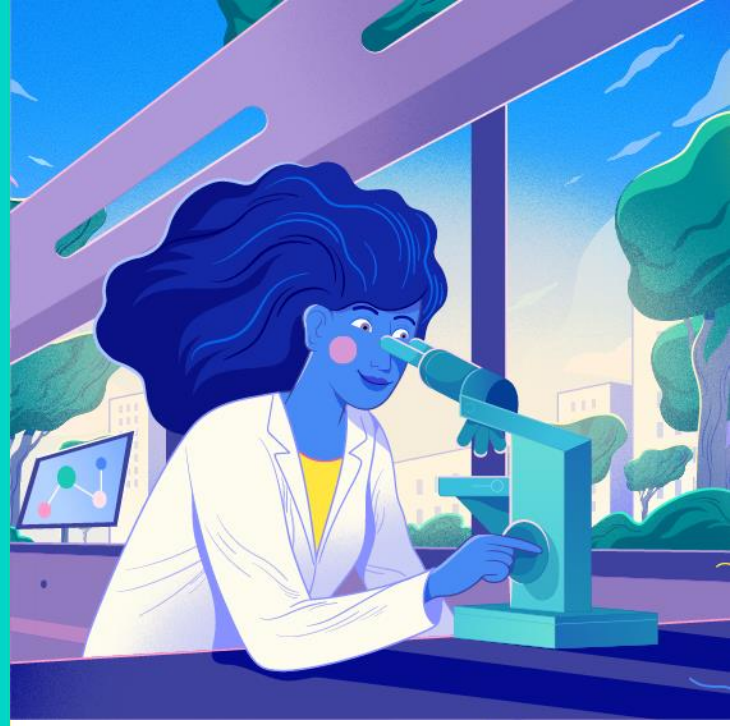
Use of Analytical chemistry in the Risk Assessment of Cosmetic and Homecare ingredients



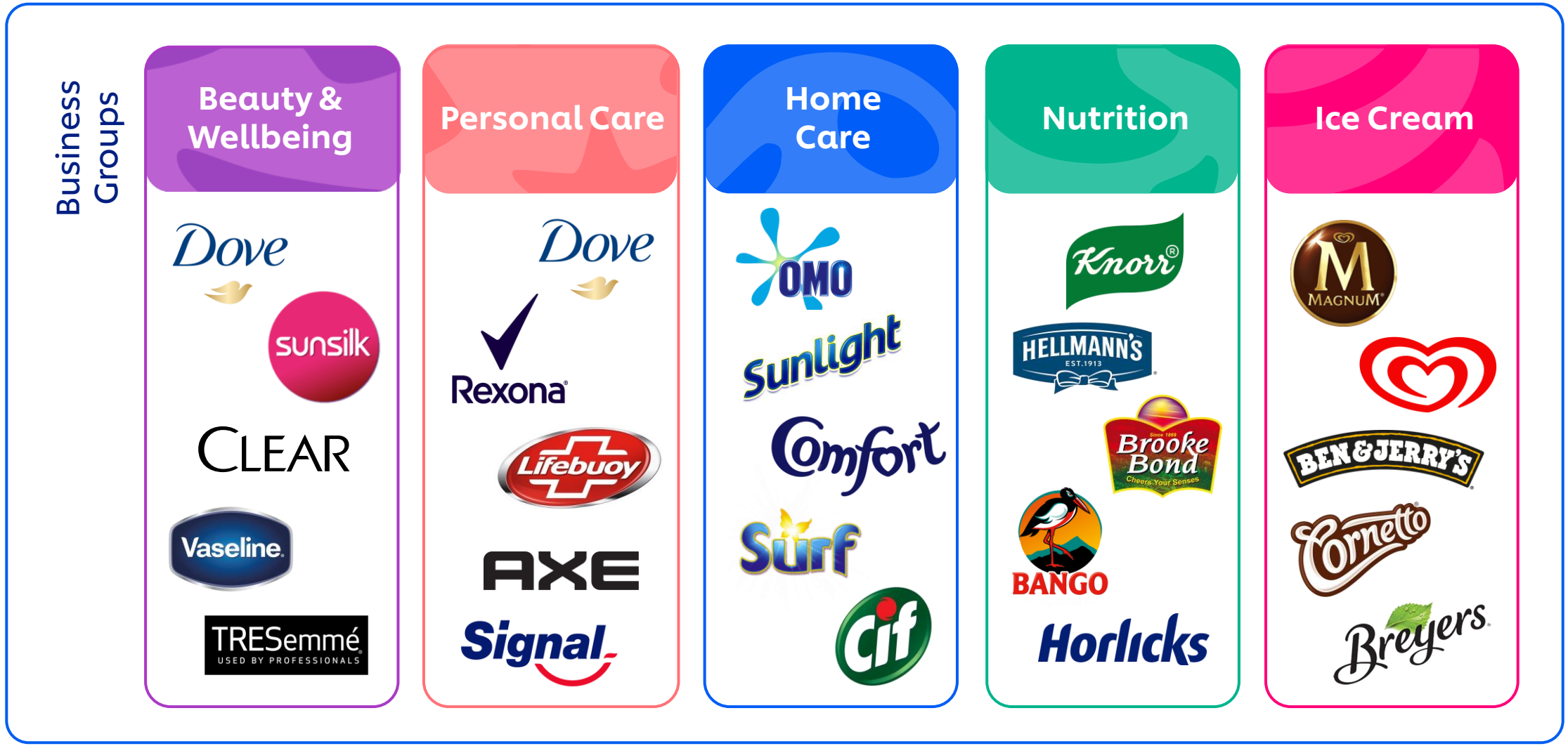
Regiane Sanches Natumi

Alexandre Teixeira

Safety & Environmental Assurance Centre (SEAC), Unilever, UK

Unilever

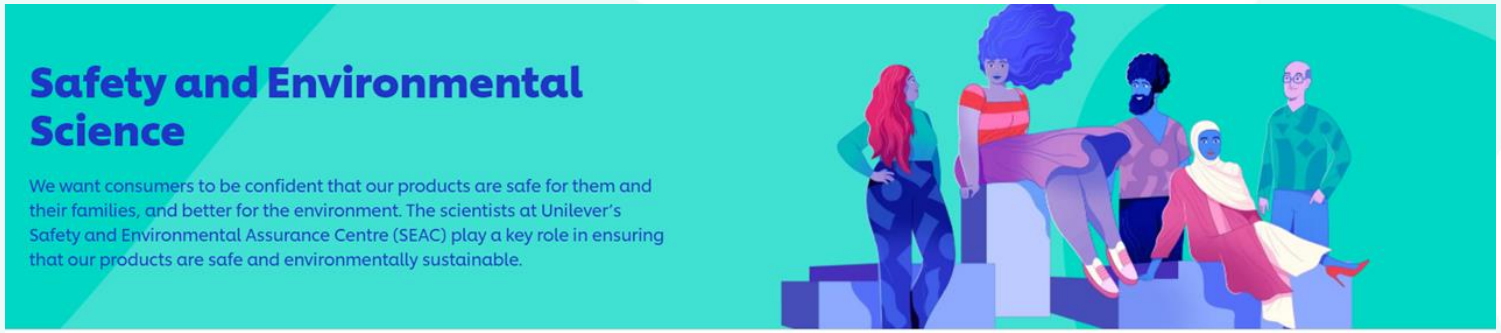
Our Five Business Groups



Note: Top Five Brands per Business Group

Unilever – Safety & Environmental Assurance Centre (SEAC)

Ensuring Unilever's Innovations & Products are Safe & Sustainable by Design



Unilever Product / Ingredient Safety Governance

- Provide scientific evidence to manage safety risks & environmental impacts

Industry-leading Safety & Environmental Sustainability Science Capability

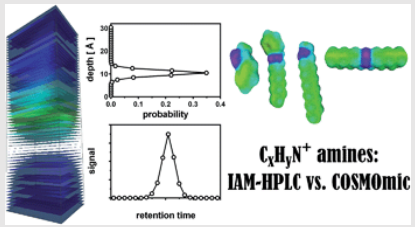
- Deploy expertise on higher risk business projects
- Collaborate with leading external research teams to develop & apply new capability
- Leverage our science & global networks for consumer trust & freedom to operate

Chemistry in SEAC

How we apply chemistry to help in the risk assessment of personal care and home care products

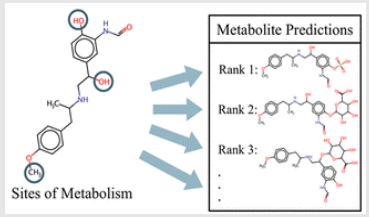
Computational/predictive chemistry

Computational modelling



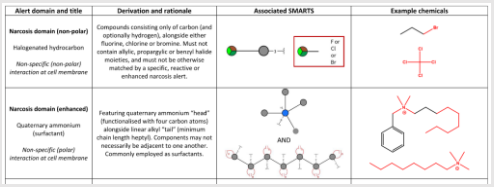
Environ. Sci.: Processes Impacts, 2016,18, 1011-1023

Metabolism prediction



Chem. Res. Toxicol. 2021, 34, 2, 286-299

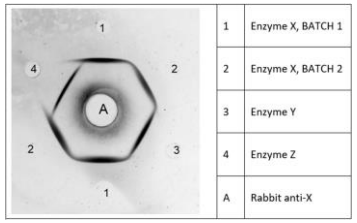
QSAR



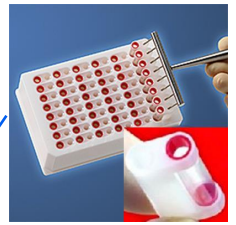
JEnviron. Sci. Technol. 2022, 56, 24, 17805−17814

Biochemistry

Enzyme/protein content/characterization



Protein binding



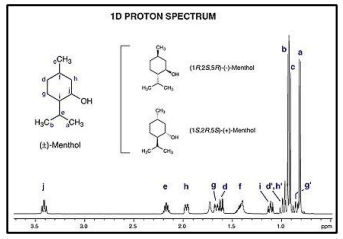
RED by Thermo Fisher Scientific Inc.

