IZSTO

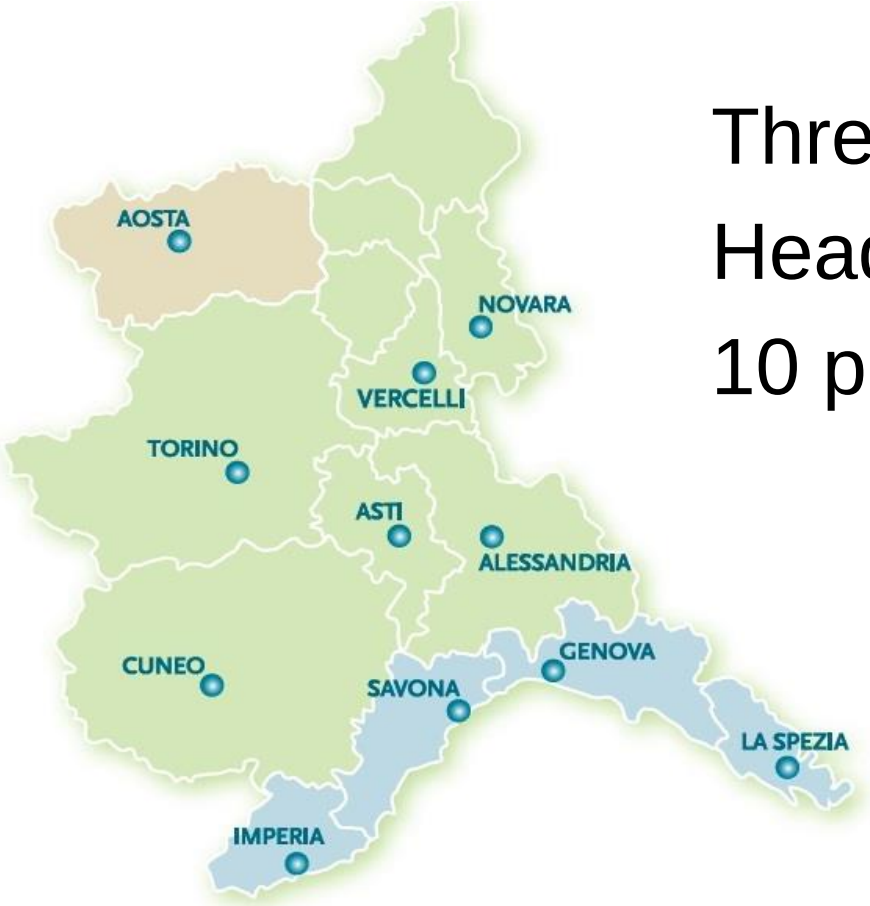
Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta

Plastic Food Contact Materials & EDC testing

Daniela Meloni
daniela.meloni@izsto.it

Network:10 Institutes in Italy

Animal health and food safety



Three Regions

Headquarter in Torino

10 peripheral laboratories

National Reference Centers & Labs

Feed control

CENTRO DI REFERENZA PER LA SORVEGLIANZA E IL CONTROLLO DEGLI ALIMENTI PER GLI ANIMALI

Beached cetacean

C.Re.Di.Ma.

