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An effect-directed strategy for characterizing emerging chemicals in food contact materials made from paper and board

Anna Kjerstine Rosenmai [a], Linda Bengtström [b], Camilla Taxvig [a], Xenia Trier [b], Jens Højslev Petersen [b], Terje Svingen [a], Mona-Lise Binderup [a], van Vugt-Lussenburg Barbara Medea Alice [c], Marianne Dybdahl [a], Kit Granby [b], Anne Marie Vinggaard [a, *]

[a] Division of Diet, Disease Prevention, and Toxicology, National Food Institute, Technical University of Denmark, Kemitorvet Building 202, DK-2800 Kgs. Lyngby, Denmark
[b] Division of Food Chemistry, National Food Institute, Technical University of Denmark, Kemitorvet Building 202, DK-2800 Kgs. Lyngby, Denmark
[c] BioDetection Systems B.v., Science Park 406, 1098 XH Amsterdam, The Netherlands

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ABSTRACT

Food contact materials (FCM) are any type of item intended to come into contact with foods and thus represent a potential source for human exposure to chemicals. Regarding FCMs made of paper and board, information pertaining to their chemical constituents and the potential impacts on human health remains scarce, which hampers safety evaluation. We describe an effect-directed strategy to identify and characterize emerging chemicals in paper and board FCMs. Twenty FCMs were tested in eight reporter gene assays, including assays for the AR, ER, AhR, PPARγ, Nrf2 and p53, as well as mutagenicity. All FCMs exhibited activities in at least one assay. As proof-of-principle, FCM samples obtained from a sandwich wrapper and a pizza box were carried through a complete step-by-step multi-tiered approach. The pizza box exhibited ER activity, likely caused by the presence of bisphenol A, dibutyl phthalate, and benzylbutyl phthalate. The sandwich wrapper exhibited AR antagonism, likely caused by abietic acid and dehydroabietic acid. Migration studies confirmed that the active chemicals can transfer from FCMs to food simulants. In conclusion, we report an effect-directed strategy that can identify hazards posed by FCMs made from paper and board, including the identification of the chemical(s) responsible for the observed activity.

1. Introduction

Food contact materials (FCMs) are materials intended to come into contact with foods, from processing equipment through to kitchen appliances and packaging. FCMs thus constitute a vast collection of products that individually can contain a large number of chemicals (Muncke et al., 2014). Humans can be exposed to these chemicals if they migrate to the food (Borchers et al., 2010), which ultimately may contribute towards causing adverse health effects. Since data pertaining to both occurrence and toxicity of a large number of chemicals that can be present in FCMs are limited, it remains difficult to assess what potential risks they may pose to human health. Among the many types of FCMs, those made from paper and board are particularly interesting in this regards, as there are still no specific EU regulations in place for these. Notably, the EU framework regulation from 2011 and 2016 do cover FCMs more broadly, stating that compounds should not transfer from FCMs into food in amounts that can adversely affect human health (EU, 2011, 2016). But since this does not adequately address specific chemical constituents, novel strategies to identify potential hazards from FCMs are needed. This means that more occurrence data needs to be collected alongside robust testing strategies designed to evaluate biological activities of the materials themselves as well as identified compounds therein.

FCMs made from paper and board can contain chemicals that have been either added intentionally as active ingredients, or that occur unintentionally as byproducts, impurities, or degradation products. Compounds may also originate from cellulose-based materials or be introduced through the recycling process.

* Corresponding author.
E-mail address: annv@food.dtu.dk (A.M. Vinggaard).

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Examples of substances detected in FCMs of paper and board are polyfluoroalkyl substances (Schaider et al., 2017), bisphenol A, phthalates (Lopez-Espinosa et al., 2007), mineral oil hydrocarbons (Lorenzini et al., 2010), and heavy metals (Conti, 1997). Some of these are suspected to cause adverse effects, for instance bisphenol A at low doses can affect anogenital distance (Christiansen et al., 2014), disturb mammary gland development (Moral et al., 2008) or behavior in offspring (Xu et al., 2010). Further, some polyfluoroalkyl substances have been reported to cause hepatomegaly, tumor induction in liver, pancreas or testis, developmental effects, and immunotoxicity (Lau, 2012). Collectively, this exemplifies that FCMs of paper and board can be chemically very complex and may contain substances with known adverse effects.

Employing classical approaches such as targeted analysis to characterize the chemical composition of the FCMs and successively testing single compounds for biological activities is therefore inadequate, as it will neither provide any information for compounds that are not explicitly known to be present in the material, nor account for the total, integrated biological activity of all the compounds present in the product– 'the cocktail effect'. To address these shortcomings, an effect-directed strategy could be applied, as exemplified in previous studies by us and others. However, although these earlier strategies were based on *in vitro* tests for genotoxicity, cell toxicity, or endocrine activity, in combination with advanced analytical chemistry to identify the active compounds in FCMs (Binderup et al., 2002; Lopez-Espinosa et al., 2007; Ozaki et al., 2004; Vinggaard et al., 2000; Weber et al., 2006), they only included a few *in vitro* endpoints or a small amount of FCM samples, or failed to fully identify the causative compounds. Thus, an improved strategy is needed to obtain good and broad toxicity profiles, as well as enhancing the identification process.