Environmental chemistry

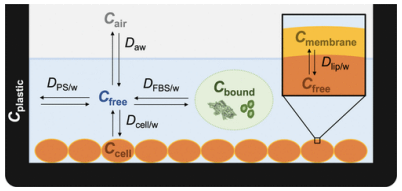
Environmental sample analyses

Analytical chemistry

Test item characterization

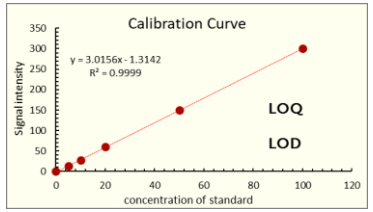


In-vitro dose confirmation



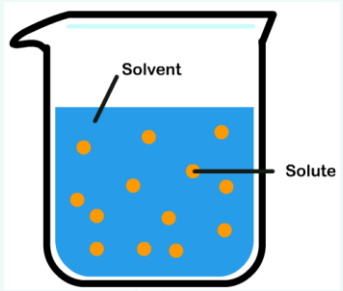
Environ. Sci. Technol. 2020, 54, 2, 1120-1127

Analytical method development



What is Analytical chemistry

The use of instruments and methods to separate, identify, and quantify matter



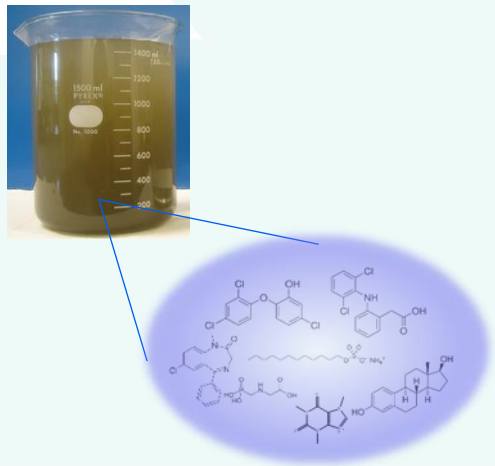
Pre treatment → **Separation** → **Identification / Quantification**

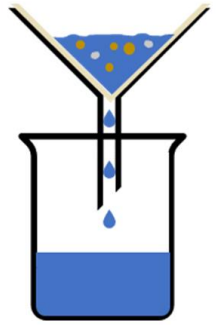
- Product
- Raw material
- Biological sample
- Environmental sample

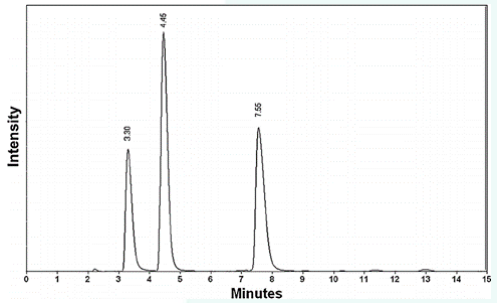
- Filtration
- Centrifugation
- Solid Phase Ex
- Liquid Phase Ex

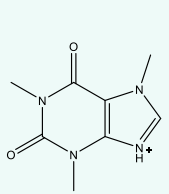
- Liquid chromatography
- Gas chromatography

- Ultraviolet/Infrared detection
- Mass Spectrometry
- Nuclear magnetic resonance

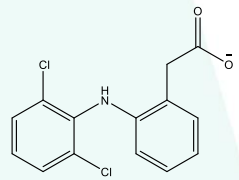




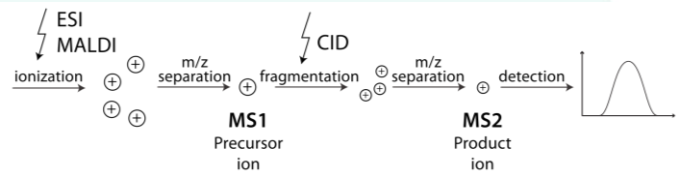




mass = 195.1 Da



mass = 294.0 Da



By Hannes Röst and M. Steiner. - Own work

Analytical chemistry in SEAC – capabilities

HPLC with UV and Fluorescence detectors

GC-MS/MS (triple quadrupole)

HPLC-MS/MS (triple quadrupole)

HPLC-MS/MS (Q-ToF)

Accredited

- GLP accredited laboratory
- Expertise in analytical chemistry is used across SEAC's consumer, occupational and environmental risk assessments

Unilever

Theme 1 – Free concentration and in-vitro dose confirmation

Cell stress panel is a screening assay consisting of 36 biomarkers representing mitochondrial toxicity, cell stress, and cell health, measured predominantly using fluorescent cellular image

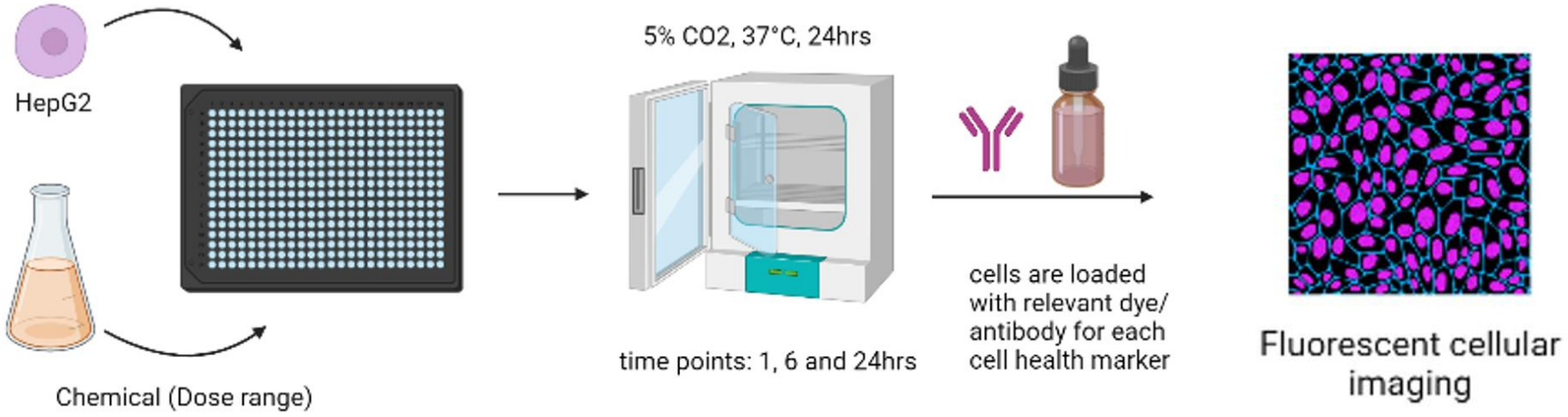
Range of biomarkers covering 10 cell stress pathways

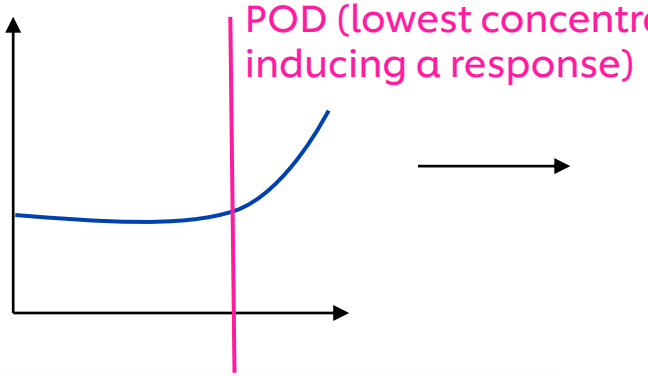
Mitochondrial toxicity: MitoSOX, PCG1α, MMP, ATP, OCR, Res Capacity
Oxidative stress: GSH, ROS, SRXN1, NRF2, HMOX1
DNA damage: pH2AX, p53
Inflammation: TNFAIP3, ICAM1, NFkB p65, IL-8
Endoplasmic Reticulum Stress: PERK, ATF4, CHOP, XBP1, BiP, ER Tracker
Metal Stress: MTF-1, Metallothionein
Heat Shock (HSP70) Hypoxia (HIF1α)
Cell Health: Cell count, Nuclear size, DNA Structure, LDH, Phospholipidosis, Steatosis, pHrodo indicator, apoptosis (caspase-3/7) & necrosis (ToPro-3)

Hatherell, S. et al. (2020) Identifying and characterizing stress pathways of concern for consumer safety in next-generation risk assessment. Toxicological Sciences, 176(1), 11-33. doi: 10.1093/toxsci/kfaa054

Unilever

Theme 1 – Free concentration and in-vitro dose confirmation





Dose response curve for each timepoint

Calculated PODs are compared with Cmax in blood

POD > Cmax in blood (high risk)

POD < Cmax in blood (low risk)