Anabolic Treatments

CIB

CENTRO DI REFERENZA PER LE ENCEFALOPATIE ANIMALI

Animal encephalopathies

CENTRO DI REFERENZA NAZIONALE PER LE MALATTIE DEGLI ANIMALI SELVATICI

Wild animal diseases

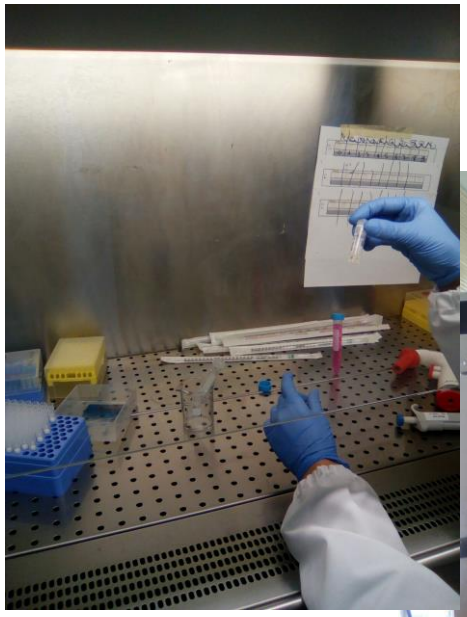
CENTRO DI REFERENZA NAZIONALE PER L'ONCOLOGIA VETERINARIA E COMPARATA

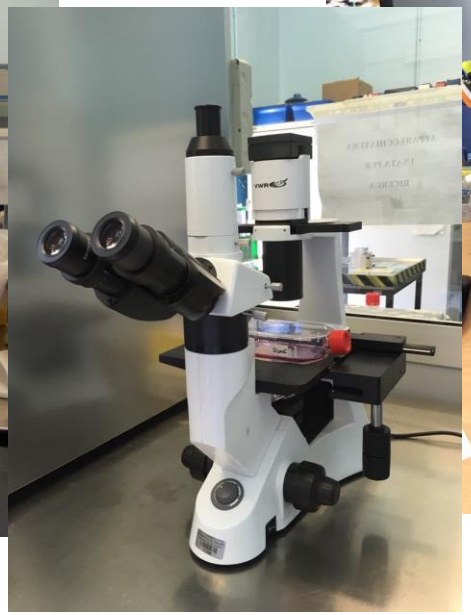
Sezione Liguria IZS PLV - Genova

Veterinary oncology

IZSTO CALUX® Laboratory

IZSTO CALUX® Laboratory equipment





Food Contact Materials scenario

- Food Contact Materials (FCM) are essential in the food manufacture, they protect food from physical, chemical and microbiological alterations and promote the product by encouraging the purchase;
- The packaging market is a highly important industrial sector. Global market value: US $400 bn* (EU €100 billion) per year;
- 70% of overall consumer packaging consumption is used for food and beverage packaging;
- Up to 100,000 substances in FCM (known, unknown) but only approximately 10 groups are currently covered in tests**;
- FCM need to be safe according to regulation, nevertheless, they represent an underestimated source of food contamination.

*Ringman j. CEPI J, 2012.

**ENVI Committee 2016

Endocrine Disrupting scenario

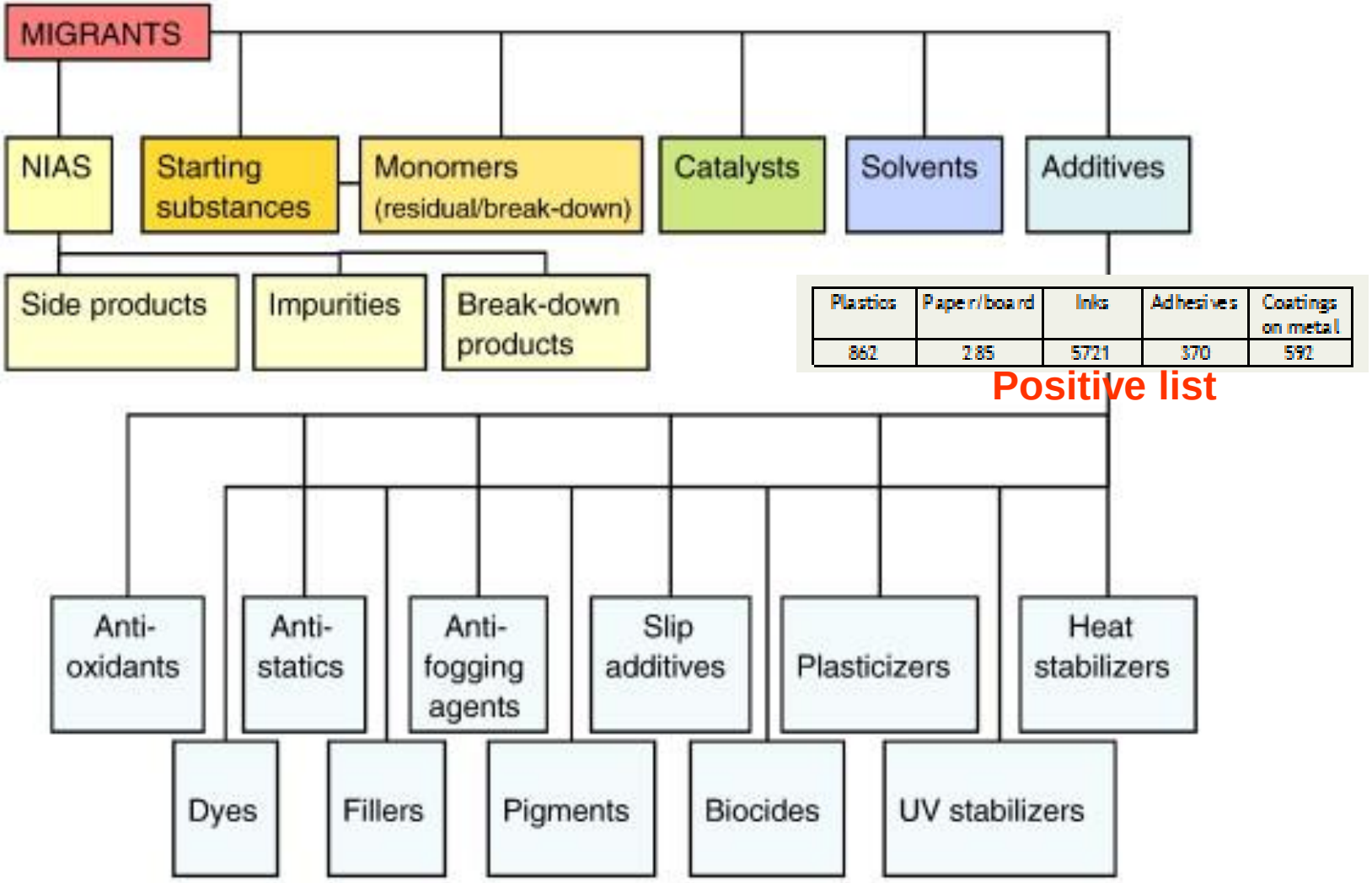
- World Health Organization (WHO) defines Endocrine Disrupting Chemicals (EDCs) as substances altering normal functions of the hormone system of living organisms causing serious dysfunctions;

- EDCs can migrate from FCM into foodstuffs;

- Many intentionally-used substances in food packaging have been identified as endocrine disruptors in biological systems (e.d. bisphenol A); non-intentionally added substances?

