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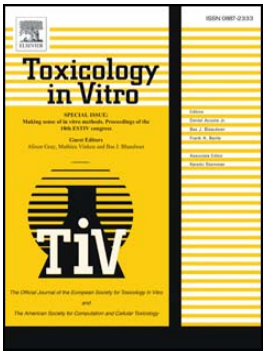

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Screening of endocrine activity of compounds migrating from plastic baby bottles using a multi-receptor panel of *in vitro* bioassays

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Abstract

Endocrine activity of 65 compounds migrating from polycarbonate replacement plastic baby bottles was assessed using *in vitro* cell based assays (reporter gene assays) involving 7 nuclear receptors, i.e. human steroid hormones receptors (oestrogen, androgen, progesterone and glucocorticoid receptors), human thyroid beta and peroxisome proliferator-activated gamma receptors, and the mouse aryl hydrocarbon receptor. The chemicals were tested at 4 concentrations ranging from 0.001 mM to 1 mM. Only twelve chemicals did not show any activity towards any of the nuclear receptors, while fifty three compounds showed a possible endocrine activity. Most of the agonistic activities were observed towards the oestrogen receptor while the PPARγ was the target for most of the recorded antagonistic activities . Agonistic activities were recorded for several phthalates, benzophenones, aromatic hydrocarbons and phenols, while compounds such as benzaldehydes, ketones and esters of fatty acid showed antagonistic activities. Thirty five chemicals were able of agonistic activities on 1 to 4 receptors and antagonistic activities were recorded for 35 compounds as well, towards 1 to 7 receptors. Sixteen compounds were able of both agonistic and antagonistic activities, but not on the same receptors, except in 2 cases for the oestrogen receptor and 4 cases for the PPARγ.

Keywords: Food contact material, Endocrine Disruptor, Endocrine Active Substance, Reporter gene assay, nuclear receptors.

Introduction

Manufactured plastic products such as food contact materials (FCM) are potential sources of endocrine active substances (EAS) (Kirchnawy et al., 2014, Mertl et al., 2014; Muncke, 2011). Migration of these EAS from plastic FCM into food can arise, and these compounds may interfere or interact with normal human hormonal activity. Multiple mechanisms of action can occur at all stages of endocrine regulation, from interference with the hormone synthesis to effects in target tissues, including mimicking or antagonizing hormones activities on nuclear receptors (Baker, 2001, Dietrich et al., 2013, EFSA, 2010, 2013; Soto et al., 2006). According to EFSA, "*Endocrine active substances are chemicals that can interact or interfere with normal hormonal activity; when this leads to adverse effects they are called endocrine disruptor"* (EFSA, 2014). Many health related problems in mammals, such as endometriosis, precocious puberty in females, reduced sperm quality, high incidences of breast, ovarian and prostate cancer, obesity and cardiovascular diseases have been associated with exposure to EAS through epidemiological studies (Diamanti-Kadaris et al., 2009, Kortemkamp et al., 2011, UNEP/WHO, 2012, Vandenberg, 2014).

Bisphenol A (BPA), probably the most studied EAS, has been approved in Europe (European Commission, 2011) as a monomer and additive in the manufacture of FCM, such as polycarbonate (PC) (used for reusable food and drink containers, baby bottles, cups, plates, etc.) and epoxy resins (used as coating in the inner side of canned food) (ANSES, 2011, Geens et al., 2014, 2012, 2011, 2010). BPA can migrate from FCM into food and beverages, as it can be present as residual starting or degradation product. Ingestion is not the only source of human exposure to BPA, but it constitutes the primary route according to the European Food Safety Agency (EFSA, 2015).

Due to potential concern for human health, BPA-containing PC was recently banned for the manufacture of baby bottles in European Union (European Commission, 2011). Subsequently, alternatives to PC such as polypropylene (PP), silicone, polyamide (PA), polyethersulphone (PES), Tritan®, etc. have emerged on the Belgian and European markets. From these replacement materials, various compounds or additives, such as plasticizers, antioxidants, UV absorbers, etc. can also migrate into food and beverages, either as residual starting products or degradation products. Although several studies on the migration of BPA from PC into foodstuffs under a variety of conditions are available (Cao and Corriveau, 2008; De Coensel et al., 2009; Kubwabo et al., 2009; Maragou et al., 2008; Nam et al., 2010), information about both the identification and the potential endocrine activity of possible migrating compounds from alternatives to PC baby bottles are scarce (Aschberger et al., 2010; Bittner et al., 2014).

During the last decade, a major effort was put into the development of methods to identify EAS (Marty et al, 2010; Soto et al., 2006). *In vitro* assays appeared to be the methods of choice to screen EAS, because they are applicable to a large number of substances, are rapid, and, last but not least, they allow to minimize the number of animals to be used (Kroese et al, 2014; Rotroff et al., 2014; Van der Burg et al., 2011).

Transactivation gene expression assays, also called reporter gene assays, are internationally recommended and validated tools (by Environmental Protection Agency of the United States as well as by the Organisation for Economic Cooperation and Development) that provide mechanistic data (Charles, 2004; Grimaldi et al., 2015a, 2015b). Indeed, a lot of EAS have been shown to interact with nuclear receptors, and for compounds for which both *in vitro* and *in vivo* data are available, a good correlation has been found between *in vitro* and *in vivo* results (Lewin et al., 2015).

Given the complexity of the endocrine system, the use of a panel of transactivation gene expression assays can help to elucidate the cellular mechanism behind endocrine activity and hormone disruption.

Until now, most research on endocrine activity has focused on the effect of compounds on oestrogen and androgen receptors (Bhattacharjee and Khurana, 2014; Blair et al., 2000; Kirchnawy et al., 2014; Kojima et al., 2004; Grimaldi et al., 2015a), but chemicals can also act on other nuclear receptors. In this study, due to the wide variety of substances that can migrate from baby bottles, a panel of cell reporter gene assays was used to test chemicals for oestrogen, androgen, progesterone, glucocorticoid, thyroid beta, peroxisome proliferator gamma and aryl hydrocarbon receptor mediated transactivation activity. These nuclear receptors cover the most important endocrine endpoints as they are involved in numerous processes such as reproduction, cellular differentiation, regulation of glucose and lipid metabolism, inflammation, immunity, etc., which explains why a hormonal disturbance could lead to a multitude of biological effects (Osimitz et al., 2012; Pereira-Fernandes et al., 2013; Zoeller et al., 2012).

This paper presents the assessment of the potential endocrine activity of 65 chemicals for which migrating from PC alternative baby bottles has previously been reported (Onghena et al., 2014, 2015; Simoneau et al., 2012) and for which genotoxicity has been recently investigated (Mertens et al, 2016). Two exposure scenarios were tested for each compound: cell exposure to a single compound for the screening of agonistic activity, and cell co-exposure to a mixture including the reference ligand and the tested compound, for the screening of antagonistic activity. Given the large number of substances to be tested in this study, it was decided to perform a screening of the endocrine activity for only 4 concentrations, starting from 1 mM, which is, according to the Organisation for Economic Co-operation and Development (OECD) guidelines (Organisation for Economic Co-operation

and Development, 2012), the highest concentration to be tested in an in vitro assay for endocrine activity. The results were processed using multivariate data analysis to investigate possible relationships among the different receptors and identify clusters among the different tested compounds. The aim was to identify compounds or group of compounds that require further investigation because of their activities as nuclear receptor agonists and/or antagonists, as well as to identify which nuclear receptors are the main targets of compounds showing an activity.

Materials and Methods

Reagent and media

Dimethylsulfoxide (DMSO) was provided by Acros organics (France). 17-β-oestradiol, 5-α-dihydrotestosterone, dexamethasone, progesterone and other refrence standards were purchased from Sigma Aldrich (USA). 2,3,7,8-TCDD was obtained from Wellington (Campro Scientific, The Netherlands). Table 1 shows the supplier of each of the 65 tested chemical.

Cell lines

The eight *in vitro* assays used in this study were based on genetically modified cell lines, allowing the assessment of the biological activity of compounds on the human oestrogen receptor (ER), human androgen receptor (AR), human progesterone receptor (PR), human glucocorticoid receptor (GR), human peroxisome proliferator-activated receptor gamma (PPARγ), human thyroid receptor beta (TRß) and the mouse aryl hydrocarbon receptor (AhR). For AhR, the H1L7.5c1 cell line was derived from the mouse hepatoma hepa1c1c7 wild-type (Van Langenhove et al. (2011)). For ER, two different cell lines were used to assess the effects on the human oestrogen receptor. The BG1Luc4E2 derived from the

human ovarian carcinoma BG-1 cell line was described in Vandermarken et al. (2015), and was named “ER1” in this paper, and the second one was the MVV-Luc derived from the human mammary gland carcinoma MCF-7 cell line, described in Willemsen et al (2004) and was named “ER2” in this paper.

For AR, PR and GR respectively, the following cell lines were used, all derived from the human mammary gland adenoma T47-D cell line : TARM-Luc, TM-Luc and TGRM-Luc. They are described in Willemsen et al. (2004). Finally, for TRß and PPARγ, the cell lines were purchased from Biodetection Systems (Amsterdam, The Netherlands). For theses bioassays, U-2 OS cells (human osteoblast) were stably transfected with human TRβ or human PPARγ2 and a luciferase reporter construct under the control of a receptor specific response element. For the readability of the results, the 8 cell lines were named according to the receptor expressed, i.e. ER1, ER2, AR, PR, GR, TRß, PPARγ, and AhR.