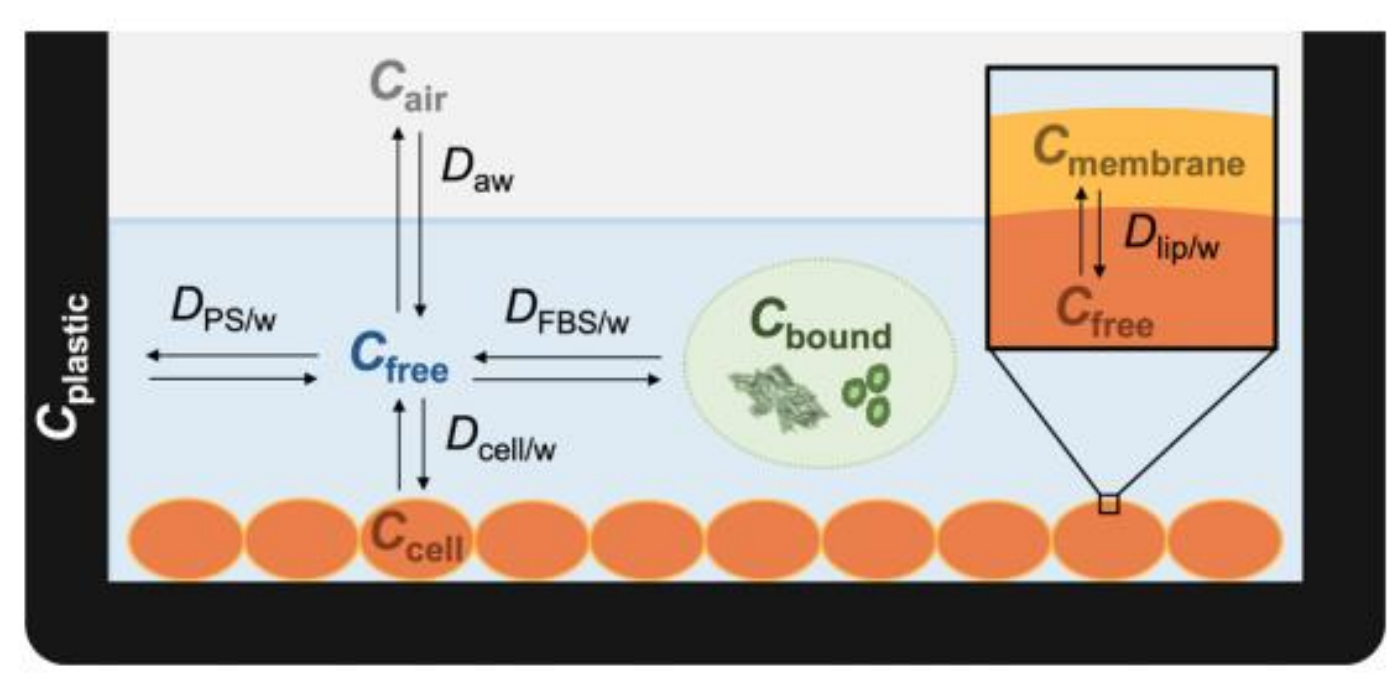
Created with BioRender.com

Hatherell, S. et al. (2020) Identifying and characterizing stress pathways of concern for consumer safety in next-generation risk assessment. Toxicological Sciences, 176(1), 11-33. doi: 10.1093/toxsci/kfaa054

Theme 1 – Free concentration and in-vitro dose confirmation

Important to derive the correct PoD. If you derive a higher PoD than the true one, then you may classify an amount as save to use, when it is not.

Cnominal = Cplastic + Cfree + Cair + Cbound



Henneberger,L. et al. Experimental validation of mass balance models for in vitro cell-based bioassays. Environ.Sci.Technol. 2020, 54, 1120-1127.

In-vitro dose confirmation

Replicate the in-vitro assay without the use of cells to quantify the concentration present in media (Cfree + Cbound)

Theme 1 – Free concentration and in-vitro dose confirmation

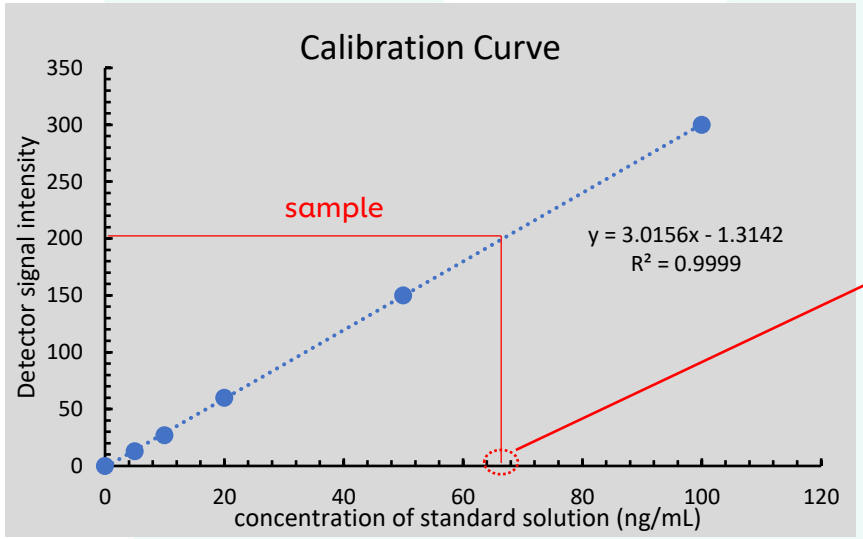
1. Method development

Separation

- Choose separation technique (LC or GC)
- Method of detection (UV, FLD,DAD, MS)
- Column
- Mobile phase

Quantification

- Select an internal standard (isotopic labelled, surrogate)
- Determine the Limit of Quantification (LOQ)
- Select the calibration range (range where the instrument has a linear response)



The concentration in our sample is approx. 67 ng/mL

Theme 1 – Free concentration and in-vitro dose confirmation

2. Method Validation

Assess if the method is fit for purpose and set acceptance criteria for future studies

Accuracy

Quality Control samples (QC) at low, mid and high level should be within 20% of nominal

Precision

6 replicates per QC, RSD should not be more than 20%

Stability of the test item

- In assay media (ambient, freezer (effect of freeze thawing), 37°C (to mimic assay conditions)
- At the autosampler
- Stored QCs should be more than 80% of the freshly prepared QCs

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Theme 1 – Free concentration and in-vitro dose confirmation

3. In-vitro dose confirmation

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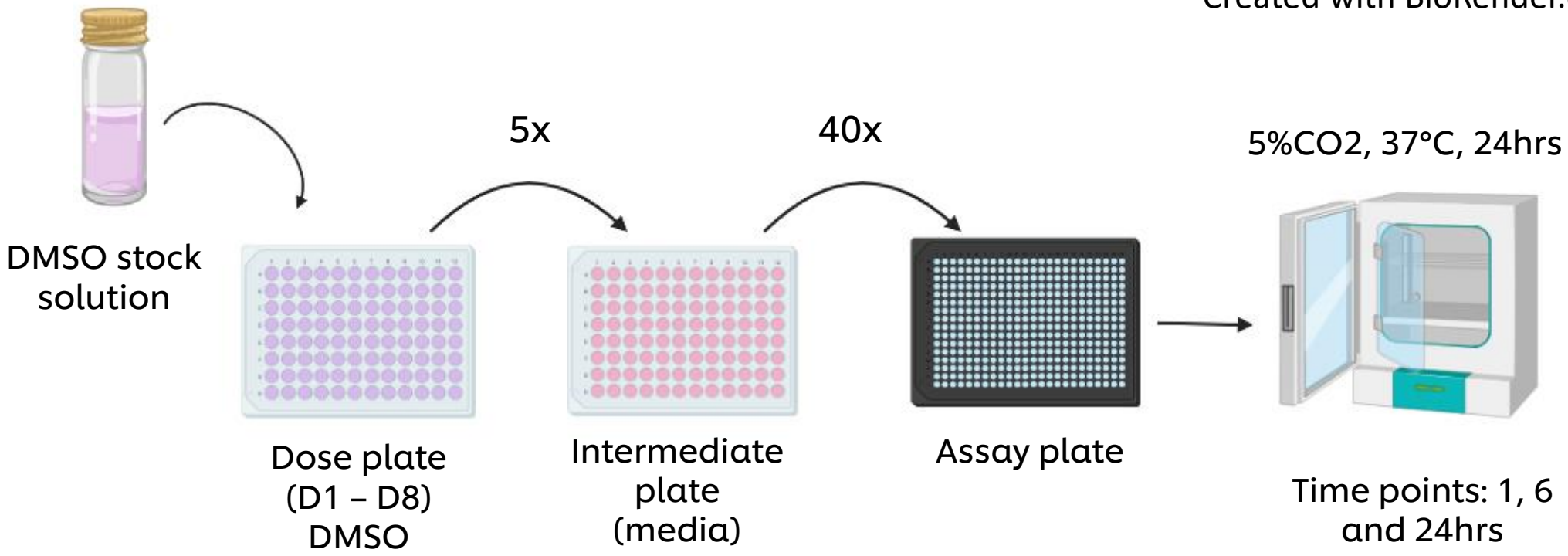


Measured value is within 20% nominal →Cnominal is used as Cfree

Measured value is not within 20% nominal → reason for the lower dose?

Theme 1 – Free concentration and in-vitro dose confirmation

What can cause a lower dose?

