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In vitro bioassays: monitoring of bioactive chemical pollutants in food and food contact materials

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Cover: The image depicts a dining table that has various colourful food packages and food items.

The cover art was created by Fanny Sjöblom (sjoblom.fanny@gmail.com)

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Abstract

We are continuously exposed to mixtures of known and unknown chemicals via our diet. The standard control program for food safety, at present, relies heavily on the chemical quantification of a limited number of chemicals with known toxic properties. However, this approach is challenged, as it is impossible to monitor the tens of thousands of chemicals that are produced and spread into the environment. There is hence a need to develop new strategies to understand the potentially hazardous effects of chemical contaminants in food and how these may affect human health.

This thesis consequently focused on developing and applying effect-based *in vitro* bioassays, which allows testing to be done in a high-throughput manner, where effects from known, unknown as well as mixtures of chemicals are taken into consideration. We specifically focused on potential toxic effects that can arise from food packages, liquid smoke flavourings and milk. We found that numerous food packaging extracts induced oxidative stress, genotoxicity, and endocrine disruption activities, while no inflammatory response was induced. Liquid smoke flavourings also revealed oxidative stress and genotoxic effects. On the other hand, several of the milk sample extracts showed aryl hydrocarbon receptor- and endocrine disruptive effects, in the form of antagonistic responses on the estrogen- and androgen receptors. In conclusion, *in vitro* bioassays were highly valuable, as they could detect the effects of complex chemical mixtures that exist within these products. The studies within this thesis show the broad applications of *in vitro* bioassays and that these can be used as an initial screening tool for chemical hazard assessment of food and food packages.

Keywords: Effect-based methods, bioanalytical tools, *in vitro* methods, food packages, paper, cardboard, milk, dairy product, smoke flavouring, food additives

In vitro bioanalyser: övervakning av bioaktiva kemiska föroreningar i livsmedel och livsmedelsförpackningar

Sammanfattning

Vi exponeras dagligen för blandningar av kända och okända kemikalier via vår kost. För närvarande består kontrollen inom livsmedelssäkerhet främst av kemisk kvantifiering av ett begränsat antal kemikalier med kända toxiska egenskaper. Detta tillvägagångssätt utmanas dock, eftersom det är omöjligt att övervaka de tiotusentals kemikalier som produceras och sprids ut i miljön. Det finns därför ett behov av att utveckla nya strategier för att förstå de potentiellt farliga effekterna av kemiska föroreningar i livsmedel och hur dessa kan påverka människors hälsa.

Denna avhandling fokuserade därför på att utveckla och tillämpa effektbaserade *in vitro* bioanalyser, vilka gör det möjligt att kartlägga effekter av kända, okända såväl som blandningar av kemikalier. Vi fokuserade specifikt på potentiella toxiska effekter som kan uppstå från livsmedelsförpackningar, flytande rökaromer och mjölk. Ett flertal extrakt av livsmedelsförpackningarna inducerade oxidativ stress, genotoxicitet och hormonstörande effekter, medan inget inflammatoriskt svar inducerades. Flytande rökaromer visade också oxidativ stress och genotoxiska effekter. Många mjölkprovsextrakt visade däremot arylkolvätereceptor- och hormonstörande effekter, i form av antagonistiska effekter på östrogen- och androgen receptorerna. Sammanfattningsvis visade *in vitro* bioanalyserna sig att vara mycket värdefulla, eftersom de kunde upptäcka effekter av komplexa kemiska blandningar som finns i dessa produkter. Studierna i denna avhandling visar hur brett *in vitro* bioanalyser kan tillämpas och att dessa kan användas som ett initialt screeningverktyg för kemiska faror i livsmedel och livsmedelsförpackningar.

Nyckelord: Effektbaserade metoder, bioanalytiska metoder, *in vitro* metoder, livsmedelsförpackningar, papper, kartong, mjölk, mejeriprodukt, rökaromer, livsmedelstillsatser

Dedication

To my family as well as friends who have cheered me on throughout this journey.

"Vilken risk något utgör för dig är inte beroende av hur mycket det skrämmer dig utan av en kombination av två faktorer. Hur farligt är det? Och hur exponerad är du för det?"
- Hans Rosling, Factfulness: Tio förklaringar till att vi har fel om världen – och varför läget är bättre än du tror

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Selin, E.***, Svensson, K., Gravenfors, E., Giovanoulis, G., Iida, M., Oskarsson, A., Lundqvist, J. (2021). Food contact materials: an effect-based evaluation of the presence of hazardous chemicals in paper and cardboard packaging. *Food Additives & Contaminants: Part A* 38:9, 1594-1607. https://doi.org/10.1080/19440049.2021.1930200

II. **Selin, E.***, Wänn, M., Svensson, K., Gravenfors, E., Giovanoulis, G., Oskarsson, A., Lundqvist, J. (2022). Hazardous chemicals in non-polar extracts from paper and cardboard food packaging: an effect-based evaluation. *Environmental Sciences Europe* 34, 85. https://doi.org/10.1186/s12302-022-00666-4

III. **Selin, E.***, Mandava, G., Vilcu, AL., Oskarsson, A., Lundqvist, J. (2022). An *in vitro*-based hazard assessment of liquid smoke food flavourings. *Archives of Toxicology* 96, 601–611. https://doi.org/10.1007/s00204-021-03190-1

IV. **Selin, E.***, Mandava, G., Karlsson, M., Lundqvist, J. (2023). Evaluation of *in vitro* bioassays as a screening tool to monitor chemical hazards in cow's milk. *Food and Chemical Toxicology* 180, 114025. https://doi.org/10.1016/j.fct.2023.114025

Papers I-IV are reproduced with the permission of the publishers. *Corresponding author.

The contribution of Erica Selin to the papers included in this thesis was as follows:

I. The main responsibility for preparing and performing the study, in addition to compiling literature and writing the manuscript with support from the co-authors. Journal correspondence was managed by her.

II. The main responsibility for preparing and performing the study, in addition to compiling literature and writing the manuscript with support from the co-authors. Journal correspondence was managed by her.

III. Partly responsible for performing the study. In addition to compiling literature, evaluating results and writing the manuscript with support from the co-authors. Journal correspondence was managed by her.

IV. The main responsibility for preparing and performing the study, in addition to compiling literature and writing the manuscript with support from the co-authors. Journal correspondence was managed by her.

Abbreviations

3R	Replace, reduce and refine
ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
AO	Adverse outcome
AOP	Adverse outcome pathway
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATPase	Adenosine triphosphatase
BEQ	Biological equivalence concentrations
BPA	Bisphenol A
CALUX	Chemically activated luciferase gene expression
CHO	Chinese Hamster Ovary
CompTox	Computational Toxicology
DEHP	Bis(2-ethylhexyl) phthalate
DHT	Dihydrotestosterone
E2	Estradiol
EC	Effective concentration
EDA	Effect-directed analysis
EFSA	European Food Safety Authority

EMA	Ethidium monoazide bromide
EPA	Environmental Protection Agency
ER	Estrogen receptor
EU	European Union
ExpoCast	Exposure Forecasting
FAF	Food Additives and Flavourings
FCA	Food contact article
FCM	Food contact material
GR-KO M1	Glucocorticoid receptor-knockout mutant 1
H4	Hickory 4
H5	Hickory 5
HLB	Hydrophilic-lipophilic balance
IC	Inhibitory concentration
KE	Key event
Keap1	Kelch-like ECH-associated protein 1
LLE	Liquid-liquid extraction
LOD	Limit of detection
MAF	Musculoaponeurotic fibrosarcoma
MIE	Molecular initiating event
MN test	Micronucleus test
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NAMs	New approach methodologies
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor erythroid 2-related factor 2

OECD	Organisation for Economic Co-operation and Development
OHF	Hydroxyflutamide
P	Phosphorylate
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-dioxins
PCDFs	Polychlorinated dibenzofurans
PPARγ	Peroxisome proliferator-activated receptor gamma
PXR	Pregnane X Receptor
QA	Quality assurance
QC	Quality control
REF	Relative enrichment factor
ROS	Reactive oxygen species
SciRAP	Science in Risk Assessment and Policy
SPE	Solid-phase extraction
tBHQ	Tert-butylhydroquinone
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TEQs	Toxic equivalents
TK6	Thymidine kinase 6
TNFα	Tumor necrosis factor α
Tox21	Toxicology testing in the 21st century
ToxCast	Toxicity Forecaster
U.S.	United States
WHO	World Health Organisation

1. Introduction

1.1 Background

Food toxicology has mainly focused on using chemical analysis to quantify individual compounds, often with known toxic properties. However, relying solely on chemical analysis poses several challenges, as the total effects of known and unknown chemicals are not covered (Escher et al., 2021a). Chemical analysis can neither detect mixture effects, resulting in that the overall chemical burden remains unknown. The tens of thousands of anthropogenic pollutants that are produced and spread into the environment can be toxic, in addition to many naturally occurring chemicals. These can exist in complex and changing mixtures, thereby highlighting the need to use an alternative and complementary approach to investigate the potentially adverse human health effects.

Effect-based methods are bioanalytical tools used to measure and detect the cellular (*in vitro*) and/or whole organism (*in vivo*) effects posed by chemicals (Brack et al., 2019). *In vitro* bioassays are a subgroup of effect-based methods, which enable complex mixtures of known and unknown chemicals to be analysed. Bioassays can use human or animal cells that have been genetically modified to amplify a response to a specific event (Escher et al., 2021a; National Research Council, 2007). However, bioassays do not only include genetically modified cells, they can also comprise of native cells (i.e. not genetically modified) and measure effects like genotoxicity and cytotoxicity (Escher et al., 2021a).

Even though bioassays as a stand-alone tool cannot quantify individual chemicals or identify the causative toxic chemicals, they instead provide a more holistic view of the complex mixtures of chemicals within a sample. Bioassays can be used together with chemical analysis, in cases when effect-directed analysis (EDA) is performed, to identify and understand the toxicity of the driving chemicals. Hitherto, studies have made great efforts in using *in vitro* bioassays for testing waste-, drinking- and surface waters (Lamoree et al., 2010; Lundqvist et al., 2019; Neale et al., 2020, 2018; Rosenmai et al., 2018), while less attention has been on food items. Therefore, in this thesis, we applied a toxicological screening to monitor chemical contaminants in food and food packages using effect-based *in vitro* methods.

The different studies in the thesis focused on; food packages, a food additive (liquid smoke flavouring products) and the food item milk. We aimed to link the dietary exposome to the potential adverse human health effects. This was accomplished by measuring molecular initiating events (MIEs) and cellular key events (KE), which closely are linked to adverse effects. The samples were extracted and toxicologically characterized using *in vitro* assays in agreement with the 21st century of toxicology testing (National Research Council, 2007).

1.1.1 Food packages

Food contact materials (FCMs) and food contact articles (FCAs) are materials as well as articles intended to come in contact with food items (European Commission, 2004). They will hereinafter be referred to as food packages in this thesis. These materials should be manufactured according to good manufacturing practices and be adequately inert to not transfer their constituents into food in amounts that could endanger human health. In addition, they should not alter the composition of the foodstuff (European Commission, 2004). Yet, this general framework directive does not give any guidance on how to address the chemical hazards of unknown substances or chemical mixtures, underlying the need to adopt a more holistic approach.

Muncke *et al.*, (2020) have previously raised concerns regarding the safety of food packaging. The reason is that chemicals, from both finished food packages and materials used for the manufacturing of food packages, can transfer into food items and subsequently be absorbed by humans.

These migrating chemicals include endocrine disruptors, which can exert their disruptive effects even at low levels (Mertl et al., 2014; Muncke et al., 2020; Rosenmai et al., 2017).

Chemicals can be intentionally- or non-intentionally added to food packages (Peters et al., 2019), as well as naturally occurring in the raw materials used to produce the food packaging. Intentionally-added chemicals, as the name suggests, refer to chemicals that are added for an intentional purpose, like increasing the shelf-life by preventing the growth of microbes or enhancing the stability of the food item by acting as a barrier. The latter oppositely denotes reaction by-products, contaminants from the recycling process and impurities formed during the production of the food package (Groh et al., 2021; Peters et al., 2019). Numerous of these substances, regardless of being intentionally- or non-intentionally added, have scarce or no toxicity data (Groh et al., 2021; Muncke et al., 2020). The present way to study how or if chemicals are transferred from the food packaging is by the following conditions: migration and/or extraction.

Migration measurements represent a more realistic scenario of chemical migration by the use of food simulants (e.g. Tenax, water or organic solvents). Extraction, on the other hand, is often described as the worst-case scenario as it is expected that chemicals will be transferred in higher quantities, for which ultrasonication-, microwave-assisted- or Soxhlet extraction can be used (Peters et al., 2019). Worst-case scenarios are of importance, as they enable emerging chemicals to be identified and larger amounts of substances will leak from the packaged item resulting in a more protective assessment. This approach was therefore applied in **Papers I** and **II**.

The Swedish Chemicals Agency received a governmental assignment to map hazardous chemicals within products as well as goods that heretofore are not yet restricted in the European Union (EU) (Swedish Chemicals Agency, 2020). One of the in-depth mappings involved investigating the occurrence of hazardous chemicals in food packages specifically made of paper and cardboard. There currently does not exist a list of approved or prohibited chemical substances intended to come into contact with food items for paper and cardboard food packaging (Swedish Chemicals Agency, 2021). Therefore, these types of food packages were of particular interest.

Together with the Swedish Food Agency and the Swedish Environmental Research Institute IVL, the Swedish Chemicals Agency conducted literature studies as well as targeted- and non-targeted chemical screening of paper and cardboard food packages. These food packages were available on the Swedish market and the assignment was conducted between the years 2017 to 2020. Non-targeted chemical analysis together with literature studies identified the chemical substances, and based on the analysis specific chemicals were prioritized. The prioritized chemicals were quantitatively analysed using target analysis (Swedish Chemicals Agency, 2021).

The food packaging extracts produced by IVL were kindly provided and used in **Papers I** and **II**. We used these extracts in our *in vitro* bioassay assessment. The testing covered a broad range of toxicity mechanisms that are of high relevance to human health.

