DB-ALM Protocol n° 154 : Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing

Skin Sensitisation & Allergic Contact Dermatitis

The Direct Peptide Reactivity Assay (DPRA) is an *in chemico* method which measures peptide reactivity of test chemicals by quantifying the depletion of synthetic heptapeptides containing either lysine or cysteine. Haptenation, i.e. the covalent binding of low-molecular weight substances (haptens) to proteins in the skin is considered a prominent mechanisms through which chemicals or their metabolites become antigenic. Therefore, information from peptide reactivity assays such as the DPRA is considered relevant for the assessment of the skin sensitisation potential of chemicals.

Résumé

The purpose of the test is to contribute to the evaluation of the skin sensitisation potential of chemicals. Haptenation i.e. the covalent binding of low-molecular weight substances ("haptens") to proteins present in skin is considered a prominent mechanism through which chemicals or their metabolites become antigenic (OECD, 2012). Therefore, information inferred from peptide reactivity assays such as the DPRA is relevant for the assessment of the skin
sensitisation potential of chemicals (Gereberick *et al.,* 2004 and 2007). Further details on the context of use and applicability domain can be found in the **Method Summary of DPRA** in DB-ALM, EURL ECVAM Recommendation (EURL ECVAM, 2013) and OECD TG No. 442C: "In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)" (OECD, 2015).

Experimental Description

Endpoint and Endpoint Measurement:

DIRECT PEPTIDE REACTIVITY: measured by quantifying the depletion of synthetic heptapeptides containing either lysine or cysteine

Endpoint Value:

% peptide depletion

Experimental/Test System(s):

Synthetic heptapeptides containing either Lysine (-Ac-RFAAKAA-COOH) or Cysteine (-Ac-RFAACAA-COOH).

Basic Procedure

Synthetic heptapeptides containing either cysteine or lysine are incubated with the test substance for 24 hours. Depletion of the peptide in the reaction mixture is measured by high pressure liquid chromatography (HPLC) using UV detection. Average peptide depletion data for cysteine and lysine are then calculated.

Status

Known Laboratory Use:

Procter & Gamble
BASF
L'Oréal

Participation in Evaluation Studies:

The DPRA has been optimised in Cosmetics Europe (former Colipa) coordinated ring-trials (Aeby *et al.*, 2010).

Participation in Validation Studies:

The DPRA protocol participated in a EURL ECVAM validation study for the assessment of its transferability and within- and between-laboratory reproducibility. The EURL ECVAM study showed that the DPRA is transferable to suitably equipped laboratories that are proficient in HPLC analysis and the results obtained demonstrated withinand between-laboratory reproducibility of 87% and 75%, respectively. On 12.12.2013 EURL ECVAM published its recommendation on the Direct Peptide Reactivity Assay (DPRA) for skin sensitisation testing (EURL ECVAM, 2013).

Regulatory Acceptance:

The test method was adopted as **OECD Test Guideline No 442C: "In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)"** (OECD, 2015). TG 442C describes an *in chemico* procedure proposed for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (UN, 2013).

Proprietary and/or Confidentiality Issues

None

Health and Safety Issues

General precautions:

General safety instructions should be followed at all times. Appropriate protective safety equipment should be worn. Unknown and coded chemicals should be considered as potential sensitising agent and toxic and must be handled with maximum care.

Abbreviations and Definitions

COLIPA	The European Cosmetics Association. European trade association of cosmetic, toiletry and perfumery industry. In 2012 became **Cosmetics Europe** .
CV	Coefficient of variance
Cys	Cysteine
DMSO	dimethyl sulfoxide
DPRA	Direct peptide reactivity assay
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
HPLC	High-performance liquid chromatography
Lys	Lysine
MW	Molecular weight
OECD	**Organisation for Economic Co-operation and Development**
SOP	Standard Operating Procedure

Last update: 21 October 2021

PROCEDURE DETAILS, 12 January 2012

Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing
DB-ALM Protocol n° 154

The protocol is based on the Standard Operating Procedure (SOP) used in the EURL ECVAM validation study on the Direct Peptide Reactivity Assay (DPRA). A validated study template is available in the **Downloads** section of this protocol in **DB-ALM.**

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Materials and Preparations

TEST SYSTEM

The DPRA is a chemistry-based assay. Nucleophile-containing synthetic peptides (cysteine peptide – Ac-RFAACAA-COOH; lysine peptide – Ac-RFAAKAA-COOH) are used to screen for skin sensitisation potential by measuring peptide depletion following incubation with allergens and non-allergens.

EQUIPMENT

Fixed Equipment

APPARATUS	SUGGESTED TYPE (or Equivalent)
Analytical Balance	Capable of accurately weighing up to 20 grams with 0.1 mg readability
Dispensing Pipets capable of delivering 250 – 750 microliters and 50 microliters	Eppendorf Research Adjustable Pipets *verify accuracy at time of use*
Liquid Chromatograph with light-excluding Autosampler capable of delivering 0.35 mL/min flow rate	Waters Alliance 2695, Waters Corp. Milford MA *Note:avoid Waters 2795 design with bottom-draw autosampler needles or adjust needle depth to avoid bottom contact*
UV Detector capable of measuring UV absorbance at 220 nm	Waters 996 Photodiode Array (preferred) Or Waters 2487 Fixed Wavelength Absorbance detector
pH meter with electrode and calibration buffers	Capable of reading +/- 0.01 pH units
HPLC Column	Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5micron; Part # 861753-902 *Alternate Column: Phenomenex Luna C18(2) 2.0 mm x 100mm x 3micron particle; Part # 00D-4251-B0*
Guard Column	Phenomenex Security Guard C18 4mm x 2mm; Part # AJO-4286
Optional: Laboratory Automated Pipetting System with appropriate tools to deliver 50– 750 microliters volumes	Beckman Biomek 2000

Consumables

CONSUMABLES	SUGGESTED TYPE (or Equivalent)
Glass Vials with Teflon or polyethylene-lined closure, nominal 4 mL capacity	Qorpak 2502T, Supplier VWR Scientific Catalog #66009-557
Glass Autosampler Vials	Compatible with Autosampler

