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Hazardous chemicals in non-polar extracts from paper and cardboard food packaging: an effect-based evaluation

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Abstract

Background: Food contact articles are used in our everyday life and information regarding the potential health hazards of migrating chemicals for humans is scarce. In this study, an effect-based evaluation of non-polar extracts of food contact articles made of paper and board was conducted with a panel of eight bioassay endpoints. These, health-relevant endpoints, included oxidative stress, inflammation, genotoxicity, xenobiotic metabolism and hormone receptor effects.

Results: In total, 62 food contact articles were pooled into 19 groups, in which articles intended to be used for similar types of food item(s) were pooled, and extracted with acetone:*n*-hexane (1:4). These were then tested in the effect-based bioassays. Bioactivities were detected for multiple materials in six out of eight assays, the two assays showing no effects were NFκB and androgen receptor agonistic response. In essence, the detection rates of the tested non-polar extracts were 72% for antagonistic effects on the estrogen receptor, 72% for antagonistic effects on the androgen receptor, 47% for oxidative stress, 28% for agonistic effects on the estrogen receptor and 33% for genotoxicity. The bioequivalent concentrations ranges in extracts of 10 mg food contact article/mL cell culture media were: for oxidative stress from 2.45 to 5.64 µM tBHQ equivalents, estrogen receptor agonistic activity from 1.66 to 6.33 ρM estradiol equivalents, estrogen receptor antagonistic activity from 1.21×10^{-3} to 4.20×10^{-3} µM raloxifene equivalents and androgen antagonistic activity 0.08–0.46 µM hydroxyflutamide equivalents. The extracts that were bioactive in multiple assays were: baking moulds, boxes for popcorn, infant formula/skimmed milk, porridge/flour mixes, pizza, fries' and hamburgers as well as packages for frozen food.

Conclusion: Non-polar extracts of food contact articles contain compounds that can activate molecular initiating events in toxicity pathways of high relevance to human health. These events included endocrine-disruptive activities, oxidative stress and genotoxicity. Effect-based methods proved to be a valuable tool for evaluating food package articles, as they can detect potentially hazardous effects of both known and unknown chemicals as well as potential cocktail effects.

Keywords: Effect-based methods, Bioanalytical tools, Food packages, Paper and cardboard, In vitro methods

Background

Food contact materials (FCMs) are used to produce food contact articles (FCAs) and other packages that are intended to come into contact with food items [1]. Via migration into food, we are exposed to a variety of chemicals that are intentionally or non-intentionally added

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to the different packing materials. Intentionally added substances (IAS) refer to the addition of chemicals for a specific purpose during the manufacturing process of the FCMs. Non-intentionally added substances (NIAS), on the other hand, are denoted as impurities of starting substances, degradation and residue products, which may have been generated during manufacturing or as a result of contamination. Manufacturing of these package materials needs to comply with good manufacturing practices and follow national as well as international legislation. This to ensure that consumer health is not compromised after intake of food containing chemicals migrating from package materials [1–4]. Still, many IAS and NIAS have inadequate or no toxicological data, and this is possibly of concern in cases of migration of undesirable chemicals into food items [5, 6].

Since the packaging material consists of a wide variety of complex mixtures, it is impossible to identify and conduct toxicity testing for all single substances. In addition, the exact chemical composition within FCAs and FCMs is not even known by the manufacturers themselves. Therefore, it has been proposed to apply effect-based methods to assess the potential presence of hazardous compounds [7, 8]. Effect-based methods integrate effects of known and unknown chemicals, in addition to cocktail effects, by the use of cultured cells. Previous studies on other environmental matrices, such as water samples, have shown that only a small fraction of biological effects observed in vitro and/or in *Vibrio fischeri* were explained by known chemicals, in certain cases as much as 99% of the effects were due to unknown chemicals or cocktail effects [9–11]. The application of effect-based methods is therefore more efficient in measuring the effects of the whole mixture and can be of great value when assessing the presence of hazardous mixtures in these types of materials.

In this study, a set of eight assays were included to cover toxicity pathways, which are relevant for human health [12]. These were: oxidative stress (Nrf2 activity), genotoxicity (micronucleus test, MN test), estrogen receptor agonistic/antagonistic effects (ER), androgen receptor agonistic/antagonistic effects (AR), aryl hydrocarbon receptor activation (AhR) and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).

In a previous study, we analysed polar extracts of FCA, made from paper and cardboard, and found that these extracts induced oxidative stress, genotoxicity, antagonistic AR, as well as AhR activity, to a high degree, while antagonistic estrogenic receptor responses were activated to a moderate extent [13]. Here, we used the same package materials as in our previous study, but instead investigated the effects of non-polar extracts by the use of effect-based methods. The extracts used in the study were a part of the governmental assignment to the Swedish Chemicals Agency, in which they conducted chemical analyses on the same extracts.

Materials and methods

Selection of food contact articles and extraction

In total, 62 materials made from paper and cardboard were obtained from bakeries, grocery stores, movie theatres, restaurants and paper companies in May and June of 2019 by the Swedish Chemicals Agency [14]. A wide range of materials was selected, including materials that are supposed to come in contact with dry or fatty food items (Table 1). The purchased packages were stored at room temperature before the extraction process and sample preparation was conducted. The 62 different types of material were pooled into 23 groups, in which similar types of materials were pooled into one group. For each group, the sample weight was approximately 1 g.

Detailed information on the extraction process and sample preparation can be found in the supplementary information (Additional file 1: S1, Sects. 2 and 3). In short, the extraction included the material as a whole, meaning that it contained also printing inks, coatings, glues, etc., which may not normally come in direct contact with the food. The samples were extracted with acetone:*n*-hexane (1:4), to retrieve non-polar chemicals, by the use of a microwave and ultrasonicator. The extracted samples were evaporated to 1 mL, centrifuged at 14 000 rpm and transferred into glass vials. Three extraction blanks were included in the study and treated in the same way as the FCA extracts, but without any packaging material [14]. The samples extracted with acetone:*n*-hexane (1:4) will hereafter be referred to as non-polar extracts, while the samples extracted in methanol in our earlier study are denoted as polar extracts [13].

Prior to bioanalysis, the 1 mL FCA extracts were evaporated to near dryness and reconstituted in 1 mL dimethyl sulfoxide (DMSO), as it is considered suitable for cell culture procedure. However, due to precipitation problems in DMSO and/or *n*-hexane, four samples were excluded (cake/pastry boxes/mats, coated paper plate, papers for wraps and boxes for cookies from supermarket). Two samples precipitated in DMSO, these were therefore again evaporated and reconstituted in *n*-hexane instead (boxes for cereals and hamburger/French fries' papers). However, Hamburger/French fries' papers extract was only tested in the Nrf2 assay, as it later precipitated in *n*-hexane.