Preparation of solutions of tested chemicals

Chemical solutions were prepared in DMSO (Acros spectroscopy grade) at a concentration of 100 mM or the solubility threshold, and then, diluted with DMSO to the final concentrations of 10 mM, 1 mM and 100 µM. The solutions were stored at -20°C in 1.5 mL brown amber vials (Chromacol). Two exposure scenarios were tested for each tested chemical: cell exposure to a single chemical, to screen for an agonistic activity and cell co-exposure to a mixture containing the reference ligand and the tested chemical, to screen for an antagonistic activity. For cell exposure to a single chemical, the tested chemical dissolved in DMSO was diluted in culture medium, such that the final concentration was 1 µM, 10 µM, 100 µM and 1 mM in culture medium containing 1% of DMSO. For the screening of the antagonistic activity, the tested chemical dissolved in DMSO was diluted as earlier described but the culture medium was beforehand supplemented with the appropriate agonistic

reference ligand at a concentration inducing 50 % of the maximal response (EC50). For both scenarios, a positive control and a calibration curve were always included to check the quality of the response of the cells. Reference agonistic ligands are those indicated in Table 2.

Reporter gene assays

The different protocols for each cell line were described in detail in literature (AhR in Van Langenhove et al., 2011; ER1 in Vandermarken et al., 2015; ER2, AR, PR and GR in Willemsen et al., 2004) or by the supplier (TRß, PPARγ from Biodetection Systems). Briefly, 24h after seeding the cells in 96 well-culture plates, culture medium was discarded and replaced by fresh medium containing the appropriate reference ligands and/or tested compounds. Depending on the cell line, cells were lysed in Triton-lysis buffer after 24 or 48 hours of exposure to the tested chemical. Next, a glow-mix solution containing the substrates of luciferase, i.e. ATP and luciferin, was added and luciferase activity was measured using a luminometer. Before cell lysis, the cells were visually inspected under the microscope to reveal morphologic anomalies indicating potential cytotoxicity. For the TRβ and PPARγ bioassays, instead, cells were incubated with 10% of 400 µM resazurin (Sigma-Aldrich, Germany) in PBS for 2h followed by measuring fluorescence, in order to simultaneously assess the cytotoxicity (O Brien et al., 2000).

Data analysis

The emitted luminescence was measured in relative light units (RLU). To normalise the values, the mean RLU-value of the highest appropriate reference ligand concentration was arbitrary set to 100% in order to express the response induced by each concentration of the

reference ligand or the tested chemical as a percentage of the maximal response (relative response).

A four parameter logistic function (Eq.(1)) was fitted to the data points (relative response as a function of the concentration) using a weighted least squares (WLS) regression (Elskens et al., 2011).

Eq. (1): $RLU = a + \frac{d \cdot x^b}{c^b + x^b}$

where a and d represent the lower and upper asymptotes respectively, c is the half maximal effective concentration and b is the slope parameter, sometimes referred as the Hill coefficient.

In order to have a first indication on the strength of the agonistic (AGO) or antagonistic (ANT) activity of the tested chemicals towards each of the 8 receptors tested, a score of "0" was assigned if no activity was detected or if cytotoxicity was observed (to a better readability, the zero is indicated by an empty space in Table 3). In Table 3, two categories were distinguished based on the level of response. For agonistic activities, the activity of compounds able to induce an increase of the relative response between 10 % and 50% were indicated as "+", while the activity of the tested compounds displaying a relative response higher than 50 %, or able to induce a full dose response curve, were indicated as "++". For antagonistic activities, the sign "+" was indicated for tested compounds showing a response decreasing for two consecutive points, with an amplitude of more than 10 %, relative to the maximal expected response for the agonistic reference ligand, while "++" was assigned to tested compounds showing a complete inhibition, and no cytotoxicity at any tested concentration. For statistical calculations, a score of "1" was considered if an activity was recorded (+ or ++). In order to avoid an erroneous interpretation of the results, when an

antagonist response is detected in parallel with a cytotoxic effect, the antagonistic effect is not accounted. In this case, a score of 0 is given.

The characteristics of each cell line were determined by performing a statistical analysis using data from repeated calibration curves (n=10) performed with the agonistic reference ligand of each receptor. Average parameter values of the logistic function were calculated, as well as the limit of detection (LoD), defined as the reference ligand concentration which gives a response corresponding to the lower asymptote value plus 3 times the standard deviation.

Statistical analysis

A statistical analysis was performed using results from the *in vitro* assays translated into a binary variable as explained above.

A principal component analysis (PCA) was performed in order to investigate possible relationships between the different types of receptors as well as correlations between receptor variables and between AGO and ANT assays results. PCA was achieved using the software Unscrambler X, version 10.3 (64 bit) of the CAMO Software AS company (Norway). The extraction procedure was based on a normalized PCA using the correlation matrix with Varimax rotation and a Kaiser normalization (Brown, 2009). The statistical tests were performed at a significance level of 5 % (p=0.05).

Results

Characterization of the reporter gene assays

Table 2 shows, for agonistic reference ligands, the characteristics of the eight cell lines used in this study, such as the relative responses corresponding to the lower and the upper asymptote, the EC50, the hill coefficient and limit of detection (LoD). When comparing both

ER cell lines, the data illustrate that ER1 is more sensitive than the ER2 cell line with a lower background, EC50 and LoD.

Screening of the tested chemicals

A qualitative assessment of agonistic and antagonistic activities of 65 compounds identified as migrating substances from alternatives to PC baby bottles (Onghena et al., 2014, 2015; Simoneau et al., 2012) was performed using a panel of 8 reporter gene assays, involving 7 different nuclear receptors. In Table 3, compounds have been grouped according to chemical classes arbitrary established, based on functional groups found in their chemical structure.

Only twelve compounds among the 65 tested did not show any agonistic or antagonistic activity. These compounds are C7 (3,5-di-tert-butylbenzoquinone), C9 (2-methylnaphatlene), C38 (naphthalene), C20 (4-methylthiobenzaldehyde), C4 (palmitic acid), C41 (ethyl stearate), C8 (2-butoxyethyl acetate), C18 (2-ethylhexyl acetate), C33 (eucalyptol), C64 (p-propylanisole), C5 (2,4-di-tert-butylphenol), and C23 (butylated hydroxytoluene or BHT).

Figure 1 shows, for each kind of assay, an example of compound showing a positive response. ~~In these graphs, only results obtained for non cytotoxic concentrations are shown.~~

Agonistic activities of the tested chemicals

Table 3 shows that none of the tested chemicals showed any agonistic activity on AR, GR or PR. A large number of compounds (35 out of 65) induced an agonistic effect on one to four receptors. Ten compounds displayed agonistic effects on the PPARγ, five on AhR and only four on TRβ, with no link with the chemical class. Most agonistic activities were recorded for ER, with 29 compounds showing agonistic activity for ER1 among which only

15 were also agonists for ER2. No compound showed an agonistic activity on ER2 and not on ER1. Based on the number of compounds with an agonistic activity on the ER, four chemical classes were of particular interest: phthalates, benzophenones, aromatic hydrocarbons and phenols. The three tested phthalates, diisobutyl phthalate (C50), dibutyl phthalate (C51) and diethylhexyl phtalate (C52), induced a relative response of more than 50 % in the ER1 assay. Two of them, i.e. diisobutyl and diethylhexylphtalate also induced an agonistic activity in the ER2 assay, but weaker than in the ER1 one. Six out of the eight aromatic hydrocarbons tested showed a positive response on ER1. Two of them, lemonene (C66) and 2,6-dimethylnaphthalene (C67), induced a stronger ER agonistic activity than the others. Only three out of the six aromatic hydrocarbons showing an activity on ER1, i.e. 2,6-diisopropylnaphthalene (C6), dimethylnaphthalene (C67) and 2,2',5,5'-tetramethylbiphenyl (C75), exhibited an agonistic activity on ER2 as well. Three of the six tested phenols, i.e. bisphenol-A (C2), bisphenol S (C49) and 4-tert-octylphenol (C73), induced a relative response higher than 50 % on both ER1 and ER2, while 4-n-nonylphenol (C88) induced a weaker activity on ER2 than on ER1. Finally, benzophenone (C22) induced a high response on both ER1 and ER2, while 4-phenylbenzophenone (C85) showed a higher response on ER1 than on ER2.

Antagonistic activities of the tested chemicals

As indicated in the material and method section, only antagonist activities associated with no cytotoxicity were considered. A large number of compounds (35 out of 65) showed an inhibitory effect on one or several receptors (Table 3). Three chemical classes appeared of particular interest: ketones, benzaldehydes related compounds, and fatty acid and fatty acid esters. All 8 benzaldehydes related compounds tested, except the 4-(methylthio)benzaldehyde (C20), induced an inhibitory effect on 3 to 6 receptors, depending on the compound. Although an antagonistic activity on PPARγ was observed for the seven tested benzaldehydes, the other

receptors for which an antagonistic activity was recorded varied for the different benzaldehydes. In addition to a strong antagonist activity on three steroid receptors (AR, GR and PR), 3,5-di-t-butyl-4-hydroxybenzaldehyde (C77) induced also a weaker antagonistic activity on three receptors: ER1, TRβ and PPARγ. Among the receptors, only the AhR seemed not to be inhibited by these compounds, except by 4-methylbenzaldehyde (C56). Four benzaldehydes seemed to be able to completely inhibit the response induced by the reference agonistic ligand of at least one receptor, i.e. 2,4,6-trimethylbenzaldehyde (C3) and 4-propylbenzaldehyde (C62), which appeared as full antagonist for ER2, GR and PR; 3,5-di-t-butyl-4-hydroxybenzaldehyde (C77) for AR, GR and PR, and finally, 3,4-dimethylbenzaldyhde (C90) for GR. Among the 12 ketones tested, 10 induced an antagonistic activity on one to 5 receptors, depending on the compound. Four fatty acid esters induced an antagonistic activity on one or several receptors as well, while from the two fatty acids tested, only stearic acid (C40) displayed a weak antagonist activity towards the AR.

