An effect-based approach for the screening of endocrine disruptors in plastic food contact materials: preliminary data

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Introduction: Food Contact Materials FCM) are essential in the food manufacture, they protect food from physical, chemical and microbiological alterations and promotes the product by encouraging the purchase. However, according to the European Food Safety Agency[1], some of the chemicals or particles that can migrate from Food Contact Materials are suspected to have Endocrine Disrupting properties[2]**.** The World Health Organization defines Endocrine Disrupting Chemicals (EDCs) as substances altering normal functions of the hormone system of living organisms and they are suspected to be associated with altered reproductive function, increased incidence of breast cancer and children developmental dysfunctions. Overall migrate of the finished packaging could be evaluated for biological effects using bioassays. Particularly, the application of reporter-gene assays (RGAs) for FCM toxicity screening analysis could represent a milestone in the characterization of the hormonally-related actions of known and unknown compounds. Here, we investigate the efficiency of a panel of CALUX® Reporter Gene Assays (Figure 1) for FCM screening purposes.

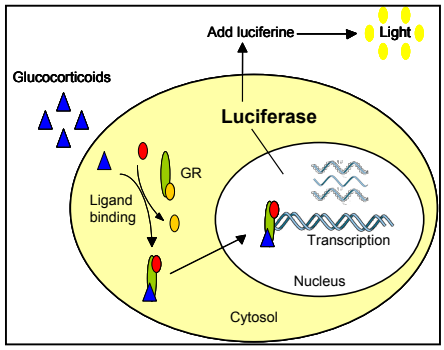

Figure 1: Principle of the GR CALUX - reporter gene assay. The signal dose increases as a result of increasing concentrations of the ligand[3].

Materials and methods:
1. Identification and characterization of the target molecules: the analysis of food packaging by means of reporter gene assays *per se* leads to a qualitative result: presence or absence of hormonal or anti-hormonal activity of glucocorticoid, androgenic and/or estrogenic nature. The impossibility of identifying the molecule (s) generating a signal makes essential a preliminary phase of characterization of known EDCs, potentially present in plastic FCM. This first part of the study has thus led to the creating of dose-response curves for known FCM, relative to the AR, ER and GR receptors;
2. Choice of target molecules: done through the evaluation of scientific literature and health legislation on EDCs and FCM. Particularly, the scientific data were interfaced with:
- The positive list of molecules included in the EU Regulation 10/2011;
- The list of "problematic" compounds from a sanitary point of view in the SIN List (ChemSec).
3. Generating dose-response curves for target molecules: the biological agonist or antagonist activity towards the estrogenic, androgenic and/or glucocorticoid receptors, of each target molecule was evaluated by preparing serial dilution of each compound in DMSO and analyzing such samples for agonist and antagonist activity by GR, AR and ER CALUX®. Ten concentrations of each compound were testedd, in a range between 0 and 4,000 ng mL-1. Graphpad Prism software (version 5.00, Graphpad Software, San Diego, CA) was used in order to calculate:

- EC50: the concentration of a molecule that produces 50% of the maximum effect (maximum luminescence). It represents the power of a molecule;
- IC50: inhibitory concentration intended as the concentration of a receptor or enzyme inhibitor necessary to inhibit 50% of the effect related to the chemical under examination;
- Relative Effect Potency (REP): calculated by comparing the action of a molecule with that of the reference compound used in the same test (EC50 reference compound/EC50 compound under examination).