Sample preparation step

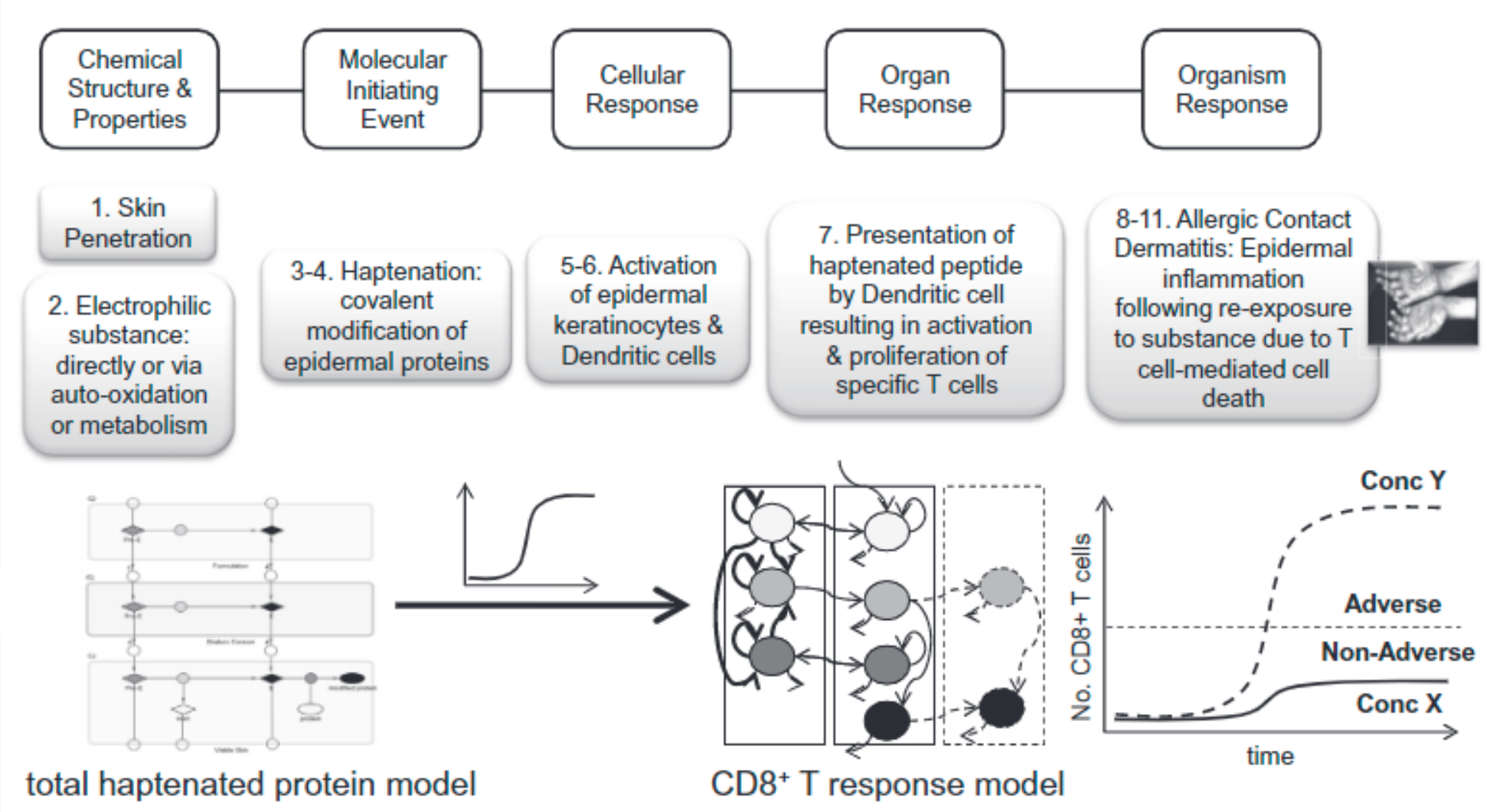
- Not considering chemical solubility in the different solvents in all the assay steps
- Not considering purity of test item
- Sample preparation error (wrong calculations, pipetting error, weighing error)

In-vitro system

- Sorption/ binding to plastic
- Evaporation
- Degradation of the test item

Unilever

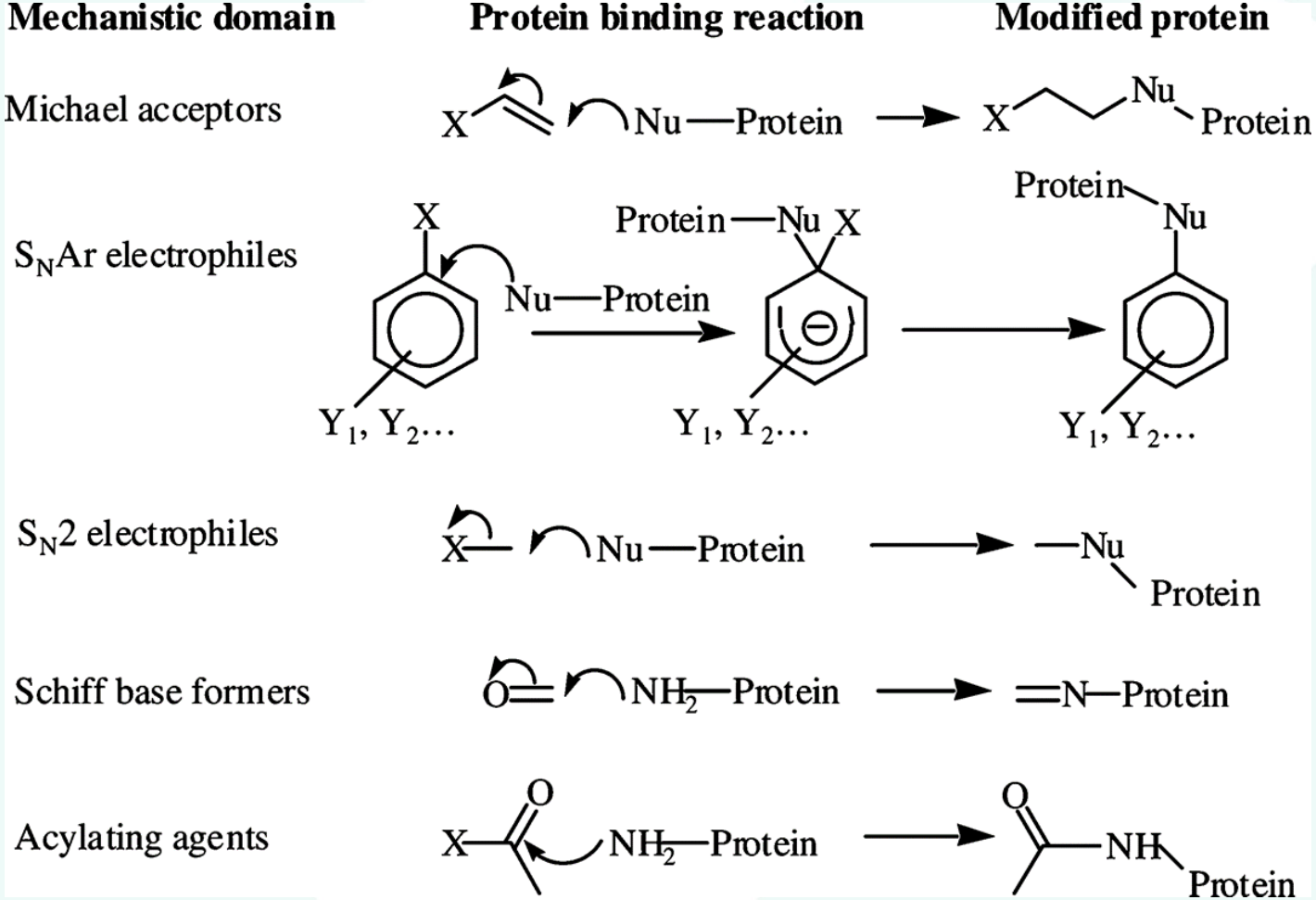
Theme 2 – Skin sensitisation



Source: Maxwell, G. et al. 2014 Applying the skin sensitisation adverse outcome pathway (AOP) to quantitative risk assessment. Toxicology in Vitro 28, 8 -12.

Theme 2 – Skin sensitisation

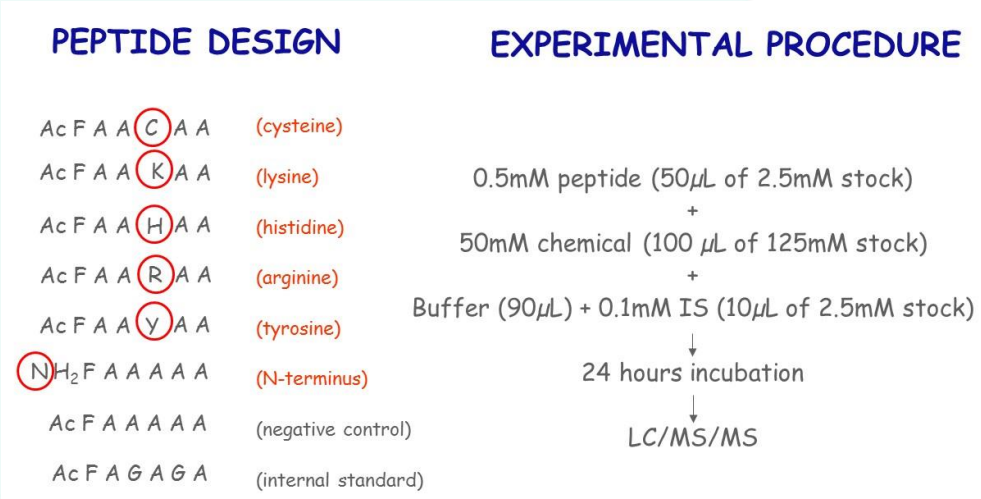
Possible Reactions Between Electrophilic Chemicals and Protein Nucleophiles



Source: Aynur O. Aptula, Grace Patlewicz, and David W. Roberts, *Chemical Research in Toxicology* **2005** *18* (9), 1420-1426

Theme 2 – Skin sensitisation

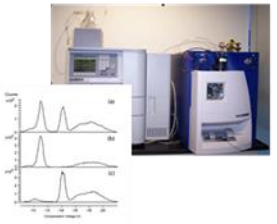
Peptide Reactivity



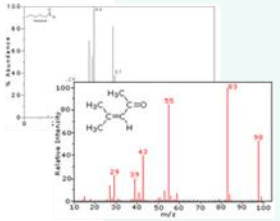
- Peptides incubated with a large excess of test chemical
- Monitor Adduct Formation by LC-MS – No detectable adducts gives a high level of confidence that the test chemical is non reactive
- Confirming the reaction mechanism useful in justifying chemicals used for read across in risk assessment
- Measure depletion of peptide over 24 hours
- Lysine (K), cysteine (C) and N-terminus peptides most relevant to skin allergy

Mix peptides and test chemical

Incubate



Analyse by LC-MS



Deduce reaction mechanism

<u>Output</u>:

