peptide**

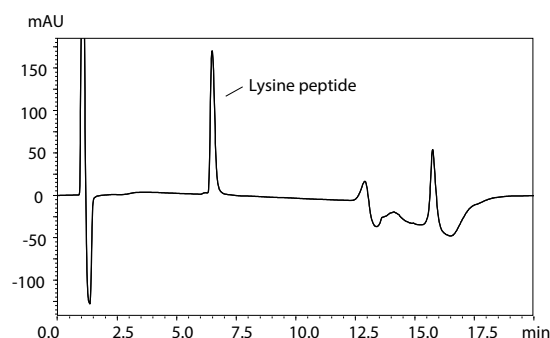
	Peptide Solution	pH7.5 Phosphate Buffer	Acetonitrile	Test Chemical Solution
Reference control	750 µL	-	250 µL	-
Test solution	750 µL	-	200 µL	50 µL
Coelution control	-	750 µL	200 µL	50 µL

**Table 2 Preparation of Sample Solutions for Lysine Peptide**

	Peptide Solution	pH10.5 Ammonium Acetate Buffer	Acetonitrile	Test Chemical Solution
Reference control	750 µL	-	250 µL	-
Test solution	750 µL	-	-	250 µL
Coelution control	-	750 µL	-	250 µL



**Fig. 1 Chromatogram of Cysteine Peptide (Reference Control A)**



**Fig. 2 Chromatogram of Lysine Peptide (Reference Control A)**

### Evaluation of Acceptance Criteria and Results of Proficiency Testing

An evaluation of acceptance criteria (Table 4) was performed from the results of the analysis sequence. We found that the criteria were satisfied for all items in this analysis.

Figs. 3 and 4 show the chromatograms of reference control C and positive control which compose the peptide standard solutions in addition to the analysis result of each test solution (data of each first analysis). Analysis of each test solution was repeated three times and the mean of each percent peptide depletion values was calculated from the analysis results (Table 5). The percent peptide depletion values indicated in the proficiency testing in Annex 2 were within the reference range.

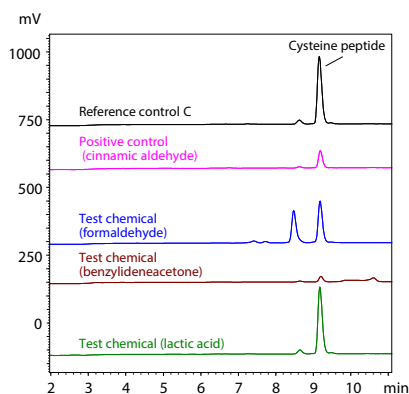


Fig. 3 Chromatograms of Sample Solutions for Cysteine Peptide

Table 3 HPLC Analytical Conditions

System	: Prominence-i
Column	: Shim-pack HR-ODS *1 (100 mm L. x 2.1 mm I.D., 3 μm)
Mobile Phase	: A: 0.1 %TFA-Water, B: 0.085 %TFA-Acetonitrile
Time Program	: B Conc. 10 % (0 min) → 25 % (10 min) → 90 % (11 min →13 min) →10 % (13.5 min →20 min)
Flow Rate	: 0.35 mL/min
Column Temp.	: 30 °C
Injection Volume	: 7 μL
Detection	: PDA220 nm

\*1 We recommend using a guard column.

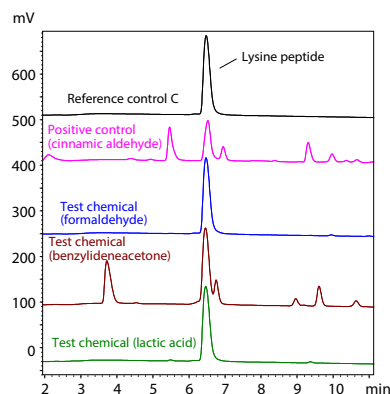


Fig. 4 Chromatograms of Sample Solutions for Lysine Peptide

Table 4 Acceptance Criteria and Results

	Cysteine Peptide	Lysine Peptide	Criteria	Judgment
Calibration curve R <sup>2</sup>	0.999	0.999	>0.990	OK
Positive control cinnamic aldehyde Mean percent peptide depletion *2 of three replicates	77.1 %	51.2 %	Cysteine peptide: 60.8 - 100 % Lysine peptide: 40.2 - 69.0 %	OK
Positive control cinnamic aldehyde Standard deviation of percent peptide depletion of three replicates	0.8 %	1.2 %	Cysteine peptide: < 14.9 % Lysine peptide: <11.6 %	OK
Reference control A Mean peptide concentration of three replicates	0.49 mmol/L	0.50 mmol/L	0.50±0.05 mmol/L	OK
Reference controls B and C Coefficient of variation of peak areas of nine replicates	3.7 %	0.3 %	<15.0 %	OK
Test chemical "formaldehyde" Standard deviation of percent peptide depletion	0.6 %	0.1 %	Cysteine peptide: < 14.9 % Lysine peptide: <11.6 %	OK
Test chemical "benzylideneacetone" Standard deviation of percent peptide depletion	0.3 %	0.3 %	Cysteine peptide: < 14.9 % Lysine peptide: <11.6 %	OK
Test chemical "lactic acid" Standard deviation of percent peptide depletion	1.8 %	0.1 %	Cysteine peptide: < 14.9 % Lysine peptide: <11.6 %	OK
Reference control C Mean peptide concentration of three replicates	0.50 mmol/L	0.51 mmol/L	0.50±0.05 mmol/L	OK

\*2 Percent peptide depletion: [1 - (peptide peak area in replicates) / (mean peptide peak areas in reference control C)] × 100

Table 5 Proficiency Testing

	Cysteine Peptide Mean Percent Depletion (%)	Range of % Cysteine Peptide Depletion listed in Annex 2 of OECD/OCDE TG 442C	Lysine Peptide Mean Percent Depletion (%)	Range of % Lysine Peptide Depletion listed in Annex 2 of OECD/OCDE TG 442C	Judgment
Formaldehyde (strong sensitizer)	41.7	30 - 60	1.7	0 - 24	OK
Benzylideneacetone (moderate sensitizer)	92.7	80 - 100	3.8	0 - 7	OK
Lactic acid (non-sensitizer)	5.5	0 - 7	1.2	0 - 5.5	OK

- The analysis performed in this article was based on the protocol recommended in the following TG 442C OECD guideline. EURL ECVAM Database Service on Alternative Methods to Animal Experimentation (DB-ALM) Protocol No. 154: Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing
- The procedure used to determine whether the percent peptide depletion value of a given substance can be obtained within the reference range for the purpose of demonstrating technical proficiency in accurately obtaining the data expected from DPRA.
- Scrum Inc.

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