MEDIA, REAGENTS, SERA, OTHERS

CHEMICALS AND SPECIAL MATERIALS	SUGGESTED TYPE (or Equivalent)
Trifluoroacetic Acid (MW=114.02) CAS N° 76-05-1	Sigma-Aldrich 299537 99+%, redistilled, for protein sequencing
Sodium Phosphate, Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$, MW=138.0) CAS N°10049-21-5	ACS Reagent Grade, Aldrich S9638
Sodium Phosphate, Dibasic Heptahydrate ($Na_2HPO_4 \cdot 7H_2O$ MW=268.0) CAS N o 7782-85-6	ACS Reagent Grade, Aldrich S9390
Ammonium Acetate ($NH_4C_2H_3O_2$ MW=77.08) CAS N° 631-61-8	ACS Reagent Grade, Sigma Aldrich 238074
Ammonium Hydroxide (NH_4OH, 28 - 30%)	ACS Reagent Grade, Sigma Aldrich 320145
Acetonitrile, HPLC Grade * CAS N° 75-05-8	HPLC Grade, Sigma Aldrich 439134
Purified Water	HPLC Grade or Millipore Milli-Q grade
Cysteine Peptide (store refrigerated) Ac-RFAACAA-COOH, MW=751.9 90-95% purity	RS Synthesis, Louisville KY, USA or JPT Peptide, Germany *Note: material contains a mixture including the peptide with one less alanine ("A") unit which may co-elute.*
Lysine Peptide (store refrigerated) Ac-RFAAKAA-COOH MW= 776.2 90-95% purity	RS Synthesis, Louisville KY, USA or JPT Peptide, Germany
Cinnamic Aldehyde, 93% purity (MW=132.16 / Positive Control) CAS N°104-55-2	Sigma Aldrich Catalog # W22861-3

*** A Note about acetonitrile:**

Some supplies of acetonitrile have had a negative impact on peptide stability (particularly cysteine). This can be assessed when starting a new batch of acetonitrile by performing the following test prior to running the assay.

- Prepare a small amount of 0.501 mg/mL cysteine peptide solution in phosphate buffer. Prepare an autosampler vial containing 750 microliters peptide solution and 250 microliters acetonitrile.
- Incubate for 24 hours.
- Visually inspect the vial for precipitation.
- Set up an HPLC run using the conditions defined in this protocol. Inject this sample every 2-3 hours for approximately 48 hours.

Compare the peak areas for each injection. The CV should be <15%.

PREPARATIONS

Media and Endpoint Assay Solutions

REAGENT	PREPARATION
100 mM Sodium Phosphate, Monobasic	Using a 1 Liter volumetric flask, dissolve 13.8 g of Sodium Phosphate Monobasic Monohydrate in purified water and dilute to final volume of 1 Liter. Store refrigerated.
100 mM Sodium Phosphate, Dibasic	Using a 1 Liter volumetric flask, dissolve 26.8 g of Sodium Phosphate Dibasic Heptahydrate in purified water and dilute to final volume of 1 Liter. Store refrigerated.
100 mM Phosphate Buffer, pH=7.5	Combine 18 mL of 0.1 M Sodium Phosphate Monobasic with 82 mL of 0.1M Sodium Phosphate Dibasic. Mix well and measure pH using a calibrated pH meter. Adjust pH to 7.5 +/- 0.05 with either the monobasic (to acidify) or dibasic (basify) solution.
100 mM Ammonium Acetate Buffer, pH=10.2	Dissolve 1.542 g of Ammonium Acetate in 200 mL purified water. Adjust the pH to 10.2 by dropwise addition of Ammonium Hydroxide using a pH meter calibrated at pH 7 & 10. Prepare fresh or use within 2 weeks.
HPLC Mobile Phase A: 0.1% (v/v) Trifluoroacetic Acid in Water	Add 1.0 mL of Trifluoroacetic acid to 1 Liter of HPLC grade Water.
HPLC Mobile Phase B 0.085% (v/v) trifluoroacetic acid in acetonitrile	Add 850 microliters of Trifluoroacetic Acid to 1 liter of HPLC grade acetonitrile.

Test chemicals pre-work preparation

Solvent Selection

Solubility of the test chemicals in a suitable solvent should be assessed before performing the actual assay. An appropriate solvent will dissolve the test chemical completely, i.e. by visual inspection the solution must not be cloudy nor have noticeable precipitate. Acetonitrile is the preferred solvent for test chemicals, however not all chemicals are soluble in acetonitrile. The following solubilization procedure for the selection of the appropriate solvent should be followed.

1. Evaluate solubility by preparing an approximately 100mM solution in **acetonitrile** . Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
2. If the test chemical is not soluble in acetonitrile, attempt to prepare a 100mM solution in **water** . Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
3. If the test chemical is not soluble in acetonitrile or water alone, attempt to prepare a 100mM solution in a 1:1 mixture of **water:acetonitrile** (this works well for many organic salts). Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
4. If the test chemical is not soluble in either acetonitrile or water, attempt to prepare a 100mM solution in **isopropanol .** Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
5. If the test chemical is not soluble in either acetonitrile, water or isopropanol, attempt to prepare a 100mM solution in **acetone or a 1:1 acetone:acetonitrile mix .** Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
6. As a last option, if the chemical is not soluble in any of these solvents, attempt to dissolve the same amount of test chemical in **300 microliters of dimethyl sulfoxide** and dilute the resulting solution with 2700 **microliters of acetonitrile** . Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less). 7. If the test chemical is not soluble in this mixture, dissolve the same amount of test chemical in **1500 microliters of dimethyl sulfoxide** and dilute the resulting solution with **1500 microliters of acetonitrile** . Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).

Note : *Water is not a good solvent choice for anhydrides due to their reactivity with water.*

Pre-weighing of test chemicals

Test chemicals are pre-weighed into clean, dry 4mL glass vials. Test chemicals will be dissolved in 3.0 mL of the appropriate solvent determined in the “Solubility Assessment” Pre-Work (see above) to prepare a 100 mM solution immediately before use. The weight of test chemical to be added to the vial is determined based on the molecular weight (“MW”) and purity. If no purity information is available, assume 100% purity.

1. Calculate the target weight of test chemical needed to prepare 3.0mL of a 100mM solution of test chemical using the formula:

$$3mL \times \frac{1\,L}{1000mL} \times \frac{100\ mmoles}{L} \times MW\left(\frac{mg}{mmole}\right) \times \frac{100}{\%Purity} = \frac{\text{MW}}{\%\ \text{Purity}} \times 30 = \text{Target Weight (mg)}$$

2. Weigh the target amount (+/- 10% of target) of test chemical directly into a glass vial and record the actual weight, identity, molecular weight and purity.
3. Tightly close each vial and store under appropriate conditions until ready to perform testing. Appropriate storage conditions for each test chemical are determined based on supplier information.

Experimental system pre-work preparation

Pre-weigh Cysteine or Lysine peptide for stock solutions (0.667 mM)

Note: Do not add buffer to the peptide solid until ready to begin the assay

Cysteine Peptide Ac-RFAACAA-COOH, 0.667 mM, 0.501 mg/mL: The assay will require approximately 800 µL/sample replicate All samples in a batch should use the identical peptide stock solution. Based on the amount of peptide stock needed, weigh an appropriate amount of peptide into a large vial or test tube.

For example, to prepare 25 mL of solution, weigh 12.5 mg of Cysteine peptide. Smaller or larger quantities may be prepared as appropriate, but the final concentration should always be 0.501 mg/mL. Record the exact weight of peptide added to the vial.