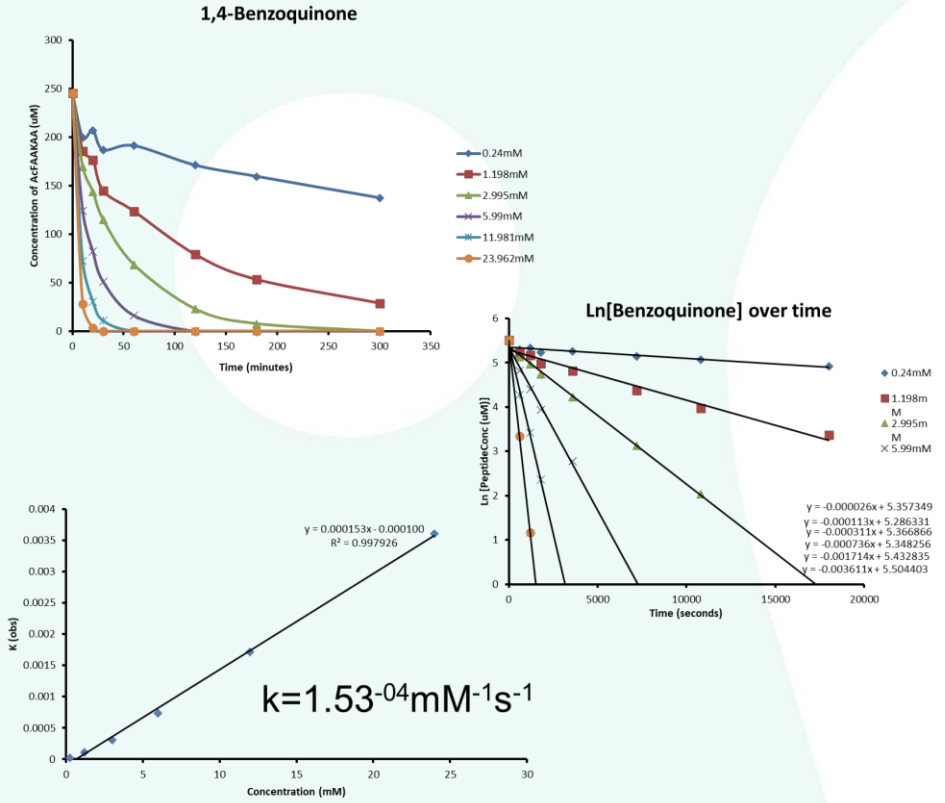
- Does the chemical react?
- Reaction mechanism
- Indication of how reactive

Source: Aleksic, Maja, et al. "Reactivity profiling: Covalent modification of single nucleophile peptides for skin sensitization risk assessment." *Toxicological Sciences* 108.2 (2009): 401-411.

Theme 2 – Skin sensitisation

Peptide Kinetics

- 96 well plate format
- 5-6 different concentrations of test chemical with each peptide
- Peptides with lysine (K), cysteine (C) and N-terminus residues used.
- Incubate at 40℃
- Time the additions so as to give 6 time-points at 10 mins – 4 hours.
- At end of incubation, fluorescent derivatising agent added to react with all remaining peptide
- Free peptide measured by fluorescence spectroscopy



Output:

- Rate of reaction with each peptide expressed as a rate constant in $mM^{-1}s^{-1}$

Theme 2 – Skin sensitisation

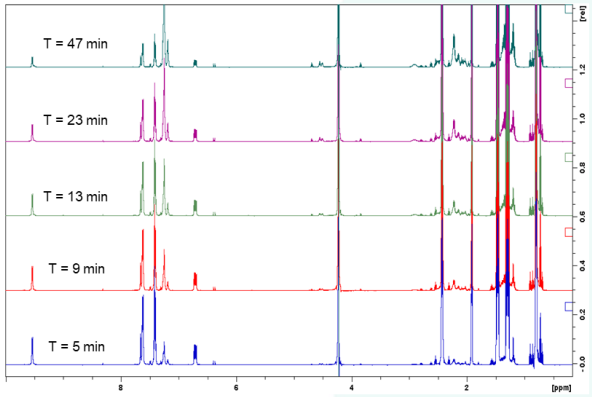
NMR Kinetics

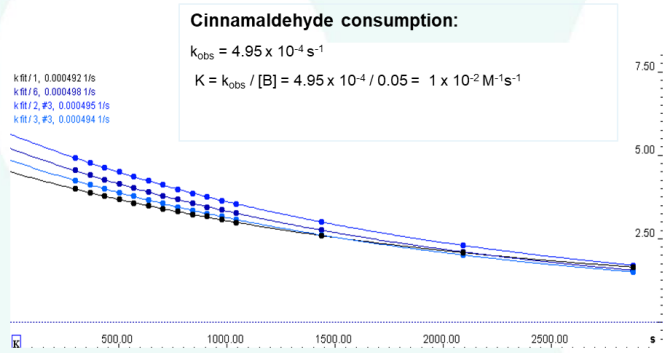
- N-butylamine and 1-butanethiol used as surrogates for amine (Lys and N-term) and thiol (Cys) protein nucleophiles
- Test chemical mixed with a large molar excess of the nucleophile
- Reactants and reaction product(s) monitored by ^{1}H NMR

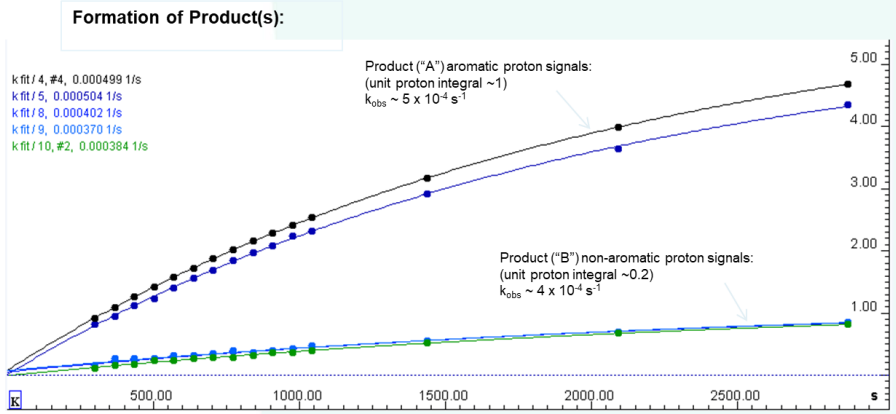
Cinnamaldehyde (5mM) plus 1-butanethiol (50mM) in 50:50 CD_3CN:Kphosphate pH9

At least two reaction products

At least two reaction products





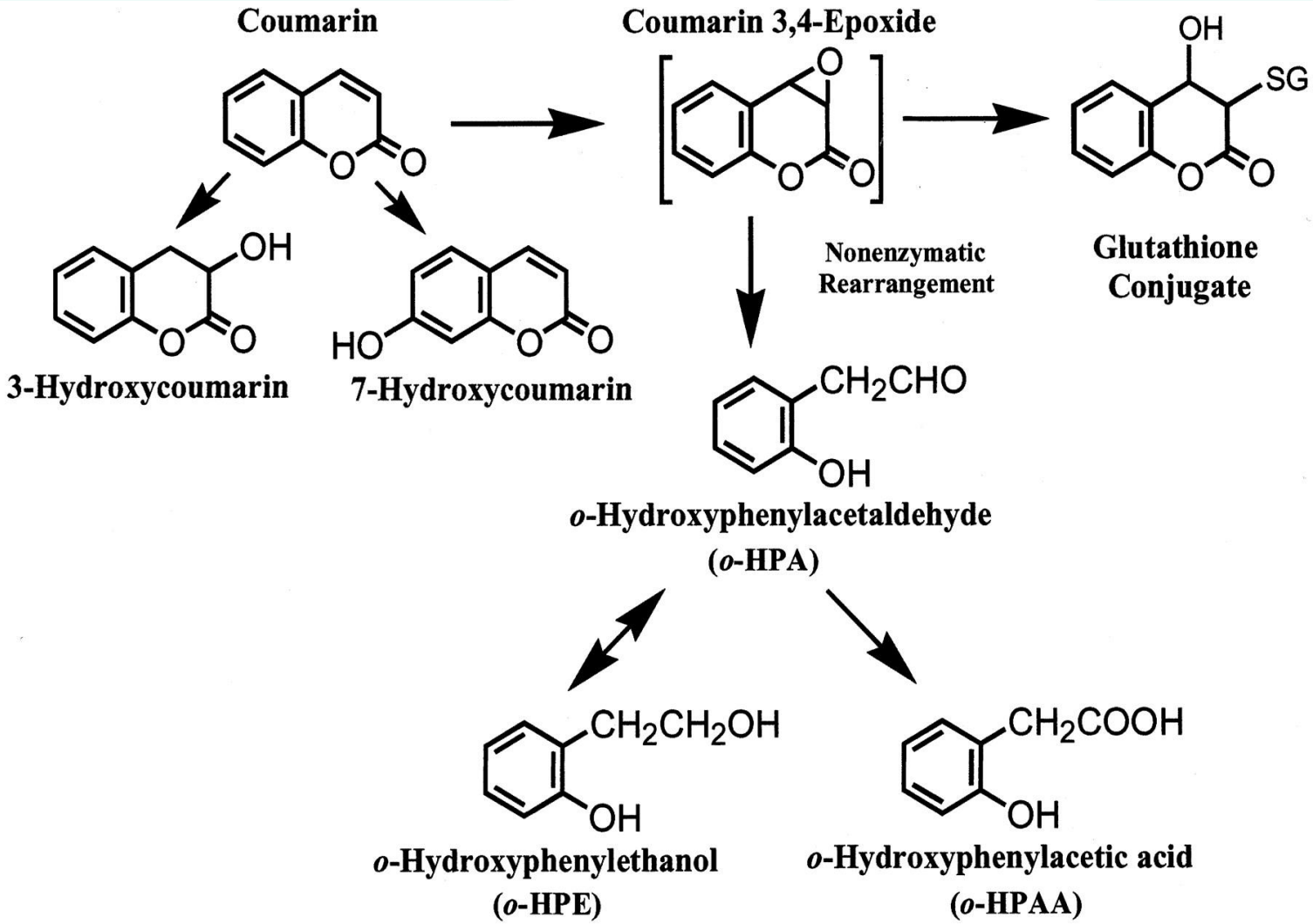


Output:

- Independent kinetic rates for multiple reaction products
- Confirmation of reaction mechanisms

Source: Sanderson, Paul N., et al. "Mechanistic understanding of molecular initiating events (MIEs) using NMR spectroscopy." *Toxicology Research* 5.1 (2016): 34-44.

