**Estrogenic activity in dry food simulants: chemical migration from paperboard packaging**

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**Introduction**

Non-inert materials, such as paperboard, can transfer chemicals into the food which are also known as migrating chemicals. Recalls of products have occurred over the years, leading to image loss of the brand and financial consequences[1,2]. NonylPhenols (NPs), BisPhenol A (BPA) and phthalates have been shown to migrate from packaging material into food[3,4,5,6,7]. These chemicals are examples of Endocrine Disrupting Compounds (EDCs). They interfere with endogenous hormone mechanisms and have been associated with many adverse health effects such as obesity, diabetes, fertility changes, prostate and breast cancer[8,9].

The objective of this study was to use the Estrogen Responsive Elements Chemically Activated LUciferase gene eXpression (ERE-CALUX) bioassay to investigate estrogenic activities in paperboard and to evaluate the impact of different aspects of this material such as the use of recycled fibers, the presence of a coating and the use of printing inks. An ERE-CALUX has the advantage that all estrogenic chemicals in a sample extract (known and unknown) can be measured and that mixture effects are taken into account. Samples were tested before they came into contact with dry food. A "worst case" scenario using a direct paperboard extraction was compared to a more realistic scenario in which extraction of the dry food simulant was applied. Estrogenic activities, from the ERE-CALUX bioassay, were directly compared to molecule-specific analysis from instrumental methods.

**Materials and methods**

Paperboard samples were kindly donated by the packaging industry before they are folded and before they come into contact with food. Regulation (EU) 10/2011 describes how to test food packaging for compliance and which food simulant should be used. For this study, poly(2,6-diphenylene oxide) or Tenax® was used as the appropriate simulant. This type of experiment is referred to as "migration", since it only involves substances that migrated from the packaging into the food simulant. Migration experiments were only performed on printed paperboard samples, since they show the highest potential of estrogenic migrating substances[10]. To obtain a "worst case" scenario, the paperboard samples were also directly extracted to determine their full estrogenic potential. An overview of this sample setup is given in Figure 1.

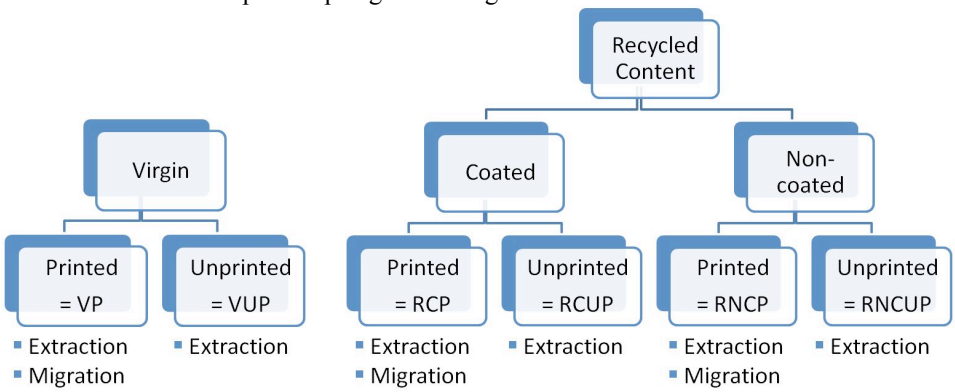


**Figure 1: Printed and unprinted samples from virgin fibers, coated recycled content fibers and non-coated recycled content fibers were prepared for extraction and migration experiments.**

Plasticizers, bis(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), were quantified in all extraction and migration samples with GC-MS. A phthalate cleanup procedure was performed on each subsample and the estrogenic activities of all raw and cleaned extracts and migration experiments were determined.

The overall estrogen activity is analyzed using the ERE-CALUX[11]. Briefly, extracts are diluted and dosed in 10 different concentrations yielding a full dose-response curve fitted with the "non-linear 4 parameter Hill function" to calculate an Effective Concentration on 50% of the curve ($EC_{50}$). The Bioanalytical Equivalent concentration (BEQ), expressed in pg 17β-estradiol (E2) equivalents per square decimeter, is calculated as the ratio of the $EC_{50}$ of the standard and the sample curve.

