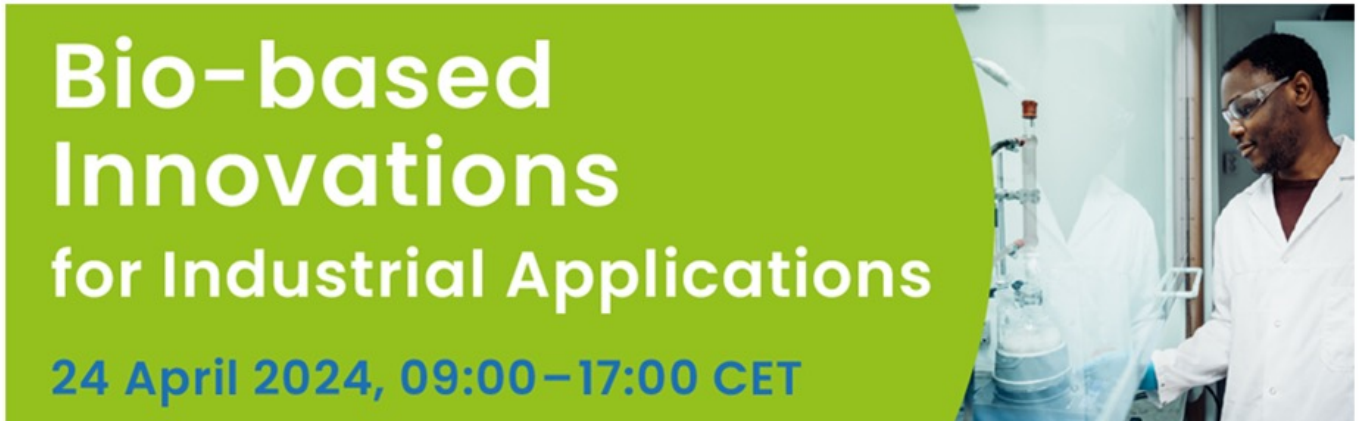

Safety and toxicity assessments and methodology

- Harrie Besselink — BDS BioDetection Systems
- Andy Booth — SINTEF

CHAMPION circular bio-based polymers

PERFECOAT

Why safety testing ?

- safeguard human health
- environmental impact
- product performance
- regulatory compliance

Early tox testing will:

- facilitate early go-no go decisions:
  - prevent time loss on (a group of) molecules with non-favourable toxicity profile
  - improved overall toxicity profile from starting materials - intermediates - final materials
- give guidance for further testing
- facilitate compliance with regulatory requirements
- market introduction of safe bio-based products

Safety testing…….how?

Chemical

?

Toxicology

Classical testing of toxicity: *in vivo* (animal) testing extrapolated to human hazard

- time-consuming
- non-ethical
- technical concerns with animal testing
- since late 1950s, search for methods to reduce or eliminate animal testing.
  “3 - Rs" - reduce
  - refine
  - replace

www.champion-project.eu

Circular High-performance Aza-Michael Polymers as Innovative materials Originating from Nature

CHAMPION
circular bio-based polymers

WP4: Toxicity and Safety Testing

Harrie Besselink

BDS
BioDetection Systems

BBI JU

Horizon 2020
European Union Funding
for Research & Innovation

Bio-based Industries
Consortium

This project has received funding from the Bio Based Industries Joint Undertaking (JU) under grant agreement No 887398. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio Based Industries Consortium.

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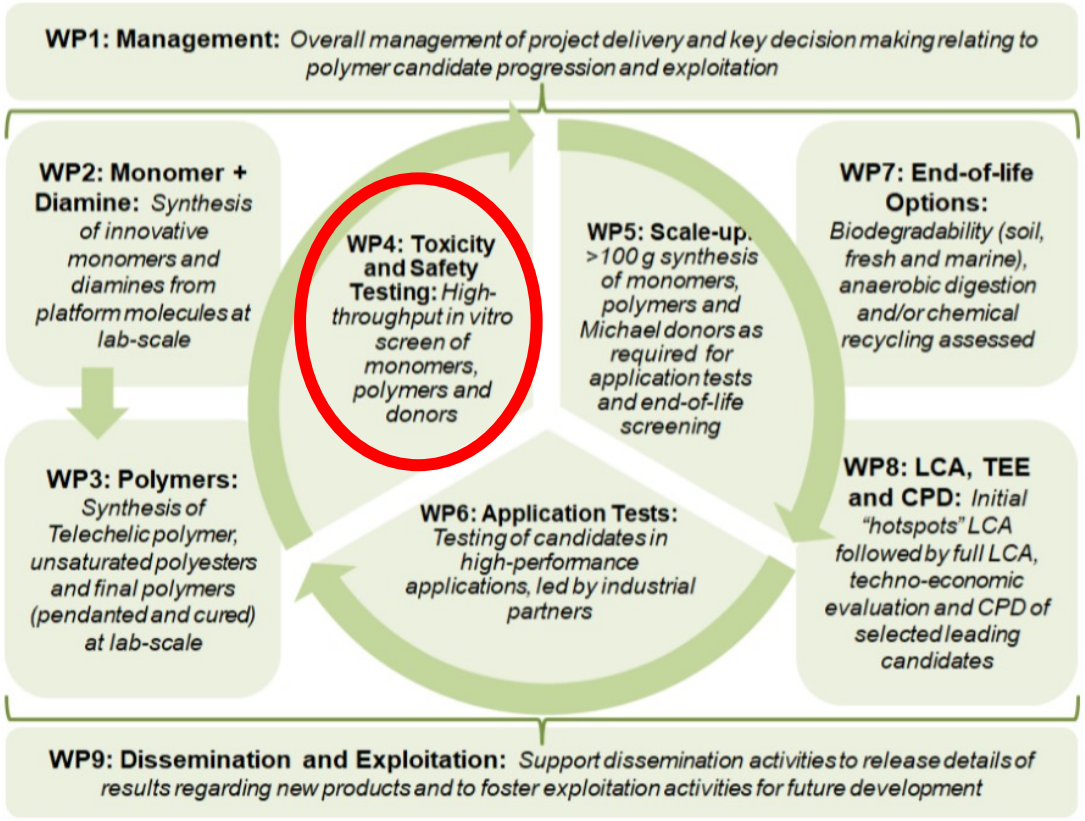
BioDetection Systems

Circular High-performance Aza-Michael Polymers as Innovative materials Originating from Nature

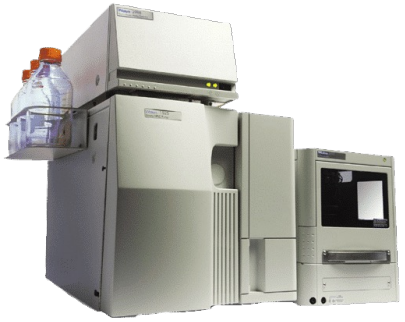
Objective

- develop novel bio-based Michael-addition polymers
- for use in home care products, structural adhesives, furniture coatings and automotive interior surfaces
- high functional qualities that cannot be met by current fossil-based products
- designed and assessed with improved end-of-life (circular by design)
- superior to current materials by ensuring that biodegradability and/or recyclability