Theme 3 – Skin metabolism



Which coumarin metabolites can be found in skin?

Source: Born, S.L. et al (2002) Identification of the cytochromes P450 that catalyze coumarin 3,4-epoxidation and 3-hydroxylation. Drug Metabolism and Disposition, 30, 483-487.

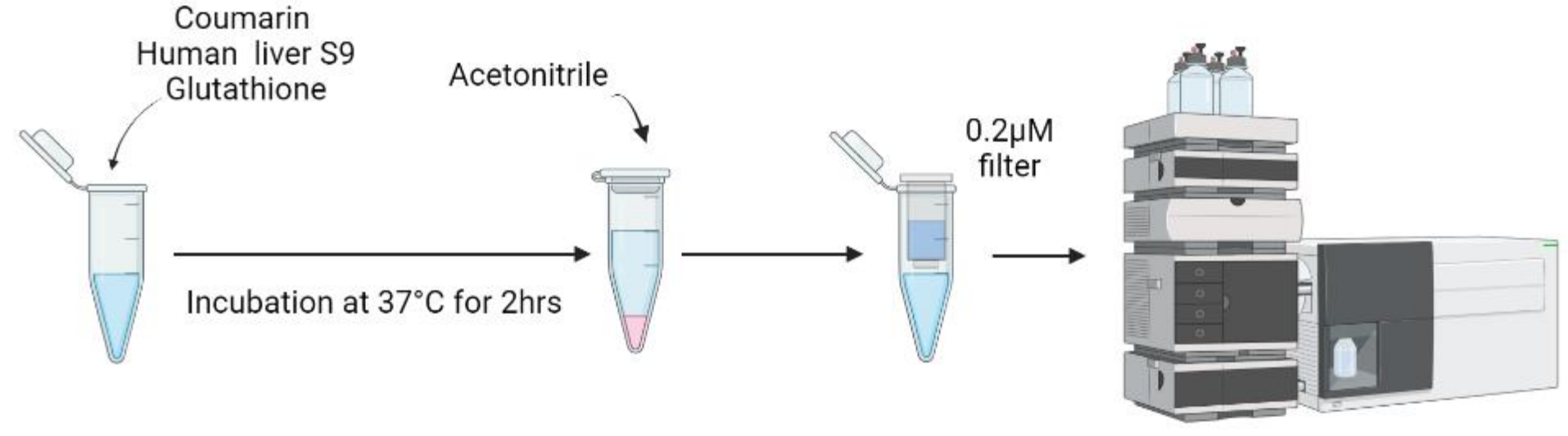
Unilever

Theme 3 – Skin metabolism

Goal : Identify potential reactive metabolites by in-vitro liver S9 incubation

- Glutathione was added to trap any reactive metabolite formed (3,4-epoxide)
- Low concentration (50µM) and high concentration of coumarin (1mM, to saturate the preferable pathway)
- Positive control for CYP activation – eugenol

Created with BioRender.com



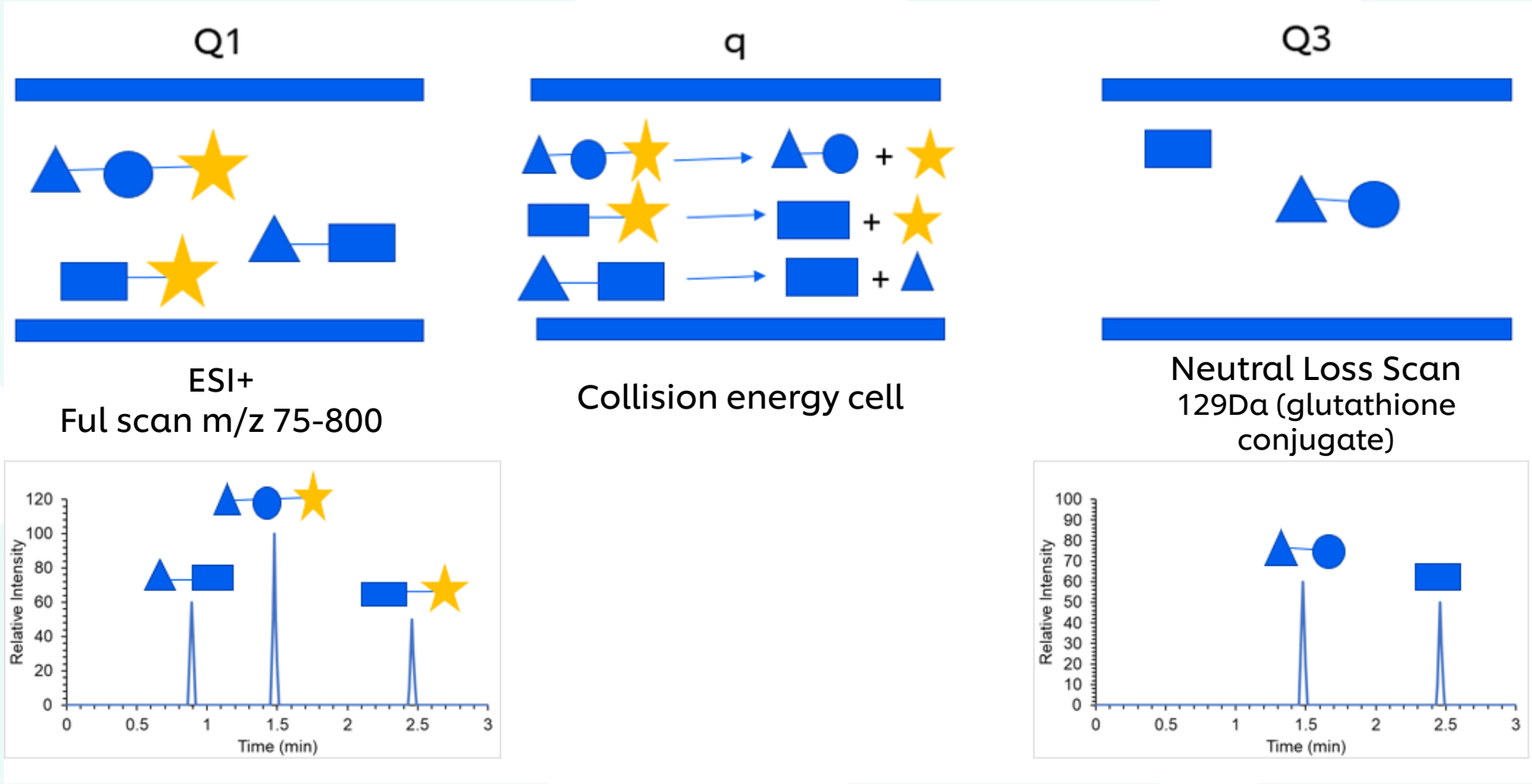
Source: Reynolds,G. et al (2021) Regulatory Toxicology and Pharmacology,127, 105075.

Theme 3 – Skin metabolism

Metabolite identification via LC-MS

Metabolites identified

- 7-Hydroxycoumarin
- Coumarin 3,4-epoxide



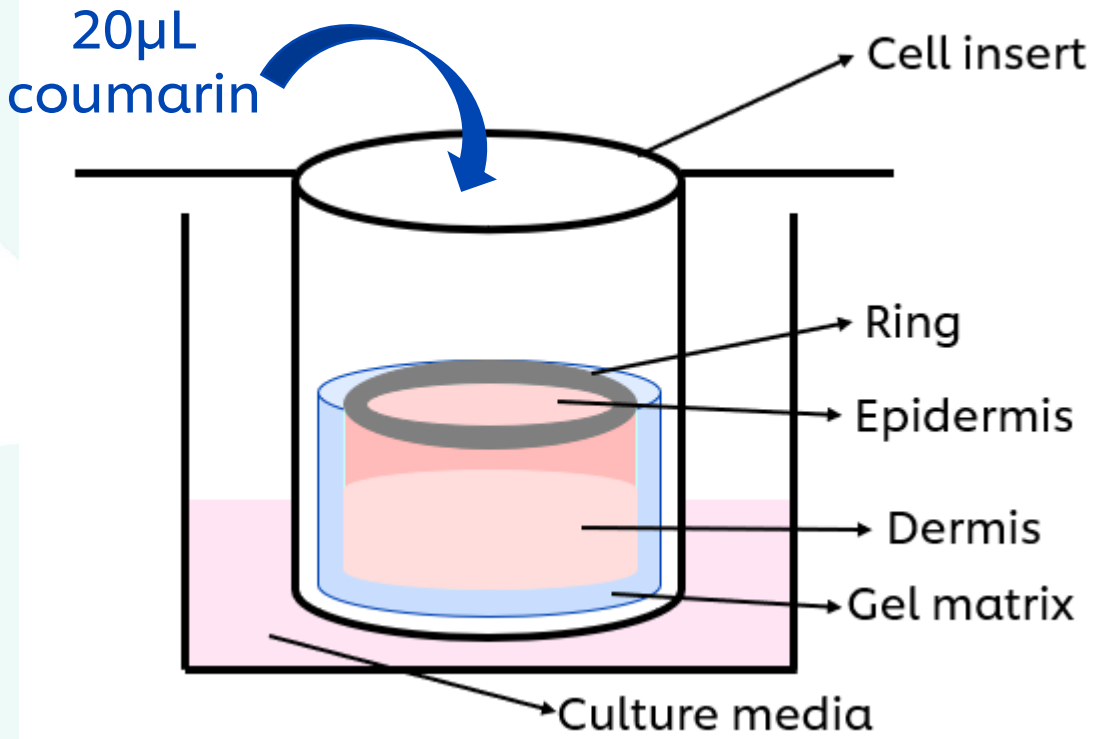
Source: Reynolds,G. et al (2021) Regulatory Toxicology and Pharmacology,127, 105075.