Lysine Peptide Ac-RFAAKAA-COOH, 0.667 mM, 0.518 mg/mL: The assay will require approximately 800µL/sample replicate. All samples in a batch should use the identical peptide stock solution. Based on the amount of peptide stock solution needed, weigh an appropriate amount of peptide into a large vial of test tube. For example, to prepare 25 mL of solution, weigh 12.9 mg of Lysine peptide. Smaller or larger quantities may be prepared as appropriate, but the final concentration should always be 0.518 mg/mL. Record the exact weight of peptide added to the vial.

Note: When starting a new lot of peptide, a small amount should be dissolved in the appropriate buffer at ~0.5 mg/mL and injected through the HPLC to verify that the chromatogram is similar to previous batches

Method

ROUTINE PROCEDURES

Preparation and handling of HPLC System

Prepare an HPLC system with a UV detector (220 nm) and mobile phase A and B described in the **Reagents** section.

1. Column Equilibration: Install a new guard column cartridge for each set of chemicals. Install the HPLC column (see Apparatus section) and equilibrate the column at 30 °C with 50% A, 50% B for at least 2 hours before use. Condition the column by running the gradient at least twice before using the column.
2. Column Storage Conditions: If the column will be stored for more than a week, fill the column with acetonitrile (without Trifluoroacetic Acid) and cap tightly. Store at room temperature.
3. System Shutdown: Following analysis, maintain a low flow (typically 0.05 mL/min) of 50% A: 50% B through the system and decrease column temperature to approximately 25 °C. If the system is to be idle for more than a week, fill the column with acetonitrile (without Trifluoroacetic acid), remove the column from the HPLC system and cap tightly and purge acid containing mobile phases from the system using a mixture of either 1:1 (v/v) acetonitrile:water or 1:1 (v/v) methanol:water.

TEST MATERIAL EXPOSURE PROCEDURES

General Planning

Suitable run sequence sizes are 1-26 test chemicals, in addition to the Positive Control and Reference Controls. If additional solvents are required, additional Reference Controls need to be prepared and the number of test chemicals will need to be reduced. This run sequence size permits the first HPLC injection to occur 24 hours after mixing the test chemical and peptide and the last HPLC injection to occur no more than 30 hours later. Appropriate controls must be included in each run sequence.

1. Label three autosampler vials for each test chemical and controls corresponding to the triplicate preparations.
2. Pre-weighing of all test chemicals and solvent selection is described in the previous section **"Test chemicals pre-work preparation"** . Do not dissolve until ready to use.
3. Pre-weighing of Cysteine and Lysine peptide is described in the previous section **"Test chemicals pre-work preparation"** . Do not dissolve until ready to use.

Test Chemical Solution Preparation

Solubility of the test chemical in the appropriate solvent is evaluated in the previous section **"Test chemicals pre-work preparation"** . 100mM solutions of test chemicals in the appropriate solvents are prepared fresh, immediately before use.

1. When ready to perform the assay, calculate and weigh out the appropriate amount of test chemical needed to prepare a 100mM solution.
2. Dissolve the test chemical by adding 3.0mL of the appropriate solvent. The resulting solution should have a test chemical concentration of 100 mM.
   Note: For test chemicals that are expensive or in short supply, it is possible to prepare smaller volumes of solutions (i.e. 1 ml), as long as the actual weight of test chemical used to prepare the 100mM stock solutions can be measured within 10% of the calculated target.
3. Mix vial to dissolve the test chemical. Slight sonication (less than 1 minute) may be used if needed. If the test chemical is not completely dissolved, do not proceed with that specific test chemical in the selected solvent.
   Re-evaluate alternative solvents (see **"Test chemicals pre-work preparation"**) to find a suitable choice.

3. Record and report the final solvent choice for each chemical.

Peptide Stock Solution Preparation

1. ***Cysteine Peptide*:** Add the appropriate amount of pH 7. 5 phosphate buffer to make a 0.667 mM solution of Cysteine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial (from above) using the equation:

$$\text{mL pH 7.5 Buffer} = \frac{\text{mg Peptide}}{0.501\ \text{mg/mL}}$$

2. ***Lysine Peptide*:** Add the appropriate amount of pH 10.2 Ammonium Acetate buffer to make a 0.667 mM solution of Lysine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial (from above) using the equation:

$$\text{mL pH 10.2 Buffer} = \frac{\text{mg Peptide}}{0.518\ \text{mg/mL}}$$

Control and Sample Preparation

Positive Control

Solution Preparation

Cinnamic aldehyde is soluble in acetonitrile. It is used as the Positive Control for the assay and is included in every assay run.

1. Calculate the target weight of cinnamic aldehyde needed to prepare 3.0mL of a 100mM solution using the formula:

$$3mL \times \frac{1\,L}{1000mL} \times \frac{100\ mmoles}{L} \times MW\left(\frac{mg}{mmole}\right) \times \frac{100}{\%Purity} = \frac{\text{MW}}{\%\ \text{Purity}} \times 30 = \text{Target Weight (mg)}$$

2. Weigh the target amount (+/- 10% of target) directly into a glass vial and record the actual weight, identity,molecular weight and purity.
3. Dissolve in 3mL of acetonitrile.

Reference Controls

Three types of "Reference Controls" are included with each study. Reference Control is a peptide solution where the test chemical is replaced by the solvent used to dissolve it.

- Reference Control A is made with acetonitrile and used to verify the accuracy of the calibration curve for peptide quantification.
- Reference Control B is made with acetonitrile and its replicates are injected in the beginning and in the end of the experimental run to verify the stability of the peptide over the analysis time.
- Reference Controls C are made with each solvent used to solubilize the test chemicals and should be included in every assay run together with the samples (see **Annex I** for example). They are used to verify that the solvent does not impact the Percent Peptide Depletion. The appropriate Reference Controls C for each chemical are used to calculate Percent Peptide Depletion (see **Data Analysis**).

Sample Preparation and Co-elution Control

Samples are prepared in triplicate for both peptides. Each assay (Cys and Lys) may be prepared concurrently (if two HPLC systems are available) or on separate days (if only one HPLC is available). One sample is prepared without peptide, to verify whether the test chemical absorbs at 220 nm and has a similar retention time as a peptide and may interfere with the data analysis (Co-elution Control).

1. Assemble the following previously prepared reagents, solvents and solutions:
   a. Peptide stock solution,
   b. Appropriate buffer (pH 7.5 phosphate buffer for Cysteine Peptide, pH 10.2 ammonium acetate buffer for Lysine peptide),
   c. Acetonitrile
   d. Test chemical solution (or solvent for Reference Controls)
2. Using 1 mL autosampler vials as containers, prepare the sample by adding the reagents in the quantity and order listed below, with gentle mixing during addition. Record the time of addition of the test chemical to the peptide solution.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM test chemical	**1:50 Ratio, Lysine Peptide** 0.5 mM Peptide, 25 mM test chemical
750 µL Cysteine peptide solution (or pH 7.5 phosphate buffer for Co-elution Controls) **200 µL Acetonitrile** **50 µL Test chemical solution (or solvent for Reference Controls)**	**750 µL Lysine peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution Controls) 250 µL Test chemical solution (or solvent for Reference Controls)**

3. Cap the vials, vortex to mix and place in the HPLC autosampler (dark) at 25 °C for 24 hours. HPLC analysis of the batch of samples should start 24 hours after the test chemical was added to the peptide solution.