1.1.2 Liquid smoke flavoring products

Smoking of food has traditionally been used as a preservation technique (Sikorski, 2004). The use of high temperatures to generate the smoke does not only provide the desired flavour, but it can also generate hazardous carcinogenic compounds. These include polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines and *N*-nitroso compounds (Sikorski, 2004). One modern technique to produce the smoke flavour, without smoking the food item itself, is by the use of the food additive liquid smoke. Liquid smoke products are prepared by pyrolysis, i.e. thermal decomposition of wood with limited oxygen, that subsequently will be condensed and fractionated (Borys, 2004; Sikorski, 2004). The final composition of the liquid smoke depends on several factors, such as the conditions used during pyrolysis, raw material, and purification- as well as the condensation method (Borys, 2004).

The Regulation (EC) No 2065/2003 specifically covers smoke flavourings intended to be used on or in food items (European Parliament, Council of the European Union, 2003). As smoke flavourings can have extensively different physicochemical properties, the regulation evaluates primary tar fraction and primary smoke condensates that together are denoted as primary products. The primary products can further be processed to yield smoke flavourings used in or on food.

The European Food Safety Authority (EFSA) issued several safety assessments of smoke flavouring primary products. It was determined that some of the products had no safety concerns, while others did (EFSA Panel on Food Contact Materials, 2012, 2011a, 2011b). In recent times, EFSA updated the guidance document for smoke flavouring primary products (EFSA Panel on Food Additives and Flavourings, FAF, et al., 2021).

The renewed document outlined that genotoxicity needs to be addressed using a tiered toxicity testing approach, where *in vitro*, *in silico* and *in vivo* data are assessed. Furthermore, reproductive and developmental toxicity testing needs to be included in the Tier I safety data for new authorizations (EFSA Panel on FAF et al., 2021, Appendix E). The evaluated smoke flavourings are largely used within the food industry. Yet, there are numerous smoke flavouring products commercially available to consumers, which **Paper III** addresses. One of the main differences between the smoke flavourings EFSA evaluated and the ones commercially available is that they use different types of wood. To distinguish these, the tested products in the thesis are referred to as liquid smoke flavourings.

Most of the existing studies have mainly focused on genotoxicity and mutagenicity (Braun et al., 1987; Hossain et al., 2013; Ohshima et al., 1989; Putnam et al., 1999). In **Paper III**, liquid smoke flavourings were evaluated with a comprehensive panel of *in vitro* methods to understand the effects of the complex mixtures within these products. Many of these chemicals have limited toxicity data and/or remain unidentified, which stresses the need for an alternative approach to evaluate their safety (EFSA Panel on FAF et al., 2021).

1.1.3 Milk

Milk and milk products are consumed worldwide. It exists limit values in milk for certain contaminants, foreign substances, toxins and other elements (European Commission, Directorate-General for Health and Food Safety, 2023). Two examples of chemical groups that are regulated by the European Commission in raw milk (i.e. unprocessed milk) as well as dairy products are the persistent organic pollutants dioxins and polychlorinated biphenyls (PCBs) (European Commission, Directorate-General for Health and Food Safety, 2023). Several EU regulations exist and cover residue substances, e.g. antibiotics and biocides, in foodstuffs such as milk (Commission

Regulation, 2010). It is imperative though to consider that milk, like any food item, also contains naturally bioactive substances, e.g. estrogen, which of course can affect the bioassay results.

Extensive research has been done on milk, which mainly focused on its composition, specific chemical groups as well as properties (Di Bella et al., 2020; Foroutan et al., 2019; Hasan et al., 2022; Karlsson et al., 2017; Mayilsamy et al., 2022; Schulz et al., 2005). Natural changes in raw milk composition have been explained by e.g. the feed, breed, management system and lactation stage (Fox and McSweeney, 1998). Concerning the feed, Schulz *et al.*, (2005) and Mayilsamy *et al.*, (2022) have for example identified dioxins and dioxin-like chemicals in milk.

Several chemical pollutants have spread into the environment, and these may potentially contaminate the feed and water that the cows consume. This can be of human health concern in the case of the consumption of meat and/or animal-based products. Both dioxins and PCBs can pollute feed and water (Mayilsamy et al., 2022; Schulz et al., 2005). We thus need test systems to detect even previously unknown substances and potential mixture effects in food, for which our **Paper IV** comes into place.

To evaluate *in vitro* bioassays as a potential screening tool for chemical contaminants in milk, milk was collected over one year. The milk samples were then screened with *in vitro* bioassays, covering wide-ranging toxicological parameters such as oxidative stress, hormonal activities and genotoxicity.

1.2 Toxicology testing in the 21st century

Toxicology testing in the 21st century (Tox21) advocates the reduction of traditional animal testing (*in vivo*) and promotes new approach methodologies (NAMs) to test chemicals in a time- and cost-efficient manner that meets the regulatory requirements (National Research Council, 2007). The paradigm shift involves the implementation of *in silico* models as well as high-throughput *in vitro* bioassays, as a screening tool before or instead of whole animal toxicity studies. *In vitro* bioassays give mechanistic information on the combined effects of hundreds to thousands of bioactive chemicals within a sample, which may predict human health-relevant endpoints. These can provide information on the MIE and KE that may be involved in the adverse outcome (AO) in humans. Furthermore, it includes the

implementation of the 3R principle (reduce, replace and refine), when animal testing is needed (National Research Council, 2007).

The new alternative testing has led to the expansion of the Toxicity Forecaster (ToxCast) program by the United States (U.S.) Environmental Protection Agency (EPA), which has led to testing thousands of chemicals (Richard et al., 2016). The U.S. EPA has additionally been involved in the Tox21 chemical library, from which the high-throughput data has been included in the ToxCast program (Richard et al., 2016). Moreover, the U.S. EPA has generated an online dashboard called Computational Toxicology (CompTox, https://comptox.epa.gov/dashboard/), which incorporates data from more than one million chemicals from multiple platforms. For example, ToxCast, Tox21, as well as chemical exposure data and prediction models (Exposure Forecasting, ExpoCast) are integrated into CompTox (Williams et al., 2017). The dashboard thereby provides information on the exposure, *in vitro* tests, physicochemical properties and environmental fate of the chemical. The databases, ToxCast and Tox21, have contributed to the expansion of adverse outcome pathways (AOPs).

1.3 Adverse outcome pathway

An AOP is a framework that connects the molecular initiating event to the AO through intermediate key events (Figure 1) (Leist et al., 2017; Organisation for Economic Co-operation and Development, OECD, 2018a). It was originally used as a tool in ecotoxicological risk assessment and has thereafter been extensively developed to relate effects on the molecular level to adverse outcomes in e.g. humans (Ankley et al., 2010).

The AOP starts with a perturbation on the molecular level called the MIE. Estrogens can for example bind to their receptors and initiate numerous cellular effects, also referred to as KE, such as decreased apoptosis, increased oxidative stress, altered hormonal signalling and DNA damage. The KE may link the different responses together to a resulting AO, like impaired reproduction, breast cancer or death (Figure 1) (Leist et al., 2017, https://aopwiki.org/aops/200). The adverse outcome can take place on an organism or population level, where zebrafish and/or daphnia can be used as alternative animal models to link specific key events to systemic reactions (Figure 1) (Escher et al., 2014). Once the link is adequately established, the

AOP may be used for regulatory applications (like in the development of test guidelines) or in risk assessment (OECD, 2018a). It can be decided that the supporting overall weight of evidence and relevant biological applicability domains (i.e. taxonomy, life stage, sex, etc.,) are sufficient to be used in a risk assessment. Conversely, knowledge gaps may be identified during this process that need to be addressed to increase the level of support of the hypothesized KE/AOP (OECD, 2018a). Either way, the use of *in vitro* test batteries promotes the development of AOP, which in turn supports the 21st century toxicology testing vision (Villeneuve et al., 2014; OECD, 2018b).

In vitro bioassays are valuable when building the AOP, together with other NAMs. The bioassays can in an efficient manner screen a large number of chemicals that cover MIE and KE of different stages within the AOP (Neale et al., 2017). These are described in detail hereafter and their location within the AOP is visualized in Figure 1. The *in vitro* data complemented with the existing tests are overall assessed when evaluating the confidence and relevance of the AOP (OECD, 2018a; Villeneuve et al., 2014). However, it is essential to consider that effects on a cellular level do not necessarily mean an adverse health response on an organism and/or population level (Escher et al., 2021b). As the biological defence- and repair system can become activated in humans and counteract the effects. Despite that, it is essential to understand how the progression from the cellular level into the organ level and ultimately organism/population response occur, which high-throughput data generated from *in vitro* methods to a certain degree can be used for (Gohlke and Portier, 2007). Furthermore, building an AOP is time-consuming and may still require the use of animals, though in a more refined manner than how they formerly have been used.

Monitoring and analysis of food safety is a challenge, but essential to protect the population from chemical hazards. The *in vitro* bioassays used in **Papers I-IV** were used to evaluate a broad range of toxicological parameters and thus filled data gaps. The methods were used as an initial strategy to screen the chemical mixtures within food packages, liquid smoke flavourings and milk. As current risk assessment cannot be done on unknown chemicals, *in vitro* methods could favourably be used in the safety assessment of complex mixtures, which was done for the samples in this thesis.

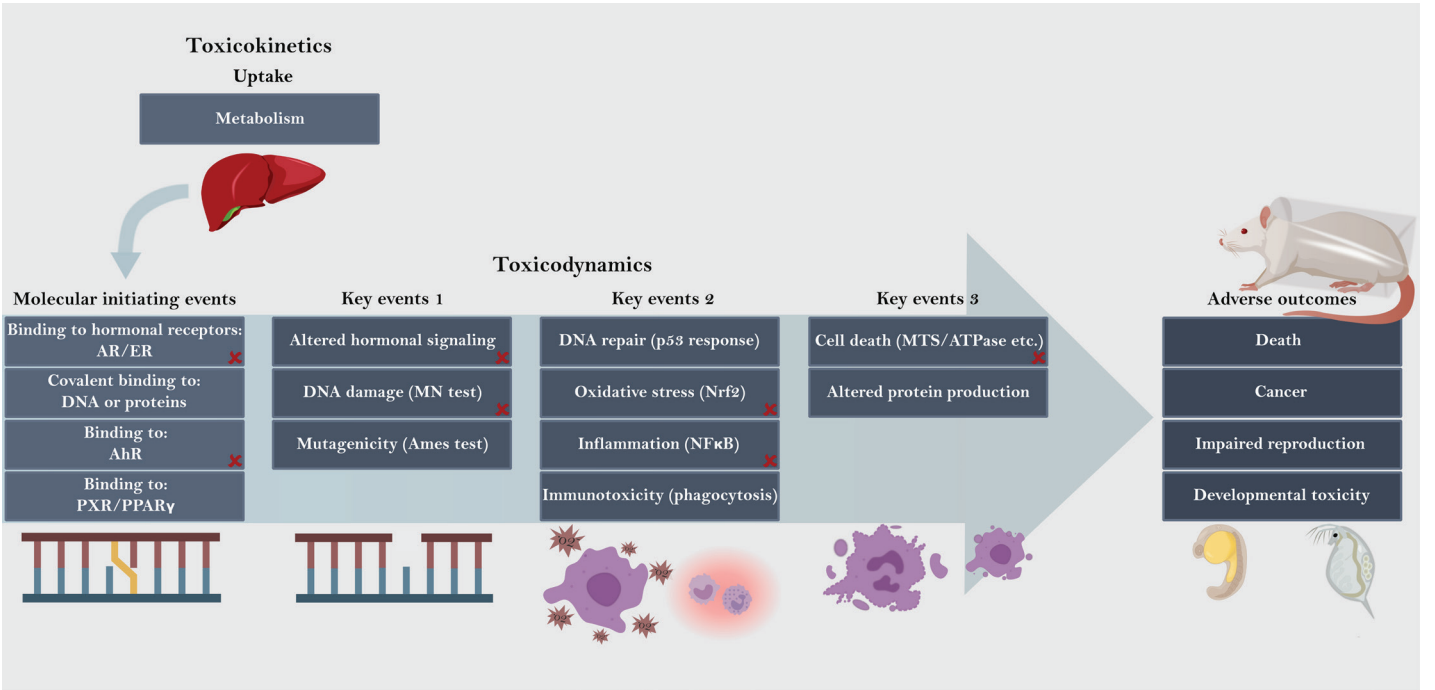


Figure 1. Overview of the adverse outcome pathway concept. The author created the image in Inkscape (v. 1.2.2), but adapted it from Neale *et al.*, (2017). The red crosses inside the text boxes illustrate the events measured, using *in vitro* bioassays, in the thesis. Abbreviations: AR = Androgen receptor, AhR = Aryl hydrocarbon receptor, ATPase = Adenosine triphosphatase, ER = Estrogen receptor, MN test = Micronucleus test, MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, NFκB = Nuclear factor kappa-light-chain-enhancer of activated B cells, Nrf2 = Nuclear factor erythroid 2-related factor 2, PPARγ = Peroxisome proliferator-activated receptor gamma and PXR = Pregnane X receptor.

2. Aims of the thesis

The overarching aim of this thesis was to develop and use *in vitro* bioassays to study the effects of hazardous substances in food and materials coming in contact with food. By using these methods it is possible to measure the biological effects of the entire complex mixtures of substances – without knowing what they are or selecting specific substances.

The specific objectives covered:

- Evaluation of hazardous effects of chemicals within food packages and explore whether polar or non-polar chemicals were driving the biological effects (**Papers I and II**)

- Study the effects of hazardous compounds in liquid smoke flavourings, and examine whether the polar or non-polar chemicals were driving the toxicities (**Paper III**)

- Investigation if *in vitro* methods are appropriate to be used as a screening tool for chemical contaminants in milk (**Paper IV**)

3. Materials and methods

In this section, the study design and methods used in **Papers I-IV** are summarized. Detailed information can be found in the supplementary information for the respective paper.

3.1 Quality control and quality assurance

To ensure that the *in vitro*-based assays retained high reproducibility, reliability and consistent performance over time, several quality control- (QC) and quality assurance (QA) steps were implemented.

One step included testing the performance of the assay, in the form of accuracy, meaning the closeness of the result to its true value (Escher et al., 2021c). It comprised testing four and six concentrations of the sample and reference compound, respectively. The selected reference compound and negative controls were used to validate each run, if the values of these appeared to be deviant the whole dataset from that run was rejected and re-analysed.