One extraction/solvent blank was dried and reconstituted in the same way as these samples and remained in *n*-hexane throughout the study, whereas the two other extraction blanks remained in DMSO (Table 1). This resulted in a total of 50 materials, instead of 62, and

Table 1 Summary of the 50 materials that were pooled into 19 food contact article groups that were included in the study

Food contact article	Printing	Number of material(s)	Solvent
Bag for cookies	Yes[a]	1	DMSO
Baking moulds	Yes[a]	1	DMSO
Board samples	No	2	DMSO
Boxes for cereals	Yes[a,c]	4	*n*-Hexane
Boxes for cookies (from manufacturer)	Yes[a]	3	DMSO
Boxes for fries' and hamburgers	Yes[a]	2	DMSO
Boxes for infant formula/skimmed milk	Yes[a]	3	DMSO
Boxes for porridge and flour mixes	Yes[a]	6	DMSO
Colored paper for baking moulds	Yes[a]	1	DMSO
Hamburger/French fries' papers	Yes[a,b]	5	*n*-Hexane
Microwave popcorn bags	Yes[a,c]	6	DMSO
Packages for frozen food	Yes[a,c]	2	DMSO
Paper for baking and baking moulds	No, but contained bleached material	5	DMSO
Paper for trays	Yes	1	DMSO
Paper plate for warm food	No	1	DMSO
Pizza boxes	Yes	2	DMSO
Pizza slice trays	Yes[a]	1	DMSO
Popcorn boxes	Yes[a]	3	DMSO
Straws	Yes[a]	1	DMSO

[a] Also contained adhesives

[b] Hamburger papers did not contain adhesives, while French fries' papers did

[c] The FCA was in contact with food prior to collection

these 50 materials were pooled into a total of 19 groups. All extracts were stored in the dark at − 20 °C until and between the analyses.

Effect-based methods

In the effect-based methods, 1 g FCA group per 1 mL solvent was diluted 100x, resulting in a starting concentration of 10 mg FCA per 1 mL cell culture media. The extracts were then diluted in a 3.3-fold dilution series, resulting in concentrations of 10, 3, 1 and 0.3 mg FCA per 1 mL cell culture media, which then were tested in quadruplicates.

For each assay run, a well-established cell line was used and a reference compound was included as a standard for validation of assay performance (Table 2). Further information on the assays can be found in the supplementary information (Additional file 1: S1, Sects. 4 to 7).

The vehicle controls consisted of DMSO or *n*-hexane for the FCA samples. An additional vehicle control consisted of water for mitomycin C (MMC), which was used as a positive control in the micronucleus test. All reference compounds were dissolved in DMSO (Table 2). Methoxychlor and tamoxifen were used as positive controls, in addition to the reference compounds, for agonistic and antagonistic estrogen receptor activity, respectively.

Data analysis

All data were evaluated using GraphPad Prism version 9.1.10 software (San Diego, California, USA). A cut-off was calculated for all bioanalytical methods, which was based on the limit of detection (LOD), to define a sample as bioactive (Table 2).

The LOD was calculated as three times the standard deviation (SD) of the vehicle control in each run, and the cut-off was the nearest integer above the LOD for agonistic response and below the LOD for antagonistic response (Table 2).

The cell viability data was normalized to the vehicle control (set to 100%) and a reduction in cell viability of more than 25% was considered cytotoxic, with the exception of the micronucleus test. For the micronucleus test, a sample was considered cytotoxic if the % ethidium monoazide (EMA)-positive event was greater than four times the vehicle control.

For Nrf2 activity, the response was calculated as fold change, as no maximum effect is reached, and was analysed using a linear regression fit [15]. The LOD was calculated as three times the SD of the vehicle control response plus one, and the cut-off was defined as an induction ratio of 1.5, which was slightly above the LOD.

The agonistic assays were normalized to the vehicle control, followed by normalization to the % max

Table 2 Summarization of detailed information regarding the different endpoints

Endpoint	Reference compound Concentration	Cut-off (%)	Cell line
Androgen receptor			
Agonism	Dihydrotestosterone (DHT) 0.001–1000 ρM	5	AR-EcoScreen GR-KO M1
Antagonism	Hydroxyflutamide (OHF) 0.0001–10 μM	70	
Aryl hydrocarbon receptor	2,3,7,8-Tetrachlorodibenzodioxin (TCDD) 0.01–1000 ρM	10	DR-EcoScreen
Estrogen receptor			
Agonism	Estradiol (E2)[a] 0.4–367 ρM	15	VM7Luc4E2
Antagonism	Raloxifene (Ral)[a] 0.1–25 nM	70	
MN test	N/A[b]	Statistically significant (p-value < 0.05)	TK6
NFκB	Tumor necrosis factor α (TNFα) 0.2–50 ng/mL	10	HepG2-NFκB
Nrf2	tert-Butylhydroquinone (tBHQ) 0.8–25 μM	1.5[c]	MCF7 AREc32

[a] Methoxychlor and tamoxifen were used as positive controls for agonistic and antagonistic estrogen receptor activity, respectively

[b] MMC was used as a positive control at concentrations 100 and 200 nM

[c] The cut-off is expressed as fold change for Nrf2

effect of the standard. The antagonistic responses were instead normalized to the unspiked vehicle controls, followed by normalization of the vehicle control with spiked vehicle control. Standard curves, of the reference compounds, for the agonistic and antagonistic responses were fitted using a four-parameter non-linear regression curve fit (log-logistic).

The effect concentration (EC), inhibitory concentration (IC) and effect concentration induction ratio 1.5 ($EC_{IR1.5}$) were calculated for the respective reference compound and further used to calculate bioanalytical equivalent concentration (BEQ) for the samples.

BEQ renders a concentration of a well-established reference compound relatable to the effect of a sample. In accordance with Escher et al. [16], the BEQ was calculated by the formula:

$$\mathrm{BEQ} = \frac{\mathrm{EC}_x \text{ or } \mathrm{EC}_{\mathrm{IR1.5}} \text{ or } \mathrm{IC}_{30}(\text{reference compound})}{\mathrm{EC}_x \text{ or } \mathrm{EC}_{\mathrm{IR1.5}} \text{ or } \mathrm{IC}_{30}(\text{sample})},$$

$x = 5, 10 \text{ or } 15.$

The micronucleus formation was analysed by a one-way ANOVA with Dunnett's multiple comparison test. Bioactivity was defined by retrieving a p-value below 0.05.

Results and discussion

Cell viability

Cell viability was measured in all cell lines to ensure that each assay was conducted under non-cytotoxic conditions (Table 2). None of the non-polar extracts were cytotoxic after 24 h exposure, which was defined by the cut-off value of 75% cell viability (Additional file 1: Figs. S1–5). Additionally, cytotoxicity testing of the micronucleus test using EMA dye revealed that none of the exposure concentrations exceeded the cut-off of 4-fold %EMA-positive events of the vehicle control (Table 3).