Note: For each set of control/sample triplicates, the replicate vials should be prepared individually, using the same solutions .

Standard Preparation for the Determination of the Calibration Curve

Standards are prepared in a solution of 20% Acetonitrile:Buffer

Note : Samples will have a mixture of 25% solvent:buffer. This difference does not adversely impact the chromatography or stability of the samples and standards

Using serial dilution, prepare standards of the peptide stock solution covering the range from 1 - 0.0167mM.

1. **Prepare approximately 10 mL of dilution buffer** by mixing 8 mL of buffer (pH 7.5 phosphate buffer for Cysteine peptide, pH 10.2 ammonium acetate buffer for Lysine peptide) with 2 mL of acetonitrile.
2. **Prepare the initial standard, "STD1"** at 0.534 mM by diluting 1600 µL of the peptide stock solution (at 0.667 mM) with 400 µL acetonitrile.
3. **Dilute 1.0 mL of standard STD1 with an equal volume of dilution buffer and continue** in a serial manner to give standards with nominal concentrations noted below. Include a blank of dilution buffer as STD 7.

	STD1	STD2	STD3	STD4	STD5	STD6	STD7 (Dilution Buffer)
mM Peptide	**0.534**	**0.267**	**0.1335**	**0.0667**	**0.0334**	**0.0167**	**0.000**

Serial Dilution Procedural Details

1. Label 5 glass vials (nominal 2-5 mL volume) with codes STD2 – STD6.
2. Add 1.00 mL of dilution buffer to vials STD2 through STD6
3. Transfer 1.00 mL of Standard STD1 to vial STD2. Mix with minimal air entrainment 4. Transfer 1.00 mL from vial STD2 to vial STD3. Mix with minimal air entrainment
5. Continue in a similar manner for standards STD4 through STD6.
6. Transfer standards to autosampler vials, cap the vials and place in the HPLC autosampler (dark) at 25 °C for 24 hours before analysis.

ENDPOINT MEASUREMENT

HPLC analysis

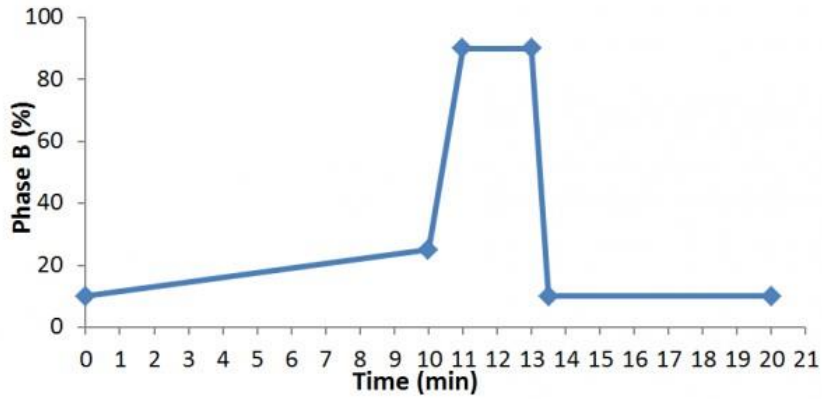
1. Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the weak and strong solvents and a column temperature of 30ºC. The HPLC analysis is performed using a flow of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials.
2. Inject equal volumes of each standard, sample and control. The injection volume may vary according to the system used (typically in the range from 3-10 µL). On some systems, 10µL injection volumes lead to unacceptably broad peaks and smaller injection volumes need to be used. Absorbance is monitored at 220 nm. If using a Photodiode Array detector, absorbance at 258 nm should also be recorded.
3. Re-equilibrate the column under initial conditions for at least 7 minutes.

Note: The 7 minute re-equilibration time was determined using a Waters 2695 HPLC system. Other systems may require more or less re-equilibration time due to system mixing volume. Shorter equilibration times will be acceptable if peak retention times are stable.

HPLC Conditions

Column	Preferred Column: Zorbax SB-C18 2.1mm x 100 mm x 3.5 micron Agilent Part Number 861753-902 Alternate Column: Phenomenex Luna C18(2) 2.0 mm x 100mm x 3 micron particle (Part # 00D-4251-B0 may require flowrates of 0.3mL/min) or any other C18 column that demonstrates acceptable peak resolution. *Note: Both columns are semi-micro scale and require careful connections to minimize extracolumn peak broadening. Waters 2695 Alliance HPLC systems provide suitable peak shapes. Semi-micro HPLC systems may improve peak resolution and it may be possible to decrease analysis time on other systems.*
Column Temperature	30 ºC
Sample temperature	25 ºC
Detector	Photodiode Array detector or Fixed Wavelength Absorbance detector with 220 nm signal for quantitation
Injection Volume	~7 µL (Volume varies according to the HPLC system. If peaks are too broad, the volume should be decreased) Set the autosampler needle depth to avoid drawing sample from the bottom of the vial.
Run Time	20 minutes
Flow Conditions	**Time** **Flow** **%A** **%B** 0 min 0.35 mL/min 90 10 10 min 0.35 mL/min 75 25 11 min 0.35 mL/min 10 90 13 min 0.35 mL/min 10 90 13.5 min 0.35 mL/min 90 10 20 min end run

Graphic representation of the gradient



Note:

Visually inspect samples prior to HPLC analysis. Generally, precipitation is not a problem. However, if a precipitate is observed, this should be noted in the data reporting template. Samples may be centrifuged at low speed (100-400 xg) in the vial to force precipitate to the bottom of the vial as a precaution, since large amounts of precipitate may clog the HPLC tubing or columns. ***Precipitate formation and removal must be recorded and reported.***

Filtering samples or use of high speed polypropylene centrifuge tubes to remove precipitate has not been evaluated and may lead to loss of peptide through adsorption, therefore this is not recommended.

Prepare two separate analysis sequences, based on the example below:

1. Calibration standards, Reference Controls A and Co-elution Controls
2. Stability of Reference Controls over analysis time (Reference Controls B) and sets of replicates (Reference Controls

2. Stability of Reference Controls over analysis time (Reference Controls B) and sets of replicates (Reference Controls C, Positive Control and test chemicals).

The first analysis sequence can be timed to complete prior to the end of the 24 hour incubation and the second sequence should be timed to assure that the injection of the first sample starts 24 (+/-2) hours after the test chemical was mixed with the peptide solution. Alternatively, since there is no chemical reaction occurring in the calibration standards, Reference Controls and Co-elution controls, the first analysis sequence can be timed to run shortly after assay setup is complete rather than directly before the second analysis sequence.