Figure 2 illustrates the number of compounds displaying agonistic and antagonistic activities per receptor. The ranking of the receptors based on the number of compounds displaying an activity is as follows (number of reacting compounds are indicated between brackets):

- agonistic activities : ER1 (29) > ER2 (15) > PPARγ (10) > AhR (5) > TRβ (4) > AR – PR – GR (0),
- antagonistic activities: PPARγ (20) > TRβ (15) > PR (14) > ER2 (12) > GR (11) > AR (8) > ER1 (6) > AhR (3).

Correlation between agonistic and antagonistic activities of migrating compounds

The results of the screening experiments were further analysed with PCA. In order to remove scale effects between variables, a correlation matrix was used. It is based on

tetrachoric correlations that are more specific for binary data, as used in the present study (Kolenikov and Angeles, 2004). For this analysis, there were initially 16 variables (agonistic and antagonistic activities tested on 8 different receptors), but since no compound showed an agonistic activity on AR, PR and GR, thirteen variables remained for PCA, i.e. TR-AGO, PPAR-AGO, AhR-AGO, ER1-AGO, ER2-AGO, TR-ANT, PPAR-ANT, AhR-ANT, ER1-ANT, ER2-ANT, AR-ANT, PR-ANT, GR-ANT.

Following Kaiser's rule (Jolliffe, 1986), four principal components were selected for interpreting the data. These new components explain about 89 % of the total variance of the system. The variable plot (Figure 3) shows the factorial plan corresponding to the first two axes. These components are usually responsible for the bulk of the variance. The first component (PC1) axis explains 40.8% of the variation and the second component (PC2) axis 24.4%. The coordinates of the original variables in the plane express their correlations with the new principal components. Variables carrying similar information or varying in a comparable way are grouped together, i.e. they are correlated. When negatively correlated, they are positioned on opposite sides of the plot origin, in diagonally opposed quadrants. The further away a variable lies from the origin, the closer it is near the correlation circle, the stronger the influence of that variable has on the PCA model. There is a clear segregation of variables along PC1 axis with PC1 lower than zero for AGO screening tests and PC1 higher than zero for ANT screening tests. This suggests that compounds identified as agonists are generally not antagonists of the same receptor.

The tetrachoric correlation matrix indicates that there are positive correlations (r_{tet} = 0.74 - 0.92) for the ANT screening tests between receptors ER1, AR, GR and PR (and particularly for ER1 and PR, which share nearly the same arrow in Figure 3) and between receptors PPARγ and TRβ (r_{tet} = 0.74). Strong positive correlations are also found between TR-AGO and AhR-ANT (r_{tet} = 0.86) and between ER1-AGO and ER2-AGO (r_{tet} = 0.98)

(Figure 3). These correlations are significant at $p < 0.01$ as indicated by the Pearson Chi^2 and reflect the fact that there is some overlap between the responses recorded in the different transactivational assays, i.e. several nuclear receptors are activated (or inactivated) by the same chemicals (see discussion).

To better visualize clusters between agonists and antagonists, a K-median clustering method based on Euclidean distance was applied. Results are summarized in Figures 4 and 5, respectively.

Clustering of compounds displaying an agonistic activity

The 35 compounds which showed an agonistic activity in any of the eight assays were clustered into four levels (Figure 4). In each level, circles display the code of the compounds showing an activity on the receptor mentioned, as well as on receptors mentioned in the circles of the n-1 level, which are linked by arrows. The compounds mentioned in level 1 displayed an agonistic activity on only one receptor, while, for example, at the 4^{th} level, compound n°6 (DIPN) displayed an agonistic activity on 4 receptors, i.e. TRβ, PPARγ, ER2 and ER1. Each compound is only mentioned once in Figure 4, and the level where it is mentioned corresponds to the number of receptors towards which it displays an agonistic activity. Seventeen compounds appeared to be selective agonists, i.e. they interacted specifically with a single receptor. Among these, eleven compounds were selective agonists of ER1 (and eighteen were able to activate both ER1 and ER2), three showed an agonistic activity with PPARγ only, two with AhR only and one with the TRβ only. None of the compounds was selective agonist for ER2. If we group the 18 compounds showing an activity on both ER1 and ER2, it remains eleven non-selective agonists interacting with two to four receptors.

In Figure 4, compounds displaying both agonistic and antagonistic activities are indicated in bold. From the 35 compounds showing an agonistic activity, 19 displayed also antagonistic activities, but generally on different receptors.

Clustering of compounds displaying an antagonistic activity

Figure 5 shows the clustering of the 35 compounds showing an antagonist activity, and for which no signs of cytotoxicity were observed on the tested cell line. They are clustered into seven levels. Thirteen compounds (see level 1 in Figure 5) appeared as selective antagonists, i.e. they showed an antagonistic activity for a single receptor. Among these, four compounds were selective antagonist of TRβ, four other compounds of PR, two of PPARγ, one of ER2, one of AhR and another one of AR. Twenty two compounds were found to be non-selective antagonists interacting with minimum two and maximum seven receptors (dispatched in levels 2 to 7 in Figure 5). Most of them showed antagonistic activities on PPARγ (20 compounds), TRβ (15 compounds) and PR (14 compounds). As in Figure 4, compounds displaying both agonistic and antagonistic activities are indicated in bold in Figure 5.

Discussion

In this study, we have tested 65 chemicals, previously identified as migrating substances from alternatives to PC baby bottles (Simoneau et al., 2012; Onghena et al., 2014, 2015), using a panel of 8 reporter gene assays, involving 7 different nuclear receptors. To our knowledge, the number of reports of this kind is scarce, i.e. on the screening of the endocrine activity of a large number of structurally diverse chemicals on eight different receptor transactivational assays. The study of Hamers and co-workers (2006) is another example of

study using a panel of multiple bioassays for testing brominated flame retardants. Other groups have published results for a large number of chemicals, these screening studies towards the endocrine activities of chemicals were always limited to one or two different receptors (Blair et al., 2000; Kojima et al., 2004; Roy et al., 2004; Takeuchi et al., 2006).

From the 65 tested chemicals, 12 substances were found to display no activity towards any of the receptors. As the receptors included in this study cover most of the endocrine endpoints, it can be concluded that these substances are unlikely to act through a mechanism of action involving nuclear receptor. As endocrine disruptors can act through other mechanisms of action than direct interaction with nuclear receptors (such as metabolic activation or effects on synthesis or metabolism of hormones) it cannot be firmly concluded than those substances showing no activity on nuclear receptors in this study are not potential EAS.

Fifty three of the 65 tested chemicals showed an activity on one or several nuclear receptors. Thirty five compounds showed an agonistic activity on at least one receptor and the same number of compounds showed an antagonistic activity, 16 compounds displaying both activities on at least one receptor, but not on the same receptors, except in 2 cases for ER and 3 cases for PPARγ.

No agonistic activity was observed towards the androgen, glucocorticoid and progesterone receptors (AR, GR and PR), indicating that the tested compounds are unlikely to induce endocrine effects by mimicking androgen, glucocorticoid or progesterone hormones.

Most agonistic activities were observed for the estrogenic receptor, as 29 of the 35 compounds displaying an agonistic activity were able to activate the oestrogen receptor. In the current study, the oestrogenic (as well as the anti-estrogenic) activity of the tested chemicals was evaluated in two different cell lines. Results in both cell lines differed as remarkably

more substances (29 versus 15) displayed agonistic activity towards ER1 than towards ER2. For the antagonistic effects, it was the opposite, as much more compounds were shown to be active in the ER2 assay than in the ER1 (12 versus 6). As shown in Table 2, the BG1Luc4E2 cell line (ER1) is more sensitive than the MVV-Luc one (ER2) with a lower background and lower EC50, consequently, weak estrogenic compounds could be detected in the ER1 cell line, and not in the ER2 one, explaining the differences reported here in ER agonistic activities. Also, xenoestrogens can bind to different receptors subtypes, either the human estrogen receptor alpha (ERα) or beta (ERβ). Both hERα and hERβ are arranged according to the modular domain structure of the steroid/thyroid hormone superfamily of nuclear receptors (Henley et al., 2005). Although the endogenous oestrogen, 17β-oestradiol (E2) shows similar affinity for both ER subtypes, with a Kd for ERα and ERβ of 0.70 nM and 0.75 nM respectively (Zhu et al, 2006), some ligands show a higher affinity for hERα, i.e. genistein or display an agonistic activity on hERα and an antagonistic activity on hERβ, i.e. chlordecone (Le Maire et al., 2010). The ratio between α and β receptors in both cell lines is still unknown, but even if it was, the cellular context needs also to be taken into account, because the expression of certain coregulators may affect nuclear receptor transcriptional responses. According to Sotoca and co-workers, the cell response varies according to the receptor ratio, the cellular context and the species and tissue of origin of the cells (Sotoca et al, 2012), which possibly explain why, in this study, more compounds show an activity in the BG1Luc4E2 cell line assay (ER1) than in the MVV-Luc one (ER2).