In our previous study on polar extracts from the same FCAs, a few extracts were cytotoxic at the highest concentration tested [13]. Other studies have investigated cytotoxicity of FCAs by using resazurin assay, RNA synthesis inhibition, membrane damage, total protein content (TPC), colony-forming ability (CFA), *Vibrio fischeri*, sperm spermatozoan motility inhibition test and other methods, as summarized by Severin et al. and Groh et al. [8, 17–20]. Some of these studies reported no or similar cytotoxicity between water and ethanol extracts, whereas others found higher cytotoxicity in ethanol extracts compared to water [20]. However, to our knowledge, no study has used such a non-polar

Table 3 Genotoxicity results of the tested non-polar extracts

Sample	Concentration	MN		EMA	Statistical significance
		% ± SD	Fold change ± SD	% ± SD	
Vehicle control	1%	0.25 ± 0.05	1.00 ± 0.20	3.63 ± 0.78	–
Bag for cookies	10 mg/mL	0.44 ± 0.05	1.73 ± 0.21	2.90 ± 0.23	N/S
Packages for frozen food	10 mg/mL	0.43 ± 0.20	1.71 ± 0.77	3.14 ± 0.42	N/S
Boxes for fries' and hamburgers	10 mg/mL	0.62 ± 0.10	2.42 ± 0.38	4.63 ± 1.17	****
MMC	100 nM	1.63 ± 0.19	5.10 ± 0.61	5.36 ± 1.29	****
	200 nM	3.95 ± 0.32	12.34 ± 1.00	6.61 ± 1.32	****

The number of technical repeats (*n*) was 4 for both samples and vehicle controls. The data show the mean ± SD of two individual runs

N/S 'not significant' samples

****Indicate a *p*-value of < 0.0001

solvent to investigate potential cytotoxic effects, during the extraction procedure, as in our study.

Nrf2 activity

Oxidative stress was evaluated as Nrf2 activity using the stably transfected cell line MCF7 AREc32. In total, 9 out of 19 samples showed an activation of Nrf2 activity after 24 h of treatment, as defined by the cut-off level of 1.5 induction ratio (Fig. 1, Table 4). Seven samples were bioactive only at the highest concentration tested (10 mg/mL), and two samples (boxes for cereals and bag for cookies) were bioactive at 3 and 1 mg/mL, respectively.

The highest activity was observed for packages for frozen food, but this specific sample was only bioactive at the highest concentration tested (Fig. 1B). Bag for cookies induced oxidative stress from 1 to 10 mg/mL in a dose-related manner (Fig. 1B).

tBHQ was used as the reference compound for oxidative stress and retrieved an $EC_{IR1.5}$ value of 3.1 µM (Additional file 1: Table S1, Fig. S10A). tBHQ equivalents for the bioactive samples ranged from 2.45 to 5.64 µM for extracts at 10 mg/mL (Additional file 1: Table S2).

Compared to our previous study with the polar extracts, the induction of oxidative stress was less potent and the efficacies were lower for non-polar extracts [13]. Activities were observed at higher concentrations and the corresponding induction ratios were lower in the present study. The most pronounced difference in activities was seen for boxes for cereals, which had an induction ratio of 1.3 at 10 mg/mL for the non-polar extract (Fig. 1), while the polar extract induced Nrf2 activity to an induction ratio of 8.9 [13]. Rosenmai et al. [21] also investigated Nrf2 activities of ethanol FCM extracts made of paper and cardboard, in which 80% of the extracts induced Nrf2. In agreement with our studies, Nrf2 activity was induced by hexane, methanol/water (1:1) and ethanol extracts of pizza boxes and boxes for cereals, suggesting that both polar and non-polar extracts are inducing the oxidative stress response [13, 21].

Micronuclei formation (genotoxicity)

Genotoxicity was measured in form of micronuclei formation using TK6 cells. Three samples were tested, at the highest concentration of 10 mg/mL, and these were: bag for cookies, packages for frozen food and boxes for fries' and hamburgers. These samples were chosen as they showed among the highest oxidative stress induction ratio and oxidative stress is being reported to be one of the potential mechanisms of genotoxicity [22].

The micronuclei formation was assessed after 24 h of exposure. All three extracts increased the % of MN compared to the vehicle control, but the extract from boxes for fries' and hamburgers was the only sample that caused a statistically significant increase in micronuclei formation (Table 3). Both concentrations of MMC caused a statistically significant increase in micronuclei formation (Table 3).

Paper and cardboard FCMs and FCAs have previously been tested for genotoxicity by Rec assay with *Bacillus subtilis*, Ames test, Comet assay, BlueScreen, p53 activation, γH2AX and micronuclei test [13, 19, 21, 23–26]. In our previous study, all four studied polar extracts (boxes for cereals, pizza boxes, cake/pastry boxes/mats and boxes for infant formula/skimmed milk) increased the formation of MN at the highest concentration tested (10 mg/mL) [13]. Pizza boxes were the sample with the highest efficacy, reaching 25% micronuclei events. Positive genotoxic effects have also been reported for ethanol-extracted virgin and recycled FCMs, made of paper/cardboard, with the Rec assay [24]. Of all the tested virgin FCMs 19% exerted genotoxicity, while 75% of all tested recycled extracts were genotoxic. Besides using the Rec assay, Ozaki et al. also used the Comet assay for eight paper/cardboard materials and found that six of