An additional step was determining the sensitivity, i.e. the assay's ability to respond to a compound (Escher et al., 2021c). It involves the determination of the limit of detection (LOD) and in our laboratory, the cut-off, meaning the determination of whether a sample was bioactive or not, was set based on the LOD. The cut-off value was often set as the even number above the LOD for the agonist and below the LOD for the antagonist. Moreover, other practical aspects included, if possible, using the same lot of materials (media, cartridges, serum, etc.,), especially during the sample preparation.

Another important consideration was to ensure that the reporting of the results in the *in vitro* studies was done systematically and transparently. Therefore, the web-based tool 'Science in Risk Assessment and Policy' (SciRAP, www.scirap.org) was applied, when applicable (Roth et al., 2021). The tool contains checklists with different criteria, where the methodological- and reporting quality are evaluated. Examples of criteria to be judged were the test system, cell culture conditions as well as funding and competing interest.

All experiments were conducted at least twice with the same principle results, on independent days. The cell passage number did not exceed passage 35 to ensure that the assay performance was not affected.

3.2 Experimental design

The experimental setup is summarized in Figure 2**.** The first step was sample preparation, which covered clean-up and extraction. Sample preparation is essential, as it captures the chemical of interest. Additionally, it ensures that the sample processing itself does not induce any activity and matrices of the samples that may interfere with the reporter gene assays are removed (Escher et al., 2021d).

Extraction in **Papers I-IV** depended on the sample item itself, but overall liquid-liquid extraction (LLE), solid phase extraction (SPE), microwave-assisted extraction and/or ultrasonication were used to capture a wide range of chemical mixtures. To confirm that the sample preparation itself did not induce any effects, extraction blanks were included.

In **Papers I** and **II**, food contact materials/articles were extracted by microwave-assisted extraction, followed by ultrasonication. Methanol was used during the extraction to investigate if polar chemicals were driving the hazardous effects, while acetone:hexane (1:4) was used to study non-polar chemical effects. The study was designed to mimic a worst-case scenario to reveal the toxicological effects that may be of relevance. The initial selection process consisted of 62 food packages made out of paper and cardboard, which were pooled into 23 groups. Similar types of materials were pooled into one group and each group weighted roughly 1 g (Selin et al., 2022b, 2021).

Paper III focused on liquid smoke flavourings. Initially, non-extracted liquid smoke flavourings were tested. These were solely filtered before being used in *in vitro* assays. In addition, we tested extracts of the products, partly by SPE and partly by LLE. By comparing the SPE and LLE, we were able to investigate if it was polar or non-polar chemicals that caused the toxicity. Polar chemicals were extracted with ethyl acetate, whereas hexane was used to extract non-polar substances. For this paper, ten commercially available liquid smoke flavourings were chosen and produced from apple, hickory, mesquite, oak or pecan wood (Selin et al., 2022a).

For the last study, **Paper IV**, clean-up of the milk samples was performed first by precipitation of proteins with acidified acetonitrile. Thereafter, SPE with the Oasis PRiME hydrophilic-lipophilic balanced (HLB) cartridge was applied to remove additional matrices, like fats, proteins, salts and phospho-lipids. The use of the cartridge has proven to retrieve high recoveries of acidic, basic and neutral compounds (Huang et al., 2015). In this paper, we collected conventional and organic semi-skimmed- as well as raw milk for one year, resulting in 32 milk samples in total (Selin et al., 2023).

Upon extraction, all samples were enriched to increase the concentration of the chemicals present in the samples, which thereby increased the sensitivity of the analysis. The extracts were then studied using *in vitro* bioassays.

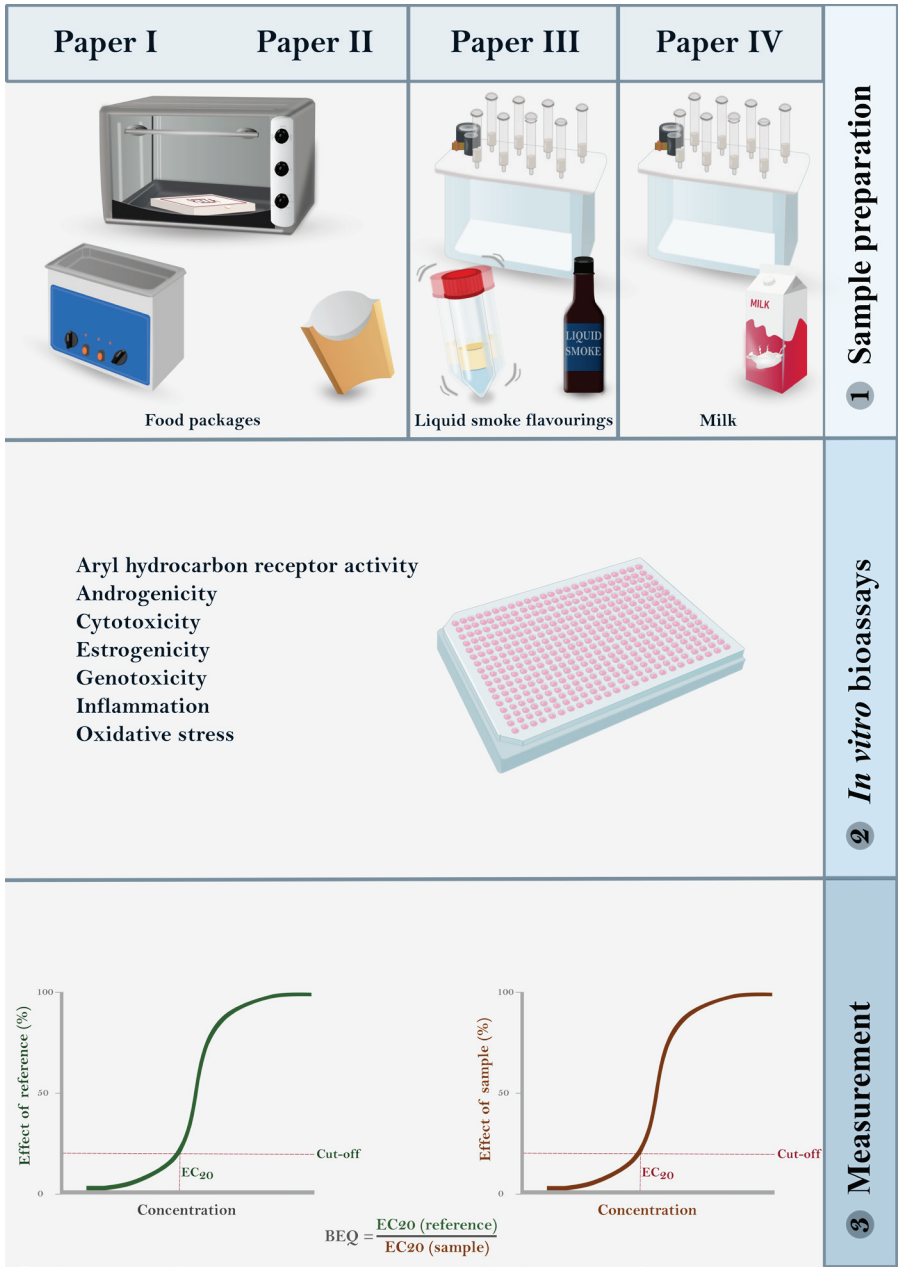


Figure 2. Schematic layout outlining the experimental design. EC_{20} denotes the concentration causing 20% of the max effect. The author created the image in Inkscape (v. 1.2.2). Abbreviations: BEQ = Biological equivalence concentrations and EC = Effect concentration.

3.3 Cell cultures

In this thesis, the aryl hydrocarbon receptor (AhR) response was measured using the mouse hepatocellular carcinoma cell line Hepa1c1c7, called DR-EcoScreen cells. It is stably transfected with the pIND-DCDR7 luciferase plasmid, holding seven copies of the dioxin-responsive elements (Kojima et al., 2018; Takeuchi et al., 2008). The benefits of using the DR-EcoScreen cells are that they are highly sensitive and allow the detection of dioxin-like compounds in addition to other ligands.

Androgen receptor responses (agonistic and antagonistic) were quantified using the Chinese Hamster Ovary (CHO) cell line with glucocorticoid receptor-knockout mutant 1 (GR-KO M1). The AR-EcoScreen GR-KO M1 cell line is stably transfected with a plasmid containing six androgen response elements and a luciferase gene. The expression of luciferase is under control of the binding to the human androgen receptor (OECD, 2020; Zwart et al., 2017). This cell line screens androgens with high selectivity in a reliable manner.

The human adenocarcinoma cell line VM7Luc4E2 identified estrogen agonistic- and antagonistic activities. The cell line has been stably transfected with the pGudLuc7.ERE plasmid comprising of four estrogen-responsive elements and a luciferase gene (OECD, 2021). It expresses ERα as well as ERβ to some extent, allowing a broad range of estrogenic compounds to be detected.

The oxidative stress assay was conducted using the MCF7 AREc32 cells derived from a human breast carcinoma cell line. This cell line has been stably transfected with a pGL3-8xARE plasmid containing a luciferase reporter gene linked to eight copies of the rat glutathione S-transferase antioxidant-responsive elements (Wang et al., 2006). Nrf2-mediated oxidative stress has shown to be one of the pathways that is highly activated by a great number of chemicals and is thus a good candidate to use when studying oxidative stress (Martin et al., 2010).

Genotoxicity was measured with the mammalian lymphoblastoid thymidine kinase 6 (TK6) cells by investigating the micronuclei (MN) formation in the cytoplasm by flow cytometry (Bryce et al., 2013; OECD, 2016).

With its high sensitivity as well as specificity, it is valuable for studying DNA damaging effects (Bryce et al., 2013; Pinter et al., 2020).

HepG2-nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a human hepatocellular carcinoma cell line stably transfected with the NFκB luciferase reporter gene (Signosis, Inc.). The NFκB signalling has essential roles in several biological processes, like inflammation and immune responses. As NFκB is being ubiquitously expressed and impacts several important biological functions, it implies the importance of including it as a toxicological parameter (Brasier, 2006).

All cells were maintained in culture medium at 37˚C with 5% CO_2 in an incubator. The medium was renewed every second to third day, and cells were not used for more than 35 passages, with a few exceptions for TK6 cells.

3.4 Exposures

After cell seeding, cells were incubated for 24 h to become adherent to the 384-well plates. Cells were then exposed to the samples for an additional 24 h to explore the toxicological hazards, which for example included estrogenic-, androgenic- and oxidative stress responses. Lastly, all the activities were quantified using a luminometer allowing dose-response curves to be retrieved (Figure 2).

For genotoxic effects, 96-well plates were used instead and cells were both plated as well as exposed for 24 h on the same day, followed by staining and measurement on the second day on the flow cytometer.

3.5 *In vitro* bioassays

A panel of bioanalytical *in vitro* methods representing important MIE and KE that are closely linked to AO are summarized in Table 1.

The reference compounds used were: 2,3,7,8-tetrachlorodibenzodioxin (TCDD, AhR activity), dihydrotestosterone (DHT, agonistic androgen response), hydroxyflutamide (OHF, antagonistic androgen response), estradiol (E2, agonistic estrogen receptor response), raloxifene (antagonistic estrogen

receptor response), tumour necrosis factor α (TNFα, inflammatory response) and tert-butylhydroquinone (tBHQ, oxidative stress).

The positive controls were 10% DMSO (cytotoxicity), methoxychlor (agonistic estrogen response), tamoxifen (antagonistic estrogen response) and mitomycin C (genotoxicity).

The concentrations for milk samples were expressed differently than the other tested samples, namely as relative enrichment factor (REF), which is unitless. It accounts for both the enrichment factor during the sample preparation and the dilution factor in the bioassays. Milk samples were 100× enriched during the sample preparation and 100× diluted in the *in vitro* bioassays, resulting in the highest concentration of 1. The highest REF was calculated as the enrichment factor at SPE × 0.01 (100× diluted in the cell medium). REF below 1 denotes diluted samples.

Table 1. Toxicological parameters and their corresponding cell line. Abbreviations: AhR = Aryl hydrocarbon receptor, KE = Key event, MIE = Molecular initiating event, MN = Micronuclei, NFκB = Nuclear factor kappa-light-chain-enhancer of activated B cells and Nrf2 = Nuclear factor erythroid 2-related factor 2.

Toxicological parameter		**Cell line**
MIE		
AhR activity	Agonism	DR-EcoScreen
Androgenicity	Agonism	AR-EcoScreen GR-KO M1
	Antagonism	
Estrogenicity	Agonism	VM7Luc4E2
	Antagonism	
KE		
Cytotoxicity		All cell lines mentioned in this table
Genotoxicity	MN formation	TK6
Inflammation	NFκB	HepG2-NFκB
Oxidative stress	Nrf2	MCF7 AREc32

3.5.1 MIE

In principle, the activation of the nuclear receptors (estrogen-, androgen-, and aryl hydrocarbon receptors) are alike and will be summarized hereafter.

In the case of AhR, a ligand like TCDD binds to AhR causing chaperons to dissociate from the receptor, resulting in the ligand-receptor complex to translocate to the nucleus. Within the nucleus, AhR can associate with the AhR nuclear translocator (ARNT) and recruit co-activators, thereby enabling the binding to dioxin-responsive elements on the DNA sequence (Bock, 2019). This affects the expression of genes involved in biotransformation, the immune system, microbial defence and reproduction to name a few (Bock, 2019).

For the steroid hormone receptors (estrogen and androgen), a specific agonist, mix of agonist/antagonist or antagonist can directly bind to the receptor, allowing the receptor to release from its chaperone proteins and bind to a DNA-specific sequence in the nucleus. Thereby increasing or decreasing the transcription of the hormonal-regulated responsive genes (OECD, 2021, 2020; Welboren et al., 2009). The increase and/or decrease may result in adverse endocrine disruptive effects. Both the estrogen- and androgen receptors have critical widespread roles in for example reproduction, foetal development, cancer progression and cell proliferation (Davey and Grossmann, 2016; Welboren et al., 2009).

Cells that have been stably transfected with a luciferase gene under the control of specific responsive elements (i.e. reporter gene cell lines), will upon ligand binding to the receptor, activate the receptor. This causes cells to transcribe and translate the reporter gene. In this thesis, the luciferase gene was chosen as the reporter gene. After the substrate luciferin is added, the luciferase enzyme converts luciferin into a bioluminescent product (light) that is measured with luminescence. The light produced is proportional to the luciferase enzyme (OECD, 2021, 2020) (Figure 3).