- Due to inherence technical and methodological difficulties in the safety assessment of FCM combined wth knowledge gaps, comliace with regulation may be currently not achievable.

What can migrate from packaging to food?



Plastics	Paper/board	Inks	Adhesives	Coatings on metal
862	285	5721	370	592

Positive list

Muncke J, 2008.

Challenges for risk assessment

- Is the paradigm RISK = EXPOSURE x EFFECT valid?
- Exposure assessment:
  - Which chemicals migrate from FCM (NIAS, mixtures, nanoparticles)?
  - Leaching ratios and thus actual exposure of consumers?
  - Food simulants do always predict worst-case leaching?
  - Combination of food consumption and/or FCM recycle scenarios with migration?
  - Ratio of food mass to contact area?
  - Exposure to substances leaching into dry foods?
- Effect assessment:
  - What is the toxicity of a given substance, of mixtures, of NIAS?
  - How relevant are low levels of chemicals migrating from FCM?
  - A changing population poses new challenges for chemical effect assessment?

Challenges for enforcement

- Is the material safe?
- What material to test?
- What substances to determine, NIAS?
- Where to start?
  - Declaration of compliance;
  - Supporting docs;
  - Limited product information avaliable to inspectors.
- Enforcements campaigns by Member States;
- What measures can be taken if non-compliance is found?

Challenges for compliance

- Is the material safe?
- What material to test?
- What substances to determine?
- Where to start?
  - Legislative guidance;
  - National regs;
  - EFSA guidance;
  - Risk assessment (mixture effects!).
- NIAS
  - Database generation;
  - Analysis (?)
- Busness operator: demonstrate compliance.

Challenges for testing - 1

- Safety assessment of FCM is currently ensured by testing single substances;
- Regulations require safety assessment for all migrating substances, including NIAS and mixtures, hence new approaches are needed;
- Testing the overall migrate or extract from finished FCM by means of *in vitro* bioassays is an option;
- Further development *of in vitro* bioassays procedures and workflow optimization are necessary.

Challenges for testing - 2

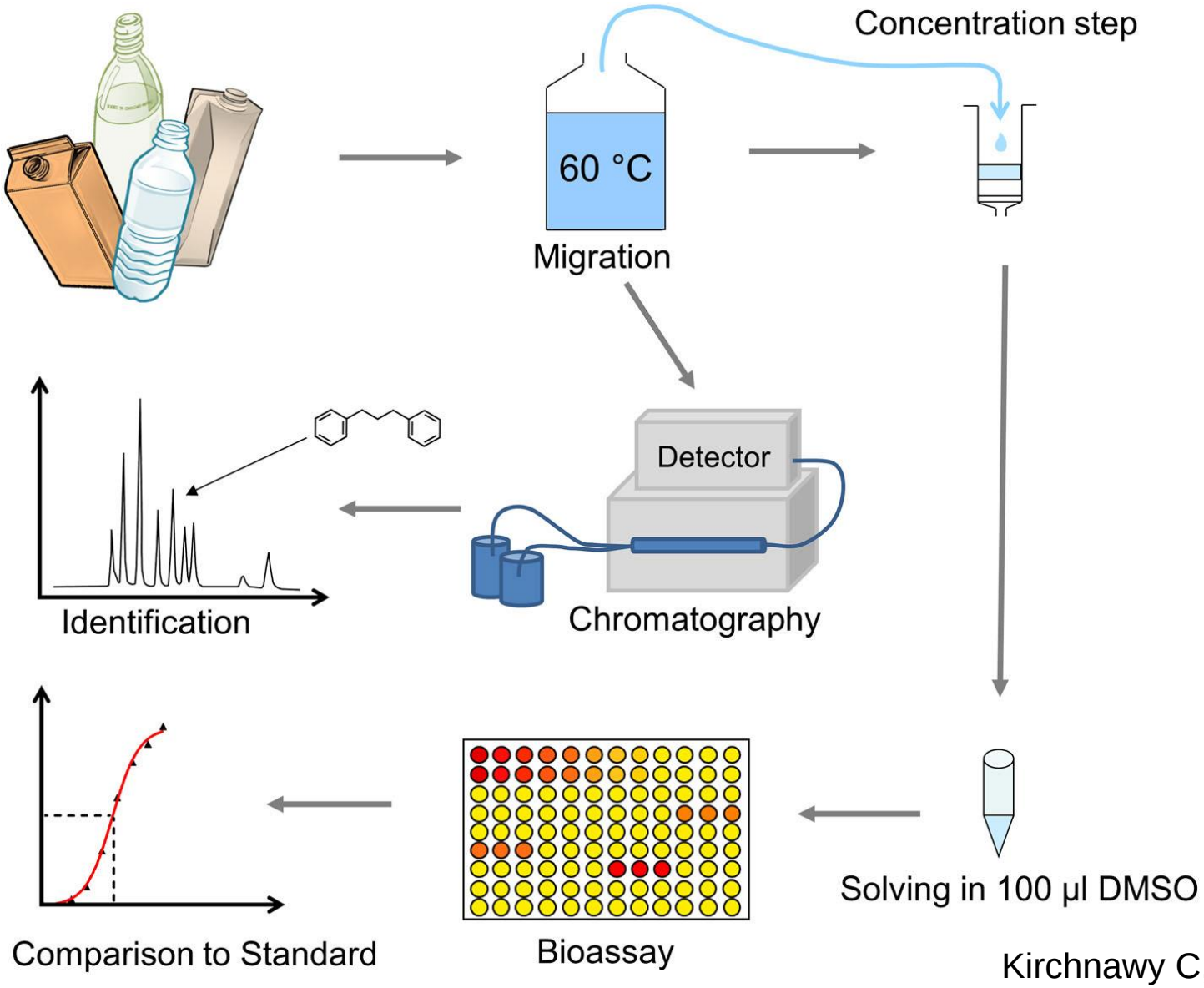
EFSA 2016 guidelines:

- Tiered approach to toxicity testing;
- Additional studies on specific endpoints and *in vitro* studies on endocrine effects;
- Read-across approach.

Experimental design

1. Selection of known or potential endocrine disrupters with authorized use in FCM in the EU;
2. Characterization of biological activity of each compound by ER, AR, GR CALUX® (in DMSO);
3. Selection of the most biologically active compounds;
4. ER CALUX® responce of selected molecules as measured in simulants;
5. Set up of extraction methods for different food simulants;
6. ER CALUX® responce of GC-MS field positive samples;
7. ER CALUX® responce of finished FCM.

Analitycal flow chart



Kirchnawy C, 2013

Selection of known or potential endocrine disrupters from positive list

- A number of molecules are reported to have hormonal activity according to bibliography, positive list of molecules included in the EU Regulation 10/2011 and SIN List. They are frequently used as additives and/or monomers in plastic FCM across the EU. We selected 32 compounds.

Characterization of biological activity of compounds by ER, AR, GR CALUX®

- Agonist or antagonist activity towards the estrogenic, androgenic and/or glucocorticoid receptors, was evaluated by AR, ER and GR CALUX® on serial dilutions of compounds in DMSO;
- Ten concentrations evaluated in a range between 0 and 4,000 ppm (estrogenic activity: up to 10 ppm);
- Calibration curves were analyzed using the Graphpad Prism software (version 5.00, Graphpad Software, San Diego, CA), determining EC50, IC50 and Relative Effect Potency (REP).

Biological activity of compounds by ER, AR, GR CALUX®

CAS n.# #chemical abstracts service number	Compound	Activity		
75-21-8	Ethylene oxide			
80-05-7	Bisphenol A			
620-92-8	Bisphenol F			
80-09-1	Bisphenol S			
84-74-2	Dibutyl phthalate (DBP)			
85-68-7	Benzyl butyl phthalate (BBP)			
88-24-4	2,2′-Methylenebis(4-ethyl-6-tert-butylphenol)			
88-99-3	Phthalic acid			
92-88-6	4,4′-Biphenol			
94-13-3	Propylparaben			
98-54-4	4-tert-Butylphenol			
99-76-3	Methylparaben			
100-42-5	Styrene			
103-23-1	Bis(2-ethylhexyl)adipate			
106-44-5	p-Cresol			
106-46-7	1,4-Dichlorobenzene			

ER acivity		Anti-AR activity		Anti-GR activity	
	Strong		Strong		
	Medium		Medium		Weak
	Weak		Weak		

Biological activity of compounds by ER, AR, GR CALUX®

CAS n.	Compound	Activity		
106-89-8	1-Chloro-2,3-epoxypropane			
108-46-3	Resorcinol 1,3-dihydrxybenzene			
117-81-7	Bis(2-ethylhexyl) phthalate			
119-47-1	2,2′-Methylenebis (4-methyl-6-tert-butylphenol)			
120-47-8	Ethylparaben			
121-79-9	Propyl gallate			
121-91-5	Isophthalic acid			
131-53-3	Dioxybenzone			
131-56-6	2,4-Dihydroxybenzophenone			
131-57-7	Oxybenzone			
301-02-0	Oleamide			
599-64-4	4-Cumyl phenol			
611-99-4	4,4′-Dihydroxybenzophenone			
10043-35-3	Boric acid			
25013-16-5	Butylated hydroxy-anisole			
26761-40-0	Diisodecyl phthalate			