**Results and discussion:**
The extraction samples are graphically presented in Figure 2, where printed and unprinted paperboard samples of all 3 fibers were tested. All virgin samples had BEQs of < 1000 pg E2 eq./dm2 whereas recycled content fibers had BEQs higher than the virgin paper samples (BEQs of < 3000pg E2 eq./dm2). A two factor ANOVA, with paperboard and printing impact, confirmed this significant difference due to fiber origin, but no significant difference was observed between printed and unprinted samples of the same fiber.

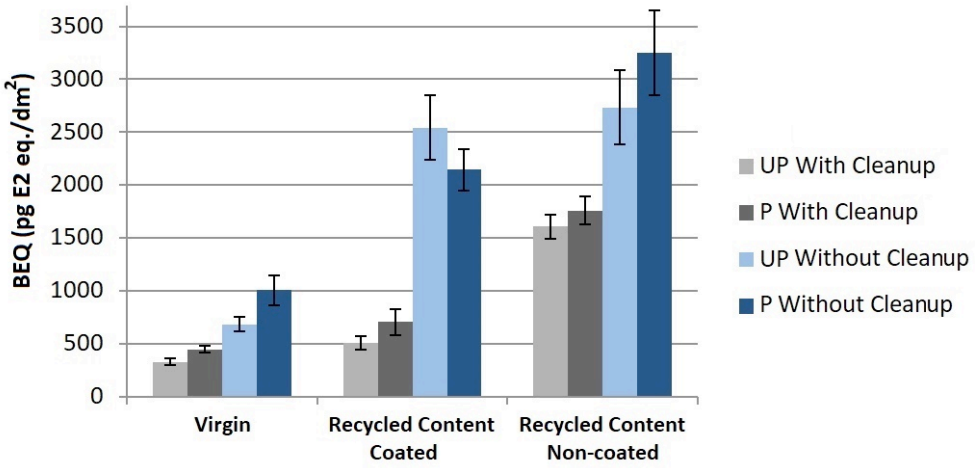


**Figure 2: Bioanalytical equivalent concentrations (BEQ) without blank correction expressed in pg E2 equivalents per dm2 of extraction experiments samples with and without a phthalate cleanup procedure. UP stands for unprinted and P for printed samples. Values are expressed as the mean ± standard deviations of triplicate determinations.**

The extractions and migrations of all printed samples are compared in Figure 3. As expected, the amount of estrogenic compounds migrating to Tenax® is lower than the compounds present in the "worse-case" total extract samples since these samples would contain all of the extractable compounds and not just the ones able to migrate. Additionally, only the food contact side was in contact with Tenax® for migration experiments and not the non-food contact side while both sides are simultaneously extracted for the "worst case" scenario.

The results for the plasticizers in extraction and migration experiment samples are presented in Table 1. It is clear that paperboard samples with recycled content fibers show the highest overall phthalate content. The migration experiments for virgin fibers are not interpretable because of the high estrogenic activity of the Tenax® blank samples. Recycled content printed fibers, coated and non-coated, show similar levels of DEHP and DBP for total extracts and migrations. BBP is not detected in the virgin fibers and its amount in recycled content fibers was lower than about 10% of the levels of DEHP and DINCH in the sample extracts. The most abundantly present compound was DINCH, however, concentration-response analysis revealed that DINCH had no estrogenic activity in the ERE-CALUX bioassay.