Results

1. Choice of target molecules: identification of 32 molecules (Table 1), equipped with documented hormone activity and currently used as additives and/or monomers for the production of plastic FCM in the EU.
2. Estrogenic activity: evaluated by ER CALUX. The biological activity of each molecule was estimated by referring to a calibration curve modelled on ten concentrations of E2 between 0 - 27 ng L-1. Twenty molecules from the 32 tested showed affinity for estrogenic receptors (Figure 2). This biological activity was thousands of times lower than that of the reference compound (REP), despite this, given the high sensitivity of the assay, 14 molecules were able to induce 50% of the maximum brightness (EC50), in a range between 0.11 and 1.99 mg L-1 (Table 2). The remaining compounds exhibited a more limited but easily appreciable biological activity.
3. Anti-estrogenic activity: evaluated by the ER CALUX. E2 was added to EC50 in the medium of exposure, so the ability of each compound to reduce its biological activity was studied. The biological activity was related to a calibration curve generated by ten tamoxifen points between 0 - 371.5 ng mL-1. None of the compounds showed antagonistic activity towards estrogenic receptors.
4. Androgenic activity: studied by AR CALUX. The biological activity was established by referring to a calibration curve gained by ten concentrations of DHT between 0 - 2.9 ng mL-1. None of the tested compounds showed agonist activity against androgen receptors.
5. Anti-androgenic activity: evaluated by AR CALUX. DHT was added to EC50 in the exposure medium, so the ability of each compound to reduce its biological activity was evaluated. The activity was evaluated by referring to a calibration curve created by ten dosges of flutamide ranging from 0 - 2.762 ng mL-1. Fifteen molecules, out of the 32 tested, showed anti-androgen activity; at least half of the compounds analyzed showed a biological activity very close to that of the reference compound (REP). These molecules caused a 50% reduction in the maximum fluorescence (IC50), in a range of 0.34 to 6.80 mg L-1.
6. Glucocorticoid activity: by GR CALUX. The biological activity was evaluated by referring to a calibration curve set by ten points of dexamethasone included between 0 - 39 ng mL-1. None of the tested compounds showed agonist activity towards glucocorticoid receptors.
7. Anti-glucocorticoid activity: by the GR CALUX test. In the medium of exposure, dexamethasone was added to EC50, so the ability of to reduce its biological activity was evaluated. The activity was evaluated by referring to a calibration curve obtained by the analysis of ten dosages of RU486 between 0 - 43 ng mL-1. Six molecules, showed a low anti- GR receptor activity, but, generally, all the compounds reveal to be thousands of times less active than the reference molecule.

Table 1. Target molecules and biological prooperties when tested by CALUX® assayes

CAS Registry Number	Compound	Biological 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	
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	

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

Legenda

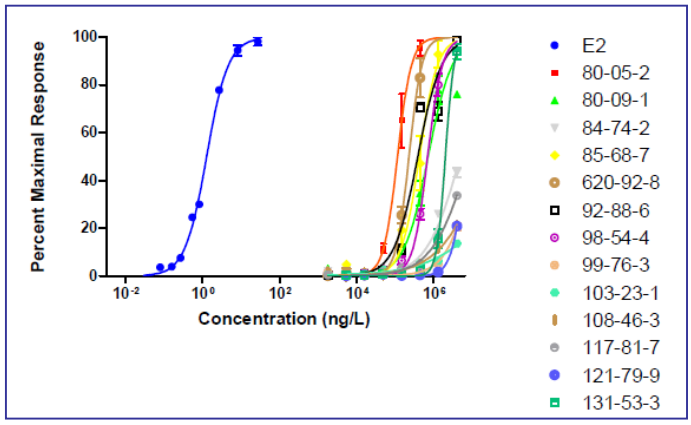

Figure 1. Calibration curves of some molecules with affinity for estrogenic receptor.

Table 2. 14 molecules induce 50% of the maximum brightness (EC50), in a range between 0.11 and 1.99 mg L-1 by ER CALUX test

CAS Registry Number	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

The European Union, through its legislative acts, intends to reduce, eliminate or avoid public health risks and therefore to prevent harmful and/or unfit food for human consumption to be placed on the market[4]. To date, official controls on FCM adopt a "target-based" approach. Many migrating compounds remain unidentified. There is a need for information on identity/quantity of chemicals leaching into food, human exposure, and long-term impact on health. In a context of new toxicology paradigms, the development of RGAs, could lead to a quickly, reliably and repeatable effect based assessment of the biological activity of FCM.

Acknowledgements:

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References

1. EFSA (2013). Scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment EFSA Journal 2013; 11 (3):3132.
2. Behnisch P (2012). Human cell based screening tests (CALUX) for the quantitative analysis of plastic additives and complex mixtures of food packaging materials. Poster, 6th Bio Detectors workshop 2012, , Amsterdam, the Netherlands.
3. Muncke J (2009). Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source? Science of The Total Environment, 407 (16); 4549-4559.
4. Comm. Reg. (EU) N.°10/2011 of 14/1/2011. On plastic materials and articles intended to come into contact with food. 2011R0010 - EN - 30.12.2011 - 002.00.