Example HPLC Sample Analysis Sequences

(A more specific analysis sequence can be found in the Annex I)

STD1 **STD2** **STD3** **STD4** **STD5** **STD6** **STD7(Dilution Buffer)** **Reference Control A, rep 1** **Reference Control A, rep 2** **Reference Control A, rep 3**	***Calibration Standards and Reference Controls*** *Verify linearity of response* *Verify precision and accuracy of pipetting* *System Suitability: r ^{2} >0.990* *Mean peptide concentration of Reference Controls A = 0.50 +/- 0.05 mM*
Co-elution Control 1 **Co-elution Control 2** **Co-elution Control 3** **Co-elution Control n**	***Co-elution Controls*** *Verify co-elution of test chemicals with peptide*
Reference Control B, rep 1 **Reference Control B, rep 2** **Reference Control B, rep 3**	***Reference Controls*** *Verify stability of Reference Controls over analysis time (see also below)*
Reference Control C, rep 1 §, † **Cinnamic Aldehyde, rep 1** **Sample 1, rep 1** **Sample 2, rep 1** **Sample 3, rep 1** **Sample n, rep 1**	***First set of replicates*** *Note : Start first set of replicates 24 +/- 2 hours after peptide:test chemical mixing.*
Reference Control C, rep 2 § **Cinnamic Aldehyde, rep 2** **Sample 1, rep 2** **Sample 2, rep 2** **Sample 3, rep 2** **Sample n, rep 2 .**	***Second set of replicates***
Reference Control C, rep 3 § **Cinnamic Aldehyde, rep 3** **Sample 1, rep 3** **Sample 2, rep 3** **Sample 3, rep 3** **Sample n, rep 3 †**	***Third set of replicates*** *For each solvent used, the mean of the peptide concentrations of the three appropriate Reference Controls C = 0.50 +/- 0.05 mM*
Reference Control B, rep 4 **Reference Control B, rep 5** **Reference Control B, rep 6**	***Reference Controls*** *Verify stability of Reference Controls over analysis time:* *CV of peptide peak areas of the nine Reference Controls B and C in acetonitrile must be < 15.0%*

§ *Three replicates for Reference Controls C should be included in the analysis sequence for each solvent which is used to dissolve test chemicals. These should be run with the Samples and are used to separately calculate the Percent Peptide Depletion in each solvent and verify that they do not impact the Percent Peptide Depletion.*

† *The difference in time between the first injection of the first replicate and the last injection of the last replicate should not exceed 30 hours.*

Data Analysis

The concentration of peptide is determined in each sample from absorbance at 220 nm, measuring the peak area of the appropriate peaks and calculating the concentration of peptide using the linear calibration curves derived from the standards (STD1 to STD7).

Note: The Cysteine peptide includes other peaks that elute near the peptide of interest (see Annex II). Refer to the example chromatogram for appropriate integration of the peak.

The percent depletion of peptide is determined in each sample measuring the peak area and dividing that by mean peak area of the reference controls.

1. Integrate the appropriate peaks and determine peak area for standards, samples and controls. The peak area of each integrated peak must be reported. The peaks should be consistently integrated "valley to valley". There may be some instances when this is not practical, but should be appropriate for most chromatograms.
2. Generate a linear calibration curve based on the concentration of standards (equal weighting) and the peak area. Suitable calibration curves will have an r 2 >0.990. For Reference Controls A calculate the mean peptide concentration, SD and CV. The mean should be 0.50 +/- 0.05 mM. Values outside of this range may indicate a pipetting or sample preparation error. The peptide concentration of Reference Controls A and C (see HPLC analysis sequence in the **Endpoint Measurement** section) must be reported.
3. For the nine Reference Controls B and C in acetonitrile calculate the mean peptide peak area at 220 nm for, SD and CV. The CV must be < 15.0%.
4. For the three Reference Controls C calculate the mean peptide peak area at 220 nm for each solvent used.
5. Calculate the mean peptide concentration (mM) for the three Reference Controls C for each solvent used, SD and CV. The mean should be 0.50 +/- 0.05 mM.
6. UV absorbance is a general detection method and interfering peaks may occur. If there is uncertainty regarding the identity of the peak, verify the UV absorbance spectrum and retention time are consistent with the Reference Control C injections.
7. For the Positive Control and for each test chemical, calculate the Percent Peptide Depletion in each replicate from the peptide peak area of the replicate injection and the mean peptide peak area in the three relevant Reference Controls C (in the appropriate solvent), by using the following formula. The Percent Peptide Depletion of every injected Positive Control and test chemical replicate must be reported. Moreover, the mean Percent Peptide Depletion of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection}}{\text{Mean Peptide Peak Area in Reference Controls } C}\right)\right] \times 100$$

Acceptance Criteria

Run Acceptance Criteria

All criteria must be met for the whole run to be considered valid. If these criteria are not met, the run must be repeated for all test chemicals.

System Suitability:

Calibration Linearity r 2 > 0.990

Mean peptide concentration of Reference Controls A = 0.50 +/- 0.05 mM

Positive Control:

The mean Percent Peptide Depletion value of the three replicates for cinnamic aldehyde must fall within the ranges reported in the following table (based on 95% Tolerance Intervals):

	Percent Cysteine Depletion		Percent Lysine Depletion	
Positive Control	**Lower Bound**	**Upper Bound**	**Lower Bound**	**Upper Bound**
Cinnamic aldehyde	60.8	100.0	40.2	69.4

Maximum Standard Deviations for Positive Control replicates:

Standard Deviation for Percent Cysteine Depletion must be < 14.9%

Standard Deviation for Percent Lysine Depletion must be < 11.6%

Stability of Reference Controls over analysis time:

CV of peptide peak areas for the nine Reference Controls B and C in acetonitrile must be < 15.0%.

Test Chemical Acceptance Criteria

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

Maximum Standard Deviation of sample replicates:

Standard Deviation for Percent Cysteine Depletion must be < 14.9%

Standard Deviation for Percent Lysine Depletion must be < 11.6%

Reference Controls in the analysis sequence:

For each solvent used, the mean of the peptide concentrations of the three appropriate Reference Controls C = 0.50 +/- 0.05 mM

Data Acceptance Criteria

Presence of precipitate

If precipitation occurs immediately when peptide and test chemical are mixed, this should be recorded and caution should be used in interpreting the data.

If precipitate occurs at the end of the 24 hrs incubation period, this should be recorded and the sample analysed, after centrifugation to settle the precipitate at the bottom to avoid clogging up the HPLC system.

Co-elution of test chemical with peptide

In cases where a test chemical co-elutes with the lysine peptide, the Cysteine 1:10-only prediction model can be used. In cases where the test chemical co-elutes with the cysteine peptide and the peptide peak can not be integrated, a determination of reactivity can not be made based on the Percent Depletion data from the lysine reaction alone, and the data should be reported as “inconclusive”. If the peak for the cysteine peptide can be integrated, follow the instructions below when determining and reporting an estimated Percent Peptide Depletion.