3.5.2 KE

For the results from the *in vitro* bioassays to be reliable, it is necessary to verify that the cells used for the analysis have sufficient high viability.
To make sure of this, cytotoxicity is measured. It ensures that the concentrations used are not cytotoxic due to interference with e.g. the cell membrane function and structure (Escher et al., 2021b).

One of the assays used to investigate cytotoxicity was the colorimetric method [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (OECD, 2018b). The principle of the assay is that metabolically active cells can reduce the MTS tetrazolium compound to the soluble formazan product. The proportion of formazan product corresponds to viable cells and is measured in the form of absorbance (OECD, 2018b; Promega, 2023a; Riss et al., 2013). This method was used for all cell lines, except TK6- and VM7Luc4E2 cells, which instead utilized ethidium monoazide bromide (EMA) dye as well as the adenosine triphosphatase (ATPase) assay, respectively.

The fluorescent nucleic acid dye EMA labels necrotic and late-stage apoptotic cells (Avlasevich et al., 2011; Riedy et al., 1991). The stain will crosslink to the nucleic acid of dead and dying cells with compromised membrane integrities after exposure to light. One of the major benefits of it, compared to other viability stains like propidium iodide, is that it will not leak out of the cells as it crosslinks with the DNA. Furthermore, gating of the dead- and dying-cell population can easily be distinguished by EMA, which is not possible to do using solely forward- and side-scattering properties on the flow cytometer (Riedy et al., 1991).

The ATPase assay is a bioluminescent test that quantifies the total ATP content in active cells (Promega, 2023b). It contains a luciferin substrate, ATPase inhibitors to stabilize the ATP and a lytic component that lyses the cells to release ATP. The cellular ATP released from the lysed cells will activate luciferin, which drives the luciferase reaction that produces photons of light. The light emitted is correlated with the ATP content and the number of metabolically viable cells can be measured via luminescence (Promega, 2023b; Riss et al., 2013). The measurement detects the ATP content of a cell population and does not provide single-cell information (OECD, 2018b).

Besides cytotoxicity, key events were measured in the form of oxidative stress, inflammation and DNA damage. Activation of these is triggered by many cellular stressors. For example, oxidation, DNA damage, electrophiles and hypoxia, can cause a sensor molecule to release from the transcription factor. The transcription factor can thereby translocate to the nucleus and bind to specific responsive elements on the DNA that trigger gene expression

(Simmons et al., 2009). The induction of key events does not only necessarily induce one type of mechanism, it can during e.g. DNA damage activate a variety of sensors, transducers and effectors forming diverse complex signal cascades.

The Nrf2 pathway, measuring oxidative stress response, can be initiated by different sorts of stimuli or inducers. These include electrophilic compounds and reactive oxygen species (ROS) that will phosphorylate (P) both Nrf2 and its anchoring Kelch-like ECH-associated protein 1 (Keap1) (Simmons et al., 2009; Wang et al., 2006). As the electrophilic compounds will induce a conformational change in Keap1, Nrf2 is prevented from being degraded and promotes trafficking to the nucleus. Once in the nucleus, Nrf2 forms a heterodimer with transcriptional regulatory proteins like the small musculoaponeurotic fibrosarcoma (MAF) family. Resulting in binding to antioxidant-responsive elements that can drive the transcription of genes involved in biosynthesis and detoxification processes. The luciferase produced, from stably luciferase transfected cells, corresponds to the Nrf2 activity (Figure 3).

One transcription factor that regulates inflammation is the NFκB. After an inducer like lipopolysaccharides or ROS is introduced, the repressor kinase IκB becomes phosphorylated and degraded. Allowing NFκB to become activated (Brasier, 2006). Activation results in NFκB entering the nucleus and binding to its response element on the promoter region of target genes. Resulting in increased expression of genes involved in inflammation, cardiovascular regulation and proliferation (Brasier, 2006).

Last, genotoxicity was ascertained by scoring the formation of micronuclei on a flow cytometer. The method utilizes two stains, the EMA- and SYTOX® Green stains (Avlasevich et al., 2011; Bryce et al., 2013; Litron Laboratories, 2018). The former labels late-stage apoptotic and necrotic cells, as previously mentioned. Cells are thereafter lysed and stained with the latter nucleic acid dye SYTOX® Green, which labels all chromatin. It differentiates liberated nuclei from the micronuclei based on the DNA-dye fluorescence intensities. In this way, healthy cells (EMA negative/SYTOX positive) cells can be distinguished from the dead or dying cells (EMA positive/SYTOX positive), which will be gated out (Avlasevich et al., 2011).

This robust test method thus concurrently measures cytotoxicity and genotoxicity. It can quantify both clastogens and aneugens, in cell lines like TK6 and CHO-K1, thus providing a comprehensive overview of chromosomal damage (Bryce et al., 2013).

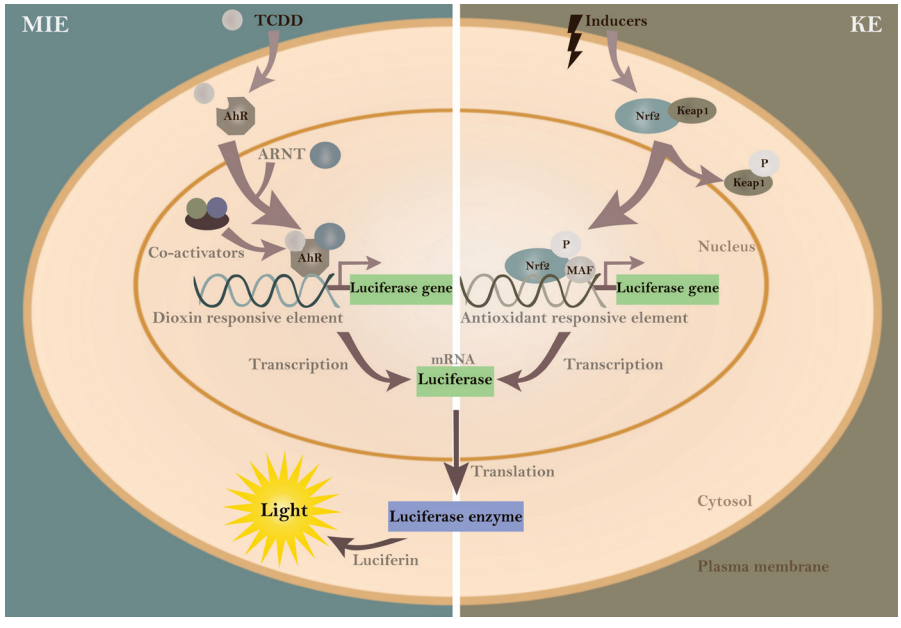


Figure 3. Activation of a MIE (left) and KE (right), namely the aryl hydrocarbon receptor and oxidative stress measured by Nrf2 activity, respectively, using reporter gene cell lines. The illustration was created by the author in Inkscape (v. 1.2.2), but it was inspired by Allard and Kopish (2008), Kojima *et al.*, (2018), Zhao *et al.*, (2021) and Larigot *et al.*, (2018). Abbreviations: AhR = Aryl hydrocarbon receptor, ARNT = Aryl hydrocarbon receptor nuclear translocator, KE = Key event, MIE = Molecular initiating event, Nrf2 = Nuclear factor erythroid 2-related factor 2, P = Phosphorylate and TCDD = 2,3,7,8-Tetrachlorodibenzo-p-dioxin.

3.6 Data evaluation

The data obtained from the micronucleus test was statistically evaluated in the software GraphPad Prism (San Diego, California USA) using One-Way ANOVA with Dunnett's multiple comparison tests. P-values <0.05 were defined as statistically significant. The acceptability criteria were ensured to be fulfilled according to the OECD guideline (2016).

For the remaining assays, the standard curves of the reference compounds were retrieved by fitting the data to a four-parameter non-linear regression or linear regression curve fit, depending on the toxicological parameter (Escher et al., 2018, 2014). Moreover, samples were classified as bioactive based on the cut-off limit, and the cut-off limit was set based on the LOD as previously mentioned (Escher et al., 2018). If a sample was considered bioactive, the biological equivalence concentrations (BEQ) were calculated to relate the effect of the sample to the corresponding concentration of a well-established reference compound (Escher et al., 2021e, 2018).

4. Results and discussion

The main results from **Papers I-IV** are summarized and discussed in the subsequent sections. Further details can be found in the individual papers and their supplementary information.

4.1 Food packages

There is a need to evaluate the toxicological effects of food packages, as chemicals may migrate from the package item into the foods and only limited information about the chemicals within the food packages exist. Therefore, in **Papers I** and **II**, MIE and KE were evaluated. A few of the MIE and KE will be focused on hereafter.

MIE

None of the samples caused androgen receptor agonistic activity, while many of the same types of food package extracts were bioactive (9 or 13 samples) in the estrogen- and androgen receptor antagonistic assays, regardless of being extracted with a polar or non-polar solvent (Table 2).

A few materials were more potent in the non-polar fraction like baking moulds, whereas other packages e.g. boxes for fries' and hamburgers in the AR antagonistic assay were more potent in the polar fraction. Antagonistic activities of the androgen receptor have formerly been reported in multiple paper and cardboard food packaging extracts (Rosenmai et al., 2017). For one of the samples, namely the sandwich wrapper, effect-directed analysis was performed by Rosenmai *et al.*, (2017) and the two paper resin acids, abietic acid and dehydroabietic acid, were identified to be the likely causative drivers of androgen receptor antagonism.

Another study identified higher concentrations of bis(2-ethylhexyl) phthalate (DEHP) in a black-printed sample with antagonistic activity, compared to non-bioactive non-printed or other colour printed samples (Kejlová et al., 2019).

The chemical analyses performed in the governmental assignment, using the same samples as in **Papers I** and **II**, showed that both polar- and non-polar extracts contained phthalates like DEHP (Swedish Chemicals Agency, 2021).

The agonistic activity of the estrogen receptor was less pronounced (3 or 5 samples were bioactive). Chemicals that could explain the estrogenic effects include benzophenones, certain phthalates and bisphenol A (BPA) as these previously have been identified as the drivers of estrogenicity (Lopez-Espinosa et al., 2007; Rosenmai et al., 2017; Vinggaard et al., 2000). Each of these chemicals was identified in at least one food package group in this study (Swedish Chemicals Agency, 2021).

KE

The toxicological evaluation of the food package extracts revealed that none of the food packages exerted an inflammatory response. On the other hand, both polar and non-polar extracts induced oxidative stress and genotoxicity (Table 2). Several of the extracts were active in the oxidative stress assay, regardless of being extracted with a polar or non-polar solvent. The polar extracts were predominately more potent than the non-polar extracts. One of the most visible examples of this is for boxes for porridge and flour mixes, where the lowest observed effect concentration was 0.3 mg food package/mL cell culture media for the polar extract and 10 mg/mL for the non-polar extract (Table 2).

In agreement with our results, Rosenmai *et al.*, (2017) observed oxidative stress activity in 80% of the ethanol-extracted paper and cardboard food packages. The causative chemical(s) of oxidative stress is currently not known, our results indicate that polar chemicals are in general more potent. However, as we did not conduct an effect-directed analysis it is not possible to identify the compounds causing the oxidative stress response. Nonetheless, Escher *et al.,* (2013) have formerly shown that oxidative stress is a very responsive assay and that only a small fraction (<0.1%) of the observed effects was explained by the detected chemicals in water samples.

Table 2. Heatmap of no observed effect concentrations (NOEC) and lowest observed effect concentration (LOEC) of the polar and non-polar food package extracts for the majority of the *in vitro* bioassays. The concentrations are expressed as mg food package per mL cell culture medium. The table is reproduced from (Selin et al., 2022b) with modifications made in Inkscape (v. 1.2.2). Abbreviations: AR = Androgen receptor, ER = Estrogen receptor and N/A = Not Applicable.

Assay	Oxidative stress		Genotoxicity		AR				ER			
					Agonism		Antagonism		Agonism		Antagonism	
Food contact article	Polar	Non-polar	Polar	Non-polar	Polar	Non-polar	Polar	Non-polar	Polar	Non-polar	Polar	Non-polar
Bag for cookies												
Baking moulds												
Board samples												
Boxes for cereals												
Boxes for cookies (from the supermarket)												
Boxes for cookies (from the manufacturer)												
Boxes for fries' and hamburgers												
Boxes for infant formula/skimmed milk												
Boxes for porridge and flour mixes												
Cake/pastry boxes/mats												
Coloured paper for baking moulds												
Hamburger/French fries' papers												
Microwave popcorn bags												
Packages for frozen food												
Paper for baking and baking moulds												
Paper for trays												
Paper plate (coated)												
Paper plate for warm food												
Papers for wraps												
Pizza boxes												
Pizza slice trays												
Popcorn boxes												
Straws												
∑Bioactive samples/assay	13/23	9/19	4/4	1/3	0/23	0/18	13/23	13/18	3/23	5/18	9/23	13/18

N/A | Not tested | NOEC = 10 mg/mL | LOEC = 10 mg/mL | LOEC = 3 mg/mL | LOEC = 1 mg/mL | LOEC = 0.3 mg/mL

The half-cross denotes the sample was cytotoxic but bioactive, and was thus not included in the total sum of bioactive sample/assay. Interpret with caution. The white box denotes 'N/A', meaning that these samples had precipitation problems and were consequently excluded from being tested. NOEC means that no concentration showed activity.

The importance of studying food packages and the benefits of using in vitro methods to do so

One way to evaluate the hazards of unknown and known chemical mixtures in food packages is by the use of *in vitro* bioassays, which previously has been proposed by Muncke, (2009). Endocrine-disrupting chemicals that are known to exist in food packages include phthalates (diethyl phthalate, dibutyl phthalate, diisodecyl phthalate, etc.,), phenols (4,4′-biphenol, BPA, etc.,) and benzophenones (4,4′-dihydroxybenzophenone, 2,2-dihydroxy-4-methoxybenzophenone). These can be intentionally added, e.g. in the form of monomers (like bisphenols) or additives (such as phthalates or benzophenones) that are used to retrieve a specific property of the material (Muncke, 2009). However, issues arise when migration occurs. Vinggaard *et al.*, (2000) found that xenoestrogens migrated from kitchen rolls. Besides intentionally added substances, non-intentionally added substances are also present in food packages as previously stated (Groh and Muncke, 2017). They are often unknown, can be produced during different stages of the manufacturing/production processes and are difficult to control (Groh and Muncke, 2017). As the final food package consists of a mixture of known and unknown chemicals that can act in an antagonistic or agonistic manner, it is essential to study the effect(s) of the food package as a whole. This is where effect-based *in vitro* bioassays can add new knowledge and discover completely new hazards. The use of bioassays provides a mechanistic insight into the chemical mixtures.