Beside the oestrogen receptor, three other nuclear receptors were activated by several chemicals in this study: the PPARγ (10 agonistic compounds), the AhR (5 agonistic compounds) and the TRβ (4 agonistic compounds).

If we look at the variety of chemicals analysed in this study, four classes were of particular interest in the agonistic assays. These were phthalates, benzophenones, aromatic hydrocarbons and phenols. Phthalates are used as plasticizers in the manufacture of polymers in order to increase the plasticity or fluidity of the material. If not bound to the polymer chain, plasticizers may migrate from the plastic material into the food, causing contamination (Gimeno, et al., 2014; Bi et al., 2013). Risk assessments on phthalates have been done by different groups of experts (e.g. EFSA, 2005). Based on several studies with experimental animals and human exposure data, concerns were expressed on reproductive and developmental toxicity and the possible endocrine disrupting potency of phthalates (Heudorf, et al., 2007). Previous *in vitro* studies have suggested that the three phthalates tested in this study demonstrated a lower efficiency and potency than E2 (Legler et al., 2002; Nishihara et al., 2000;) and are poor estrogenic binders (Blair et al., 2000). This seems to be confirmed in our study. Beside oestrogenic activities, dibutylphthatalate and diisobutylphthalate showed an agonistic activity on PPARγ, which is also in line with literature (Pereira-Fernandes et al., 2013). According to the *in vitro* study of Pereire-Fernandes and co-workers, these two phthalates exhibited a weak obesogenic activity which would suggest that these compounds could be an environmental factor in the obesity development (Pereira-Fernandes et al., 2013). In addition, antagonistic activities were observed towards the ER, PPARγ and TRβ. The phthalates did not show an activity towards the other nuclear receptors. Interestingly, diethylhexylphtalate (C52) was the only compound from the list of the 65 tested chemicals showing both a "high" agonist activity (score of "++") and a "high" antagonist activity (score of "++") on the same receptor, i.e. the ER from the cell line derived form human ovarian cells (ER1 cell line), while in the ER2 cell line (derived from human mammary gland tissue), it shows a low agonist activity (score of "+") and a high antagonist activity (score of "++"),

demonstrating again the importance of the cell context on the agonist or antagonist activity of the test compounds towards nuclear receptors.

Another large group of dietary toxicants are the aromatic hydrocarbons. Many of them are suspected to be carcinogenic and have been shown to exhibit endocrine and developmental toxicity (Kummer et al., 2008) and cause alteration in sperm quality, affecting the male reproductive function (Kisin et al., 2015). These aromatic hydrocarbons were a major group of chemicals tested in this study. They are used as flavour and fragrances agents, antioxidant, etc. An important fraction of aromatic hydrocarbons group is made up of naphthalene and its derivatives. Some studies have reported adverse health effects in human and animals, such as haemolytic anaemia in infants born from mothers exposed to naphthalene during pregnancy, a decrease of body weight and lethargy of the female rabbit, an increase of female mice mortality, or a decrease of body weight and fetotoxicity when mice are exposed to this compound via gavage (U.S. Environmental Protection agency, 1998). In our study, naphthalene (C38) and its derivatives did not show any agonistic activity, but the 2,6-diisopropylnaphthalene (DIPN, C6) and the 2,6-dimethyl naphthalene (C67) induced an estrogenic response. This type of response for the DIPN was previously shown by another research group, using a yeast model (Vinggaard et al., 2000). DIPN also displayed an agonistic activity on the PPARγ and TRβ receptor. To our knowledge, activities on PPARγ and TRβ of DIPN have not previously been reported.

It has been demonstrated, in numerous studies, that phenolic compounds induce a variety of toxic and physiological effects. In the present study, BPA, BPS, n-4-nonylphenol, p-tert octylphenol and 2,4-di-tert-butyl phenol were evaluated for their endocrine activity. These compounds have similar chemical features to BPA and induce an estrogenic effect except for 2,4-di-tert-butylphenol. These four chemicals are known EAS and may cause adverse health effects in humans and wildlife (Kuruto-Niwa et al., 2005). Based on animal

studies, a panel of experts from EFSA considered that BPA causes adverse effects on mammary glands, liver and kidney. The possible effects on nervous, reproductive, immune, metabolic and cardiovascular system are not considered to be adverse by the panel at present, but they can not be totally excluded (EFSA, 2015). As BPS displays similar metabolism and mechanism of action *in vitro* than BPA, it could also have similar adverse effects *in vivo*. The two other estrogenic compounds, nonylphenol and octylphenol, cause adverse reproductive and developmental effects in animal models (Avdogan and Barlas, 2006; Bian et al., 2006; Chitra et al., 2002). Benzophenones, a group of chemicals also largely studied, are used as UV absorbers and like phthalates, they are not chemically bound to the polymeric structure and consequently, they can easily migrate into the food or beverage. In *in vivo* studies, administration of benzophenone induced a slight increase in uterine weight in the uterotrophic assay, suggesting that benzophenone has a estrogenic activity (Chemicals Evaluation and Research Institute, 2001). However, the scientific panel of EFSA, which performed a risk assessment on benzophenone, considered these effects as limited (EFSA, 2009). For 4-phenylbenzophenone, also an agonistic activity on the TRβ receptor was observed. Such an activity has not been previously observed, and indicates a possible disrupting effect of the 4-phenylbenzophenone on the functions regulated by thyroid hormones.

Interestingly, fatty acid and fatty acid esters show also agonistic activities mainly on ER. This is in line with results from literature, as according to Kato and co-workers (1989), arachidonic acid, palmitic acid, stearic acid, oleic acid, and docosahexaenoic acid are able to bind to the estrogen, progesterone, androgen, and glucocorticoid receptors on binding sites different from those of the endogenous steroids. On the other hand, linoleic acid was shown to be able of binding to the estrogen receptor and to stimulate estrogen inducible genes (Liu et al, 2004) while Suzuki and co-workers (2008) suggested that saturated fatty acids can exhibit estrogenic activity.

For antagonism, activities were recorded on all the nuclear receptors included in the study, but most activities were recorded towards the PPARγ : from the 35 compounds showing an antagonistic activities, 20 were able to antagonize the PPARγ. Three chemical classes were also of particular interest: benzaldehydes, ketones and esters of fatty acid. Benzaldehydes are endogenous compounds of numerous plants and of various species of insects for chemical defence and as pheromones. They are used principally as flavour and fragrances agents. They play a growing role in the marketing of food and beverage packaging. They mask unpleasant odours, e.g. in PVC, where these compounds are used as fragrances to cover the smell of sulphur. Results show that all benzaldehydes examined in this study induced an inhibitory effect on more than one receptor, except for 4-(methylthio)benzaldehyde (C20), which displayed no activity. Ketones and esters of fatty acid, such as benzaldehydes, are used principally as flavour and fragrances agents or can be present as degradation products of additives used in polymer manufacture (e.g. camphor). A lack of information still exists regarding the endocrine disrupting potential associated with these chemical classes, but the screening performed in this study seems to indicate possible endocrine activity through inhibition of nuclear receptors activity.

PCA showed that there was an overlap between activities of chemicals towards the various receptors, indicating that several substances exhibited both agonistic and antagonistic activities, but not on the same receptors (except in 2 cases for ER and 3 cases for PPARγ).

This analysis also showed a positive correlation between receptors, e.g. between PPARγ and TRβ. Indeed, 11 compounds which were antagonists of PPARγ were also antagonists of TRβ, and seven of them were benzaldehydes, ketones or esters of fatty acid. These two receptors are involved in metabolism and adipogenesis process as well as in diseases such as obesity, diabetes and cancers. They share a conserved DNA-binding domain

and form both a heterodimer with retinoid X receptor (Lu and Cheng, 2010). Although it has been demonstrated that through various mechanisms both can affect diverse biological functions, nothing has yet been published on common ligands. Another strong correlation observed in this study was the one between TR-AGO and AhR-ANT. AhR is an intracellular ligand-dependent transcriptional factor involved in regulation of biological response to planar aromatic hydrocarbons. These receptors are expressed in all tissues (Tsay et al., 2013). In its simplest form, a signalling pathway may be defined as a signalling molecule interacting with an effector and a set of co-effectors, inducing the transcription of a battery of genes which share a specific response element, leading to various physiological effects. In reality, the signalling pathway is connected to other receptors via multiple cross-talks (Pascussi et al., 2008). The inhibition of the AhR-ERα crosstalk induced by AhR ligand was observed in MCF7 and T47D cells according to Safe and collaborators. Our results show that the antiestrogenic effect of 4-propylbenzaldehyde could be the consequence of an AhR-ER crosstalk.

Our *in vitro* study demonstrated that many chemicals migrating from alternatives to PC baby bottles are possible endocrine active substances. It is important to note that on one hand, the concentrations of chemicals used in the assays are higher than realistic blood or tissue concentrations under normal human exposure conditions. On the other hand, humans can be exposed to a mixture of different chemicals and endogenous hormones as well, that can result in additive and synergic effects.