ER acivity — Strong, Medium, Weak

Anti-AR activity — Strong, Medium, Weak

Anti-GR activity — Weak

Color meaning

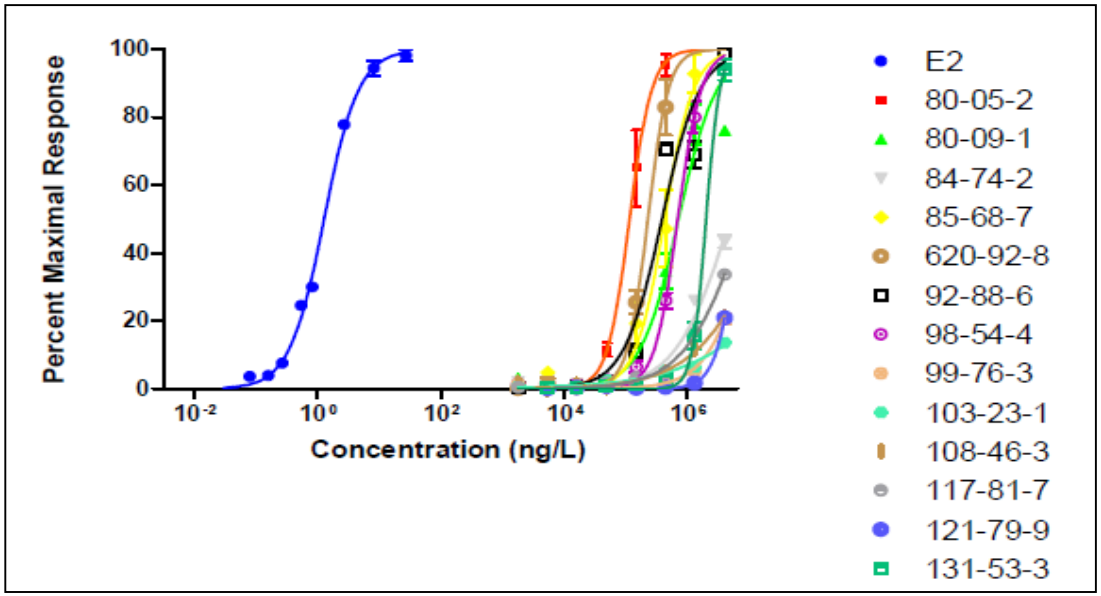
ER acivity (EC50)	**Strong ≤ 1ppm**	
	Medium ≤ 10 ppm ≥	
	Weak ≥ 10 ppm	
Anti-AR activity (IC50)	**Strong ≤ 1ppm**	
	Medium ≤ 4 ppm ≥	
	Weak ≥ 4 ppm	
Anti-GR activity (IC50)	**Weak**	

Active compounds by ER CALUX®

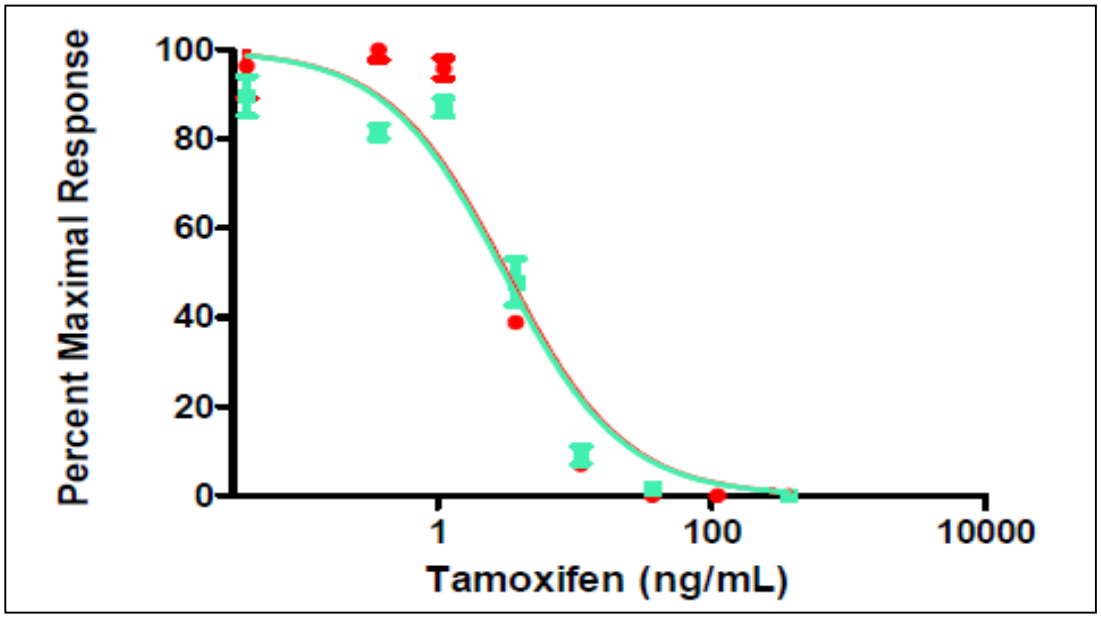
CAS n.	Compound	EC50 mg L-1 (ppm)	REP
80-05-7	Bisphenol A	0,11	1,11E-05
620-92-8	Bisphenol F	0,23	5,61E-06
80-09-1	Bisphenol S	0,71	1,78E-06
84-74-2	Dibutyl phthalate (DBP)	4,88	2,61E-07
85-68-7	Benzyl butyl phthalate (BBP)	0,42	3,01E-06
92-88-6	4,4′-Biphenol	0,38	3,31E-06
94-13-3	Propylparaben	0,46	2,77E-06
98-54-4	4-tert-Butylphenol	0,69	1,83E-06
99-76-3	Methylparaben	12,24	1,04E-07
103-23-1	Bis(2-ethylhexyl)adipate	187,90	6,76E-09
108-46-3	Resorcinol 1,3-dihydrxybenzene	23,99	5,29E-08
117-81-7	Bis(2-ethylhexyl) phthalate	8,36	1,52E-07
120-47-8	Ethylparaben	1,33	9,55E-07
121-79-9	Propyl gallate	6,95	1,83E-07
131-53-3	Dioxybenzone	1,99	6,38E-07
131-56-6	2,4-Dihydroxybenzophenone	0,49	2,60E-06
131-57-7	Oxybenzone	1,34	9,48E-07
599-64-4	4-Cumyl phenol	0,15	8,47E-06
611-99-4	4,4′-Dihydroxybenzophenone	0,29	4,38E-06
50-28-2	17β-estradiol	1,27E-06	1