1. ***Negative depletion values***
   If the Percent Peptide Depletion is < - 10.0%, it should be considered that this may be a situation of co-elution, inaccurate peptide addition to the reaction mixture or just baseline "noise." If this happens, the coelution controls (test chemical alone chromatograms and 220/258 ratio) should be carefully analyzed. If the peptide peak appears at the proper retention time and has the appropriate peak shape (see examples on page 22), the peak can be integrated. In this case, there may just be baseline noise causing the peptide peak to be bigger or there may be some co-elution/overlap in retention time of the peptide and test chemical. The peptide peak is visible and can be integrated. The calculated %-depletion should be reported as an "estimate." If this was only an issue for lysine, use the "cysteine-only" prediction model. If this is an issue with cysteine or both cysteine and lysine, use the table on page 20 of the SOP.

   If the peak does not have the proper shape due to complete overlap in retention time of the test chemical and peptide and can not be integrated, calculation of Percent Peptide Depletion is not possible. If this is an issue for lysine, use the "cysteine-only" model. If this is an issue for cysteine or both cysteine and lysine, the data must be reported as **"inconclusive"** .
2. ***Co-elution Controls***
   If a chemical (Co-elution Control) absorbs at 220 nm and has a similar retention time as a peptide (Reference Control) (overlap of "valley to valley" integration periods), then co-elution of the test chemical with the peptide should be reported. In order to assure that baseline noise is not being identified as interference, the "interfering" chemical peak should have a peak area that is >10% of the mean peptide peak area in the appropriate Reference Control.

   The chromatograms of the reaction mixtures should also be inspected in case of possible co-elution to verify if the peaks of the chemical and the peptide are indeed not baseline separated. If co-elution occurs with cysteine peptide alone or with both peptides, proper integration and calculation of Percent Peptide Depletion is not possible. The data should be recorded as **"interference"** for that peptide.
3. ***Peak purity indicator: area ratio 220/258***
   When a Photodiode Array detector is used, co-elution of chemical and peptide may be explored by looking at the UV spectrum at 258nm and calculating the area ratio of 220/258. This value should be consistent over all samples and standards for a pure peptide peak and thus gives a measure of peak purity. For each sample a ratio in the following range would give a good indication that co-elution purity. For each sample a ratio in the following range would give a good indication that co-elution has not occurred:
   90%<Mean Area ratio of control samples <110% .

   However, calculation of peak purity (area ratio of 220/258) in test samples might not always be possible, particularly if the test chemical is highly reactive with the peptide, leading to very small peaks.

4. ***Co-elution with reactivity and estimated depletion values***
   In some instances, a test chemical may have an overlapping retention time with either of the peptides, and be reactive with that peptide. This can make the peak area of the peptide appear to be larger than it really is, therefore the calculated percent depletion may be **underestimated** .

   In cases where the overlap in retention time between the test chemical and peptide is not complete, the underlying peaks can be deconvoluted and percent depletion can be calculated and recorded with a notation of " **co-elution – percent depletion estimated** ".

Prediction Model

The mean value of the Percent Cysteine Peptide Depletion and Percent Lysine Peptide Depletion is calculated for the Positive Control and for each test chemical. Negative depletion values should be considered as “Zero” when calculating the mean.

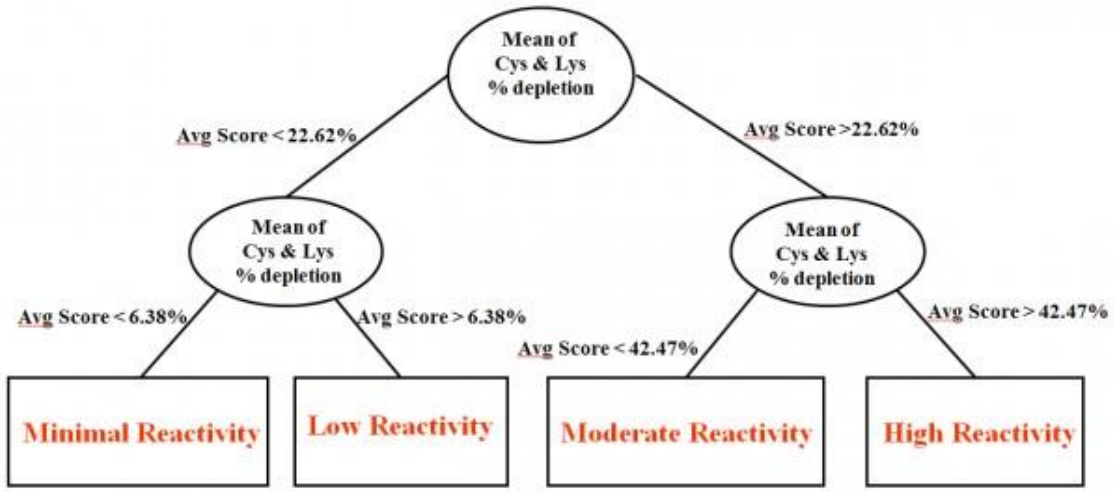
Before applying the prediction model, the experimental data need to be evaluated with care regarding the possibility of co-elution. Table below lists the different scenarios possible and recommended approach:

Mean depletion values	No co-elution	Co-elution with Cysteine alone or Cysteine and Lysine	Co-elution with Lysine only
Less than 6.38%	Minimal Reactivity	Inconclusive	Apply Cysteine-only prediction model
Between 6.38% and 22.62%	Low Reactivity	≥ Low Reactivity	Apply Cysteine-only prediction model
Between 22.62% and 42.47%	Moderate Reactivity	≥ Moderate Reactivity	Apply Cysteine-only prediction model
More than 42.47%	High Reactivity	High Reactivity	Apply Cysteine-only prediction model