Despite the potential usefulness of reporter gene assays to detect possible endocrine active substances, there are some limitations, and therefore, a lack of response in a cell based assay does not mean that the compound has no endocrine activity, as already mentioned

above. The major limitation is that this type of assays does not allow a direct extrapolation to an *in vivo* response, because *in vivo* pharmacokinetic and pharmacodynamics interactions, as well as biotransformation process (metabolisation), are lacking in these *in vitro* models (Zacharewski, 1998). Indeed, the *in vitro* activity of certain substances is not confirmed *in vivo*, or some activities are differently quantitated *in vitro* and *in vivo*, as it was observed for example for ethinylestradiol, which was shown 25 times more estrogenic (in terms of relative potency to E2) *in vivo* than *in vitro* (Henneberg et al., 2014). Also, certain xenobiotics exert their activity through mechanisms that are independent of nuclear receptors (Zacharewski, 1997). A well-known case is phthalates which display adverse effects on the male reproductive function, including hypospadias, cryptorchidism, reduced testosterone production and decrease sperm counts. These antiandrogenic effects are not the consequences of an antagonism towards the androgen receptor, but are due to a disruption of the androgen biosynthesis, as revealed by animal phthalate exposure studies (Lottrup et al, 2006). Xenobiotics can induce also species, tissues, and cell specific responses, as it was shown for tamoxifen which differently affects breast and ovarian tissues (Zacharewski, 1998).

The reporter gene assays used in the present study are however useful tools to identify modes of action and for identifying potential endocrine active substances and thus potential endocrine disruptors.

Conclusion

The screening for possible endocrine activity of compounds identified in literature as possible migrants from non polycarbonate plastic baby bottles was performed using seven human nuclear receptors (oestrogen, androgen, glucocorticoid, progesterone, thyroid beta and peroxisome proliferator-activated gamma receptors and the mouse aryl hydrocarbon). Most of the agonistic activities were observed towards the oestrogen receptor, and most of the

antagonistic activities towards the PPARγ, but responses were recorded towards all the seven nuclear receptors. The diversity of the targeted nuclear receptors shows the importance of using a panel of several in vitro assays when screening for endocrine activities.

From the 65 compounds tested, a "short list" of 12 compounds was shown to be of low concern, as they did not show any activity towards the nuclear receptors. The group of the 53 remaining compounds require further investigation in order to confirm their endocrine activity *in vivo.*

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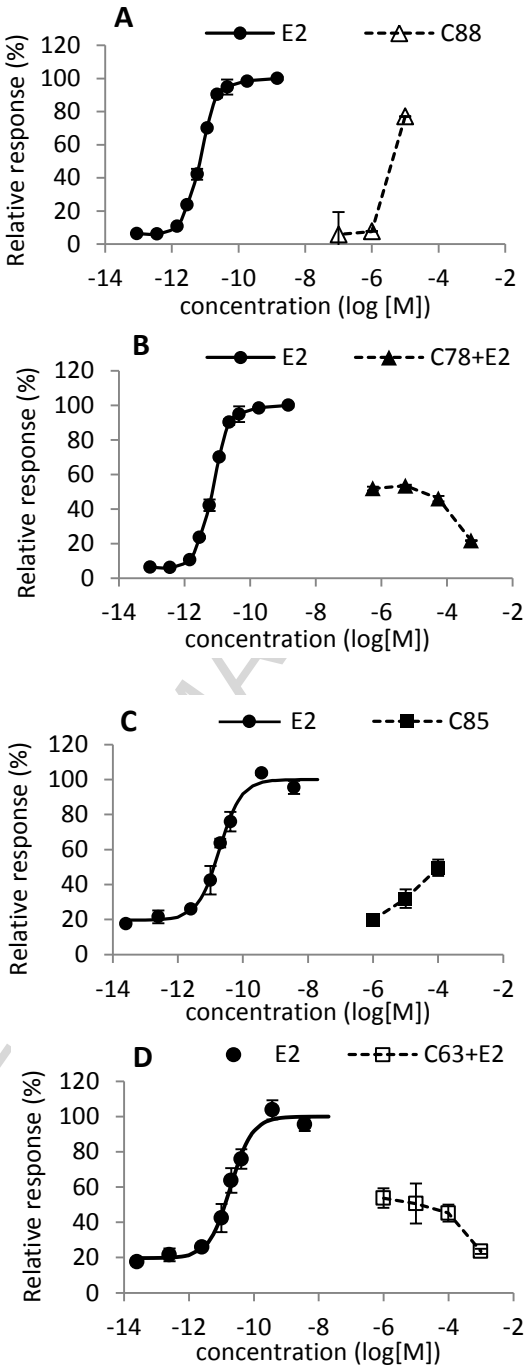