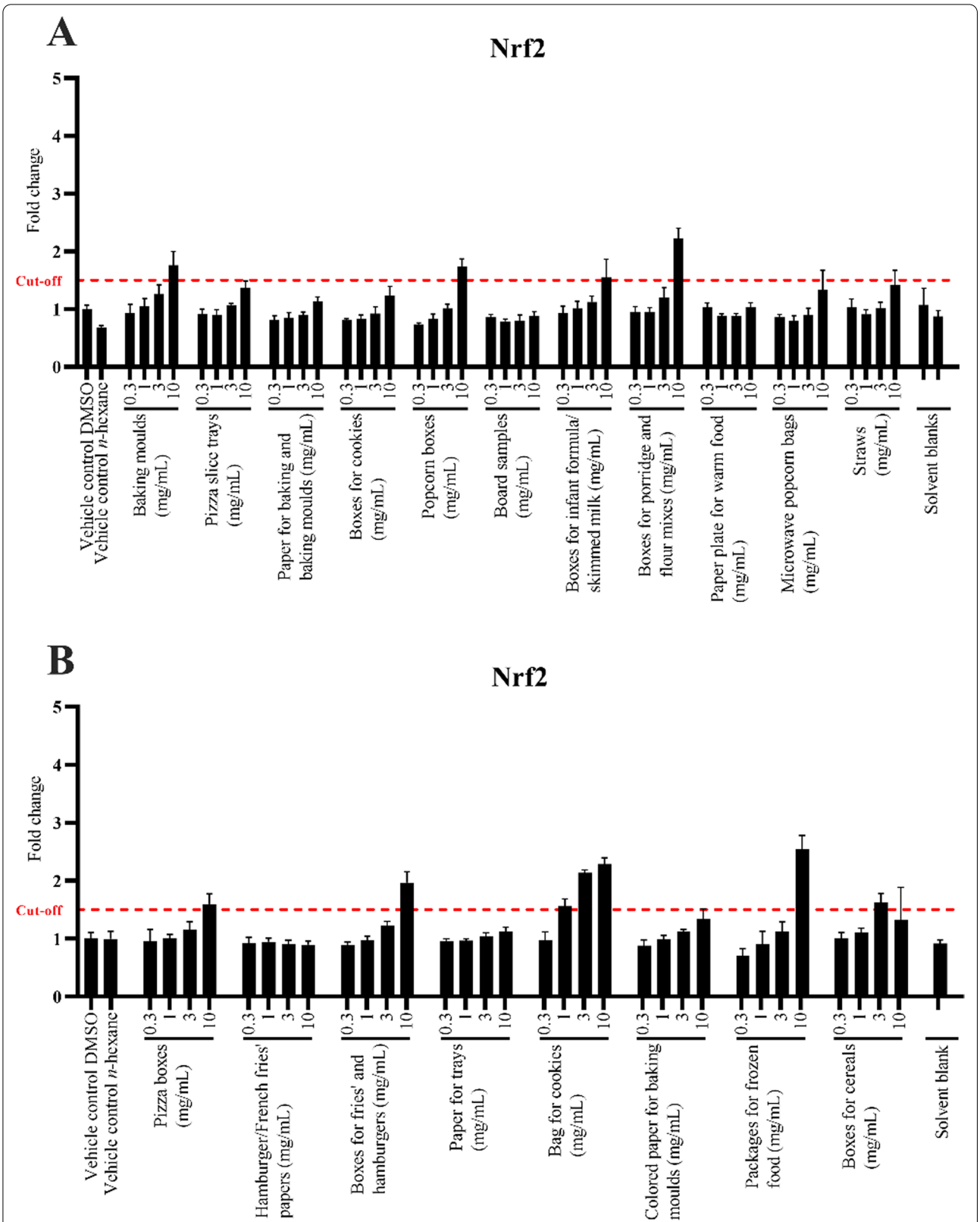

Fig. 1 Nrf2 activity (fold change compared to the vehicle control) in MCF7 AREc32 cells after 24 h exposure to FCA extracts (**A**, **B**). The number of technical repeats (*n*) was 8 for the vehicle controls and 4 for the samples (mean ± SD)

Table 4 Bioactivities of polar and non-polar extracts. Colour-coded heatmap summarizing the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) of polar and non-polar FCA extracts activities for the majority of bioactive assays

Assay	Nrf2		Genotoxicity		AR Antagonism		ER Agonism		ER Antagonism	
Food contact article	**Polar** mg/mL	**Non-polar** mg/mL	**Polar** mg/mL	**Non-polar** mg/mL	**Polar** mg/mL	**Non-polar** mg/mL	**Polar** mg/mL	**Non-polar** mg/mL	**Polar** mg/mL	**Non-polar** mg/mL
Bag for cookies	10	1	-	10	10	10	10	10	10	3
Baking moulds	3	10	-	-	10	3	10	10	10	3
Board samples	10	10	-	-	10	10	10	10	10	10
Boxes for cereals	1	3	10	-	10	10	10	10	10*	10
Boxes for cookies (from supermarket)	3	N/A	-	N/A	10	N/A	10	N/A	10*	N/A
Boxes for cookies (from manufacturer)	3	10	-	-	10	10	10	10	3	10
Boxes for fries' and hamburgers	3	10	-	10	3	10	10	10	10	10
Boxes for infant formula/skimmed milk	3	10	10	-	10	10	10	10	10	3
Boxes for porridge and flour mixes	0.3	10	-	-	10	10	10	10	10*	10
Cake/pastry boxes/mats	3	N/A	10	N/A	10	N/A	10	N/A	10	N/A
Colored paper for baking moulds	10	10	-	-	10	10	10	10	10	10
Hamburger/French fries' papers	10	10	-	N/A	10	N/A	10	N/A	10	N/A
Microwave popcorn bags	10	10	-	-	10	10	3	3	10	10
Packages for frozen food	10	10	-	10	10	10	10	10	10	10
Paper for baking and baking moulds	10	10	-	-	10	10	10	10	10	10
Paper for trays	10	10	-	-	10	10	10	10	10	10
Paper plate (coated)	0.3	N/A	-	N/A	10	N/A	10	N/A	10*	N/A
Paper plate for warm food	10	10	-	-	10	10	10	3	10	10
Papers for wraps	10	N/A	-	N/A	10	N/A	10	N/A	10	N/A
Pizza boxes	1	10	10	-	10	10	10	1	10	3
Pizza slice trays	3	10	-	-	10	10	10	10	10	10
Popcorn boxes	3	10	-	-	10	10	10	10	10	10
Straws	10	10	-	-	10	10	10	10	10	10
∑ Bioactive samples/assay	13/23	9/19	4/4	1/3	13/23	13/18	3/23	5/18	9/23	13/18

NOEC = 10 mg/mL | LOEC = 10 mg/mL | LOEC = 3 mg/mL | LOEC = 1 mg/mL | LOEC = 0.3 mg/mL

-: denotes samples that were not included in the assay

N/A: 'not applicable', meaning that these samples had precipitation problems and were therefore excluded from being tested in the study

*: denotes that the sample was cytotoxic, but bioactive, and was thus not included in the total sum of bioactive sample/assay. Interpret with caution

the paper/cardboard materials also induced a genotoxic response, in which three of these were made of virgin materials [24]. Later on, Ozaki et al. identified dehydroabietic acid and abietic acid to be the possible causative genotoxic drivers, which are resins acids that can be used during different processes in paper and packaging production [27]. Furthermore, water-extracted raw paperboard material intended for wet food, named starting paperboard, increased the phosphorylation of the DNA double-strand marker γH2AX and p53 marker in both HepG2 and HepaRG cell lines [26]. The paperboard end products, meaning paperboard retrieved from the recycling of the starting paperboard, increased the expression of p53 and γH2AX markers, although the latter marker only showed effects in the HepG2 cell line. A statistically significant increase in DNA damage using the Comet assay (%tail intensity) was only observed at the highest concentration tested (2 mg/mL) for the starting paperboard extract in the HepG2 cells and end product paperboard extracts in the HepaRG cells [26].