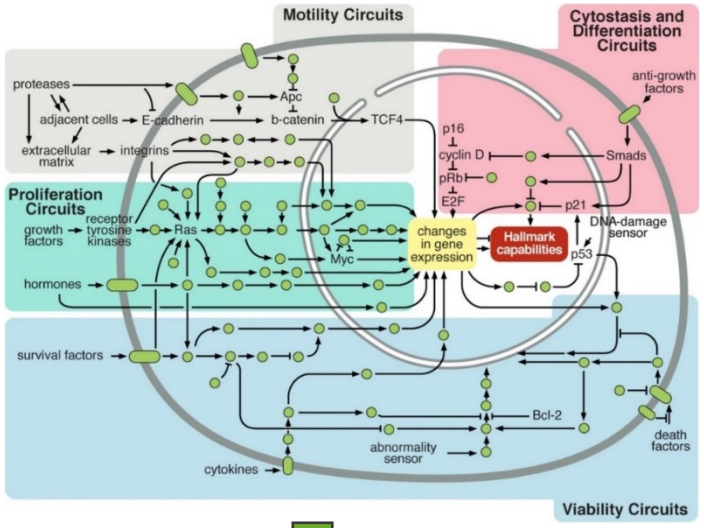


Aim: assess the safety of monomers, polymers and products (coatings, adhesives,…)

Chemical analysis vs biological analysis



Safety testing……. cellular pathway-based approach

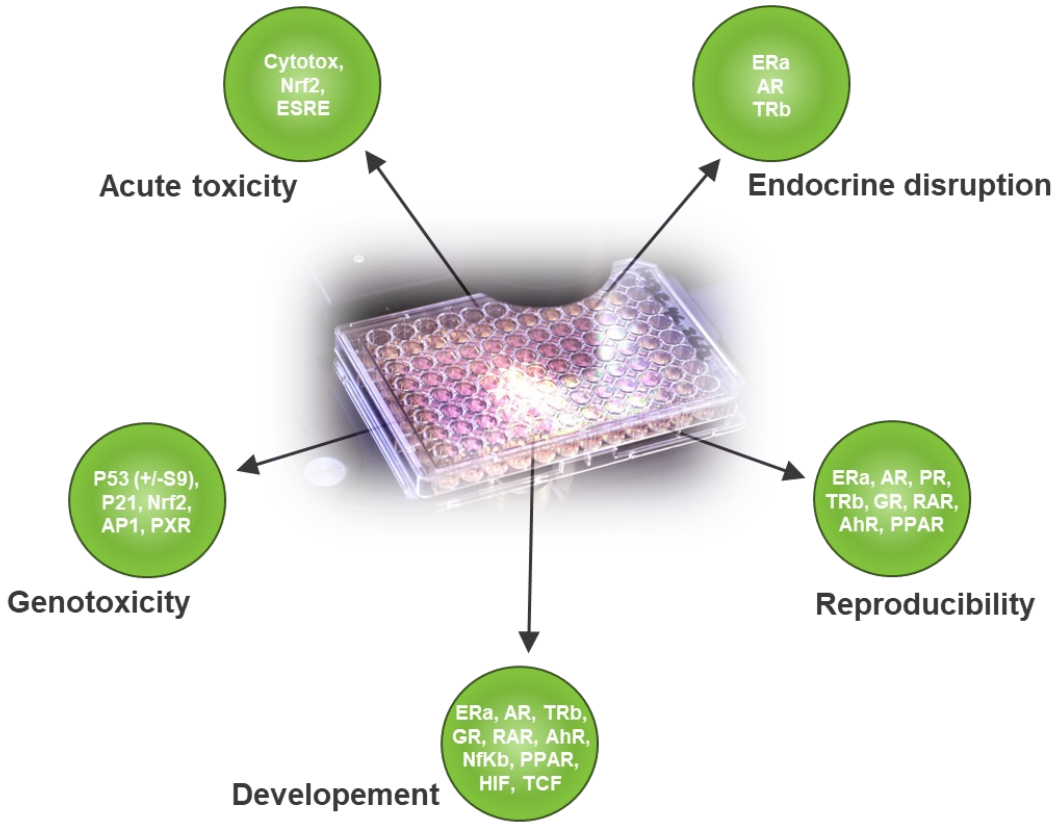


Cellular pathways

Assay development

Nuclear receptor assays	Stress pathway assays
ERa CALUX (ago/anta)	Hif1a CALUX
AR CALUX (ago/anta)	TCF CALUX
PR CALUX (ago/anta)	ESRE CALUX
GR CALUX (ago/anta)	NFkB CALUX
TRb CALUX (ago/anta)	Nrf2 CALUX
RAR CALUX (ago/anta)	p21 CALUX
LXR CALUX (ago/anta)	p53 CALUX
PPARa CALUX (ago/anta)	cytotox CALUX
PPARd CALUX (ago/anta)	
PPARg CALUX (ago/anta)	
AhR CALUX	

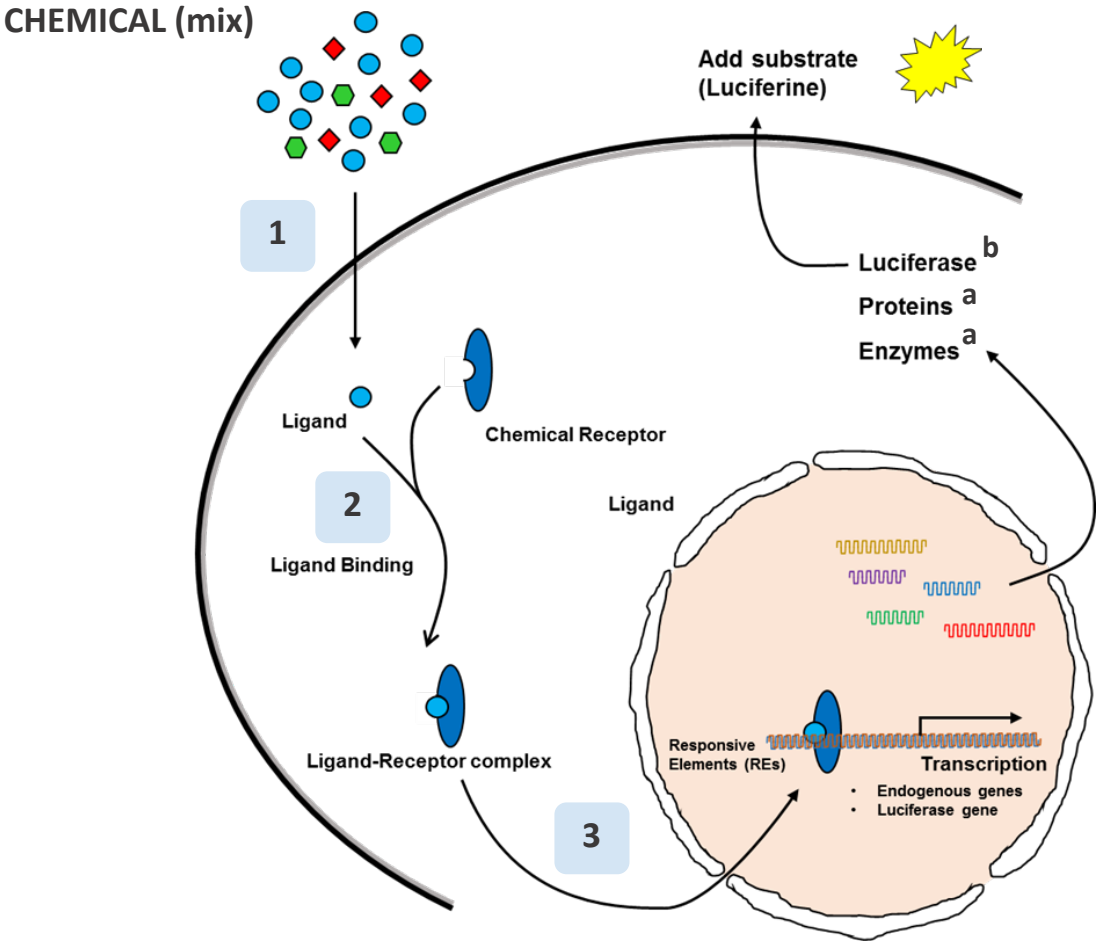
Link (groups of) assays to toxicological endpoints