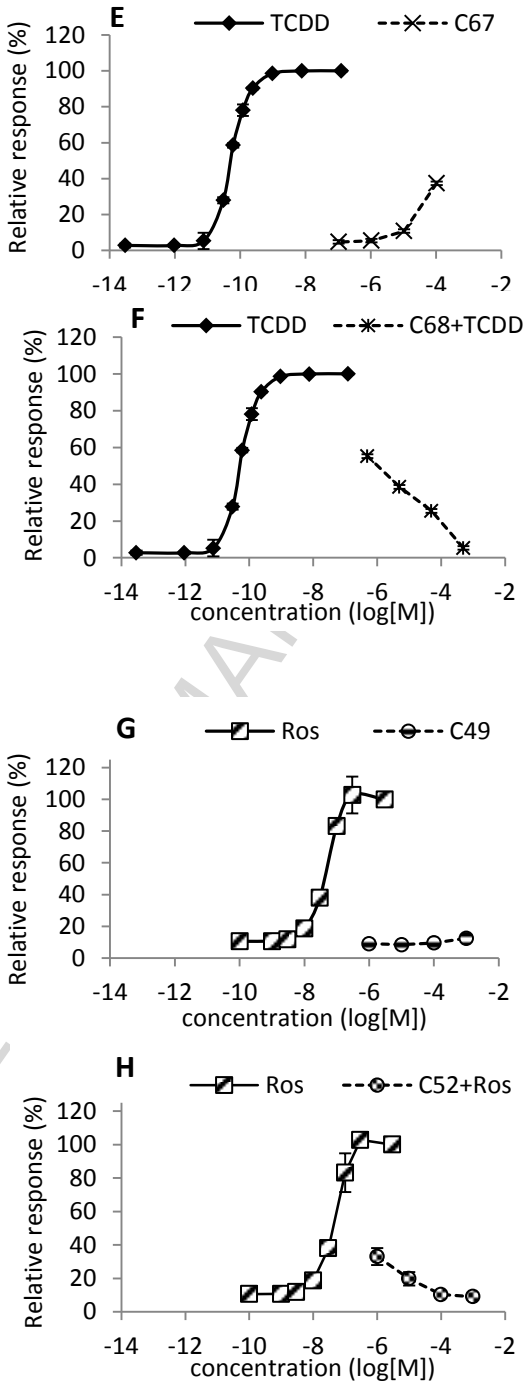

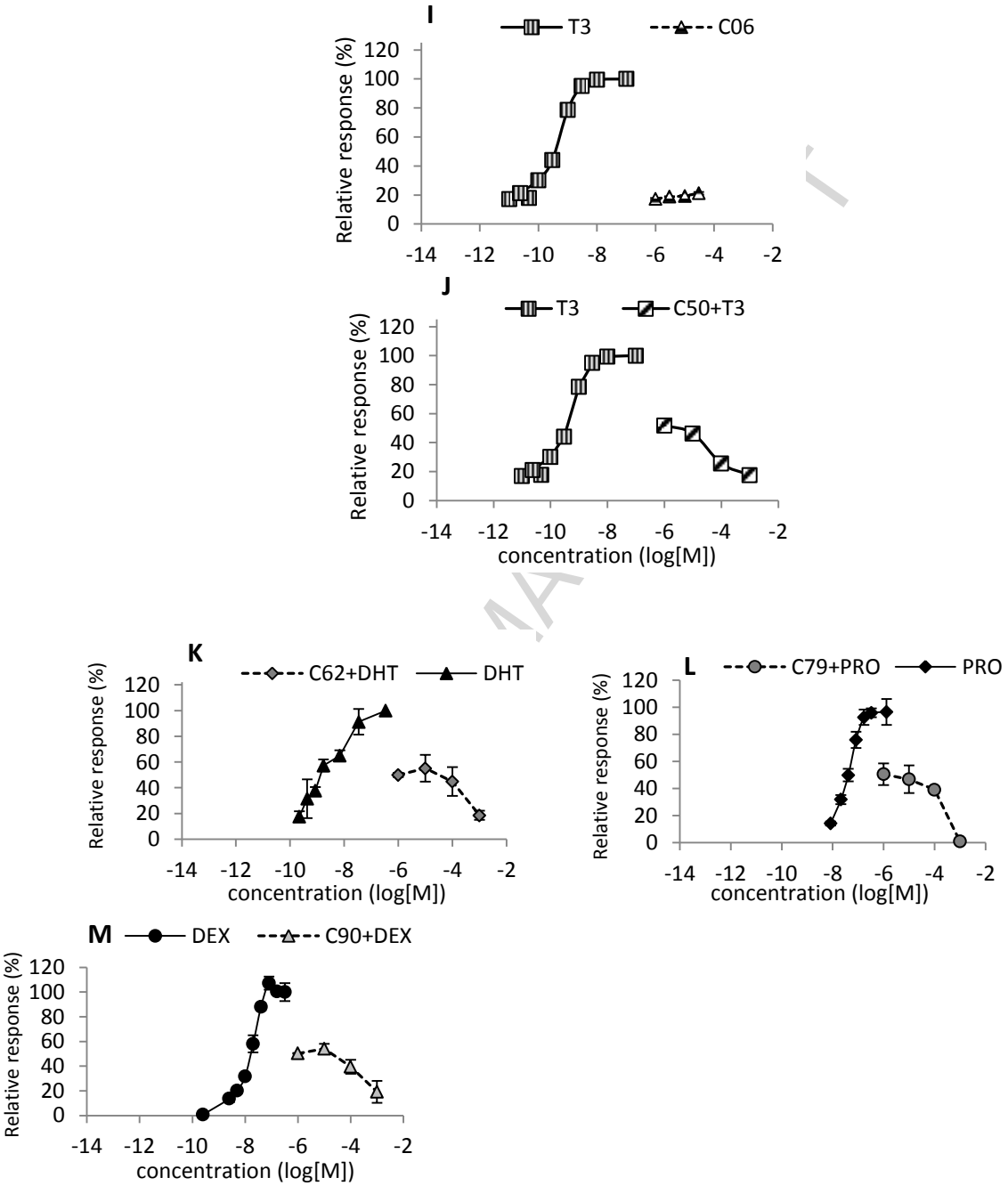

Figure 1 : Examples of endocrine activities recorded in the reporter gene assays used in this study. (**A**) Agonist activity of 4-nonylphenol (C88) in ER1 assay; (**B**) Antagonist activity of Azacyclotridecan-2-one (C78) in ER1 assay; (**C**) Agonist activity of 4-Phenylbenzophenone (C85) in ER2 assay; (**D**) Antagonist activity of 2-Undecanone (C63) in ER2 assay; (**E**) Agonist activity of 2,6-dimethyl naphthalene (C67) in AhR assay ; (**F**) Antagonist activity of 2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one (C68) in

AhR assay; (**G**) Agonist activity of bisphenol S (C49) in PPARγ assay; (**H**) Antagonist activity of diethylhexylphtalate (C52) in PPARγ assay; (**I**) Agonist activity of diisopropylnaphthalene (C6) in TRβ assay; (**J**) Antagonist activity of diisobutylphthalate (C50) in TRβ assay; (**K**) Antagonist activity of 4-propylbenzaldehyde (C62) in AR assay; (**L**) Antagonist activity of Tetradecanoic acid, ethyl ester (C79) in PR assay; (**M**) Antagonist activity of 3,4-dimethylbenzaldehyde (C90) in GR assay. For each compound, four concentrations were tested. For agonistic activities, only concentrations of test compounds showing no cytotoxicity are shown. For antagonistic activities, only compounds showing no cytotoxicity at any concentration were taken into account. E2: 17-β-estradiol; TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxine; Ros: Rosiglitazone; T3: triiodothyronine; DHT: 5-α-dihydrotestosterone; PRO: progesterone; DEX: dexamethasone. Relative response: percentage of the maximum luciferase activity obtained with the agonist reference standard. Results are expressed as mean ± SD (n = 3 independent experiments).

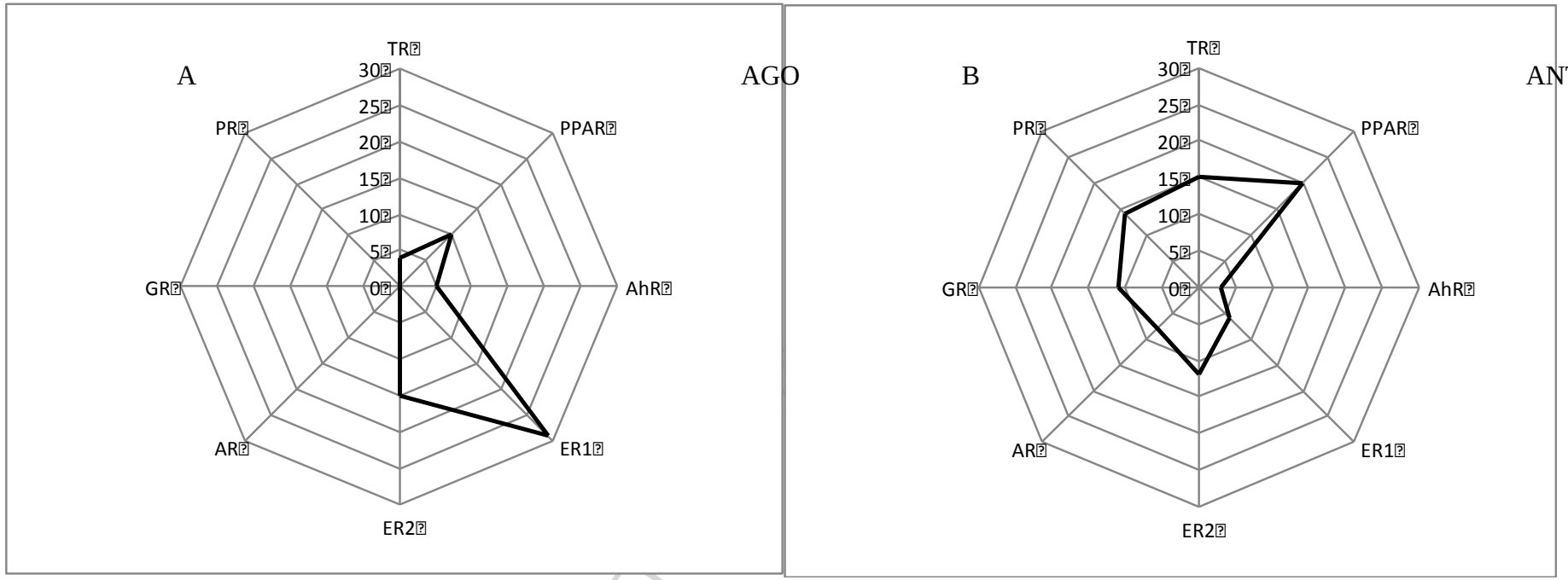

Figure 2: Number of compounds displaying an agonistic (A, AGO) or an antagonistic (B, ANT) activity, per receptor, according to data of Table 4. ER (1 or 2) : human oestrogen receptor; AR : human androgen receptor; PR : human progesterone receptor; GR : human glucocorticoid receptor; PPARϒ : human peroxisome proliferator-activated gamma receptor; TRβ : human thyroid ß receptor; AhR : mouse aryl hydrocarbon receptor.

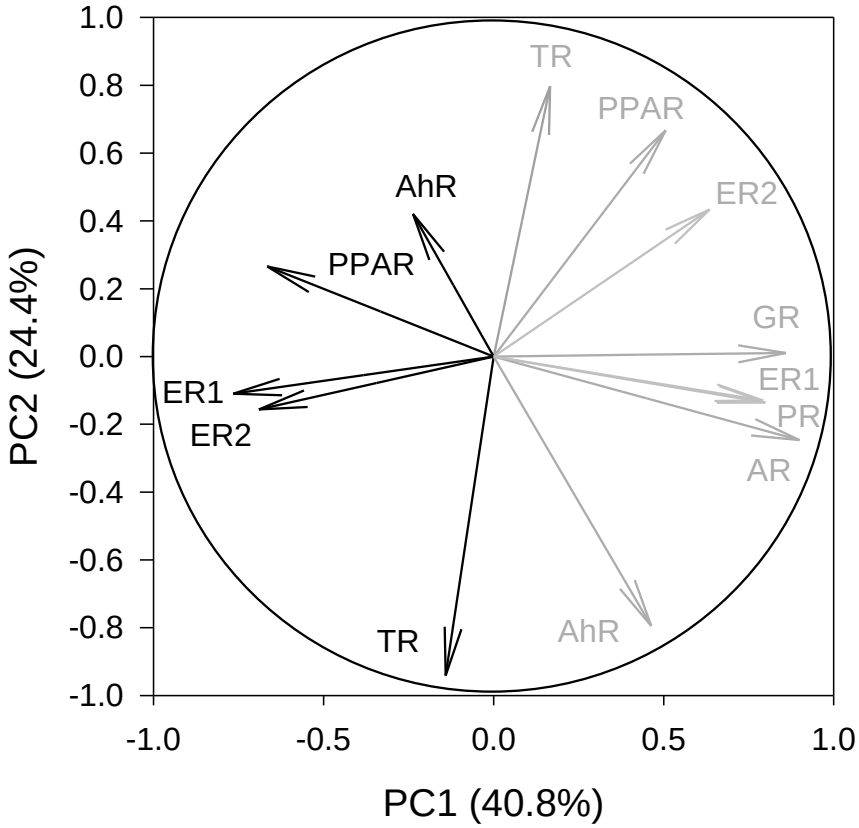

Figure 3: Principal component analysis (PCA) performed on 13 variables: projection of the variables on the factorial plan (PC1 (40.8%) x PC2 (24,4%)) (see text for details). Black arrows : variables corresponding to agonistic activities , grey arrows : variables corresponding to antagonistic activities. ER (1 or 2) : human oestrogen receptor; AR : human androgen receptor; PR : human progesterone receptor; GR : human glucocorticoid receptor; PPAR : human peroxisome proliferator-activated gamma receptor; TR : human thyroid ß receptor; AhR : mouse aryl hydrocarbon receptor.