The MN test also revealed significant formation of micronuclei of the end product extracts in the two human hepatic cell lines HepG2 and HepaRG at the highest concentration tested. The authors hypothesized that the genotoxic effects may be explained by contaminants during the recycling processes or the addition of additives [26].

Another study displaying positive responses included ethanol extracts of paper and cardboard, where 2/20 extracts were genotoxic in the Ames test. These materials came from a microwave pizza tray and popcorn bag [21]. However, no genotoxic responses have also been observed for ethanol extracts made of virgin and recycled paper in the Ames test, regardless of the inclusion of a metabolism step in the test (S9) [19]. Additionally, no genotoxic response was seen for the food grade carton in the BlueScreen assay when Tenax was used as a food simulant [25], or water as well as ethanol extracts in the Ames test and Comet assay [23].

Estrogen receptor activity

Estrogen receptor agonistic and antagonistic activities were assessed in the stably transfected VM7Luc4E2 cell line.

For the agonistic assay, 5 out of 18 samples were bioactive, as defined by the cut-off limit of 15% of the max

effect of estradiol (Additional file 1: Fig. S6). Of these extracts, paper plate for warm food, microwave popcorn bags and pizza boxes were bioactive at lower concentrations as well. Paper plate for warm food exhibited the highest estrogenic effect of 61% at a concentration of 10 mg/mL. The bioequivalent concentrations for the bioactive samples, expressed as 17β-estradiol equivalents (E2EQ), ranged from 1.66 to 6.33 ρM for extracts at 10 mg/mL (Additional file 1: Table S2). The non-linear dose regression of E2 resulted in an EC_{15} value of 1.4 ρM (Additional file 1: Fig. S10B, Table S1). The positive control methoxychlor obtained an agonistic estrogenic effect of 146% (*data not shown*).

The antagonistic estrogen receptor response was also measured and samples causing an activity below 70% max effect of raloxifene were defined as bioactive (Fig. 2). In total, 13 out of 18 samples were bioactive in a dose-related manner, with the majority of the extracts being bioactive at the highest concentrations tested (Fig. 2). Baking moulds, pizza boxes and boxes for infant formula/skimmed milk exhibited the highest efficacies in the antagonistic assay. The bioactivities of the samples corresponding to bioequivalent concentrations of raloxifene (RalEQ) ranged between 1.21×10^{-3} and 4.20×10^{-3} µM at 10 mg/mL (Additional file 1: Table S2). The reference compound Ral obtained an IC_{30} value of 0.001 µM (Additional file 1: Fig. S10C, Table S1). The positive control tamoxifen caused a 36% antagonistic estrogenic effect (*data not shown*).

Similar to the current study, only a few polar package material extracts induced estrogenic agonistic response in the former study [13]. Both the polar and non-polar extracts microwave popcorn bags and colored paper for baking moulds were bioactive in the agonistic assay. Several of the packages also induced antagonistic activities, such as pizza slice trays, popcorn boxes and boxes for infant formula/skimmed milk, which only were bioactive at the highest concentration tested. Importantly, even though none of the extracts were defined as cytotoxic there is a risk that antagonistic activity is related to an undetected cytotoxic effect.

Previous studies have observed estrogenic responses in board and paper. Rosenmai et al. observed agonistic ER activity in 9 out of 20 ethanol-extracted FCMs [21]. Paperboard with water-soluble print, paperboard with UV print and the pizza box showed the most pronounced agonistic activity, with LOEC values ranging from 0.1 to 0.3 cm^2 FCM/mL. Ethanol extracts made of kitchen rolls have also caused estrogenic activity in yeast estrogen screen assay, where 78% of the recyclable kitchen rolls and 18% of virgin kitchen rolls increased estrogenic activity [28]. The higher activity of recycled board FCMs was also later confirmed by Vandermarken et al. [29]. Furthermore, approximately 90% of the water-extracted paper and cardboard take-away containers displayed estrogenic activity in the E-Screen assay [30].

Vinggaard et al. identified that the 3 paper materials out of 20 tested papers, containing the highest amount of bisphenol A (BPA) (10.6–24.1 mg BPA/kg paper), also exhibited the highest estrogenic effects [28]. Additionally, Rosenmai et al. identified BPA, di-butyl phthalate (DBP) and butyl-benzyl phthalate to be the potential drivers of the agonistic estrogenic effect in the pizza box extract [21].

Antagonistic ER activity has been reported in two out of three studied food cartons in the yeast estrogen screen assay, but this could not be confirmed in the ERα CALUX assay [31]. The authors established that the antagonistic activity was specific to the yeast cells and recommend that further testing of FCMs should be done with human reporter gene assays instead [32]. The two cartons showed activity in the range from 0.1 to 10 mg 4-ortho hydroxytamoxifen equivalents/L [32]. On the other hand, very weak or no agonistic as well as antagonistic activity of acetonitrile–ultrapure water (1:1) paper extracts have been reported in the yeast estrogen test [32].

Androgen receptor activity

Androgen receptor activity was examined using the stably transfected Chinese hamster ovary (CHO) cell line AR-EcoScreen GR-KO M1.

No extracts were defined as bioactive, defined by the cut-off limit of 5% of the DHT maximum, for the agonistic assay (Additional file 1: Fig. S7). The reference compound DHT had an EC_5 value of 6.9 ρM (Additional file 1: Fig. S10D, Table S1).

Antagonistic activity was detected in 13 samples in a dose-related manner, where boxes for infant formula/skimmed milk and baking moulds obtained the highest efficacies (Fig. 3A). For several of the non-polar extracts, the effect diminished at lower concentrations, but still exerted a dose-related trend (Fig. 3). None of the extracts were detected as cytotoxic, but there is a risk that antagonistic activity is related to an undetected cytotoxic effect. OHF was used as a reference compound for the antagonistic effects and obtained an IC_{30} value of 0.1 µM (Additional file 1: Fig. S10E, Table S1). Bioactivities of the samples corresponding to bioequivalent concentrations of OHF (OHFEQ) ranged between 0.08 and 0.46 µM (Additional file 1: Table S2).