A particularly remarkable find was the genotoxic effects measured in some of the extracts, underlining the need to further assess the safety of food packages.

4.2 Liquid smoke flavorings

In the following study, **Paper III**, we applied another type of sample to the *in vitro* methods, namely liquid smoke flavorings.

In the study, ten different types of smoke flavorings were used and tested in non-extracted and extracted formats. We wanted to understand the drivers of toxicity by comparing extracts of liquid smoke flavorings produced with the more polar solvent ethyl acetate against extracts produced with the non-polar

solvent hexane. Additionally, as previously mentioned, two forms of extraction were performed; the traditional LLE and modern SPE.

The complete results for all products studied are published in **Paper III**. In the following discussion, however, we only focus on the results from two out of the ten commercially available liquid flavorings. In **Paper III**, these hickory wood liquid smoke flavorings were referred to as hickory 4 (H4) and 5 (H5). The reason why these two were chosen to be discussed is that these were among one of the few products that were extracted with both SPE and LLE, in addition to having estimated intakes expressed as tBHQ equivalents above the acceptable daily intake (ADI).

MIE

Activities tested included AhR, estrogen receptor agonistic activity as well as androgen receptor responses (agonistic and antagonistic). A few of these will be discussed hereafter.

When observing H4 and H5, only solid-phase extracted H4 was bioactive in the AhR assay, while non- and liquid-liquid extracted samples remained inactive (Fig 3 in **Paper III**). It is well-known that PAHs are generated during the formation of smoke, for which its concentrations increase with higher temperatures (Šimko, 2005). The aryl hydrocarbon receptor can be activated by various chemicals, which include PAHs (Boonen et al., 2020). The solid-phase extraction appeared to have removed the cytotoxic substances from the sample, making it possible to analyze it at higher concentrations and thus detect AhR activities which for the other extracts were hidden by cytotoxicity.

The packaging material used has been shown to impact the concentrations of PAHs (Guillén et al., 2000; Šimko, 2005). Lower concentrations of certain PAHs have been observed when storing liquid smoke flavorings in polyethylene bottles compared to glass bottles (Guillén et al., 2000). This may be explained by sorption onto the plastic. Further studies should investigate the impact of packages. Especially those made of recyclable plastic. It would also be of interest to analyze the same product if available in different packages (e.g. the same product being available in both plastic and glass bottles).

For estrogen agonistic activity, non-extracted hickory samples 4 and 5 were inactive (Fig SI-6 in **Paper III**). Upon solid phase extraction, H4 became bioactive, whereas H5 was below the cut-off limit. Hickory sample 4 showed to also be bioactive when being liquid-liquid extracted with hexane. Interestingly, hickory sample 5 was more potent when extracted with ethyl acetate than with hexane (Fig SI-6 in **Paper III**).

KE

Oxidative stress was enhanced by both H4 and H5, irrespective of being extracted or not (Figure 4). The SPE- and non-extracted samples obtained a similar induction in fold change at the highest non-cytotoxic concentration, but the activity was observed only at much higher concentrations for the SPE extracts. Interestingly, LLE with hexane showed a drastically lower response than ethyl-acetate LLE samples (Figure 4). The H4 and H5 samples, extracted with ethyl acetate, were considerably more potent than the hexane-extracted sample. It shows that the extraction procedure has a large impact on the oxidative stress activities and the polar substances appear to be the main driver of oxidative stress.

As oxidative stress is one of the multiple mechanisms that can drive genotoxic effects, we tested a few concentrations of H4 and H5 in the micronucleus test. Upon SPE, the micronuclei formation statistically significantly increased to a higher degree than the non-extracted samples (Figure 4). Though, the concentrations for the SPE samples were much higher, as cytotoxicity appeared at a lower concentration for the non-extracted samples and were thus excluded. Furthermore, the hexane LLE samples showed a significant increase in genotoxicity for nearly all concentrations for both H4 and H5 (Figure 4).

The results suggest that non-polar substances are driving the genotoxicity, even though these were tested at higher concentrations. Previous studies have confirmed DNA damaging effects in the form of increased DNA single-strand breaks (Ohshima et al., 1989), alterations in rats' pyloric glands upon oral exposure to hickory-smoke condensate (Shichino et al., 1992) and enhanced p53 response after exposure to liquid smoke flavorings (Hossain et al., 2013).

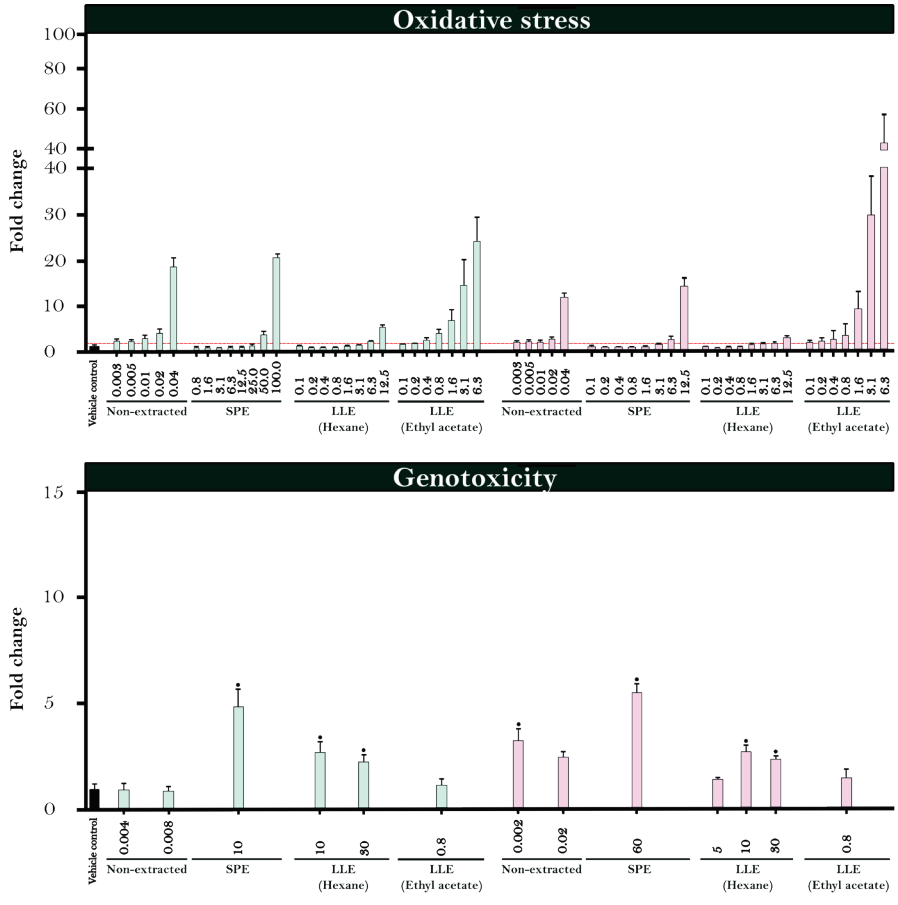


Figure 4. Oxidative stress and the genotoxic response of the commercially available liquid smoke flavour hickory from two different manufacturers. The figure is reproduced from (Selin et al., 2022a) with modifications made in Inkscape (v. 1.2.2). The mint green bars represent hickory 4, while the lilac bars signify hickory 5. Concentrations on the *x*-axis are expressed as µL liquid smoke flavouring/mL cell culture medium. Samples that were statistically significantly different from the control are indicated with an asterisk (*p value < 0.05). Abbreviations: LLE = Liquid-liquid extraction and SPE = Solid phase extraction.

Using in vitro methods as an alternative approach to understand the effects of the complex chemical mixtures of liquid smoke flavourings

The effects of the hickory liquid smoke flavourings purchased from different manufacturers varied to a large degree. This is likely dependent on factors like the raw materials used, pyrolysis process, purification method and chemical content (Budaraga et al., 2016; Sikorski, 2004).

These factors probably influence the type of bioactive substance that is present in the product, which may have potential mixture effects.

The commercially available liquid smoke flavourings used in the study had poor information on the identity and manufacturing process, compared to those registered as smoke flavourings.

Both hickory samples 4 and 5 had BEQ values above the acceptable daily intake of tBHQ for one serving size, indicating that hazardous compounds exist in these products and should be investigated further to ensure their safety. Using *in vitro* bioassays to study these effects shows great potential, as it accounts for mixture- and potential additive effects, which is what we are exposed to. It would be of great benefit if researchers could collaborate with the manufacturers of these sorts of products in order to understand how the different effects of the same liquid smoke flavour occur.

4.3 Milk

In the last paper, **Paper IV**, we investigated if effect-based *in vitro* methods could be used as a monitoring tool for chemical contaminants in milk samples. In total, 32 milk samples were collected throughout approximately one year (weeks 26 2020 to 21 2021) and consisted of both organic and conventional semi-skimmed- as well as raw milk. As no major differences between the conventional- and organic milk were observed, the results of them were combined in Table 3.

MIE

The agonistic activity of the hormonal receptors (androgen and estrogen) showed no activity throughout the year, regardless if raw- or semi-skimmed milk samples were tested (Table 3). The non-existing activity of agonistic activity is great both in regards to using effect-based *in vitro* methods as a screening tool, but also for human health.

The remaining assays, i.e. the antagonistic hormonal- and aryl hydrocarbon receptors had stronger responses. Approximately 31% to 41% of the 32 milk samples were positive in these assays (Table 3). One important aspect to consider in Table 3 is that many of the samples tested in AhR and AR were only analyzed at lower REF concentrations (<REF 1), in addition to one sample in the ER assay, as these were cytotoxic at higher REF.

As both the conventional- and organic milk samples did not show any major differences, the cytotoxic responses of these were also combined in Table 3, where the most potent cytotoxic concentration is highlighted by a symbol. Irrespective, one of the most responsive assays was AhR.

Former studies used chemically activated luciferase gene expression (CALUX) bioassay to determine the concentrations of polychlorinated dibenzo-dioxins (PCDD), polychlorinated dibenzofurans (PCDFs) and/or PCBs in milk samples (Chou et al., 2008; Mayilsamy et al., 2022). Chou *et al.*, (2008) retrieved concentrations below the previously set threshold limit of 3 ρg World Health Organisation (WHO)-toxic equivalents (TEQs)/g fat (European Commission, Directorate-General for Health and Food Safety, 2006). Mayilsamy *et al.*, (2022), conversely, retrieved values both below and above the prior set threshold limit.

Since the beginning of this year, the max level of the sum of dioxins was lowered to 2 ρg WHO-PCDD/F-TEQ/g fat (European Commission, Directorate General for Health and Food Safety, 2023). This resulted in that all of our bioactive samples were above the threshold limit. However, it is essential to consider that the BEQ calculated from the AhR assay is not directly comparable to the WHO-TEQ. The reason for this is that the BEQ values calculated from our results cover the sum of biological effects of dioxin- and dioxin-like compounds, which also are included in the WHO-TEQ system, in addition to thousands of other chemicals that can activate AhR. To understand the fraction of BEQ that constitutes the WHO-TEQ substances in the samples, a chemical analysis needs to be performed, which was not done in this study.

There are heretofore only a few studies available investigating the hormonal effects of milk, especially antagonistic (inhibitory) effects. Many studies have rathered focused on identifying specific compounds using chemical analysis (Courant et al., 2007; Mustonen et al., 2009; Steinshamn et al., 2008), shedding light on the need for further research to understand if the background levels are due to pollutants or naturally occurring substances.

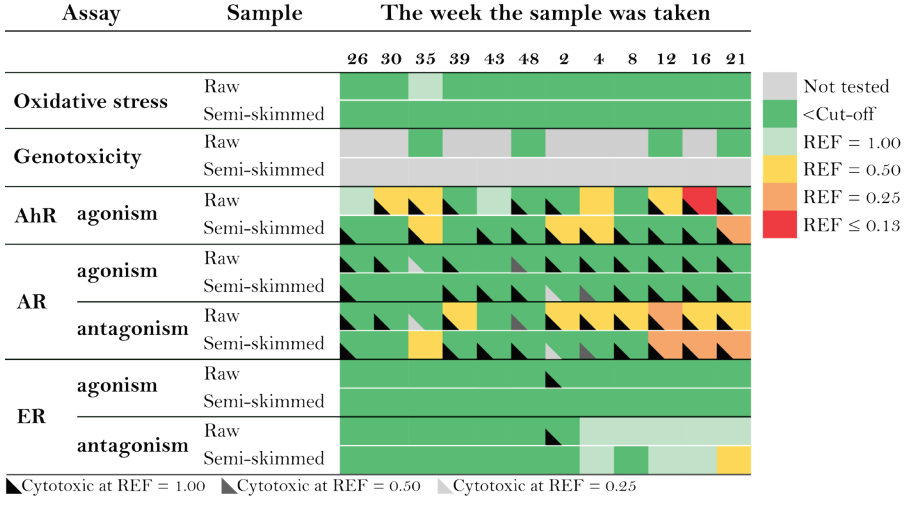
KE

No or low responses were seen for oxidative stress and genotoxicity (Table 3). Only one out of 32 milk samples induced oxidative stress (in the form of Nrf2 activity).

Rather than investigating oxidative stress *per se*, another study has reported changes in the composition of different milk samples (lipid oxidation, degradation of vitamin A, etc.,) with longer storage time as well as in clear storage bottles compared to pigmented bottles (Zygoura et al., 2004).

The general lack of background activities of oxidative stress and genotoxicity is of great benefit from a monitoring perspective, as the chance of detecting contaminants is increased. The induction of oxidative stress that was observed in one case, is also valuable from a methodological standpoint as it demonstrates that oxidative stress-causing pollutants can be captured if present within the milk.

Table 3. Heatmap presenting the effect concentrations (EC) expressed as relative enrichment factors (REF) at $EC_{IR1.7}$ (oxidative stress), EC_{10} (AhR- and AR agonism) and EC_{20} (ER agonism). The inhibitory concentrations (IC) are expressed as REF at IC_{30} (AR- and ER antagonism). The semi-skimmed- and raw milk samples were collected during weeks 26 (year 2020) to 21 (year 2021). The heatmap was created in Inkscape (v. 1.2.2). Abbreviations: AhR = Aryl hydrocarbon receptor, AR = Androgen receptor and ER = Estrogen receptor.