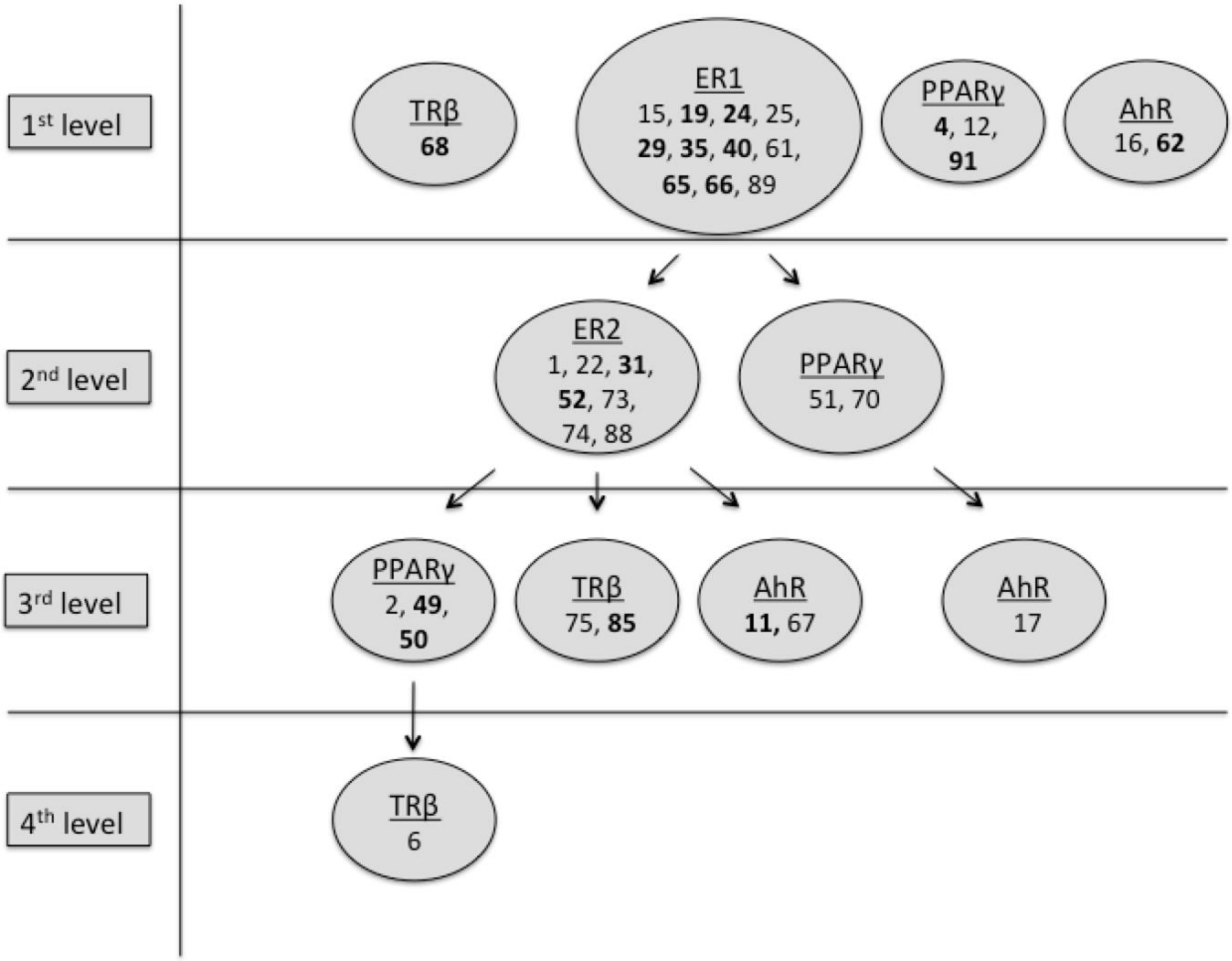

Figure 4: Clustering of compounds displaying an agonistic activity on one or several nuclear receptors. The level represents the number of receptors a given compound interacts with. ER (1 or 2) : human oestrogen receptor; AR : human androgen receptor; PR : human progesterone receptor; GR : human glucocorticoid receptor; PPARϒ : human peroxisome proliferator-activated gamma receptor; TRβ : human thyroid ß receptor; AhR : mouse aryl hydrocarbon receptor; n, substance code. The compounds showing both agonistic and antagonistic activities are indicated in bold.

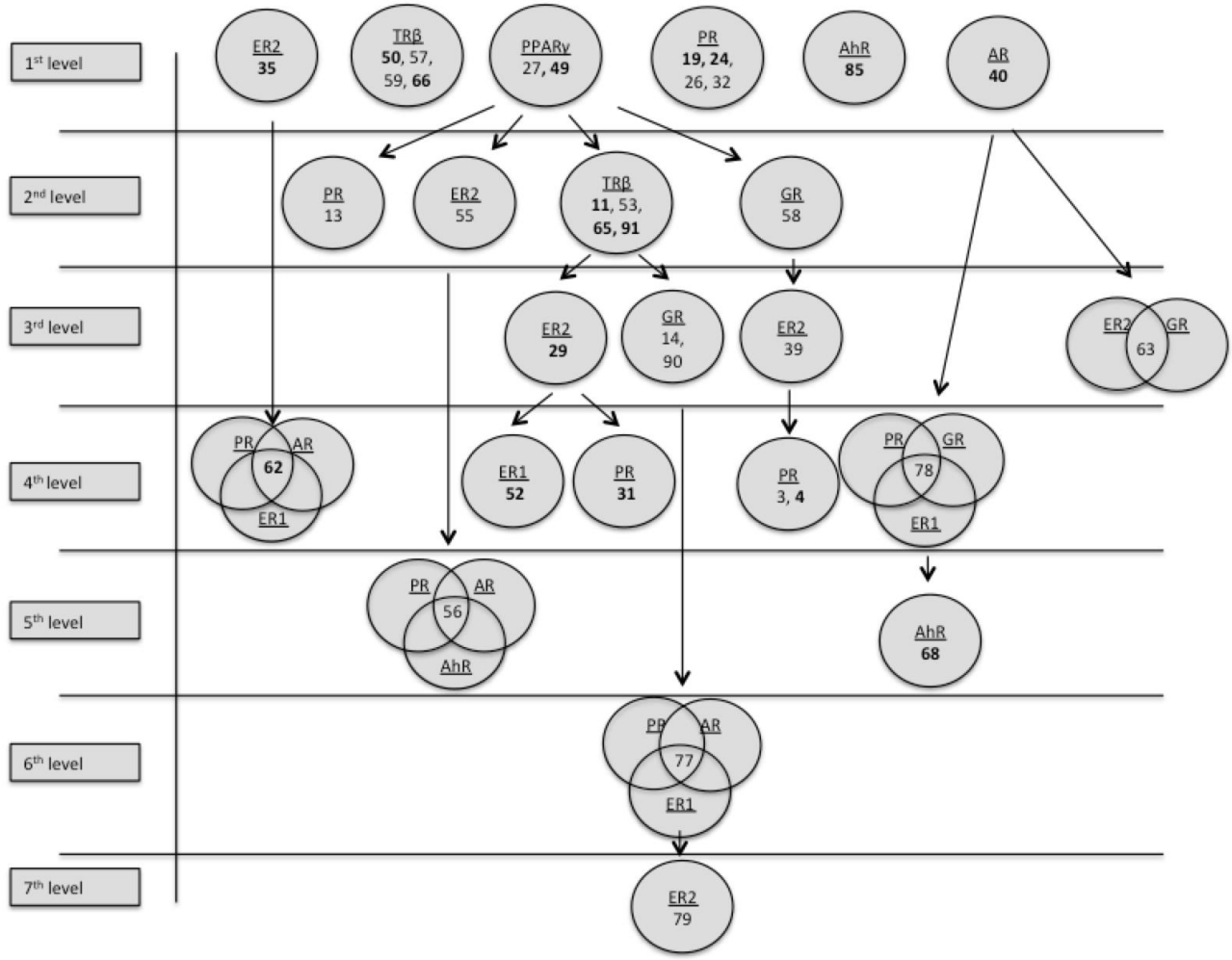

Figure 5: Clustering of compounds displaying an antagonistic activity on one or several nuclear receptors. The level represents the number of receptors a given compound interacts with. ER (1 or 2) : human oestrogen receptor; AR : human androgen receptor; PR : human progesterone receptor; GR : human glucocorticoid receptor; PPARϒ : human peroxisome proliferator-activated gamma receptor; TRβ : human thyroid ß receptor; AhR : mouse aryl hydrocarbon receptor; n, substance code. The compounds showing both agonistic and antagonistic activities are indicated in bold.