Predictions range between 75-100%

CALUX mechanism-based reporter gene assays

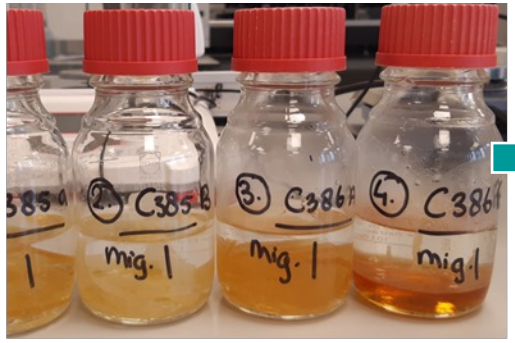
BioDetection Systems

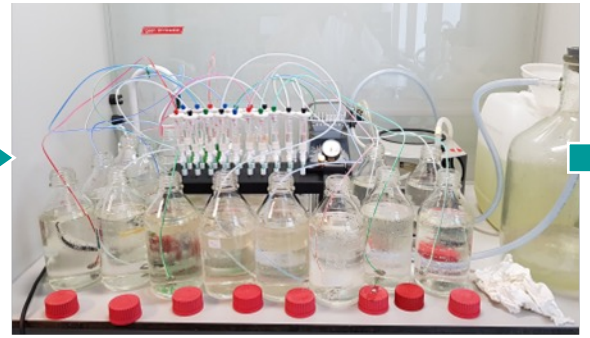


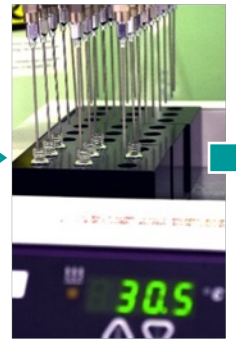
name	pathway	reference compound
DR CALUX	dioxin receptor activation	2,3,7,8-TCDD
PAH CALUX	dioxin receptor activation	benzo-a-pyrene
ER CALUX	estrogen receptor activation	17β-estradiol
ERalpha CALUX	estrogen receptor α activation	17β-estradiol
Anti-ERalpha CALUX	repression estrogen receptor α activation	tamoxifen
ERbeta CALUX	estrogen receptor β activation	17β-estradiol
Anti-ERbeta CALUX	repression estrogen receptor β activation	tamoxifen
AR CALUX	androgen receptor activation	dihydrotestosterone
Anti-AR CALUX	repression androgen receptor activation	flutamide
PR CALUX	progesterone receptor activation	progesterone
Anti-PR CALUX	repression progesterone receptor activation	RU486
GR CALUX	glucocorticoid receptor activation	dexamethasone
Anti-GR CALUX	repression glucocorticoid receptor activation	RU486
TRβ CALUX	thyroid receptor activation	T3
RAR CALUX	retinoic acid receptor activation	retinoic acid
PPARγ CALUX	PPARγ activation	rosiglitazone
PPARα CALUX	PPARα activation	GW7674
PPARδ CALUX	PPARδ activation	L165041
LXR CALUX	LXR activation	GW3965
kappaB CALUX	NFκB pathway activation	TPA
P21 CALUX	transcription of p21 inhibitor of cell cycle progression	actinomycin D
Nrf2 CALUX	activation of the Nrf2 pathway	curcumin
P53 CALUX	p53-dependent pathway activation	actinomycin D
genotox CALUX	p53-dependent pathway activation +/-S9	cyclophosphamide
TCF CALUX	wnt/TCF pathway activation	lithium chloride
AP1 CALUX	AP1 pathway activation	TPA
HIF1alpha CALUX	Hif1alpha pathway activation	cobaltous chloride
ER stress CALUX	ERSE activation leading to endoplasmic reticulum stress	tunicamycin

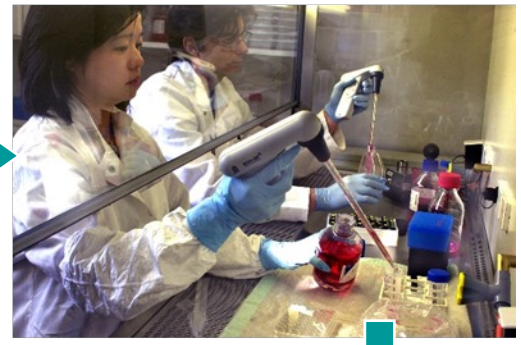
Toxicity and safety testing using effect-based bio-analysis