Monitoring of milk by utilizing in vitro methods

One interesting aspect was the seasonal variation in the antagonistic response of the estrogen receptor. No effects were observed until week 4 and it persisted until week 21. Repeated sampling needs to be done to explain the cause of the variation.

Combining *in vitro* bioassays with effect-directed analysis may allow potential causative chemicals to be identified (Brack et al., 2016). For AhR and AR (antagonistic), the activities occurred on and off throughout the year but varied quite a bit between the samples. Using a similar approach to what was mentioned above, with effect-directed analysis, may identify the driving chemicals of these activities. Still, as milk has a high turnover on the market the methods would rather be used seasonally, than daily, to capture the effects.

4.4 General discussion

4.4.1 Toxicity testing using *in vitro* bioassays

Advantages and limitations

Even though there are many benefits to using *in vitro* bioassays, there also exist pitfalls. One of these is the lack of metabolic capacity of certain cell lines. Metabolism is of importance when studying for example genotoxicity or endocrine disruptive activities (i.e. effects on the hormonal receptors), as it can result in more toxic chemicals being produced, less toxic metabolites being generated, or potentially unaltered activity (Mollergues et al., 2017). Thus, there is a possibility that false positive- or negative results are gained. Hepatic metabolism can be mimicked *in vitro* by the addition of exogenous metabolic enzyme mixtures (like S9 liver fractions) (Escher et al., 2021f; Mollergues et al., 2017). There is a risk that toxicity has been underestimated for substances needed to be bioactivated in the papers, as no S9 mixtures were added. Though, substances that efficiently can be metabolized, risk being overestimated when using metabolic components *in vitro*.

Other shortcomings include the absence of biological defense as well as repair response, in addition to cross-talks between organs/tissues, that may avert toxic effects, for which *in vivo* tests account for (Escher et al., 2014). With that being said, *in vivo* testing does not come without flaws.
Extrapolation between interspecies is for example needed to derive safe levels, which adds a level of uncertainty (Escher et al., 2021f). In case *in vitro* methods are run with human cells, such uncertainty factor does not need to be accounted for. Furthermore, human cells can for example be used instead of whole animal testing, for which results can be delivered within a

shorter time frame with lower variability and cost, as well as being more sensitive (Escher et al., 2021f).

Further disadvantages comprise of issues regarding whether cytotoxicity possibly could mask specific effects and what specific test batteries that should be measured. Chemicals can induce a range of effects and choosing the most pertinent assay can be a challenge. Additionally, using *in vitro* bioassays as a standalone tool does not answer the question of which chemical is driving the effect. But, combining bioassays with advanced chemical analysis (i.e. effect-directed analysis) may allow the causative agent to be found, which previously has been done on wastewater (Hashmi et al., 2018).

Even though several challenges exist, there are numerous benefits to *in vitro* methods. Besides rapidly being useful by screening a large number of compounds, they also provide mechanistic information on MIE and KE that ultimately can culminate in an AO. *In vitro* methods are a valuable complement to the *in vivo* methods, which show great promise in applying to a wide range of different matrices.

4.4.2 Paradigm shift

As previously mentioned, there has been an initiation of a paradigm shift from traditional animal testing to alternative test methods, which includes *in vitro* and *in silico* models. The *in silico* model can use *in vitro* data to make predictions of possible endpoints in humans and animals (Escher et al., 2021f). To be able to extrapolate the results from *in vitro* to *in vivo* environment, several factors need to be taken into consideration that relate to the toxicokinetics processes (i.e. absorption, distribution, biotransformation and extraction) of a chemical. The *in silico* test method can predict toxicities that can arise based on the chemical structure or physicochemical properties, while *in vitro* bioassays elucidate specific mechanistic effects (Escher et al., 2021f). These methods together can be used in the human risk assessment process.

The work in the thesis can be considered as the first phase in assessing the safety of the food packages and food items, where the *in vitro* methods have acted as an early warning system in order to try to understand the potential organism response.

5. Conclusion

This thesis had the overall aim of studying the potentially toxic effects of food packages and liquid smoke flavourings. Additionally, milk was tested to evaluate whether *in vitro* bioassays could be used as a screening tool for chemical pollution. A comprehensive panel of *in vitro* bioassays were used to conduct these screenings.

In **Papers I** and **II** food packages made from paper and cardboard were the main focus. Both the polar and non-polar food packaging extracts induced a range of assays like oxidative stress, genotoxicity and endocrine-disruptive activities. No response was seen for inflammation or the androgen receptor (agonistic activity). The polar extracts appeared to drive oxidative stress to a higher degree than the non-polar extracts, whereas the non-polar extracts drove the antagonistic estrogen receptor activity. Effect-based *in vitro* bioassays showed to effectively evaluate hazardous effects of known, unknown and mixtures of chemicals in food packages. Testing of food packaging using *in vitro* methods is a quick and cost-effective way to evaluate the safety, compared to *in vivo* test methods. The extraction of the food packages, meaning an exaggerated procedure where larger amounts of chemicals leak from the package, allowed problematic packages to be found. The packages of potential concern included bag for cookies, boxes for infant formula/skimmed milk, cake/pastry boxes/mats as well as pizza boxes.

Paper III instead focused on commercially available liquid smoke flavourings. Two of the ten tested flavourings, produced from hickory wood, revealed genotoxic and oxidative stress effects. The former response seemed to be driven by non-polar chemicals, while the latter appeared to be induced by polar substances. The *in vitro* bioassays were able to evaluate the

complex chemical mixtures of liquid smoke flavorings and thereby provided new mechanistic information.

Paper IV aimed to evaluate the applicability of using *in vitro* methods as a screening tool for monitoring chemical hazards in cow's milk. Both organic and conventional semi-skimmed- as well as raw milk were collected throughout one year. The antagonistic estrogen- and androgen receptor responses as well as aryl hydrocarbon receptor activity were the most responsive assays. In total, 10 to 13 of the 32 milk samples were bioactive. The remaining assays (oxidative stress, genotoxicity as well as agonistic estrogen- and androgen receptor activity) showed no or low activity. The assays proved promising results, as low or no background activity increases the chances of detecting contaminants. The more activated assays need further research to understand if the background variation is caused by pollutants, foreign substances, or natural compounds. Even though *in vitro* bioassays may not be used daily to monitor milk, due to the high turnover on the market, they can still be used seasonally or monthly to evaluate changes in trends.

Lastly, it is important to remember that findings on an *in vitro* level cannot directly be extrapolated to *in vivo* effects. Still, using *in vitro* methods can evaluate different health-relevant toxicological parameters and thereby fill data gaps. These methods are suitable to be used as an initial tool to screen complex chemical mixtures that exist within these and other products, preferably before entering the market to protect the population from unnecessary exposure to chemical hazards. The results obtained from the studies show the great and broad application of *in vitro* methods, which can give input into the chemical hazards that exist in food items and food packages.

6. Future perspectives

The studies done in this thesis have provided knowledge about the toxicities that can arise from food packages, liquid smoke flavourings and milk by the use of *in vitro* bioassays. However, future research is needed to ensure the safety of these and suggestions on how to achieve it are stated below.

Food packaging

- **Papers I** and **II** used a worst-case scenario extraction setting to detect emerging chemicals. For the bioactive samples, it would be of interest to perform a migration testing where a more realistic scenario would be used
- Understanding which chemicals that are driving the toxic effects would be beneficial (e.g. by effect-directed analysis). It would provide information regarding whether a specific chemical group is the causative agent, which possibly can be removed during the production or manufacturing of new food packages
- The general framework directive needs to be updated and guide how to address the chemical hazards of unknown or mixtures of chemicals. Additionally, a regulation for specifically paper and cardboard food packaging should be put in place, as there currently does not exist one

Liquid smoke flavourings

- The liquid smoke flavourings used in **Paper III** showed to drive a broad range of toxic effects. We therefore propose that future studies should investigate specifically genotoxic effects further. One proposal is the inclusion of liver S9 fractions when performing the genotoxicity testing, as certain chemical may only exert their toxicity after being metabolically activated
- As the bioactivities widely vary between the products, it would be beneficial to conduct collaboration with the producer of the liquid smoke flavourings. Potentially to understand the driving chemical(s) by the use of effect-directed analysis and/or performing minor changes in the manufacturing of these and see how or if the effects are altered using *in vitro* bioassays

Milk

- A follow-up study of **Paper IV** would be valuable, in order to understand if the seasonal differences in antagonistic estrogenicity would appear again, and/or if other alterations of the parameters would be seen. Additionally, as currently, only a few *in vitro* studies are available for milk, it is difficult to understand the background activities seen in e.g. AhR assay, which also suggests the need to perform a follow-up study
- Applying *in vitro* methods to diverse milk products or milk from different producers would be interesting to assess further

General aspects for all studies

- Apply whole organism tests (like zebrafish embryo testing) and other sophisticated models to study the immune-mediated effects, potentially to understand the effects on the intestinal tissue (such by the EpiIntestinal tissue model) is encouraged. These models, as an addition to the *in vitro* bioassays, would be interesting to study to get a broader understanding of the toxic effects
- Incorporating liver S9 fractions together with co-factors would be fascinating, not only for genotoxicity testing but also for the hormonal receptors (androgen- and estrogen receptors) to understand the impact of metabolic components

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Popular science summary

We are exposed to a large and increasing number of chemicals in our everyday lives, frequently through our diet. Most of the available studies focus mainly on single chemicals with known toxic properties. However, this approach is challenged as tens of thousands of chemicals are produced as well as spread into the environment with limited knowledge of the potential toxicological effects these may have on human health. As it is impossible to monitor all the chemicals through chemical analysis, there is a need to develop new methods that can investigate how these chemicals in food may affect human health.

This thesis focused on using genetically modified cell-based methods to measure toxic effects, also referred to as reporter cells. These cells are modified to respond to toxicity events to gain a mechanistic understanding of unknown, known and mixtures of chemicals. With the help of these cells, we were able to measure specific health-relevant effects, such as hormone-mediated responses, inflammation and DNA-damaging effects.

In the first and second studies of the thesis, we used the methods to study commercially available food packages. It is known that chemicals from the packaging can transfer into the food item itself, thus we wanted to investigate what sorts of potentially harmful effects could arise upon the transfer. To investigate the mechanisms further, we investigated if water- or fat-soluble chemicals were driving the toxicities. Our results demonstrated that none of the chemicals within the food packages induced inflammation, while both water- and fat-soluble chemicals caused oxidative stress, meaning increased radical formation.

Several of the food packages affected the hormone receptors, which response highly differed between the package items. The studies highlighted that cell-based methods can be used as a valuable tool to monitor the presence of chemical contaminants in food packaging.

The third study focused on applying cell-based methods to liquid smoke flavourings, which add smoke flavour to the food without actually smoking the food item. We were able to see that the toxicities varied to a large degree depending on the product and flavouring, some products lacked activity while others exhibited toxicities to a high degree in the tests. Additionally, we wanted to see if water- or fat-soluble chemicals were driving the toxic effects, in a similar fashion that was done for the food packages. It was revealed that fat-soluble chemicals had more DNA-damaging effects, whereas water-soluble chemicals increased radical formation and general toxicity. The method allowed us to test the complex mixtures of different liquid smoke flavourings, and it emphasised that more studies are needed to evaluate the safety of liquid smoke flavourings.

The fourth and last study of the thesis, aimed to evaluate if the cell-based methods could be used as a screening tool to monitor chemical hazards in commercially available Swedish cow milk. For several of the methods, like oxidative stress and DNA damaging effects, we saw very little activity, while other methods like effects on the hormonal receptors exerted more activity. Overall, the study showed great promise in regards to using cell-based methods to detect potentially hazardous chemicals within cow's milk.

To conclude, this thesis was designed to apply and develop cell-based methods to assess the toxicity of food items and packages. The results revealed the broad range of toxicity mechanisms that these exert and the methods allowed us to study the biological effects of complex mixtures of chemicals, rather than studying single chemicals. Applying these methods could act as an early warning system to detect potentially hazardous chemicals and thereby ensure the safety of food as well as food packages.

Populärvetenskaplig sammanfattning

Vi exponeras dagligen för ett stort antal kemikalier, ofta genom vår kost. De flesta tillgängliga studier fokuserar främst på enskilda kemikalier med kända toxiska egenskaper. Detta tillvägagångssätt utmanas dock eftersom tiotusentals kemikalier produceras och sprids ut i miljön med begränsad kunskap om vilka potentiella toxikologiska effekter dessa kan ha på människors hälsa. Eftersom det är omöjligt att övervaka alla kemikalier genom kemisk analys finns det behov att utveckla nya metoder som kan undersöka hur kemikalier i livsmedel kan påverka människors hälsa.

Denna avhandling fokuserade på användandet av celler som är genetiskt modifierade, även kallade reporter-celler, för att mäta toxiska effekter. Dessa celler har modifierats för att svara på hälsorelevanta effekter som till exempel inflammation, hormon-medierade och DNA-skadande effekter.

Den första och andra studien i avhandlingen använde reporter-celler för att undersöka kommersiellt tillgängliga matförpackningar. Det är välkänt att kemikalier från förpackningen kan överföras till själva livsmedlet, därför ville vi undersöka vilka potentiella skadliga effekter som kan uppstå på cellnivå efter extraktion av livsmedelsförpackningar. För att få ytterligare mekanistisk information undersökte vi om vatten- eller fettlösliga kemikalier drev toxiciteten. Våra resultat visade att inga kemikalier i livsmedelsförpackningarna inducerade inflammation, men både vatten- och fettlösliga kemikalier orsakade oxidativ stress, vilket innebär ökad radikalbildning. Utöver detta, påverkade flera av matförpackningarna hormonreceptorerna, där effekten av kemikalierna drastiskt skiljdes mellan de olika förpackningsartiklarna. Studierna visade att cellbaserade metoder är ett värdefullt verktyg

för att övervaka förekomsten av kemiska föroreningar i livsmedelsförpackningar.