Table 1: Overview of tested chemical migrating compounds

number	Chemical	CAS	supplier	Purity
1	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (TXIB)	846-50-0	sigma	>98.5%
2	Bisphenol A	0-05-7	sigma	>99%
3	2,4,6-Trimethylbenzaldehyde	87-68-3	sigma	98%
4	2,4-Dimethylbenzaldehyde	5764-16-6	sigma	>90%
5	2,4-Di-tert-butylphenol	6-76-4	sigma	99%
6	2,6-Diisopropylnaphthalene (DIPN)	4157-81-1	sigma	>99%
7	3,5-Di-tert-butylbenzoquinone	19-22-2	sigma	98%
8	2-Butoxyethyl acetate	12-07-2	sigma	99%

9	2-Methylnaphthalene	1-57-6	sigma	97%
11	Octyl methoxycinnamate	466-77-3	sigma	98%
12	2-Ethylhexyl acrylate	03-11-7	sigma	98%
13	Isophorone	8-59-1	sigma	97%
14	4-Ethylbenzaldehyde	748-78-1	sigma	>97%
15	4-tert-Butylcyclohexyl acetate	2210-23-4	sigma	99%
16	Oleamide	01-02-0	sigma	>99%
17	Methyl oleate	12-62-9	sigma	99%
18	2-Ethylhexyl acetate	03-09-3	sigma	>99%
19	□-methylstyrene	8-83-9	sigma	99%

20	4-(Methylthio)benzaldehyde	446-89-7	igma	95%
22	Benzophenone	19-61-9	igma	>99
23	Butylated hydroxytoluene (BHT)	28-37-0	igma	>99%
24	Camphor	6-22-2	igma	96%
25	Cyclododecene	501-82-2	igma	>96%
26	Cyclohexanone	08-94-1	igma	>99.5
27	2-Isopropyl-5-methylcyclohexanone	0458-14-7	igma	≥97%
29	Isopropyl laurate	0233-13-3	igma	*
31	Methyl laurate	11-82-0	igma	99.5%
32	Erucamide	12-84-5	igma	97%

33	Eucalyptol	70-82-6	sigma	99%
34	Palmitic acid	7-10-3	sigma	>99%
35	Methyl palmitate	12-39-0	sigma	>97%
38	Napthalene	1-20-3	sigma	99%
39	N-Butylbenzenesulfonamide	622-84-2	sigma	99%
40	Stearic acid	7-11-4	sigma	>95%
41	Ethyl stearate	11-61-5	sigma	>97%
49	Bisphenol S	0-09-1	sigma	≥98%
50	Diisobutyl phthalate	4-69-5	sigma	99%
51	Dibutyl phthalate	4-74-2	sigma	99%
52	Bis(2-ethylhexyl)	17-	sig	≥96%

	phthalate	81-7	ma	
53	3,3,5-Trimethylcyclohexanone	73-94-9	igma	98%
55	Acetophenone	8-86-2	igma	≥99%
56	4-methylbenzaldehyde	04-87-0	igma	≥97%
57	2-phenyl-2-propanol	17-94-7	igma	97%
58	Fenchone	787-20-4	igma	≥98%
59	Ethyldiglycol acetate	12-15-2	igma	99%
61	□-terpineol	8-55-5	igma	≥90%
62	4-Propylbenzaldehyde	8785-06-0	igma	97%
63	2-Undecanone	12-12-9	igma	99%
64	p-Propenylanisole	180-	igm	≥99.5%

		23-8	a	
65	Butoxyethoxyethyl acetate	24-17-4	ig ma	≥99.2%
66	limonene	2-52-4	ig ma	≥99%
67	2,6-Dimethylnaphtalene	8804-88-8	ig ma	≥90%
68	2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	0396-80-2	he mos	98%
70	Oxacyclotridecan-2-one	47-05-7	ig ma	98%
73	4-tert-Octylphenol	40-66-9	ig ma	97%
74	Cedrol	7-53-2	*ig ma*	≥99%
75	2,2',5,5'-Tetramethylbiphenyl	075-84-1	he mos	98%
77	3,5-di-tert-Butyl-4-hydroxybenzaldeh	620-98-0	he mos	*

	yde			
78	Laurolactam	47-04-6	sigma	98%
79	Ethyl myristate	24-06-1	sigma	99%
85	4-Phenylbenzophenone	128-93-0	sigma	99%
88	4-n-nonylphenol	04-40-5	sigma	≥96%
89	Dicyclopentyl(dimethoxy)silane	26990-35-0	CI	98%
90	3,4-dimethylbenzaldehyde	973-71-7	sigma	98%
91	Irganox 1010	683-19-8	sigma	98%

* Purity not specified by the manufacturer

Table 2: Parameters of the reporter gene assays.

Parameter	ER1	ER2	AR	GR	PR	PPA R	TR	AhR
Upper asymptote (%)	97±1	99±1	99±2	99±1	101±2	95±6	99±1	93±4
Hill coefficient	1.5±0.1	1.3±0.4	0.9±0.2	1.4±0.4	2.0±0.3	1.7±0.2	1.1±0.2	1.9±0.1
EC_{50} (M)	8.3±1.6 10^{-12}	17±5 10^{-12}	1.8±0.6 10^{-9}	4.4±0.9 10^{-8}	3.3±0.9 10^{-7}	6.4±0.2 10^{-8}	4±1 10^{-10}	5.5±0.7 10^{-12}
Lower asymptote (%)	7±14	28±5	5±10.5	1.4±0.1	3±1	7±23	15±5	3±1
Limit of detection (M) oD	1.0 10^{-12}	5.0 10^{-12}	4.1 10^{-11}	2.2 10^{-9}	5.9 10^{-8}	1.2 10^{-8}	5.8 10^{-11}	0.8 10^{-12}

Mean ± standard deviation (n=10 calibration curves). ER (1 or 2) : human oestrogen receptor; AR : human androgen receptor; PR : human progesterone receptor; GR : human glucocorticoid receptor; PPARϒ : human peroxisome proliferator-activated gamma receptor; TRβ : human thyroid ß receptor; AhR : mouse aryl hydrocarbon receptor. The following chemicals were used as reference ligands : ER1 & ER2 : 17-β-oestradiol; AR : 5-α-dihydrotestosterone ; GR : dexamethasone ; PR : progesterone ; PPARϒ : Rosiglitazone ; TRβ : Triiodothyronine ; AhR : 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Table 3: Overview of the activation or inhibition of steroid and non-steroid hormone receptors by chemicals

	Tested compound	Chemical classes	Agonistic effect								Antagonistic effect							
			R1	R2	R	R	R	Rβ	PARγ	hR	R1	R2	R	R	R	Rβ	PARγ	hR
13	Isophorone	Ketones																
24	CAMPHOR		+												+			
26	Cyclohexanone																	
27	2-Isopropyl-5-methylcyclohexanone																	
53	3,3,5-Trimethylcyclohexanone																	
55	Acetophenone																	
58	Fenchone													+				
63	2-Undecanone											+	+	+				
68	2,6-DI(T-BUTYL)-4-HYDROXY-4-METHYL-2,5-CYCLOHEXADIEN-1-ONE							+			+		+		+			+
70	Oxacyclotridecan-2-one																	
78	Laurolactam										+		+	+	+			

6	2,6-Diisopropylnaphthalene (DIPN)	**Aromatic hydrocarbons**																
19	α-METHYLSTYRENE																	
25	Cyclododecene																	
66	LEMONENE		+													+		
67	2,6-Dimethylnaphtalene		+						+									
75	2,2',5,5'-Tetramethylbiphenyl																	
3	2,4,6-Trimethylbenzaldehyde	**Benzaldehydes**										+		+	+			
4	2,4-DIMETHYLBENZALDEHYDE																	
14	4-Ethylbenzaldehyde																	
56	4-Methylbenzaldehyde																	
62	4-PROPYLBENZALDEHYDE											+	+		+			
77	3,5-Di-t-butyl-4-hydroxybenzaldehyde												+	+	+			
90	3,4-Dimethylbenzaldehyde													+				
16	Oleamide	**Fatty acids and fatty**																
17	Methyl oleate								+									
	ISOPROPY																	

29	L LAURATE	**acid**	+									+					+	
31	METHYL LAURATE	**esters**	+													+		
35	METHYL PALMITATE											+						
40	STEARIC ACID																	
79	Ethyl myristate											+	+	+	+	+	+	
50	DIISOBUTYL PHTALATE		+													+		
51	Dibutyl phthalate	**Phthalic acid esters**	+															
52	DIETHYLHEXYLPHTALATE		+								+	+					+	
1	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate		+															
11	OCTYL METHOXYCINNAMATE		+													+	+	
12	2-Ethylhexyl acrylate	**Other esters**																
15	4-Tert-butylcyclohexyl acetate																	
59	Ethyldiglycol acetate																	
65	BUTOXYETHOXYETHYL ACETATE		+															
2	Bisphenol A (BPA)	**Phenols**	+	+														
49	BISPHENOL S (BPS)		+	+														
73	4-Tert-		+	+														

	octylphenol																	
88	4-n-nonylphenol		+															
22	Benzophenone		+	+														
85	4-PHENYLBENZOPHENONE	**Benzophenones**	+															
57	2-Phenyl-2-propanol																	
61	α -Terpineol	**Alcohols**																
74	Cedrol		+	+														
32	Erucamide																	
39	N-Butylbenzenesulfonamide	**Amides**												+				
89	Dicyclopentyl(dimethoxy)silane	**Silane**																
91	IRGANOX 1010																	

ER (1 or 2) : human oestrogen receptor; AR : human androgen receptor; PR : human progesterone receptor; GR : human glucocorticoid receptor; PPARϒ : human peroxisome proliferator-activated gamma receptor; TRβ : human thyroid ß receptor; AhR : mouse aryl hydrocarbon receptor. The assessment of the activity of the chemical was based on the relative response of the cell line, expressed as a percentage of the maximal response induced by the reference agonistic ligand. "+" = agonistic or antagonistic effect (respectively: increase of the relative response of more than 10 % or decrease of the relative response for two consecutive points of more than 10 %); "++": for agonistic effect : relative response > 50 %, for antagonistic effect : complete inhibition of the cell response. Empty space = no effect. Compounds indicated in UPPER CASES showed both agonistic and antagonistic activities.

Highlights

- Endocrine disrupting activity of 65 compounds migrating from polycarbonate replacement plastic baby bottles was assessed.
- Most of the agonistic activities were observed towards the human oestrogen receptor (29 compounds)
- Most of the antagonistic activities were observed towards the PPARγ (20 compounds)
- Thirty five chemicals were able of agonistic activities on 1 to 4 nuclear receptors
- Antagonistic activities were recorded for 35 compounds as well, towards 1 to 7 nuclear receptors