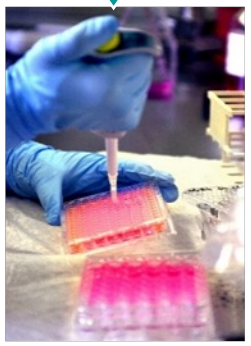
BioDetection Systems

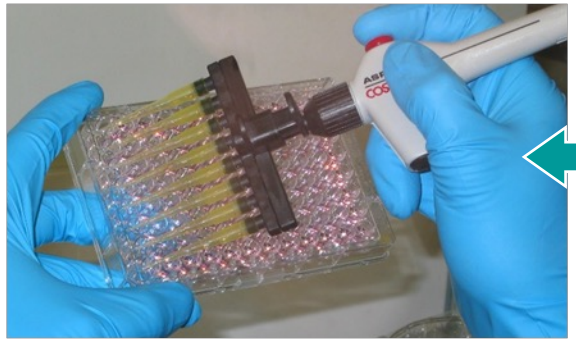


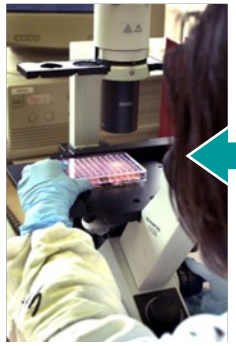


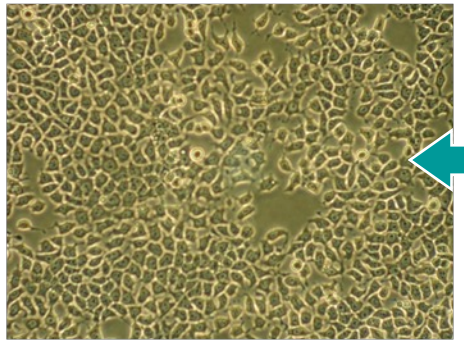


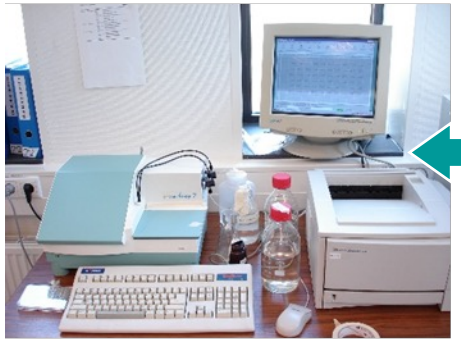












Horizon 2020 European Union Funding for Research & Innovation

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PERFECOAT

Safety testing in a context of polymer application

(amines/diols, Michael acceptors, (unsaturated)polymers and Aza-Michael polymers)

activity low > high

	Code	Owner	Cytotox20%	ERa	AR-anti	PR-anti	TRb	TRb-anti	PXR	PPARa	PPARa-anti	PPARg	PPARg-anti	PPARd	PPARd-anti	HepG2-AhR	Hif1a	TCF	AP1	ESRE	Nrf2	p21	p53 GENTOX	chemical class
	Assay ref		-4.1	-9.8	-5.3	-8.4	-7.0	-4.4	-4.4	-7.0	-4.2	-5.1	-6.8	-5.1	-5.7	-8.0	-2.6	-0.3	-6.7	-4.6	-2.8	-5.9	-5.9	
Amines / diols	N7S	WR	-0.5	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	06. WP2 amines
	N8S	WR	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	06. WP2 amines
	N9S	WR	-0.6	>	>	>	>	>	-1.6	>	>	>	>	>	>	>	>	>	>	>	>	>	-0.4	06. WP2 amines
	D18a	UoY	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	08. WP3 diols
	D23a	AVA	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	-0.9	>	>	08. WP3 diols
Michael acceptors	M19	WR	-2.7	>	>	>	>	>	-3.2	-2.4	>	>	>	>	>	>	>	>	-3.2	-2.7	-3.2	>	-3.4	09. WP3 Michael acceptors
	M20	WR	>	>	>	>	>	>	-1.5	>	>	>	>	>	>	>	>	>	>	-3.0	-1.0	>	>	09. WP3 Michael acceptors
	M21a	WR	-1.7	>	>	>	>	>	-2.2	>	>	>	>	>	>	>	>	>	-2.6	-1.7	-2.6	>	-2.0	09. WP3 Michael acceptors
	M21b	WR	-2.5	>	>	>	>	>	-2.5	>	>	>	>	>	>	>	>	>	-3.0	>	-3.0	>	-2.8	09. WP3 Michael acceptors
	M21c	WR	-2.0	>	>	>	>	>	-2.5	>	>	>	>	>	>	>	>	>	-3.0	>	-3.0	>	-2.2	09. WP3 Michael acceptors
Unsaturated polyester	P46l	VTT	-2.4	>	>	>	>	>	>	>	>	>	-2.5	>	>	>	>	>	-3.0	>	-2.9	>	-3.1	10. Unsaturated polyester
	P46n	VTT	-2.4	>	>	>	>	>	>	>	>	>	-2.5	>	>	>	>	>	-3.7	>	-3.5	-2.5	-3.0	10. Unsaturated polyester
	P46p	VTT	-1.5	>	>	>	>	>	>	>	>	>	-2.5	>	>	>	>	>	-2.6	>	-2.7	-1.5	-2.2	10. Unsaturated polyester
	P46q	VTT	-1.6	>	>	>	>	>	>	>	>	>	-2.5	>	>	>	>	>	-2.6	>	-3.0	-1.5	-2.2	10. Unsaturated polyester
	P64	STAHL	-2.2	>	>	>	>	>	-4.7	>	>	>	-4.0	>	>	>	>	>	-2.3	-2.4	-3.4	>	-2.0	10. Unsaturated polyester
Aza-Michael polymers	C94a	UoY	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	-0.5	>	>	-0.5	>	>	11. Aza-Michael polymers
	C95a	UoY	>	>	>	>	>	>	-1.7	>	>	>	>	>	>	>	>	>	>	>	>	>	>	11. Aza-Michael polymers
	C103a	UoY/UNI	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	11. Aza-Michael polymers
	C116a	UoY	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	-0.5	>	>	11. Aza-Michael polymers
	C117a	UoY	-1.6	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>	-2.5	>	>	11. Aza-Michael polymers

Low bioactivity of amines/diol candidates

Reactive Micheal acceptors show relatively high bioactivity

Unsaturated polyesters show relatively high bioactivity

Resulting Aza-Michael polymers show low bioactivity

Integrate testing data with physico-chemical data and establish structure-activity relationships

BioDetection Systems

SAR table

Compound	MW	Linear	Branched	Acceptor type	No. acceptor	Adduct	Effect
Monomers and diluents							
A6#	low	•		a	low		-
A17#	low	•		b	low		
A12	low	•		a	low		
A16	low	•		c	low		
A22	low	•		b	low		
M15	low		•	d	medium		
M19	low	•		d	low		
(pre)Polymers							
M13	high	•	•	d	medium		
M21b	middle	•		d	low		
M21c	middle	•		d	low		
P22b	high	•		a	high		
P23c	high	•		c	high		
P23i	high	•		c	high		
P46c	middle	•		c	medium		
aza-Michael polymers							
C103a	high	•		c		•	-
C118a	high	•		a		•	-
C119a	high	•		a		•	-
C61a	high	•		a		•	
C64a	high	•		c		•	
C94a	high	•		c		•	
C95a	high	•		c		•	

Aza-Michael acceptors exhibited much more activity than aza-Michael polymers

Monomers and diluents:
activity higher for candidates with more acceptor groups.
activity higher for candidates with an acrylate as acceptor.