Den tredje studien tillämpade cellbaserade metoder på flytande rökaromprodukter, vilka tillför röksmak till mat utan att röka livsmedlet. Vi kunde se att toxiciteten varierade mellan de olika produkterna och smaksättningen, där vissa produkter inte orsakade någon aktivitet medan andra uppvisade toxicitet i hög grad. Dessutom ville vi undersöka om vatten- eller fettlösliga kemikalier drev de toxiska effekterna, på ett liknande sätt som undersöktes för matförpackningarna. Vi kunde se att fettlösliga kemikalier orsakade mer DNA-skadande effekter, medan vattenlösliga kemikalier ökade radikalbildningen och allmän toxicitet. Metoderna gjorde det möjligt att testa de komplexa blandningarna av olika rökaromprodukter. Däremot behövs flera studier för att utvärdera säkerheten hos dessa produkter.

Den fjärde och sista studien i avhandlingen inriktade sig till att utvärdera om cellbaserade metoderna kan användas som ett screeningverktyg. Detta för att övervaka potentiella kemiska föroreningar i kommersiellt tillgänglig svensk komjölk. För ett flertal metoder, såsom oxidativ stress och DNA-skadande effekter såg vi lite eller ingen aktivitet, medan andra metoder som effekt på hormonreceptorerna påvisade mer aktivitet. Sammantaget visade studien mycket lovande resultat av att använda cellbaserade metoder för att uppräcka potentiellt farliga kemikalier i komjölk.

Sammanfattningsvis utformades denna avhandling för att tillämpa och utveckla cellbaserade metoder för att bedöma toxikologiska effekter i livsmedel och livsmedelsförpackningar. Resultaten visade att dessa utlöser många toxikologiska effekter och metoderna tillåter oss att studera effekter av komplexa blandningar av kemikalier, snarare än att bara fokusera på enskilda kemikalier. Tillämpning av dessa metoder skulle kunna fungera som ett tidigt varningssystem för att upptäcka potentiellt farliga kemikalier och därigenom försäkra oss om säkerheten för såväl livsmedel som livsmedelsförpackningar.

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I

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Food contact materials: an effect-based evaluation of the presence of hazardous chemicals in paper and cardboard packaging

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ABSTRACT

Food contact materials (FCMs) can contain hazardous chemicals that may have the potential to migrate into food and pose a health hazard for humans. Previous studies have mainly focused on plastic materials, while data on packaging materials made from paper and cardboard are limited. We used a panel of cell-based bioassays to investigate the presence and impact of bioactive chemicals on human relevant endpoints like oxidative stress, genotoxicity, inflammation, xenobiotic metabolism and endocrine system effects in extracts made from paper and cardboard. In total, 23 methanol extracts of commonly used paper and cardboard available on the Swedish market were extracted as a whole product using methanol to retrieve polar substances, and tested at concentrations 0.3–10 mg/mL and 0.2–6 mg/mL. At the highest concentration bioactivities were observed in a high proportion of the samples: oxidative stress (52%), genotoxicity (100%), xenobiotic metabolism (74%), antiandrogenic- (52%) and antioestrogenic receptor (39%). Packages of potential concern included cake/pastry boxes/mats, boxes for infant formula/skimmed milk, pizza boxes, pizza slice trays and bag of cookies. It should be noted that the extraction for packages like cake/pastry boxes can be considered exaggerated, as the exposure usually is shorter. It can be hypothesised that the observed responses may be explained by inks, coatings, contaminants and/or naturally occurring compounds within the material. To summarise, an effect-based approach enables hazard identification of chemicals within FCMs, which is a valuable tool for ensuring safe use of FCMs.

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Food packaging materials; effect-based strategy; paper and board; *In vitro* analysis; bioassays; hazard identification

Introduction

Food contact materials (FCMs) are defined as materials intended to come into contact with food. These materials should be stable against varying temperatures, ensure prolonged shelf-life of foods, as well as protect against biological and chemical contaminations. They can be made from a range of materials such as plastic, glass, paper, cork and paperboard (Simoneau 2016).

Chemicals present in FCMs can either be intentionally added for a specific function or non-intentionally added. Non-intentionally added substances (NIAS) can originate from breakdown products, chemical interactions with the food item and the package material, or from contaminants (Peters et al. 2019). Examples of chemicals in FCMs are primary aromatic amines, mineral oil hydrocarbons, plasticisers (e.g. phthalates, adipates, terephthalates etc.), and bisphenol A (BPA), all of which have the potential to cause adverse health effects in humans (Lopez-Espinosa et al. 2007; Lorenzini et al. 2010; Campanella 2015). These chemicals have mainly been studied in plastic packaging materials and it is important to investigate other types of packaging materials, such as paper and cardboard (Campanella 2015; Severin et al. 2017; Park et al. 2018; Schweighuber et al. 2019).

There is currently no harmonised legislation within the European Union (EU) for chemical safety of paper and cardboard FCMs. The EU framework regulation states that FCMs should be in compliance with good manufacturing practice and not transfer their substances in amounts that could negatively affect human health or alter the food itself (EU 2004; EU

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Table 1. Food packaging materials (FCMs) tested.

FCM	Printing	Purchased at	No. of pooled samples
Baking moulds	Yes	Supermarket	1
Pizza slice trays	Yes	Store	1
Paper for baking and baking moulds	No, but contained bleached material	Manufacturer/bakery/supermarket	5
Boxes for cookies	Yes	Manufacturer	3
Popcorn boxes	Yes	Movie theatre/store	3
Cake/pastry boxes/mats	Yes	Bakery	5
Board samples	Yes	Manufacturer	2
Boxes for infant formula/skimmed milk	Yes	Manufacturer	3
Boxes for porridge and flour mixes	Yes	Manufacturer	6
Paper plate for warm food	No	Restaurant	1
Paper plate (coated)	Yes	Store	1
Boxes for cereals	Yes	Supermarkets	4
Boxes for cookies	Yes	Supermarket	3
Microwave popcorn bags	Yes	Supermarkets	6
Straws	Yes	Movie theatre	1
Pizza boxes	Yes	Restaurant	2
Papers for wraps	Yes	Restaurants	3
Hamburger/French fries' papers	Yes	Restaurants	5
Boxes for fries' and hamburgers	Yes	Restaurant	2
Paper for trays	Yes	Restaurant	1
Bag for cookies	Yes	Bakery	1
Coloured paper for baking moulds	Yes	Manufacturer	1
Packages for frozen food	Yes	Supermarket	2

2011; EU 2016). Still, the framework does not regulate specific substances, and this is a limitation since migration of chemicals from the finished product containing inks, additives and adhesives may affect human health (Muncke 2010; Koster et al. 2015).

There is limited knowledge on toxicity of chemicals present in the environment. Toxicity testing of water samples has demonstrated that unknown compounds account for up to 99.1% of the biological effects for certain endpoints when tested in mammalian *in vitro* systems or in the bacterium *Vibrio fischeri* (Escher et al. 2013; König et al. 2016; Neale et al. 2020). Effect-based *in vitro* methods can be used to assess these effects. Our study did not focus on identifying chemicals, as known and unknown NIAS may exert the identified effects and these may not be identified by targeted chemical analyses.

Prior studies have reported effects on the aryl hydrocarbon- (AhR), oestrogen- (ER) and androgen (AR) receptors, as well as oxidative stress responses and genotoxicity by certain paper and cardboard FCMs (Bengtström et al. 2014; Rosenmai et al. 2017; Severin et al. 2017). This indicates that these endpoints are relevant human health outcomes to examine further when assessing the safety of FCMs.

There are a limited number of studies that use effect-based methods to identify bioactive chemicals in FCMs. Therefore, we used an effect-based approach in this study to evaluate the presence of hazardous chemicals in a large number of commonly used FCMs made of paper and cardboard. This panel of bioassays represented toxicity pathways of high relevance to human health. The endpoints investigated were oxidative stress (Nrf2 activity), genotoxicity (micronucleus test), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκβ) signalling, oestrogen (ER), androgen (AR) and aryl hydrocarbon receptor (AhR). Sixty-seven commonly used paper and cardboard FCMs available on the Swedish market were chosen, and included materials like pizza boxes, microwave popcorn bags and fastfood packaging.

Material and methods

Sample preparation and extraction

A total of 67 food packages made from paper and cardboard were purchased by the Swedish Chemicals Agency from a variety of sources (e.g. supermarkets, bakeries and restaurants) in 2019 (Table 1). The selection of FCM samples were based on sales statistics of purchased materials on the Swedish consumer market (Kemikalieinspektionen 2020).

The sample extraction procedures are described in the Supplementary information (Section SI-1). Briefly, samples were cut into small pieces, with inner and outer surfaces containing inks, glue,

coatings, lacquers, coatings, etc., consisting of approximately 1 g of material, except for the extract paper for baking and baking moulds which received a weight of 0.6 g. The 67 materials were categorised into groups and similar materials (like pizza boxes) were pooled together and this resulted in a total of 23 extracts (Table 1). Each pool of materials contained in total 1 g, except for baking and baking moulds. The samples were placed in Teflon-coated test tubes and microwaved for 20 min at 80°C in 15 mL of methanol, before being transferred into glass tubes. An additional extraction by ultrasonication was performed for 15 min in the Teflon-coated tubes using an additional 10 mL of methanol (Alin and Hakkarainen 2012; Melski et al. 2003). Thereafter, the pooled extracts were evaporated under a nitrogen stream to 0.5 mL, and thereafter diluted with 0.5 mL ultrapure water (Milli-Q ®) to obtain a final volume of 1 mL. The extraction procedure allowed water-soluble chemicals and to some extent fat soluble substances to be extracted. Three solvent blanks were prepared following the same extraction procedure as for the FCMs, but without material.

Bioassays

All extracts were analysed in quadruplicate in the bioassays mentioned in Table 2. Each bioassay used an established cell line and comprehensive information of the bioassays are available in Supplementary information (Section SI-1).

The vehicle control was the solvent methanol MeOH/Milli-Q water (1:1), which samples were dissolved in. The standards were dissolved in DMSO due to low solubility in MeOH/MQ water, and therefore DMSO was included as an additional vehicle control. Information on standards for respective assay are shown in Table 2. Mitomycin C (MMC), tamoxifen and methoxychlor were used as positive controls for genotoxicity, antioestrogenic activity and agonistic oestrogen activity, respectively.

Data analysis

All sample results and positive controls were normalised to the activity of the vehicle control(s), which was set to 1 for reporter gene assays and 100% for cell viability assays. The standard curves for the nuclear-receptor bioassays were created on a four parameter non-linear regression sigmoidal curve fit using GraphPad Prism 8 Software (San Diego, California USA).

The effect concentration (EC_{50}) and inhibitory concentration (IC_{50}) were calculated from the four parameter regression curve, as previously described by Escher et al.(2018) (Table SI-1).

For the transcription factor Nrf2, the standard curve was fitted by linear regression using GraphPad Prism 8 Software. An effect concentration corresponding to an induction ratio of 1.7 was calculated for Nrf2 activity, as no maximum response exists (Table SI-1) (Escher et al. 2014). Micronuclei results were analysed in quadruplicate with

Table 2. Summary of bioassays and respective endpoints.

Endpoint	Cell line	Standard Concentration	Added treatment Concentration
Oxidative stress – Nrf2 activation (Lundqvist et al. 2019)	MCF7 AREc32	Butylhydroquinone (tBHQ) 0.8–25 µM	N/A
Genotoxicity – Micronucleus (MN) events (OECD 2016a)	TK6	N/A	N/A
Agonistic ER activity (OECD 2016b)	VM7Luc4E2	Oestradiol (E2) 0.4 to 367 ρM	N/A
Antiestrogenic activity (OECD 2016b)	VM7Luc4E2	Raloxifen (Ral) 0.1–24.5 nM	E2 0.1 nM
Agonistic AR activity (OECD 2016c)	AR-EcoScreen GR-KO M1	Dihydrotestosterone (DHT) 0.001–1000 ρM	N/A
Antiandrogenic activity (OECD 2016c)	AR-EcoScreen GR-KO M1	Hydroxyflutamide (OHF) 0.0001–10 µM	DHT 200 ρM
Xenobiotic metabolism – AhR activation (Rosenmai et al. 2018)	DR-EcoScreen	2,3,7,8-Tetrachlorodibenzodioxin (TCDD) 0.01–1000 ρM	N/A
Inflammatory response – NFκβ activation (Lundqvist et al. 2019)	HepG2-NFκβ	Tumour necrosis factor α (TNFα) 0.2–50 ng/mL	N/A
Cytotoxicity (MTS/ATPase*/%EMA**)	All mentioned above	N/A	N/A

* ATPase was only used for VM7Luc4E2 cells ** % EMA was only used for TK6 cells

GraphPad Prism 8 using one-way ANOVA followed by Dunnett's multiple comparison test. *P*-values <0.05 were considered statistically significant.

For all bioassays, the classification of a sample as bioactive was based on a cut-off limit, which was calculated as 1 plus 3 times the standard deviation (SD) of the normalised vehicle control. The cut-off limit for the antioestrogenic and antiandrogenic tests were calculated as 1 minus 3 times the SD of the normalised vehicle control. More information of the cut-off values are presented in Table SI-1. The cut-off value for cytotoxicity was set at 75% of cell viability compared to the vehicle control for all assays, except for the micronuclei test. For this, the cut-off limit of cytotoxicity was set at fourfold increase in EMA-positive events compared to the vehicle control. The bioanalytical equivalent concentration (BEQ) was calculated for the highest concentration for each bioactive extract based on the linear range of the extract's concentration-response and dose–response curves of the assay-specific standard (Table SI-2).

Results and discussion

Cell viability

FCM extracts were evaluated for cytotoxicity in MCF7 AREc32, HepG2-NFκβ, AR-EcoScreen GRKO M1, DR-EcoScreen and VM7Luc4E2 cell lines at concentrations from 0.3 to 10 mg food packaging material/mL of cell medium, to ensure that specific toxic responses were evaluated under non-cytotoxic conditions (Figure SI-1 – 5). The extract from baking and baking moulds was tested at concentrations from 0.2 to 6 mg food packaging material/mL of cell culture medium because of technical reasons. Samples causing a cell viability of <75% were defined as cytotoxic. Any sample that was cytotoxic is represented by hashed grey-black bars in the graphs for each endpoint. As these samples were cytotoxic, although not severely, the results for those exposure concentrations should be interpreted with care. All other extracts were found to be non-cytotoxic (Figure SI-1 – 5).