Inducing 50% of maximum brightness (EC50), in a range between 0.11 to 1.99 mg L-1

ER CALUX® analysis



ER CALUX® activity

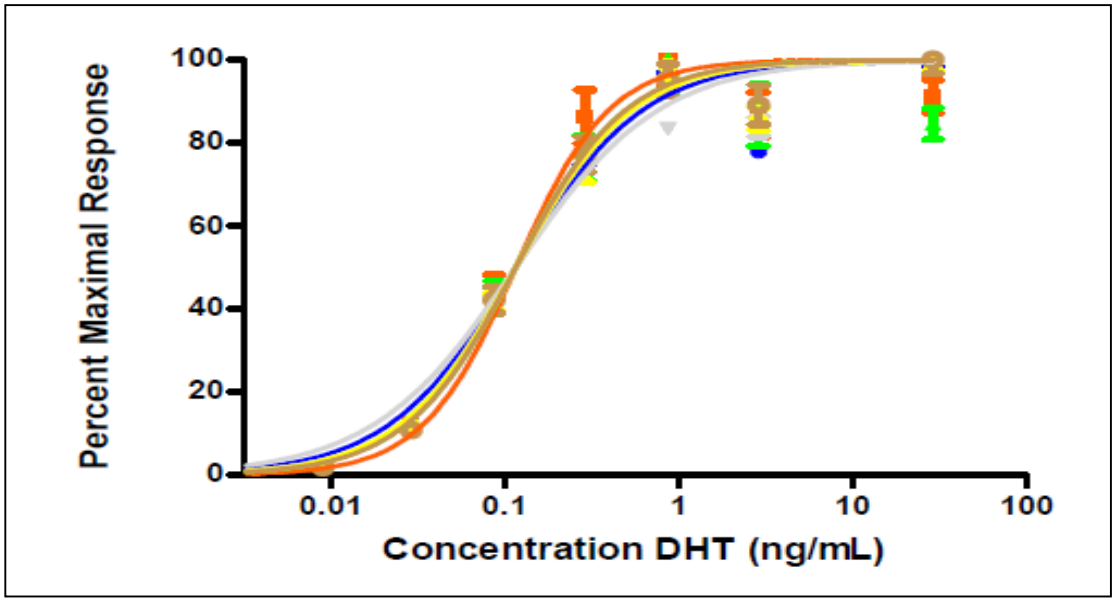


Anti - ER CALUX® activity

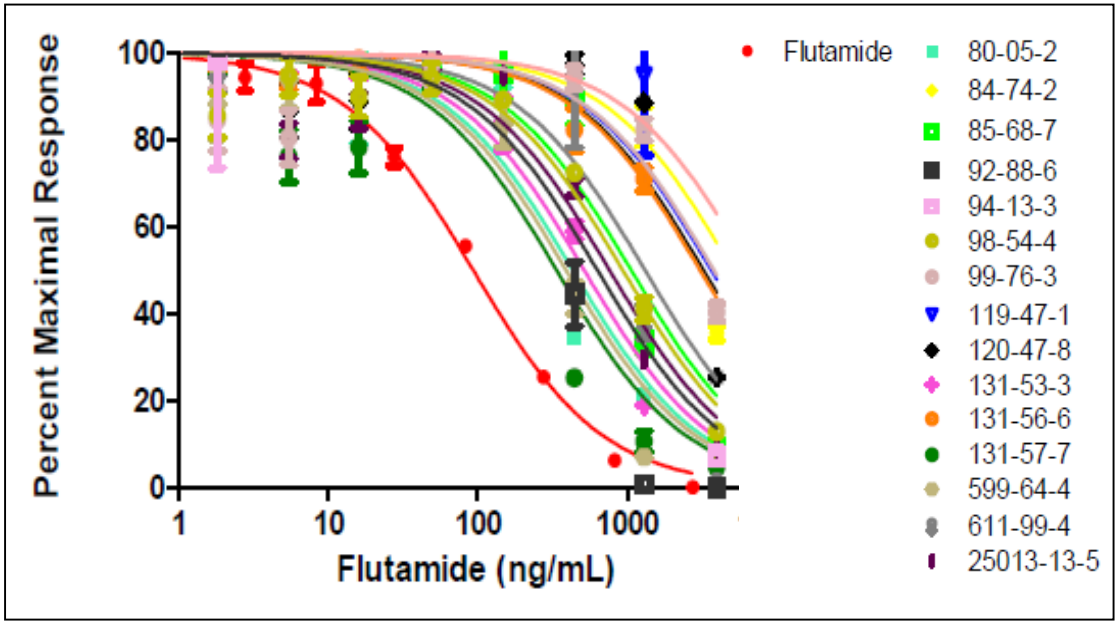
Anti - Androgenic compounds by AR CALUX®