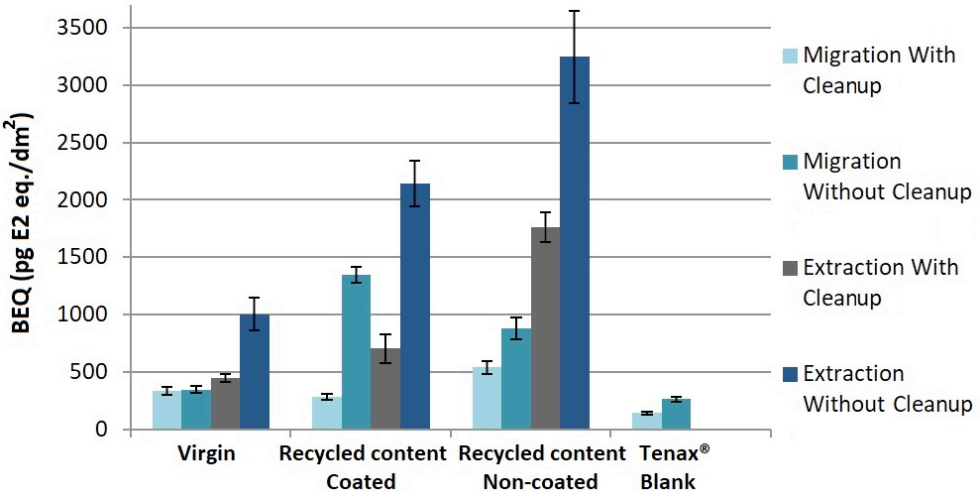


**Figure 3: Bioanalytical equivalent concentrations (BEQ) without blank correction expressed in pg E2 equivalents per dm2 of printed extraction and migration experiments with and without a phthalate cleanup procedure. Values are expressed as the mean ± standard deviations of triplicate determinations.**

| Sample | DEHP (µg/dm²) | DBP (µg/dm²) | BBP (µg/dm²) | *Total phthalates (µg/dm²)* | DINCH (µg/dm²) |
|---|---|---|---|---|---|
| **Extractions** | | | | | |
| V UP | 1.2 | 0.4 | 0 | *1.7* | 19.1 |
| V P | 1.9 | 1.3 | 0 | *3.3* | 33.7 |
| RC UP | 76.1 | 29.6 | 6.4 | *112* | 98.7 |
| RC P | 61.3 | 20.8 | 8.4 | *90.5* | 124 |
| RNC UP | 97 | 40.8 | 10.1 | *148* | 146 |
| RNC P | 104 | 46.2 | 11 | *161* | 157 |
| ACN Blank | 0.4 | 0.3 | 0.2 | *0.8* | 0 |
| **Migrations** | | | | | |
| V P | 3.3 | 3.4 | 0 | *6.5* | 0 |
| RC P | 43 | 16.1 | 3.1 | *62.2* | 77.4 |
| RNC P | 131 | 46 | 18 | *195* | 144 |
| Tenax® Blank 1 | 4.2 | 2.2 | 0.4 | *6.8* | 0 |
| Tenax® Blank 2 | 1.8 | 1.6 | 1.5 | *4.9* | 7.3 |

**Table 1: GC-MS results for bis(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) in µg/dm² for all migration and extraction paperboard samples and blanks for Tenax® and acetonitrile (ACN). Average relative standard deviations (RSD) of 18%, 10%, 11% and 10% for DEHP, DBP, BBP and DINCH respectively. V stands for virgin fibers, RC for recycled content coated and RNC for recycled content non-coated samples. P for printed and UP for unprinted.**

To compare the migration results to current legislation, the values obtained here are expressed per kg simulant and are multiplied by a factor 6, as described in Regulation (EU) 10/2011. The Specific Migration Limit (SML) is currently only valid for migration experiments for plastic food contact materials, but is used here as a reference value for paperboard migrations. The results for recycled content fibers with average Tenax® blank subtraction are presented in Table 2. The DBP results suggest that there might be a problem with the specific migration from recycled content non-coated, since the migration concentration is close to the limit set for plastic food contact materials. Accordingly, it might be interesting to include these compounds in future monitoring. DINCH, BBP and DEHP show values below the SML for the recycled content samples and thus would not be expected to represent a risk.

| Sample | DEHP (mg/kg) | DBP (mg/kg) | BBP (mg/kg) | DINCH (mg/kg) |
|---|---|---|---|---|
| RC P | 0.24 | 0.09 | 0.01 | 0.44 |
| RNC P | 0.77 | 0.26 | 0.10 | 0.84 |
| SML | 1.5 | 0.3 | 30 | 60 |

**Table 2: GC-MS results for bis(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) in mg/kg Tenax® for all migration paperboard samples with blank correction. RC stands for recycled content coated, RNC for recycled content non-coated and P for printed samples. The Specific Migration Limit (SML) of plastic food contact materials for each compound is indicated.**

**Acknowledgements:**
The VM7LUC4E2 cell line was developed by the University of California-Davis (USA) with funding from a Superfund Research Program grant (ES04699) from the National Institute of Environmental Health Sciences.

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