Prepolymers:
high activity, no relation with number/type of acceptor sites.
Higher MW prepolymers slightly less active

Bioactivity classification

- \- No activity or only cytotoxicity (CYT) at > 0.001 mg/ml
- Activity on one non-CYT assay at > 0.001 mg/ml
- Activity on >1 non-CYT assay OR at < 0.001 mg/ml
- Activity on >5 non-CYT assays OR >3 non-CYT assays at <0.001 mg/ml

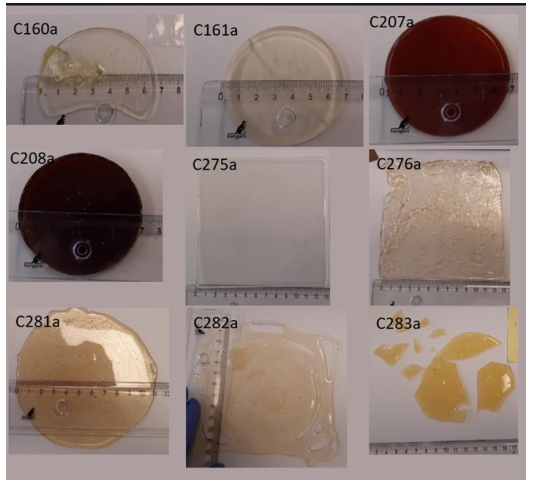
Safety tests in a context of polymer application

(solid samples for migration studies insoluble cured polymers)

BioDetection Systems

CALUX panel results for migration extracts of nine cured films

Code	Cytotox20%	ERa	TRb	PXR	PPARa	PPARg	PPARd	Hif1a	TCF	AP1	ESRE	Nrf2	p21	p53 GENTOX
Assay ref	-4.1	-9.8	-7.0	-4.4	-7.0	-5.1	-5.1	-2.6	-0.3	-6.7	-4.6	-2.8	-5.9	-5.9
C160a	-0.44	>	>	-0.4	>	>	>	>	>	-0.7	>	-1.4	>	-0.7
C161a	2.6	>	>	1.6	>	>	>	>	>	2.5	>	1.4	>	>
C207a	0.3	>	>	-0.01	>	>	>	>	>	-0.5	>	-1.0	>	-0.01
C208a	0.8	>	>	>	>	>	>	>	>	-0.2	>	-0.9	>	0.1
C275a	2.5	>	>	1.2	>	>	>	>	>	2.3	>	1.3	>	>
C276a	1.0	>	>	>	>	>	>	>	>	0.3	>	-0.2	>	0.3
C281a	2.5	>	>	1.7	>	>	>	>	>	1.7	>	1.5	>	1.2
C282a	2.2	>	>	1.2	>	>	>	>	>	1.2	>	1.5	>	1.2
C283a	1.6	>	>	>	>	>	>	>	>	1.3	>	-0.02	>	1.0



CALUX results of cured polymer film migration extract C207a, together with separate components.

Code	Cytotox20%	ERa	TRb	PXR	PPARa	PPARg	PPARd	Hif1a	TCF	AP1	ESRE	Nrf2	p21	p53 GENTOX	
C207a	0.3	>	>	-0.01	>	>	>	>	>	-0.5	>	-1.0	>	-0.01	cured film extract
N40S	>	>	>	>	>	>	>	>	>	>	>	>	>	>	amine(N)
P23	-1.9	>	>	-3.0	>	>	-1.5	>	>	-3.4	>	-3.7	>	-2.3	unsaturated polyester(P)
M15	-2.9	>	>	>	>	>	>	>	>	-3.5	>	-3.5	>	-3.5	Michael acceptor(M)

Lowest effect concentrations in LOG(mg/ml)

High Performance Bio-based Functional Coatings for Wood and Decorative Applications

Work package 6

WP6 - Safety and sustainability assessments

Month 1 – Month 31

This project receives funding from the Bio-based Industries Joint Undertaking (JU) under the European Union's Horizon 2020 research and innovation programme under grant agreement No 101022370. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio-based Industries Consortium.

WP6 Task 6.1 Chemical safety assessment

- **Objectives:**
- Feedback key chemical risk information for all candidate biopolymer coating materials (iterative 'safe by design' approach).
- Conduct leaching studies to determine components transferring from the polymer to (i) water and (ii) those that may cause skin irritation.

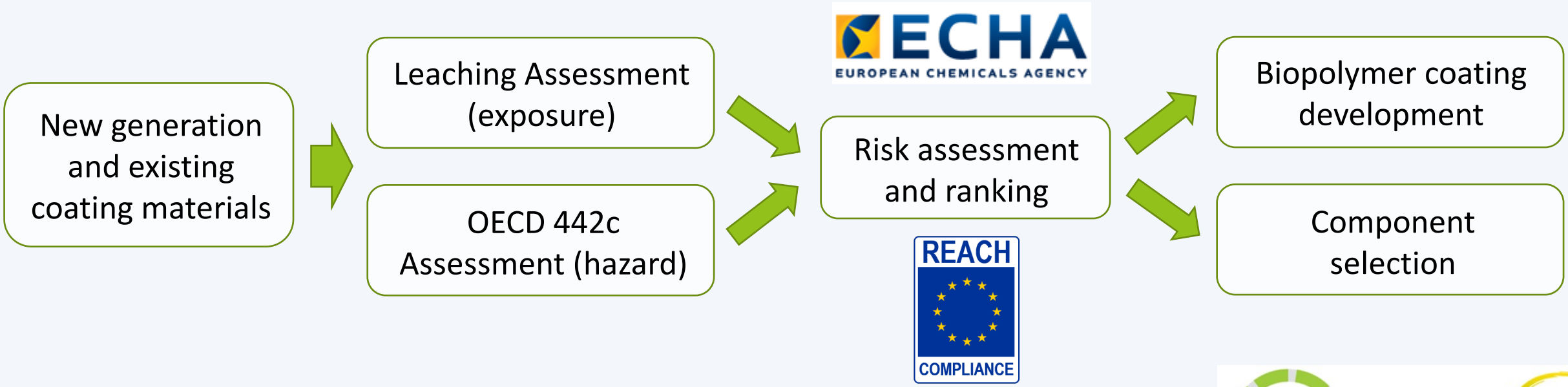
This project receives funding from the Bio-based Industries Joint Undertaking (JU) under the European Union's Horizon 2020 research and innovation programme under grant agreement No 101022370. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio-based Industries Consortium.

Task 6.1 - Chemical safety assessment

Conduct a comparison of the chemical exposure risks between the new and existing materials.

All major chemicals identified in the product leachates will be quantified and cross-referenced with the European Chemicals Association (ECHA) database and EU REACH regulation (EC 1907/2006).

Alternative will be proposed to substitute chemicals having the highest risk.