Our prior study obtained similar results, of which approximately half the polar extracts showed antagonistic effect and none of the samples showed agonistic androgenic response [13]. Rosenmai et al. have reported that ethanol-extracted package materials induced agonistic AR activity in 6 out of 20 materials, while the

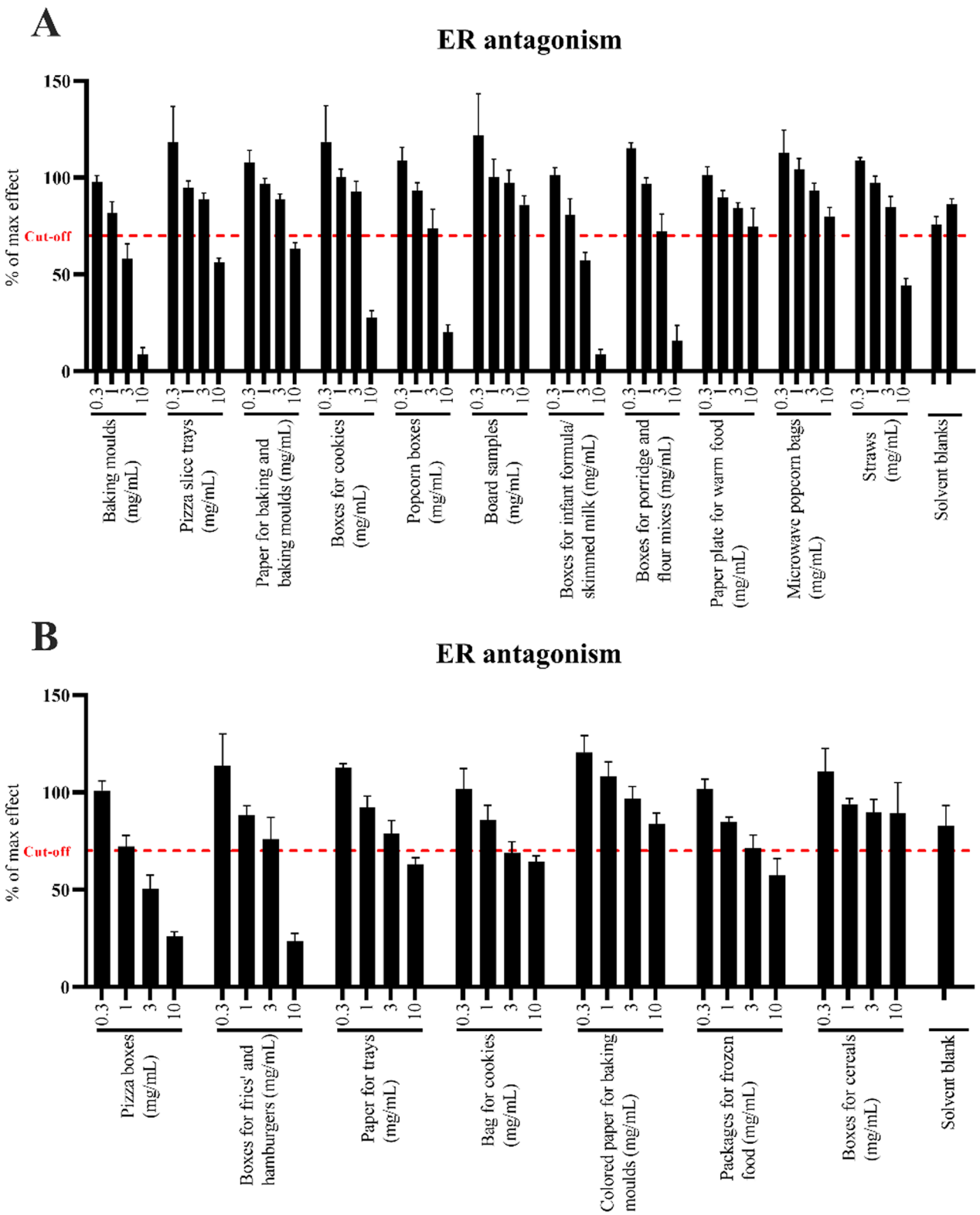

Fig. 2 ER antagonistic response (% max effect of raloxifene) in VM7Luc4E2 cells after 24 h of exposure to FCA extracts (**A**, **B**). The number of technical repeats (*n*) was 8–12 for the vehicle controls and 4 for the samples (mean ± SD). Samples with an activity below the cut-off limit were defined as bioactive