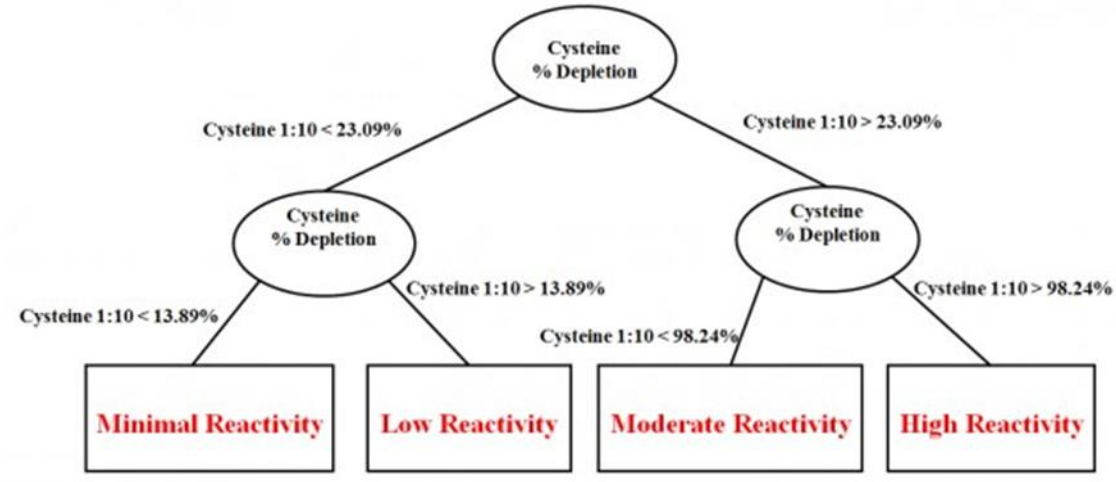
A reactivity category can be assigned to each test chemical by using the **“Cysteine 1:10/Lysine 1:50”** prediction model outlined below. In cases where a test chemical co-elutes with the lysine peptide, the **“Cysteine 1:10-only”** prediction model can be used. In cases where the test chemical co-elutes with the cysteine peptide and percent depletion can not be estimated, a determination of reactivity can not be made based on the Percent Depletion data from the lysine reaction alone. The data should be reported as **“inconclusive”.** The lysine reactivity alone does not carry enough weight to drive a lysine-only prediction model.

On the basis of the Cysteine 1:10/Lysine 1:50 Prediction Model or the Cysteine 1:10-only Prediction Model, chemicals assigned to the minimal reactivity category should be classified as non-sensitisers whereas chemicals assigned to the Low, Moderate or High reactivity categories should be classified as sensitisers.

Cysteine 1:10/Lysine 1:50 Prediction model



Cysteine 1:10-only Prediction Model



Annexes

ANNEX I

Example HPLC Analysis

Example DPRA run:

There are 5 test chemicals. Chemical 1, 2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in isopropanol. The following vials should be set up:

Analysis Sequence 1:

STD 1 STD 2 STD 3 STD 4 STD 5 STD 6 Dilution buffer blank Reference Control A, rep 1 (made with acetonitrile) Reference Control A, rep 2 (made with acetonitrile) Reference Control A, rep 3 (made with acetonitrile) Coelution Control for Chemical 1 Coelution Control for Chemical 2 Coelution Control for Chemical 3
Coelution Control for Chemical 4 Coelution Control for chemical 5

Analysis Sequence 2:

Reference Control B, rep 1 (made with acetonitrile) Reference Control B, rep 2 (made with acetonitrile) Reference Control B, rep 3 (made with acetonitrile) Reference Control C, rep 1 (made with acetontrile) Reference Control C, rep 1 (made with isopropanol) Cinnamic aldehyde, rep 1 Chemical 1, rep 1 Chemical 2, rep 1 Chemical 3, rep 1 Chemical 4, rep 1 Chemical 5, rep 1 Reference Control C, rep 2 (made with acetontrile) Reference Control C, rep 2 (made with isopropanol) Cinnamic aldehyde, rep 2 Chemical 1, rep 2 Chemical 2, rep 2 Chemical 3, rep 2 Chemical 4, rep 2 Chemical 5, rep 2 Reference Control C, rep 3 (made with acetontrile) Reference Control C, rep 3 (made with isopropanol) Cinnamic aldehyde, rep 3 Chemical 1, rep 3 Chemical 2, rep 3 Chemical 3, rep 3 Chemical 4, rep 3 Chemical 5, rep 3 Reference Control B, rep 4 (made with acetonitrile) Reference Control B, rep 5 (made with acetonitrile) Reference Control B, rep 6 (made with acetonitrile)

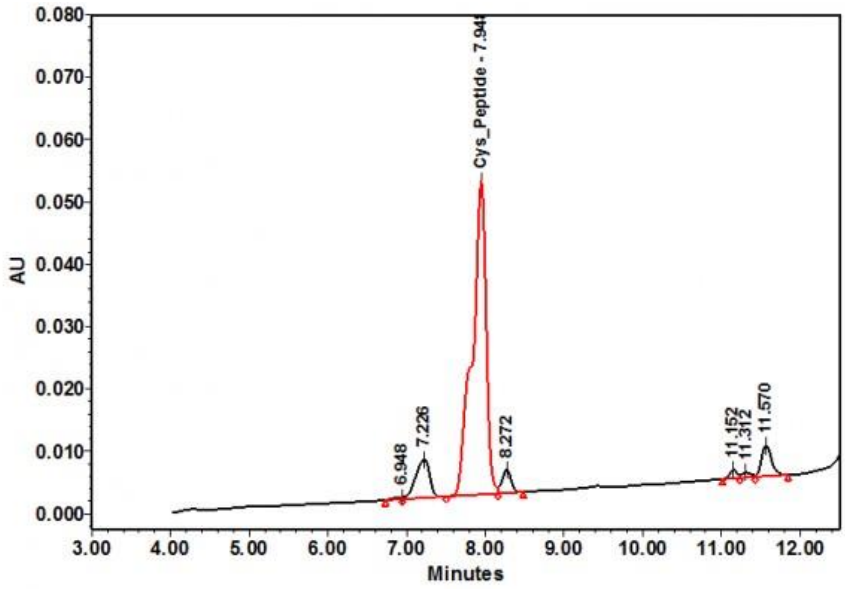
Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean peptide peak area of the Reference Control C made with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean peptide peak area of the Reference Control C made with isopropanol.

ANNEX II

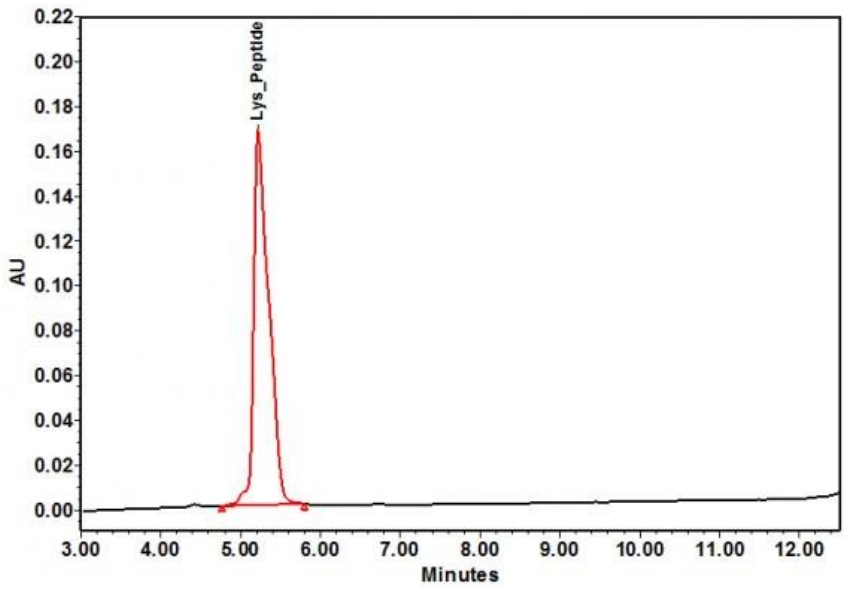
Example Chromatograms

Cysteine Peptide (Retention Time approximately 8-9 minutes)