Theme 3 – Skin metabolism

Metabolism in ex vivo human skin cultures

Goal: Determine the nature and extent of metabolism in the skin

NativeSkin® model (Genoskin)



Time points: 1, 6 and 24hrs

Samples:

- Stratum corneum was removed from the epidermis using 50 stripped tapes per biopsy
- Epidermis
- Dermis
- Cell inserts
- Rings
- Matrix gel
- Culture media

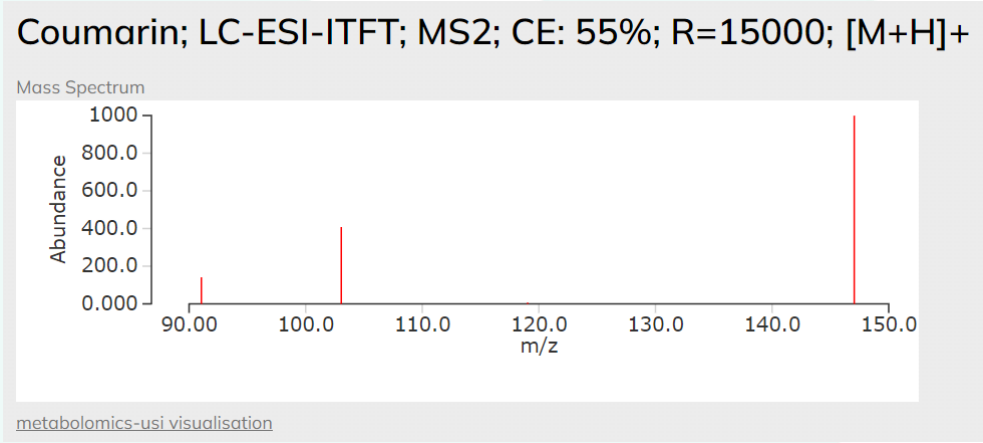
Source: Reynolds,G. et al (2021) Regulatory Toxicology and Pharmacology,127, 105075.

Theme 3 – Skin metabolism

Ex-vivo skin LC-MS

ESI +, Multiple Reaction Monitoring (MRM)

Compound	Transition	Cone energy (V)	Collision energy (eV)
Coumarin	m/z 146.97 > 103.02	66	16
7-hydroxycoumarin	m/z 162.90 > 106.99	24	20
7-hydroxycoumarin-glucuronide	m/z 339.30 > 163.00	40	30



Source: https://massbank.eu/MassBank/. Last accessed: 02/02/2023

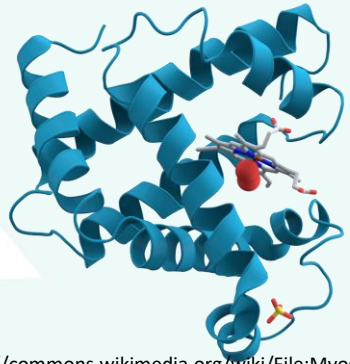
Result: Skin metabolism of coumarin is not significant, since very low concentration of 7-hydroxycoumarin was detected.

Source: Reynolds,G. et al (2021) Regulatory Toxicology and Pharmacology,127, 105075.

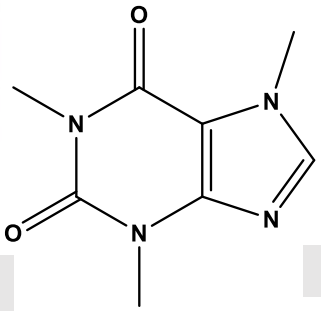
Theme 4 – Measuring partitioning parameters

PBK modelling, QSAR and bioaccumulation are essential steps in both NGRA and environmental risk assessment.

These require input of compound specific <u>partitioning parameters</u>, such as:

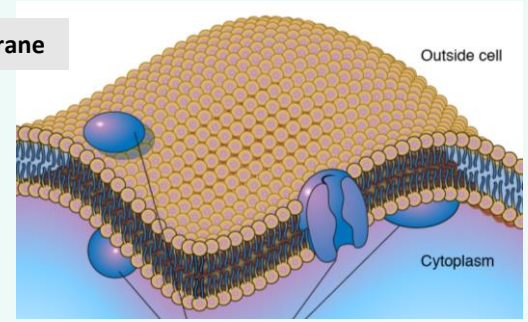


https://commons.wikimedia.org/wiki/File:Myoglobin.png



Partitioning to plasma protein

Partitioning to cell membrane



Courtesy: National Human Genome Research Institute - genome.gov

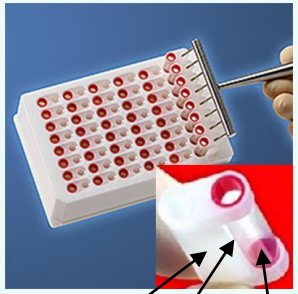
- Plasma:protein partitioning is a parameter required for PBPK modelling

- Membrane:water partitioning: is a parameter required in PBK modelling, but also for fish bioaccumulation and narcotic toxicity predictions – measured in the lab using liposomes

Theme 4 – How we measure partitioning parameters

Equilibrium dialysis

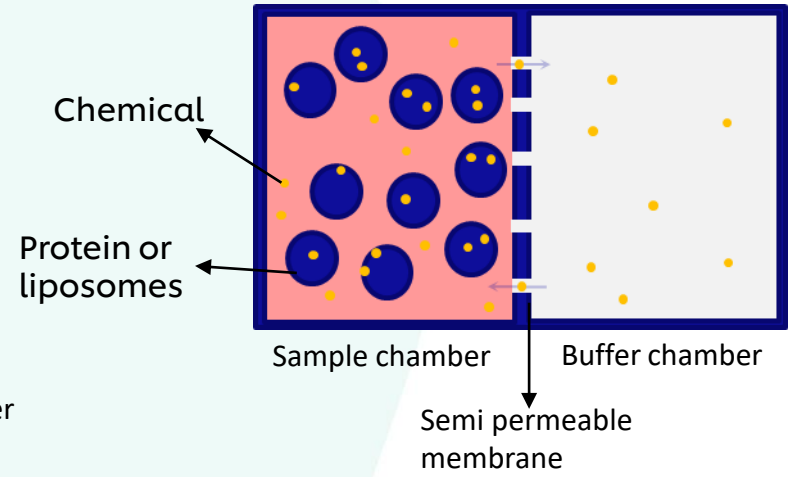
RED by Thermo Fisher Scientific Inc.

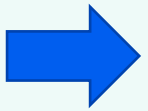


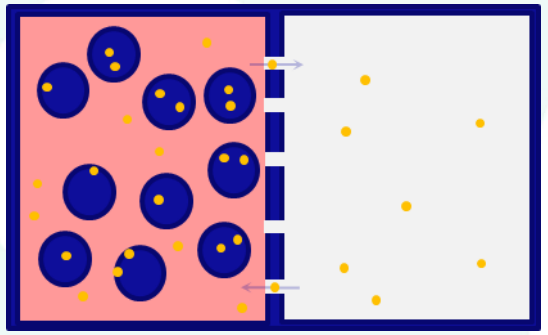
Buffer chamber

Semi permeable membrane

Sample chamber







- Chemical + protein or liposome are dosed in sample chamber
- Only chemical is small enough to pass trough membrane

By measuring how much passed into the buffer chamber we can determine how much is bound to protein (protein binding) or liposome (cell membrane binding)

We need Analytical Chemistry to quantify how much chemical is free

What is a liposome?

- A small artificial lipid bilayer that mimics the cell membrane
- Easily create in standard lab conditions
- Used to calculate membrane-water partitioning

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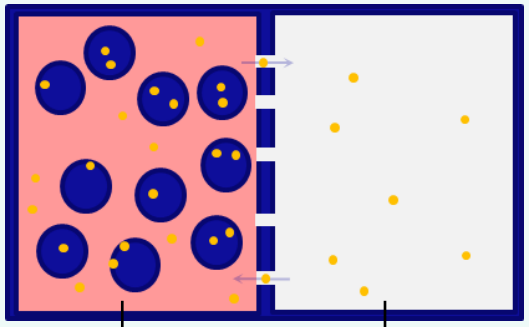
Theme 4 – How we measure partitioning parameters

Method developed and validated

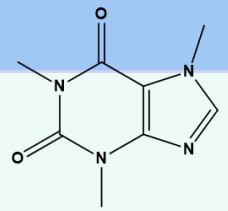


Pre treatment

- Addition of solvent and centrifugation to precipitate proteins

Separation

- Polar chemical, use Liquid chromatography



Quantification

- Mass Spectrometry for increased sensitivity

Analyse buffer chamber to determine free amount (not bound to protein)