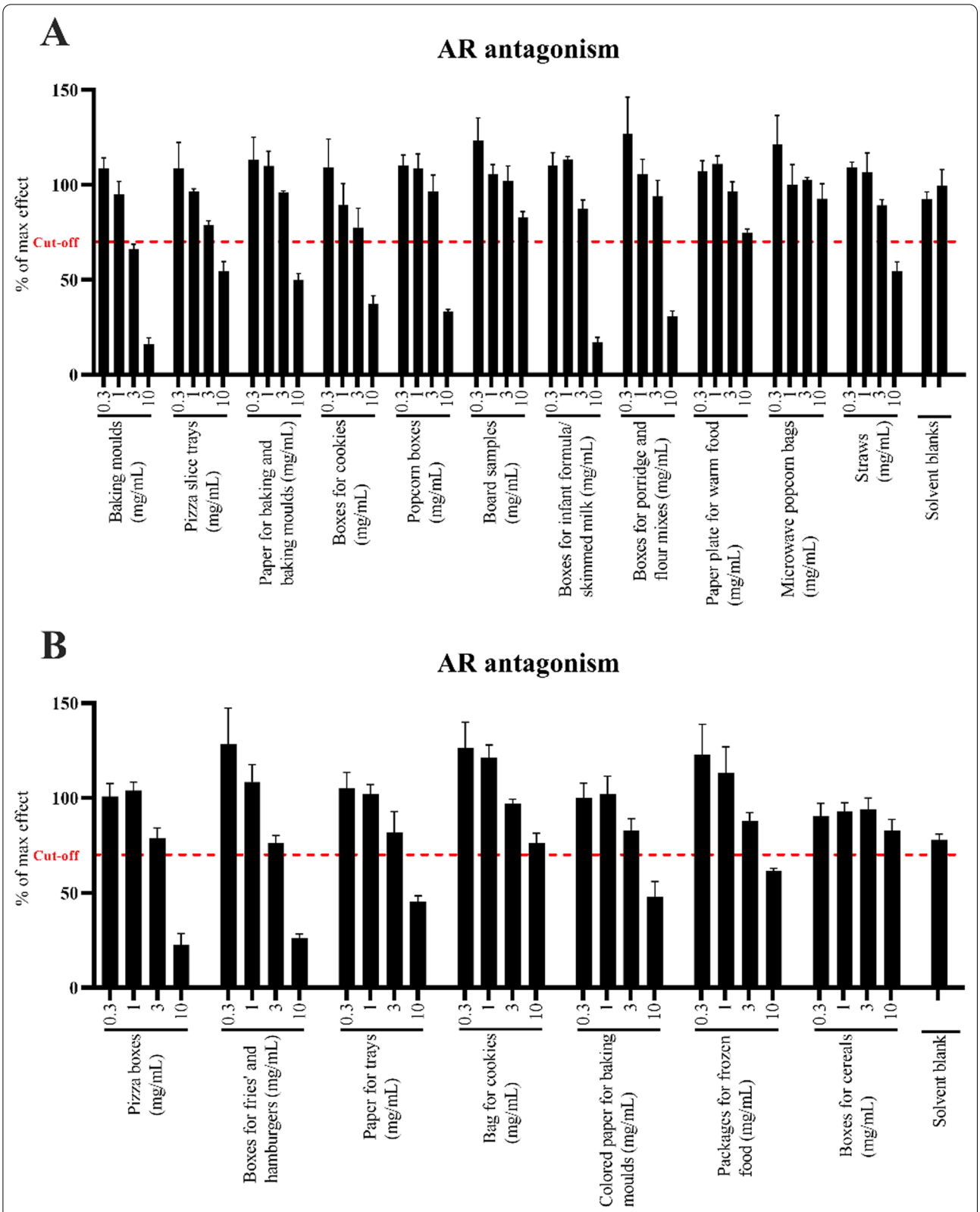

Fig. 3 Antagonistic response (% of max effect of OHF) in AR-EcoScreen GR-KO M1 cells after 24 h of exposure to FCA extracts (**A**, **B**). The number of technical repeats (*n*) was 8–12 for the vehicle controls and 4 for the samples (mean ± SD). The cut-off limit is represented by the red dotted line and samples with an activity below the cut-off limit were defined as bioactive

antagonistic AR activity was shown in 9 out of 20 extracts, with paperboard with UV print being the most potent material [21]. However, in another study 3 ethanol-extracted food cartons for milk products were tested, where no agonistic activity was detected and inconsistent result was obtained between the yeast androgen and AR CALUX assay [31]. The former assay positively detected 2/3 samples, while no activity was seen in the latter assay. The authors suggested that the inconsistent antagonistic results can be explained by the specificity of the yeast tests [31].

Kejlová et al. [32] also investigated paper and board FCMs extracted using the polar solvents acetonitrile–ultrapure water (1:1) and identified weak or no agonistic and antagonistic activity, except for one sample with black printing. Effects on the androgen as well as estrogen receptors have been suggested to be linked to phthalates, phenols, resin acids and inks, where the antagonistic mode of activity is most prominent [21, 33–35].

The concentration of bis(2-ethylhexyl) phthalate (DEHP) in the black printed sample showing antagonistic activity in Kejlová et al. study was 390 ng/g, while other concentrations of dialkyl phthalates ranged from 520 to 2400 ng/g, except for diisononyl phthalate which was below the limit of quantification. In general, the phthalates concentrations were higher in the black printed sample compared to the non-printed or other colour printed, which lacked antagonistic androgen activities [32]. The chemical analysis, conducted by the Swedish Chemicals Agency, of the paper and board extracts tested in the present investigation and in our previous study, showed that both polar and non-polar extracted FCAs contained DEHP [13, 14]. The non-polar extracted pizza boxes contained low levels of DEHP, determined semi-quantitatively [13]. An additional quantitatively chemical analysis on the same materials was performed after extraction in acetonitrile and water using an ultrasonicator and shaking for 1 h each. The pizza boxes contained among the highest amounts of DEHP compared to other materials (18.1 and 25.2 mg DEHP/kg material) [13].

Aryl hydrocarbon receptor activity

The aryl hydrocarbon receptor activity was examined by the use of the DR-EcoScreen stably transfected cell line. However, the solvent/extraction blanks exhibited a relatively strong AhR activity (64–70% of TCDD maximum), indicating that the samples have been contaminated with AhR active compounds during handling or the evaporation process of the samples. The methodological problem has not been seen before in the blanks in our laboratory, but it is worth mentioning that all samples do not reach the effect level in the blanks. This indicates that contamination does not occur in all samples or that substances with antagonistic effects inhibit the AhR activity in certain samples. New extraction/solvent blanks undergoing the same extraction procedure were tested, in addition to the solvent itself; neither of these obtained any AhR activity. The results for AhR activity should therefore be interpreted with caution and no definite conclusions of the results could be drawn (Additional file 1: Fig. S8). The standard curve of the reference compound TCDD, resulted in the EC_{10} of 0.8 ρM (Additional file 1: Fig. S10F, Table S1).

Previous studies have detected high AhR activity for methanol/water (1:1), ethanol and water extracts made from paper and board using both the DR-EcoScreen cells and H4IIE-CALUX assay [13, 19, 21, 36], where it was proposed to be caused by contamination during the manufacturing processes of the FCMs or natural chemicals within the material itself. Unfortunately, no conclusion could be drawn regarding the AhR activity in our study. Nevertheless, our results demonstrate the importance of including blanks that are treated in the same way as the samples, as it reduces the possibility of false-positive data.

NFκB activity

The NFκB activity was measured with the stably transfected human hepatoma HepG2-NFκB cells. Upon exposure to the FCA extracts, none of the samples exhibited a detectable NFκB response, defined by the cut-off limit of 10% of max effect of TNFα (Additional file 1: Fig. S9). The reference compound TNFα obtained an EC_{10} value of 8.3 ng/mL (Additional file 1: Table S1, Fig. S10G).

The lack of response was also reported in our previous study with polar FCA extracts [13], suggesting that these materials do not contain compounds that induce an inflammatory response or that other models, like the human small intestinal model EpiIntestinal, might be more suitable to measure immunological responses, as done by Kejlová et al. [32].

Bioactivities of polar and non-polar extracts

Altogether, both the polar and non-polar extraction resulted in bioactivities in form of oxidative stress, agonistic ER and antagonistic AR as well as ER for multiple FCAs [13]. No effects were detected for AR agonistic and NFκB responses. The results from both this study and our previous study [13] are summarized in a heatmap (Table 4) showing the lowest observed effect concentration (LOEC) for each extract and toxicity endpoint.

For oxidative stress, some of the same materials were bioactive both as polar and non-polar extracts (Table 4). However, marked differences in potencies were observed. The most prominent example of this was seen for boxes for porridges and flour mixes, where the LOEC was

0.3 mg/mL for the polar extracts and 10 mg/mL for the non-polar extracts (Table 4). Similar results were also seen for pizza boxes, boxes for fries' and hamburgers, boxes for cereals, boxes for infant formula/skimmed milk, popcorn boxes and baking moulds, indicating that the polar substances are the main cause of the activity in those extracts (Table 4).

Interestingly, several of the same materials were bioactive in the antagonistic AR assay for both the polar and non-polar extracts. But baking moulds extracted with the non-polar solvent was more potent and obtained a LOEC of 3 mg/mL, while the polar extracted baking mould only obtained a LOEC of 10 mg/mL (Table 4). The reverse trend in potency was seen for boxes for fries' and hamburgers, where polar substances were more potent and seemed to be driving the antagonistic AR action.

In regards to ER activity, the microwave popcorn bags retrieved a LOEC of 3 mg/mL for both polar and non-polar extracts in the agonistic assay (Table 4). The pizza box, on the other hand, exhibited the highest potency of all samples in the ER assays (LOEC: 1 mg/mL for agonism) for the non-polar extract.