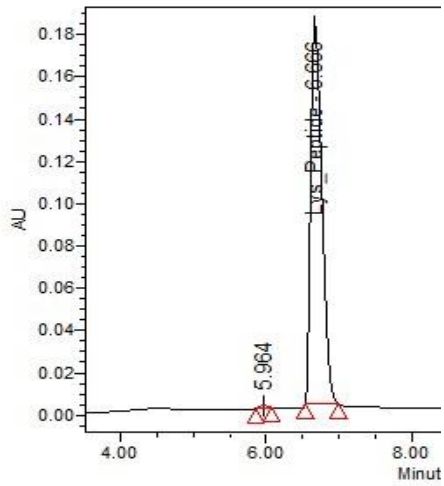
Note: The Cysteine peptide shown contains a mixture including the peptide with one less alanine ("A") unit which causes the leading edge shoulder on the peak. Both peptides react in a comparable manner and are integrated together.

Lysine Peptide (Retention Time approximately 5-6 minutes)

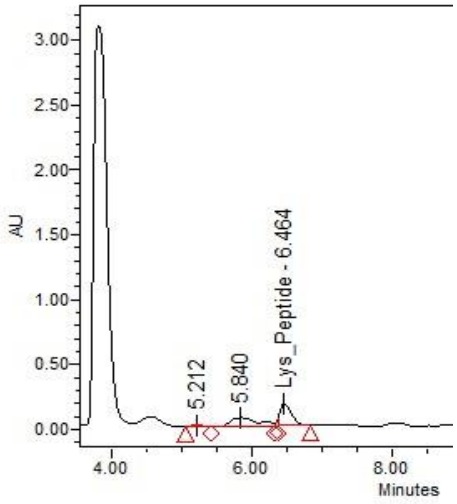


ANNEX III

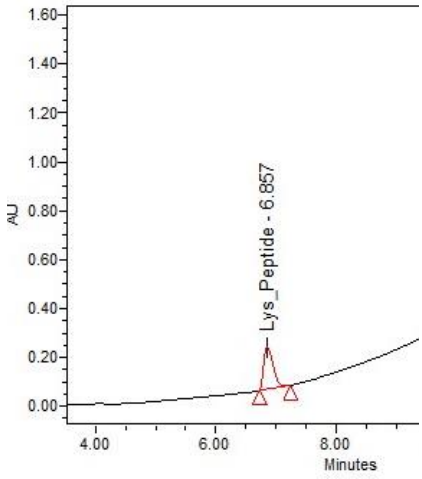
Examples of co-elution:
Situation 1: Possible co-elution or baseline noise/strange looking baseline. The peptide peak can be integrated, but should be considered an estimate.



The peptide peak is clearly visible and can be integrated. Perhaps there is some small test chemical peak with the same retention time which would make the peptide peak appear bigger and give a <-10% depletion.



The lysine peak overlaps slightly with a test chemical peak. This would be reported as possible co-elution/estimated % depletion



The peptide peak can be integrated but the baseline is not flat so this should be considered an estimate because the “area under the curve” can not be determined with complete certainty.

ANNEX IV

Data reporting checklist for cysteine and lysine peptide reactivity assay

For the system suitability the following should be reported:

- Peptide peak area at 220 nm of each Standard and Reference Control A replicate.
- The linear calibration curve should be graphically represented and the R^2 reported.
- Peptide concentration (mM) of each Reference Control A replicate.
- Mean peptide concentration (mM) of the three Reference Controls A, SD and CV.

For the analysis sequence the following should be reported:

Reference Controls:

- Peptide peak area at 220 nm of each B and C replicate.
- Mean peptide peak area at 220 nm of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean peptide peak area at 220 nm of the three appropriate Reference Controls C (for calculation of Percent Peptide Depletion).
- For each solvent used, the peptide concentration (mM) of the three appropriate Reference Controls C.
- For each solvent used, the mean peptide concentration (mM) of the three appropriate Reference Controls C, SD and CV.

Positive Control (cinnamic aldehyde):

- Peptide peak area at 220 nm of each replicate.
- Percent Peptide Depletion of each replicate.
- Mean Percent Peptide Depletion of the three replicates, SD and CV.

For each test chemical the following should be reported:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time. If precipitate was re-solubilised or centrifuged.
- Peptide peak area at 220 nm of each replicate (for systems equipped with a PDA detector the peak area at 258 nm should also be reported).
- Percent Peptide Depletion of each replicate.
- Mean of Percent Peptide Depletion of the three replicates, SD and CV
- Mean of Percent Cysteine and Percent Lysine Depletion values.
- Reactivity class. Co-
- elution.

Bibliography

- Aeby, P., Ashikaga, T., Bessou-Touya, S., Schepky, A., Gerberick, F., Kern, P., Marrec-Fairley, M., Maxwell, G., Ovigne, J.-M., Sakaguchi, H., et al. (2010)
  Identifying and characterizing chemical skin sensitizers without animal testing: Colipa's research and method development program. *Toxicology In Vitro 24, 1465–1473*
- EURL ECVAM (2013)
  EURL ECVAM Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing. *JRC Scientific and Policy reports.* **Link to document** (last access 20.01.2014)
  European Union Reference Laboratory for Alternatives to Animal Testing
- Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., and Lepoittevin, J.-P. (2004) Development of a peptide reactivity assay for screening contact allergens.
  Toxicological Sciences 81, 332–343
- Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G., and Lepoittevin, J.-P. (2007)
  Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach.*Toxicological Sciences 97, 417-427*
- Maxwell, G., Aeby, P., Ashikaga, T., Bessou-Touya, S., Diembeck, W., Gerberick, F., Kern, P., Marrec-Fairley, M., Ovigne, J.-M., Sakaguchi, H., et al. (2011)
  Skin sensitisation: the Colipa strategy for developing and evaluating non-animal test methods for risk assessment.*Alternatives to Animal Experimentation (ALTEX) 28, 50-55*
- OECD (2015)
  OECD Test Guideline No. 442D: *In Vitro* Skin Sensitisation ARE-Nrf2 Luciferase Test Method. Adopted on 4thFebruary 2015. **Link to document** (last access: 05.06.2015)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2012)
  OECD Series on Testing and Assessment No. 168: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. **Link to Part 1** , **Link to Part 2** (last access 20.08.2013)
  OECD Environmental Health and Safety Publications
- United Nations (2013)
  Globally Harmonised System of Classification and Labelling of Chemicals (GHS); New York and Geneva. Fifth revised edition. **Link to document** (last access 15.01.2014)