Analyse sample chamber to determine total amount of chemical dosed

$$Fraction\ bound\ to\ protein = 1 - \frac{free\ amount}{total\ amount}$$

$$Caffeine\ PPB \approx 35\%$$

J Pharm Sci. 1982 Dec;71(12):1415-8

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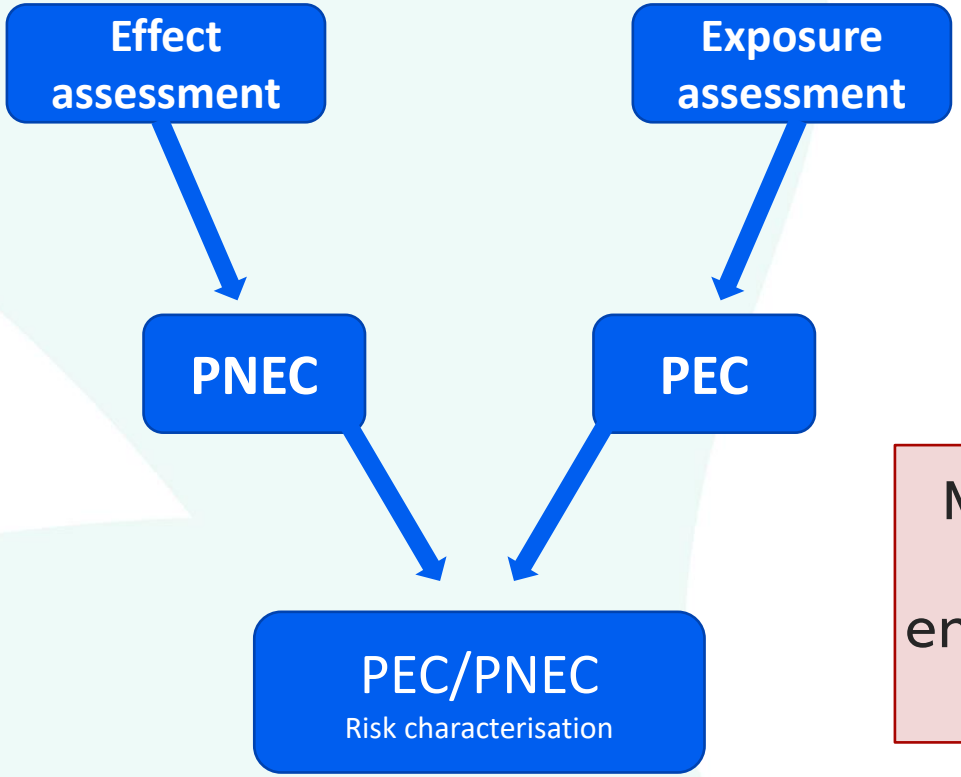
Theme 5 – Analytical Chemistry to support Environmental Risk Assessment of chemicals

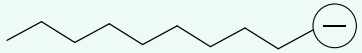


Tools available for exposure assessment:

- Modelling
- Measurement

Measurements of the concentration of the compound(s) of interest in the relevant environmental matrices are performed using **analytical chemistry** techniques

Theme 5 – measuring the concentration of a anionic surfactant used in homecare entering the environment from a WWTP



Surfactant X

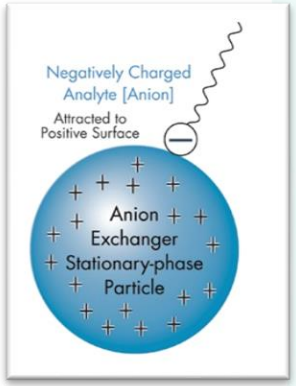
Step 1: Collect effluent wastewater

Step 2: Sample pre-treatment

- Filter the sample with filters of 0.2µm pore size to remove solid materials and microorganisms

Step 3: Sample concentration

- The concentration of Surfactant X is expected to be low in effluent so a sample concentration step is required
- For this we use a Anion Exchange Solid Phase Cartridge which is made of a positively charged phase that will attract our negatively charged surfactant

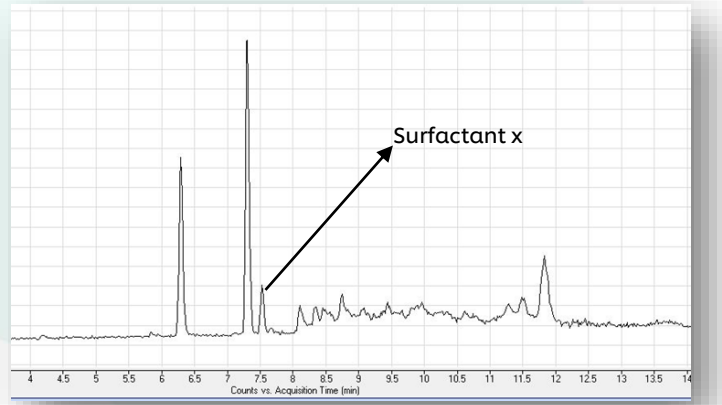


https://www.waters.com/

- This will clean our sample further by removing unwanted chemicals and concentrate it 100x

Step 4: Separation and Identification

- Apply a HPLC-MS (liquid chromatography with Mass Spectrometry detection) method to separate and quantify the compound
- The resulting chromatogram is complex, but because the Mass Spectrometer provides a mass value for each peak we are able to determine which one corresponds to our compound

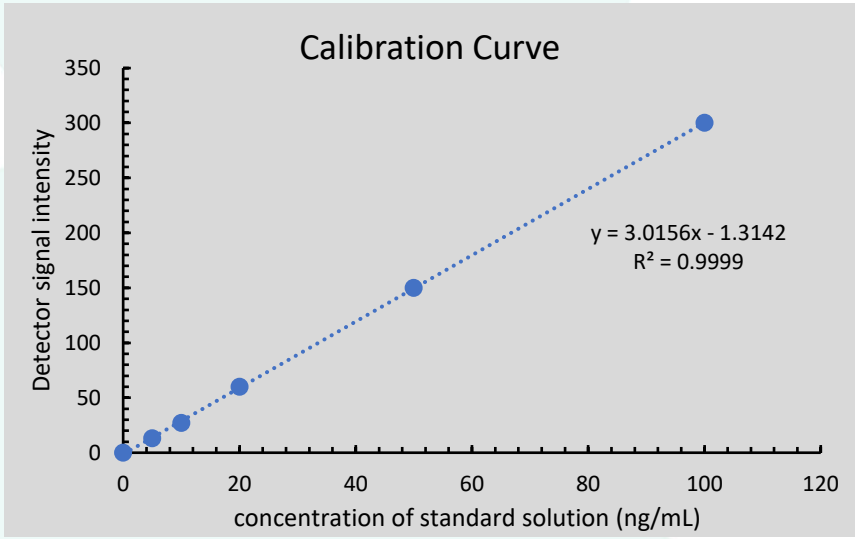


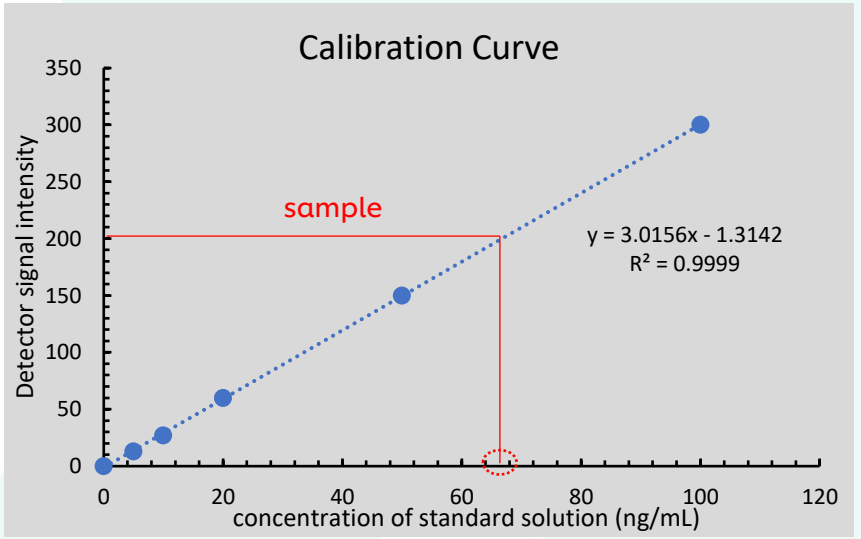
Theme 5 – measuring the concentration of a anionic surfactant used in homecare entering the environment from a WWTP

Step 5: Quantification

In order to determine how much Anionic surfactant X is in our sample, we need to perform a quantification experiment. This is typically achieved by running an external standard calibration line

1. Using a pure standard of to prepare solutions of known concentration
2. Analyse these standard solutions together with your sample with the same HPLC-MS method.
3. Plot the intensity of the response (this can be the peak height or area) against the concentration of the standard to create a calibration curve
4. Once we determine the signal intensity for in our sample we can use this calibration curve as a refence to determine it's concentration in wastewater (red line)





The end – Thank you for listening

Thank you to:

Sandrine Spriggs

Suzanne Martin

Richard Cubberley

Nicola Haywood

Samera Rafiq

Ian Sanders

- Questions ?

Check out some of our research:

- **Experimental validation of mass balance models for in vitro cell-based bioassays**. Henneberger,L. et al. Environ.Sci.Technol. 2020, 54, 1120-1127
- **Glutathione metabolism in the HaCaT cell line as a model for the detoxification of the model sensitisers 2,4-dinitrohalobenzenes in human skin –** Spriggs, S et al Toxicol Lett 2015 Aug 19;237(1):11-20
- **Predicting the phospholipophilicity of monoprotic positively charged amines -** Droge, ST. et al. Environ. Sci.: Processes Impacts, 2017,19, 307-323
- **Determination of Protein Haptenation by Chemical Sensitizers Within the Complexity of the Human Skin Proteome –** Parkinson, E. et al. Toxicol Sci. 2018 Apr; 162(2): 429–438
- **Monitoring and modelling of siloxanes in a sewage treatment plant in the UK –** van Egmond, R. et al. Chemosphere. 2013 Oct; 93(5): 757-65
- **Biodegradation Kinetics of Fragrances, Plasticizers, UV Filters, and PAHs in a Mixture—Changing Test Concentrations over 5 Orders of Magnitude –** Birch, H. et al. *Environ. Sci. Technol.* 2022, 56, 1, 293−301

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