The higher potency of the non-polar extract was also seen in the antagonistic ER assay (Table 4). The results indicate that non-polar substances are driving the ER agonistic and antagonistic effects, but the former was less pronounced.

The Swedish Chemicals Agency performed chemical analyses on the same extracts used in this study, in which they identified substances that exist in printing inks (phthalates, 1,2-cyclohexane-dicarboxylic acid, dinonyl ester; DINCH), plasticizers (phthalates, DINCH), impurities of recyclable materials (phthalates, DINCH, mineral oils, bisphenols, polycyclic aromatic hydrocarbons) and coatings (PFAS) [14]. Chemicals that could explain estrogenic effects are bisphenol A (BPA) and their analogues, benzophenones and certain phthalates [13, 21, 28, 30]. Each of these substances were identified by chemical analysis in at least one FCA group in the present study [14].

The same FCAs were also quantitatively measured after extraction in acetonitrile and water using an ultrasonicator and shaking for 1 h each. BPA was for example then detected in pizza boxes and boxes for infant formula/skimmed milk at concentrations of 18.3–22.0 mg/kg material and 8.2–11.5 mg/kg material, respectively [14]. These package materials were amongst those containing the highest amount of BPA. In pizza boxes, the mean concentration corresponds to a concentration of 0.2 µg BPA/mL extract in the bioassay (0.9 µM). CompTox Chemicals Dashboard bioactivity data for BPA generated two activity concentrations (AC_{50}) values of 0.4 µM and 19.6 µM for agonistic ER activity in VM7 cells [37]. The Organisation for Economic Co-operation and Development (OECD) test guideline 455 reported an EC_{50} value of 0.5 µM in the VM7Luc4E2 cell line [38]. Thus, the ER agonistic activities in polar-extracted pizza boxes may partly be explained by the detected concentration of BPA.

Additionally, the polar and non-polar extract from boxes for infant formula/skimmed milk showed among the highest AR antagonistic activity. This specific sample contained BPA in amounts ranging from 8.2 to 11.5 mg /kg, which corresponds to an average concentration of 0.09 µg BPA/mL in the bioassay (0.4 µM) [14]. In CompTox Chemicals Dashboard, BPA was reported as both active and inactive for AR antagonistic activities [37]. BPA was active for antagonistic activity in for example the human breast cancer cell line MDA-kb2 (AC_{50}: 10.8 µM and 80.1 µM) [37]. The OECD test guideline 458, on the other hand, used BPA as a positive control for antagonistic effects in the AR-Ecoscreen cell line and reported log IC_{30} values from − 7.52 to − 4.48 M (0.03–33.11 µM) [39].

Based on the OECD test guideline, we suggest that antagonistic AR activities in the infant formula/skimmed milk polar extract might partly be explained by BPA.

Migration of chemicals from FCAs and FCMs into food items depends on several factors: physicochemical properties of the chemical, temperatures, exposure to light, composition of the food item itself and storage time [40]. In this study, we used a design that utilized a worst-case scenario extraction and in the future it would be interesting to use a less extensive extraction method or conduct migration testing on the same FCAs that were positive in the extraction experiment. Another aspect for the future would be to consider the potential loss of volatile compounds at evaporation of extracts, which might impact the final results.

As chemical migration from package material to food item may occur, it is necessary to evaluate the safety for the consumers. It has been proposed that effect-based bioassays could be a valuable tool to monitor the presence of these types of hazardous chemicals in FCAs and FCMs, aiming to safeguard the population from exposure to such compounds via food contamination [7, 8]. Of high concerns is the presence of genotoxic activities. A few of the materials that showed genotoxic abilities were polar-extracted pizza boxes and non-polar extracted boxes for fries' and hamburgers. These specific samples also induced oxidative stress, which may be associated with genotoxicity (Table 4) [12]. The endocrine-disruptive effects were often only detected at the highest concentration. Although the results from the study only reflect what migrated from the package material and not in the food item, interaction with food constituents may also have an impact on the adverse health effects [41].

Conclusions

This study utilized a panel of eight effect-based methods to investigate the effects of non-polar extracts made of commonly used FCAs that exist on the Swedish market. Both the AR and ER antagonistic assays detected the highest number of bioactive samples (13/18). Altogether, bioactivities were detected for multiple extracts in all assays. The exemptions were for NFκB and AR agonistic responses, where no effects were detected. The detection rates of all studied extracts were the following: 47% for oxidative stress, 33% for genotoxicity, 72% for antagonistic hormonal activities and 28% for ER agonistic response.

For oxidative stress, the effects seemed to mainly be driven by polar chemicals, while non-polar substances seem to drive the ER antagonistic response. Non-polar chemicals appeared to have low ER agonistic effects. To conclude, the usage of effect-based methods proved to be useful in evaluating the presence of hazardous compounds in FCAs made of paper and cardboard.

Abbreviations

AC: Activity concentration; AhR: Aryl hydrocarbon receptor; AR: Androgen receptor; BPA: Bisphenol A; BEQ: Bioanalytical equivalent concentration; DBP: Di-butyl phthalate; DEHP: Bis(2-ethylhexyl) phthalate; DHT: 5α-Androstan-17β-ol-3-one; DINCH: 1,2-Cyclohexane-dicarboxylic acid, dinonyl ester; DMSO: Dimethyl sulfoxide; E2: β-Estradiol; EC: Effect concentration; ER: Estrogen receptor; EMA: Ethidium monoazide bromide; FCAs: Food contact articles; FCMs: Food contact materials; LOD: Limit of detection; IAS: Intentionally added substances; IC: Inhibitory concentration; IR: Induction ratio; MeCl: Methoxychlor; MMC: Mitomycin C; MN: Micronucleus test; NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NIAS: Non-intentionally added substances; Nrf2: Nuclear factor erythroid 2-related factor 2; OECD: Organisation for economic co-operation and development; OHF: Hydroxyflutamide; Ral: Raloxifene hydrochloride; TAM: Tamoxifen; tBHQ: Tert-butylhydroquinone; TCDD: 2,3,7,8-Tetrachlorodibenzo-dioxin; TNFα: Tumor necrosis factor alpha.

Supplementary Information

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Additional file 1. Additional materials S1 (Sects. 1–7), additional tables S1, S2 and additional figures S1–S10.

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Author contributions

ES and MW conducted the effect-based methods and evaluated the data of the FCA extracts. ES was also responsible for study design and writing the manuscript. GG was responsible for the study details, sample handling, extraction and sample distribution. KS, EG, AO and JL contributed to the study design. All authors contributed to disseminating the results and critically reviewing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Detailed information and additional data are available in the supplement (Additional file 1). Further information will be provided upon request from the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that competing interest that may be considered is that JL and AO are owners of the company BioCell Analytica Uppsala AB which offers effect-based testing services, mainly to the water sector.

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