CAS number	Molecola	IC50 mg L-1 (ppm)	REP
80-05-7	Bisphenol A	0.43	0.21
84-74-2	Dibutyl phthalate (DBP)	5.15	0.02
85-68-7	Benzyl butyl phthalate (BBP)	1.07	0.08
92-88-6	4,4′-Biphenol	0.63	0.14
94-13-3	Propylparaben	6.80	0.01
98-54-4	4-tert-Butylphenol	0.94	0.10
99-76-3	Methylparaben	3.82	0.02
119-47-1	2,2′-Methylenebis (4-methyl-6-tert-butylphenol)	3.68	0.02
120-47-8	Ethylparaben	3.25	0.03
131-53-3	Dioxybenzone	0.52	0.17
131-56-6	2,4-Dihydroxybenzophenone	3.08	0.03
131-57-7	Oxybenzone	0.34	0.26
599-64-4	4-Cumyl phenol	0.39	0.23
611-99-4	4,4′-Dihydroxybenzophenone	1.32	0.07
25013-16-5	Butylated hydroxy-anisole	0.76	0.2
13311-84-7	**Flutamid**	**0.09**	**1**

AR CALUX® analysis



AR CALUX® activity

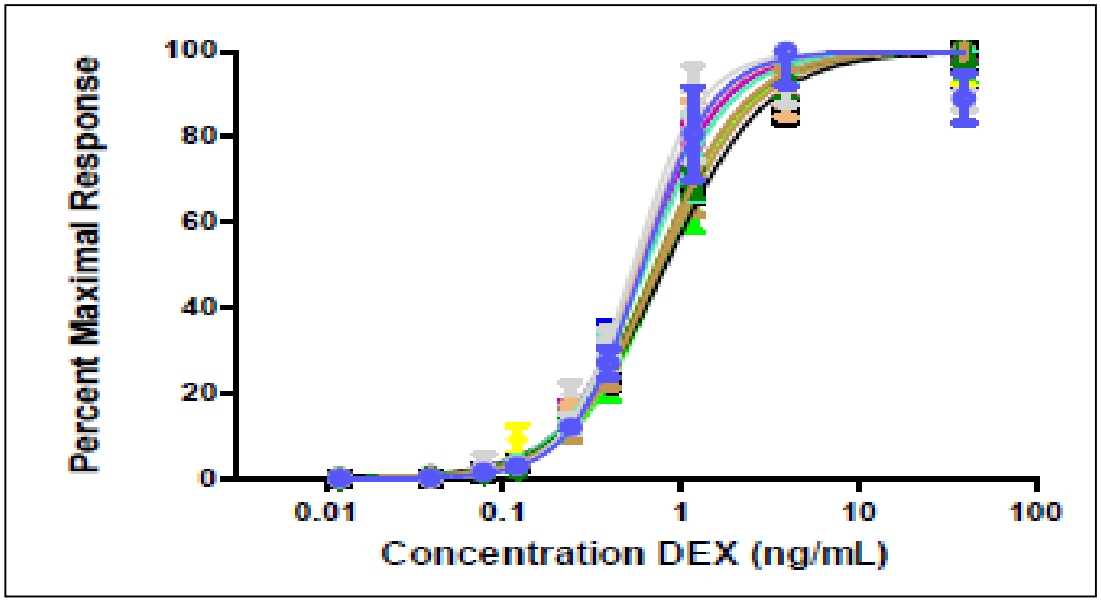


Anti - AR CALUX® activity

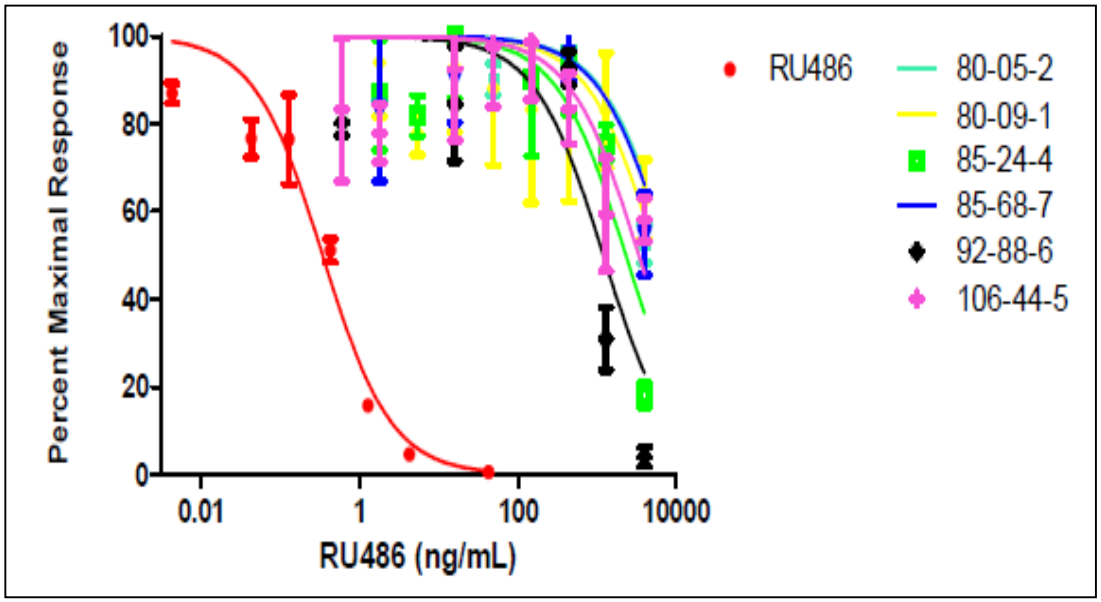
Anti - Glucocorticoid compounds by GR CALUX®