To enhance existing testing procedures of FCMs made from paper and board, we aimed to develop an effect-directed strategy that combines a broad panel of *in vitro* assays with state-of-the art analytical chemistry. This was done to better facilitate the identification of potential problematic paper and board FCMs, but focused specifically on improving the identification of potentially hazardous compounds. As a proof-of-principle, twenty FCMs of paper and board were partly analyzed by the effect-directed analysis to identify biological activities, of which two FCMs were subjected to the entire step-by-step procedure attempting to identify biologically active constituents.

2. Materials and methods

2.1. Strategy work-flow

The strategy for FCMs of paper and board includes ten steps from extract preparation to identification of compounds with biological activity and determination of migration of these (Fig. 1).

2.2. Test compounds and chemicals

Chemicals used for producing extracts and fractions are described elsewhere (Bengtstrom et al., 2014). All aqueous solutions were prepared using ultrapure water obtained from a Millipore Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). HPLC-MS grade formic acid and a water solution of 25% ammonium hydroxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC TOF-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Standards for the LC-qTOF method: Di-*n*-butyl phthalate (DBP) (CAS: 84-74-2) (99%), deuterated di-*n*-butyl phthalate (d_4-DBP) (CAS: 93952-11-5) (>98%), benzyl-butyl phthalate (BBP) (CAS: 85-68-7) (99%), di-isobutyl phthalate (DiBP) (CAS: 84-69-5) (99%), bisphenol A (BPA) (CAS: 80-05-7) (99%), methylparaben (CAS: 99-76-3) (99%), bisphenol A diglycidyl ether (BADGE) (CAS: 1675-54-3) (95%), perfluorooctanoic acid (PFOA) (CAS: 335-67-1) (95%), abietic acid (AA) (CAS: 514-10-3) (75%), dehydroabietic acid (DHAA) (CAS: 1740-19-8) (95%), isorhamnetin (CAS: 480-19-3) (99%) and rhamnetin (CAS: 90-19-7) (99%) were all obtained from Sigma-Aldrich and 4-oxo-retinoic acid (CAS: 150737-18-1) (98%) were obtained from Santa Cruz Biotechnology, TX, USA. Stock solutions for *in vitro* testing of DBP, BBP, DiBP, BPA, AA, DHAA, isorhamnetin, rhamnetin, and 4-oxo-retinoic acid were prepared in DMSO at 40–50 mM.

2.3. Quantitative structure–activity relationship (QSAR) screening of FCM compounds

A QSAR screen was performed for 2076 known FCM compounds. Initially, a consolidated list of 4041 unique compounds – including additives, monomers, solvents, photo-initiators, dyes, and pigments – was compiled using two publicly available sources: (Council_of_Europe, 2009) and (Federal_Office_of_Public_Health, 2011). Of these, in-house structural information was available for 2076 compounds; the final number included in the QSAR screen consisting of a combination of models for genotoxic carcinogenicity, mutagenicity, developmental toxicity, and endocrine activity. Detailed information on the performance of the individual models, the applied decision algorithms, and the method for preparation of the structure set have been described previously (Wedebye et al., 2015). According to validation results, the applied models have prediction accuracies of 70–85%.

2.4. FCM sample selection and production of extracts

Twenty paper and board FCM samples were obtained from retailers or manufacturers (Table 1). The selection criteria were a) consideration regarding starting material of the FCM (i.e. virgin *vs* recycled), b) the presence of printing inks, c) the intended conditions of use, and d) the type of food used in contact with the material.

The FCM extracts and fractions were prepared as previously described (Bengtstrom et al., 2014). Briefly, double-sided extraction of the FCMs (37–112 dm^2) was performed in 650 mL ethanol for 4 h under reflux, before successively evaporated to an average concentration of 32.8 ± 9.8 dm^2/mL. The two FCM extracts S3 and S7 were subjected to the entire strategy, starting with fractionation by HPLC under both alkaline and acidified eluent conditions. Reproducibility of the extraction method has been published previously (Bengtstrom et al., 2014).

2.5. In vitro testing of extracts, fractions, and identified compounds

In vitro tests were performed using eight reporter gene assays: Androgen receptor (AR), Estrogen receptor (ER), Aryl hydrocarbon receptor (AhR), Peroxisome proliferator-activated receptor γ (PPARγ), Glucocorticoid receptor (GR CALUX), Retinoic acid receptor (RAR CALUX), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2 CALUX), and Transformation-related protein 53 (p53 CALUX), essentially as described previously (Piersma et al., 2013; Rosenmai et al., 2014, 2016; Taxvig et al., 2012; Van der Linden et al., 2008; Vinggaard et al., 2002). All assays were run in agonist mode, however the AR assay was also run in antagonist mode (0.1 nM R1881 added). To validate assay performance, positive control compounds were included: rosiglitazone for PPARγ assay (1E-6 M); 2,3,7,8-tetrachlorodibenzo-p-dioxin for AhR assay (0.5E-12 to 3E-9 M); 17β-estradiol for ER assay (0.36E-12 to 367E-12 M); R1881 (agonist)(1.2E-12 to 2.7E-9 M) and hydroxyflutamide (antagonist) (1E-9 to 5E-6 M) for AR assay; all-trans-retinoic acid for RAR CALUX