Method development – Mass-based quantification

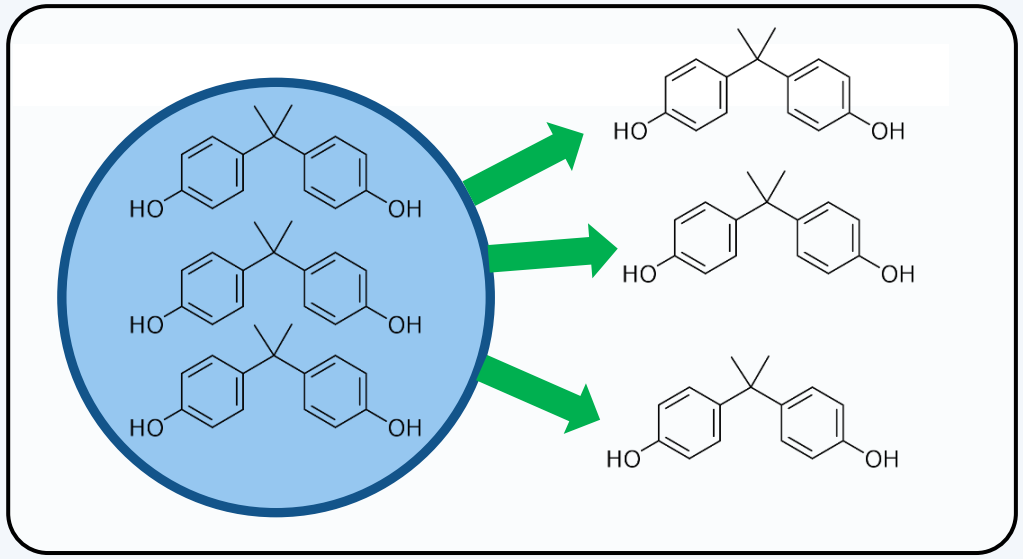
Activities:

- Chemicals/materials received are subjected to the skin sensitisation assessment (DRPA method).
- Average molecular weights (MWs) for four components were determined experimentally using LC-MS analysis.
- Further improvement of the accuracy and sensitivity of the method by developing an LC-MS/MS method for the quantification of peptides.

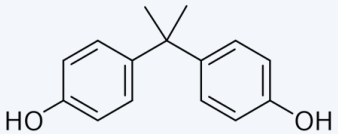
OECD 442c – Skin Sensitisation

Aqueous leaching

(Additives, residual chemicals, monomers)



Polymer material



Bisphenol A

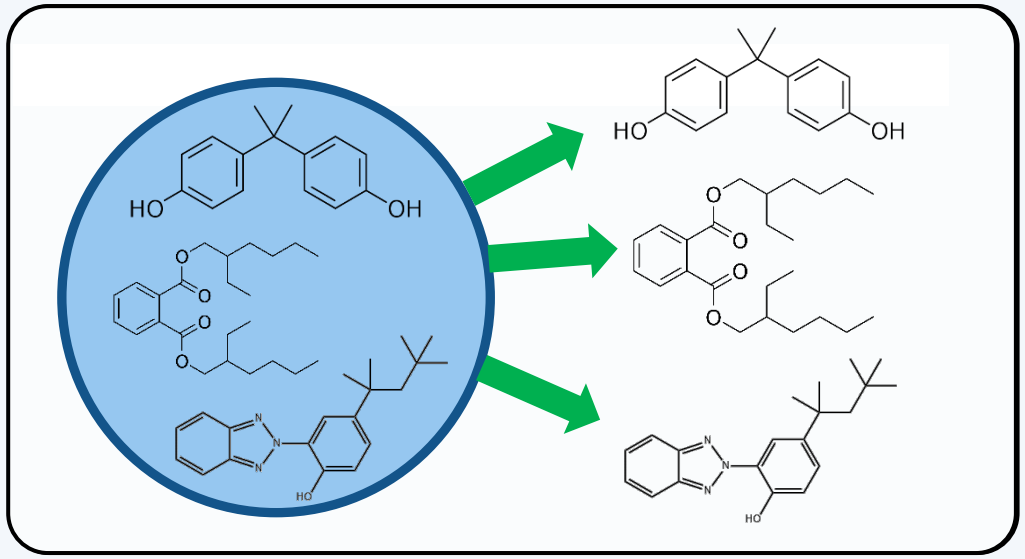
LC-MS Analysis

Target and non-target screening

OECD 442c – Skin Sensitisation

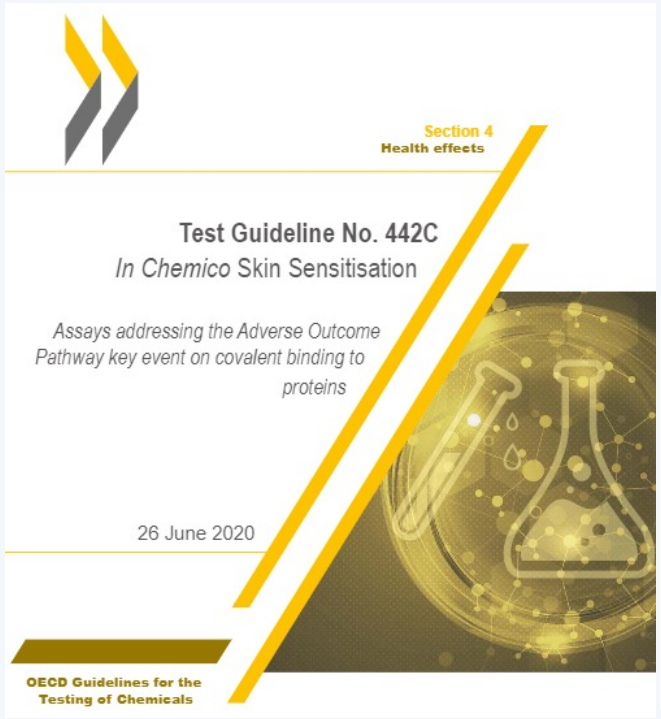
Aqueous leaching

(Additives, residual chemicals, monomers)



Challenge with chemical mixtures!

OECD 442c – Skin Sensitisation

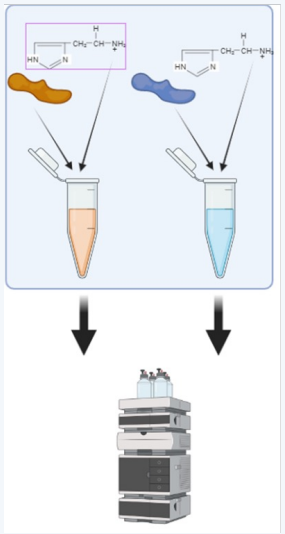


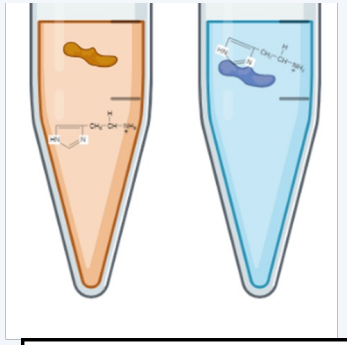
An in *chemico* procedure (Direct Peptide Reactivity Assay – DPRA) used for supporting the discrimination between skin sensitisers and non-sensitisers.