CAS number	Chemical	IC50 mg L-1 (ppm)	REP
80-05-2	Bisphenol A	8.31*	4.12E-5*
80-09-1	Bisphenol S	5.68*	6.02E-5*
85-68-7	Benzyl butyl phthalate (BBP)	7.79*	4.39E-5*
88-24-4	2,2′-Methylenebis(4-ethyl-6-tert-butylphenol)	2.32*	1.47E-4*
92-88-6	4,4′-Biphenol	1.20*	2.84E-4*
106-44-5	p-Cresol	3.38*	1.01E-4*
84371-65-3	**RU486**	**3.42E-04**	**1**

GR CALUX® analysis



GR CALUX® activity



Anti - GR CALUX® activity

GR CALUX® analysis on experimental finished FCM (from manufacturers)

Sample ID	Description	Simulant	ng EEQ/L	LOD [ng EEQ/l]	LOQ[ng EEQ/l]
BP1	A/PP Bag	3%AcOH	< LOD	0,2	0,5
VP1	PP Bowl	3%AcOH	< LOD	0,2	0,5
VP2	PP Bowl	50%EtOH	< LOQ	0,1	0,3
VE1	APET Bowl	10%EtOH	< LOQ	0,1	0,2
VE2	APET Bowl	3%AcOH	< LOD	0,2	0,5
FT1	PA/PE Thermoformable films	3%AcOH	< LOQ	0,1	0,3
BP2	PA/PE Bag 3g	3%AcOH	< LOQ	0,1	0,2
BP3	PA/PE Bag 10g	3%AcOH	< LOQ	0,1	0,3

GR CALUX® analysis on official control GC-MS non-compliant field samples

Sample ID	Description	Simulant*	ng EEQ/L	LOD [ng EEQ/l]	LOQ[ng EEQ/l]
VP3	Bowl	Isooctane	< LOQ	0,1	0,2
CA1	Paper with sticker	EtOH 95%	2,6	0,1	0,2
CA2	Paper with sticker	3%AcOH	220	0,1	0,2
VP4	PP Bowl	10%EtOH	86	0,0	0,1
VP5	PP Bowl	3%AcOH	71	0,1	0,3
VP6	PP Bowl	20%EtOH	61	0,1	0,3
VP7	PP Bowl	50%EtOH	< LOQ	0,1	0,2
VP8	PP Bowl	95%EtOH	0,11	0,0	0,0

*Specific migration

GC-MS analysis on official control non-compliant field samples (incomplete data)

ID	Description	Simulant	Diisobutyl phthalate (DIBP)	Di-n-butyl phthalate (DBP)	Bis-2-etilesilftalato (DEHP)	Bis-2-ethylhexyl phthalate (DEHA)	Acetyl tributyl (ATBC)
					mg/kg		
VP3	Bowl	Isooctane	1.7	0.5	4.4	< 0.1 - 0.5	< 0.1 - 0.5
CA1	Paper with sticker	EtOH 95%	-	-	-	-	-
CA2	Paper with sticker	3%AcOH	-	-	-	2400	2100
VP4	PP Bowl	10%EtOH	-	-	-	-	-
VP5	PP Bowl	3%AcOH	-	-	-	-	-
VP6	PP Bowl	20%EtOH	-	-	-	-	-
VP7	PP Bowl	50%EtOH	-	-	-	-	-
VP8	PP Bowl	95%EtOH	-	-	-	-	-

Summary

- The aim of the study was to characterize a group of food contact approved use compounds using ER, AR and GR CALUX® bioassays;
- This kind of studies are essential in order to screen food contact material by CALUX® bioassays;
- Plastic food packaging of different resin types were migrated by food simulants according to EC 10/2011. Migrates were concentrated by solid phase extraction and analyzed by bioassys. No reactivity emerged;
- Some official control phthalate positive samples were tested by ER CALUX® bioassay. Reactivity emerged (cautious interpretation!).

Conclusions 1

- Today's toxicological knowledge threshold concepts for unidentified food packaging migrants require thorough reconsideration and validation according to latest scientific developments;
- Effect evaluation might prove useful for risk assessment;
- *In vitro* tests give an integrated picture of various toxicological effects and may offer a robust and economic solution;
- *In vitro* tests can be used directly to highlight problematics of FCM or for screening purposes.

Conclusions 2
Future research and agreements to achieve

- Selection of solvents, time and temperature for migration procedure as well as clean-up, SPE, affinity purification;
- Efficiency and reliability of different tecniques should be investigated aiming at method optimization ensuring no esternal contamination or loss of compounds (volatiles);

Conclusions 3
Future research and agreements to achieve

- Assay selection and clear interpretation of test results: clear relatioship between *in vitro* response and *in vivo* endpoint (trigger values?);
- Define the threshold above which a follow up action should start;
- Optimized procedures and workflows should become not only standardized but widely harmonized.

Acknowledgements

Ministero della Salute

Research project: IZS PLV 03/13 RC

BDS BioDetection Systems

CAMERA DI COMMERCIO INDUSTRIA ARTIGIANATO E AGRICOLTURA DI TORINO

IZSTO

Elena Bozzetta
Danilo Pitardi
Maria C. Cavarretta
Daniela Loprevite
Marcello Freguglia

And thank you!