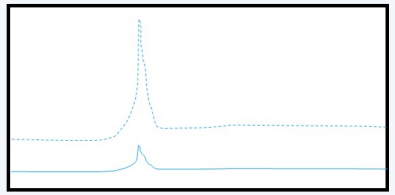
- Average molecular weight (MWs) of test chemical is required.

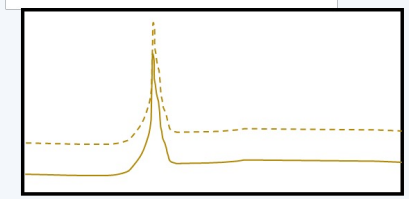
Establishment and validation

- Establishment of a skin sensitisation method consisting of a chemical procedure (Direct Peptide Reactivity Assay – DPRA) used for supporting the discrimination between skin sensitisers and non-sensitisers.









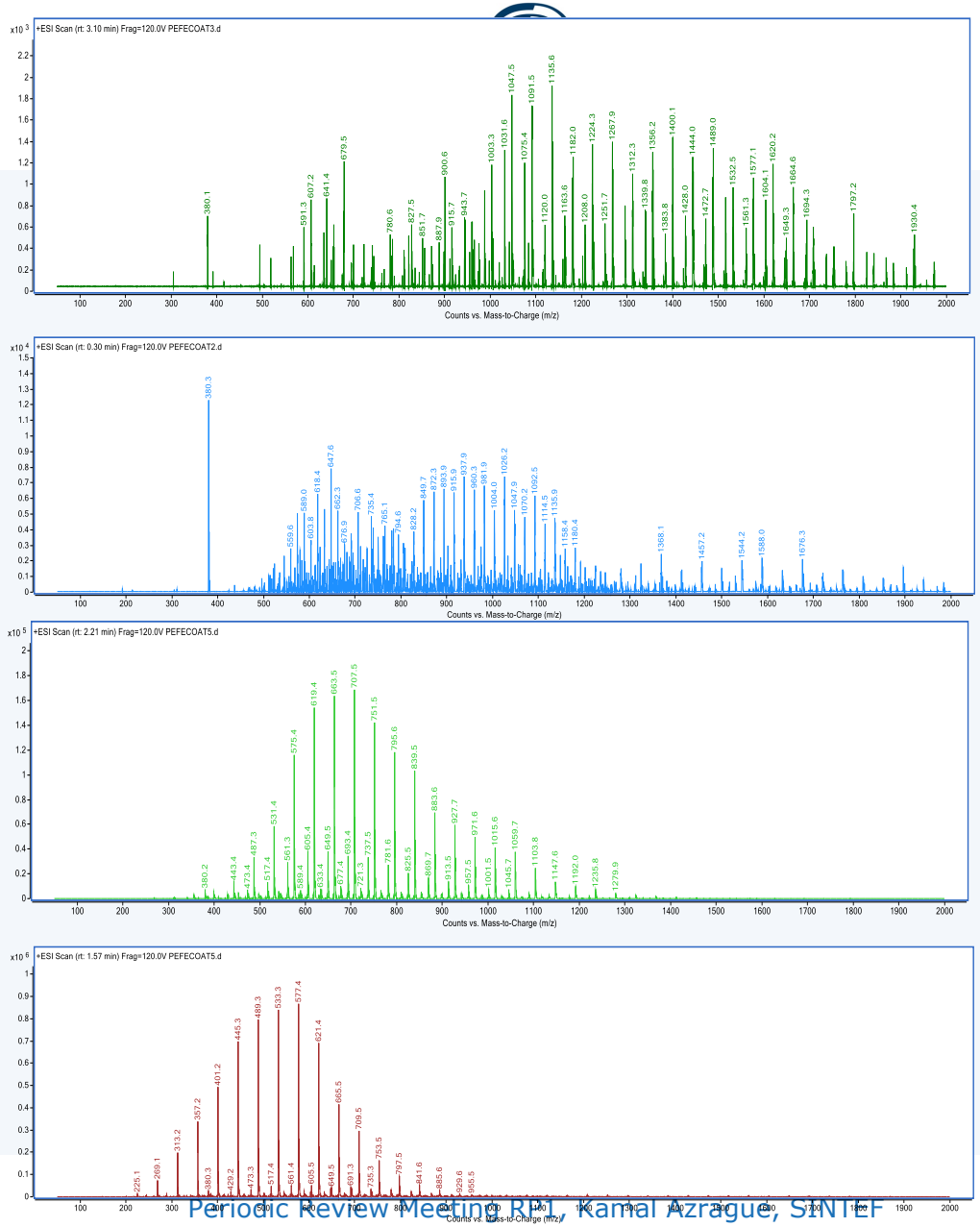
- The method tested and validated using recommended reference chemicals and is now ready for application.
- Further improvement of the accuracy and sensitivity of the method by developing an LC-MS/MS method for the quantification of peptides.

	CYS	LYS	Mean depletion	Reactivity	Model
Cinnemaldehyde 1	85.63%	72.62%	79.13%	high	Cysteine/Lysine
Vanillin 1	100.00%	inconclusive	100.00%	high	Cysteine-only
Formaldehyde 1	43.19%	2.82%	23.01%	moderate	Cysteine/Lysine
Ethylene glycol 1	97.32%	4.05%	50.69%	high	Cysteine/Lysine

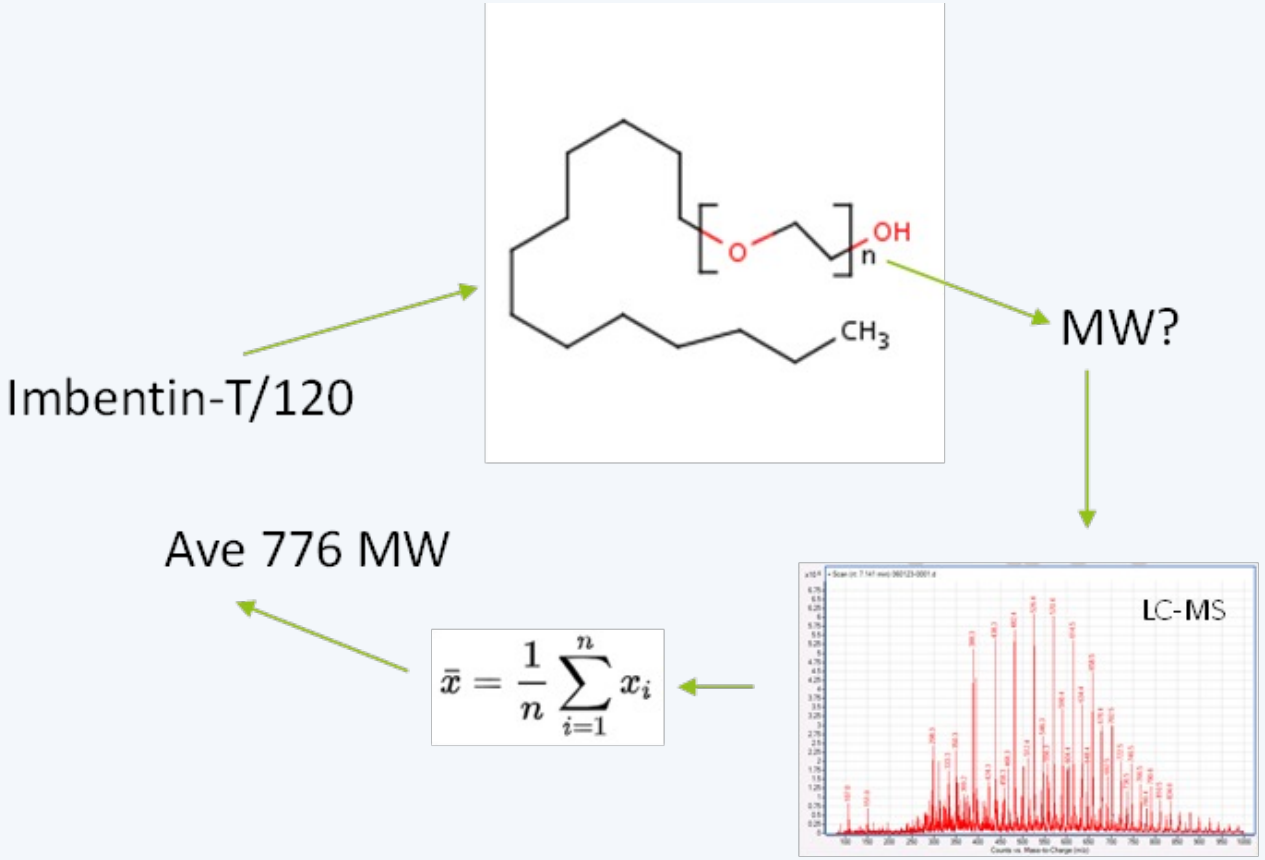
Assessment of coating components

Requires molecular weight of test chemical to be known

No.	Compound/Component	MW g/mol
1	Disponil SLS 101 Special	674
2	RHODAFAC RS/710-E	324
3	Emulsogen EPN 287	?
4	Polirol AL 1347	?
5	Imbentin-T/120	?
6	AEROSOL A-102 E	?
7	CaCO3	100
8	TiO2	80
9	Propylene glycol propyl ether	178
10	1-Hydroxycyclohexyl phenyl ketone	204
11	Trimethylolpropane ethoxylate triacrylate	av. 428



Average molecular weight determination



No.	Compound/Component	MW (g/mol)
1	Disponil SLS 101 Special	674
2	RHODAFAC RS/710-E	324
3	Emulsogen EPN 287	**ave.1210**
4	Polirol AL 1347	**ave. 866**
5	Imbentin-T/120	**ave. 776**
6	AEROSOL A-102 E	**ave. 713**
7	$CaCO_3$	100
8	TiO_2	80
9	Propylene glycol propyl ether	178
10	Irgacure 184	204
11	Trimethylolpropane ethoxylate triacrylate	ave. 428
12	Polydimethylsiloxane	**n.d.**

Results for single chemicals

- Results of the DPRA testing are in line with the other classifications.
- Review of the available standards and guidelines concerning painting test for the leaching studies.
- ISO 15181-1:2007 (Paints and varnishes: Determination of release rate of biocides from antifouling paints) was selected.
- Testing of the method and harmonisation against an internal leaching standard operating procedure (SOP) is ongoing.

Summary from the skin sensitisation testing and comparison to existing classifications

Constituent Type	Name	CAS	Classification	CLP notifications (% of all)	Effects in-vivo (REACH)	DPRA - Skin Sensitisation
Modifier	Calcium carbonate ($CaCO_3$)	471-34-1	Skin Irritant 2	Skin Irritant 2 (10%)	N	NA
Pigment	Titanium dioxide (TiO_2)	13463-67-7		Skin Irritant 2 (<0.1%)	N	NA
Defoamer	Polydimethylsiloxane	63148-62-9		Skin Irritant 2 (1.6%)	NA	NA
Coalescent	Propylene glycol propyl ether	1569-01-3	Skin Irritant 2	Skin Irritant 2 (20%)	N	Low
Photoinitiator	Irgacure 184	947-19-3		Skin Irritant 2 (0.1%)	N	Minimal
Diluent	TMPEOTA	28961-43-5		NA	Y	High
Main resin	Epoxyacrylate	55818-57-0	Skin Irritant 2	Skin Irritant 2 (6.5%)	Y	NA
			Skin Sensitizer 1	Skin Sensitizer 1 (92%)		
Surfactant	XTT sodium salt	111072-31-2	Pre-registration			High
Surfactant	RHODAFAC RS/710-E	9046-30-5	Not in the database			Low
Surfactant	AEROSOL A-102E	68954-91-6	Skin irritant	Skin Irritant 2 (82%)	NA	High
Surfactant	Imbentin-T/120	9043-30-5	Skin irritant	Skin Irritant 2 (21%)	NA	Low
Surfactant	Emulsogen EPN 287	?	Not in the database			Minimal
Surfactant	Polirol AL 1347	?	Not in the database			Moderate

Summary & Reflections

BioDetection Systems

SINTEF

- Two different methods for conducting safety assessment of new biobased chemicals and materials are presented
- There a many ways of conducting a safety assessment and these should be selected on a case by case basis
- Important to note that no single test allows a full safety assessment
- Standard methods increase the comparability of different data sets and increase robustness
- The approaches outlined here are cost effective and high throughput methods – potential for widespread use
- Neither method uses animals (*in vivo*), just cells (CHAMPION) or analytical chemistry (PERFECOAT)

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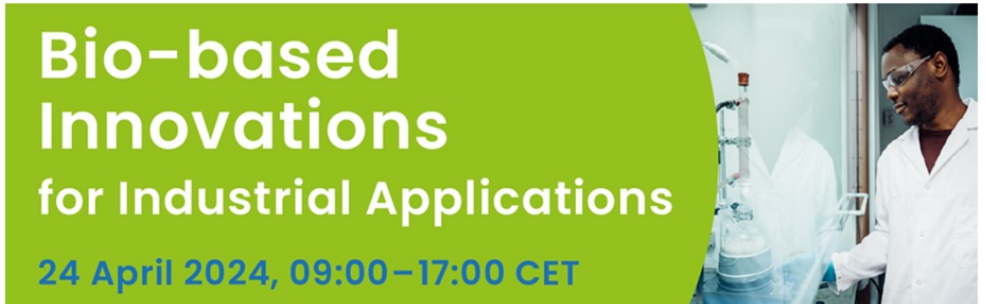

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