Oxidative stress

Oxidative stress was assessed using a stably transfected breast cancer cell line (MCF7 AREc32). This cell line contains a luciferase reporter gene that is under the control of an antioxidant responsive element (ARE), meaning that induction of ARE will result in increased luciferase activity. Activation in ARE triggers an upregulation of genes that code for enzymes and antioxidant proteins involved in the body's defence against oxidative stress.

Twelve of the FCMs had Nrf2 activity above the cut-off level of 1.7-fold activation at 10 mg/mL (Figure 1). The extracts showing the highest potency for oxidative stress were cake/pastry boxes/mats, boxes for infant formula/skimmed milk, boxes for cereals and pizza boxes. These samples displayed a 5.1–8.9-fold increase in Nrf2 activity at 10 mg/mL compared to the vehicle control. Most of the extracts that activated Nrf2 at the highest concentration also exerted an activation at the lower concentration 3 mg/mL, in a dose-related manner. tBHQ was used as the standard for the assay (Figure SI-9). The BEQ values, at non-cytotoxic concentrations, ranged from 4.2 to 28 μM tBHQ equivalents (Table SI-2).

In line with these results, Rosenmai et al. (2017) observed an oxidative stress induction for 16 out of 20 board and paper samples in the Nrf2 CALUX reporter gene bioassay. The highest activities in their study were observed for the cereal box, sausage tray, tomato punnet, imported paperboard, paperboard with water-soluble print and offset print materials.

Based on our results, the four FCMs showing the highest potency for Nrf2 activation (i.e. boxes for cereals, pizza boxes, cake/pastry boxes/mats and boxes for infant formula/skimmed milk) were selected for evaluation of genotoxicity.

Genotoxicity

Based on the results from the Nrf2 activity assay, four samples (boxes for cereals, pizza boxes, cake/pastry boxes/mats and boxes for infant formula/skimmed milk) were tested at 3 and 10 mg/mL in the *in vitro* micronucleus (MN) test using TK6 cells.

Ethidium monoazide (EMA) was used to measure cytotoxicity, in which a cut-off for cytotoxicity was set as a fourfold increase in EMA-positive events compared to the vehicle control (Bryce et al. 2013). None of the samples caused cytotoxicity (Figure 2b). All four samples induced

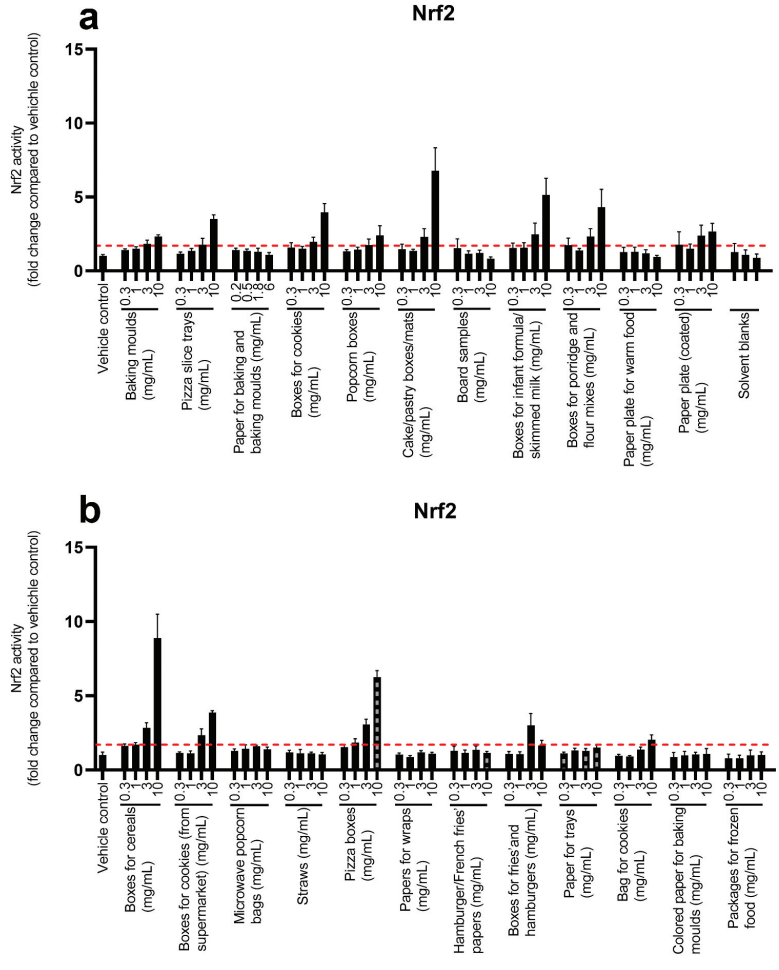

Figure 1. Oxidative stress response in MCF7 AREc32 cells exposed to FCM extracts for 24 h (a, b). The graphs represent mean ± SD of quadruplicates (n = 4) from one representative experiment, and the dotted line is the induction ratio 1.7 fold change, which was defined as the cut-off for bioactivity. The hashed grey black bars represent concentrations that were cytotoxic.

a statistically significant increase in micronucleus events at the highest concentration tested compared to the vehicle control, showing that these samples contain genotoxic compounds (Figure 2a).

The genotoxicity data were obtained from the human lymphoblastic cell line TK6 that is p53 competent and karyotypically stable, which has proven to produce more reliable results than false-positive prone rodent cell lines (Fowler et al. 2012). Compared to the classical chromosomal aberration test, where structural chromosomal damage is studied, the MN test allows detection of both structural and numerical alterations. The usage of TK6 cells has been highlighted to be both highly sensitive and specific (Pinter et al. 2020). To our knowledge, this is the first study using a micronucleus test with TK6 cells to investigate paper and board FCMs.

The possible explanation for the high micronucleus events for the pizza box may be due to the fact that cardboard is often manufactured from recycled materials containing inks. Previous studies using DNA repair Rec assay and Comet assay supported the positive genotoxic response for paperboard samples

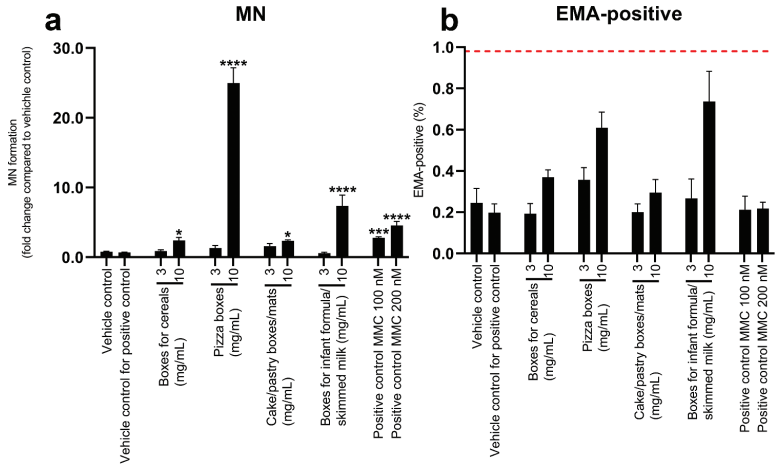

Figure 2. Micronuclei formation in TK6 cells after exposure to FCM extracts for 24 h (a) and cytotoxicity test (b) Mitomycin C (MMC) was used as a positive control at concentrations 100 and 200 nM (a,b). The graphs illustrate mean ± SD of quadruplicates (n = 4) from one representative experiment. The dotted line in graph B represent the cut-off limit determined by the manufacturer's protocol. Data was analysed using one-way ANOVA, followed by Dunnett's post-hoc test. Results that were statistical significant are indicated by asterisks (* p-value < 0.05, *** p-value < 0.001, **** p-value < 0.0001).

(Ozaki et al. 2004, 2005). Ozaki et al. (2004, 2005) suggested that the genotoxicity might be explained by potential mixture effects, unknown toxicants, paper resins and/or the amount of recycled matter. The recycled material showed a higher induction of DNA damage compared to virgin samples in their study. Still, varying results have been reported for *in vitro* genotoxicity tests and they appear to be influenced by the extraction method, material, cell type, genotoxic endpoint, dose and metabolic capacity of used cells. Additional research is needed in the use of recycled material in food packages, as there is an increased demand for its use, particularly for circular economies.

Furthermore, the genotoxic responses observed in all other materials in this study may be explained by the extensive usage of coatings or inks (Figure 2a). It is possible that genotoxic and hazardous substances exist in packages in form of ink and coatings, since these are not regulated within the EU, making it difficult to ensure that coatings and inks do not contain hazardous substances.

The ink can be mineral oil based and/or contain photoinitiators that have the ability to generate highly reactive species that covalently can bind to DNA and create DNA adducts (Szeliga and Dipple 1998; Tarnow et al. 2016). The findings of this study highlight the importance to further evaluate genotoxic substances in FCMs.

Oestrogen receptor activity

Oestrogenic response was assayed in the VM7Luc4E2 cell line, which stably expresses the luciferase gene under control of the oestrogen responsive element (ERE).

Only three extracts out of 23 samples exerted ER agonistic activity above the cut-off value (Figure SI-6). The BEQ for microwave popcorn bags was calculated to 10.6 ρM oestradiol equivalents in 10 mg FCM/mL (Table SI-2).

Antioestrogenic effects were assayed by stimulating VM7Luc4E2 cells with oestradiol in the cell culture medium and measured as a decrease in activity compared to the oestradiol-treated control (Figure 3).

Nine of the samples exerted strong antioestrogenic activity at the highest concentration tested. The effect was dose-related and higher concentrations exerted stronger antioestrogenic effects.

Raloxifen was used as a standard for antioestrogenic activity (Figure SI-10). The BEQ values were 1.9–8.2 nM raloxifen equivalents in 10 FCM mg/mL (Table SI-2). Tamoxifen at a concentration of

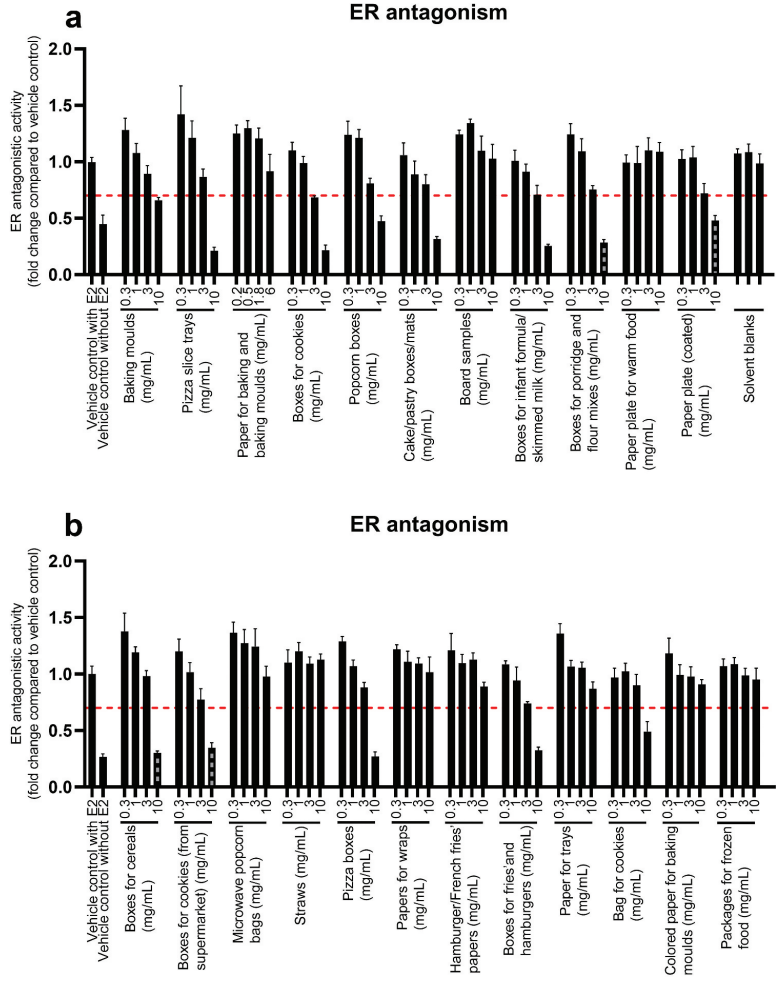

Figure 3. Antioestrogenic effects in VM7Luc4E2 cells after 24 h of exposure to FCM extracts (a,b) The graphs illustrate mean ± SD of quadruplicates (n = 4) from one representative experiment. Unspiked medium with MeOH/MQ water was used as a control for the assay (1%). The dotted line shows the cut-off limit of 0.7. Samples with an activity below the cut-off were defined as bioactive. The hashed grey black bars represent concentrations that were cytotoxic.

3.36 µM was used as a positive control for antioestrogenic activity and had a response between 0.3- and 0.6-fold change compared to the solvent control.

Both agonistic and antioestrogenic receptor effects were seen in the FCM extracts, indicating that chemicals within FCMs have multiple mechanisms of action on the oestrogen receptor. Nevertheless, the antioestrogenic response was observed in more extracts in our experiments. Similarly, activation of ER by paper for household use and food container cardboard has been reported in E-Screen, BG1luc4E2 (renamed VM7Luc4E2) and YES assays (Vinggaard et al. 2000; Lopez-Espinosa et al. 2007; Rosenmai et al. 2017). A possible explanation is the presence of bisphenol A (BPA) and certain phthalates. Exposure to chemicals like BPA and BPA analogues

have been linked to impaired ovary function as well as reduced sperm production and quality (Siracusa et al. 2018).

Effects on the oestrogen receptor could also be linked to UV-photoinitiators in printing inks that may leach from the outer carton, which previously have been observed with benzophenones (Muncke 2010). Studies on the oestrogenicity of benzophenones are however debatable, as oestrogenicity has been observed *in vitro* using MCF7 cell proliferation and YES assays, while *in vivo* uterotrophic assay and *in vitro* human ERα reporter gene assay failed to demonstrate any oestrogenicity (Muncke 2010).

Androgen receptor activity

Androgenic effects were examined with the AR-EcoScreen GR-KO M1 assay, which employs stably transfected CHO cells with human androgen receptor elements linked to a luciferase gene (Zwart et al. 2018).

No agonistic activity of the androgen receptor was exerted in any of the samples (Figure SI-7).

Antiandrogenic activities were assayed by stimulating AR-EcoScreen GR-KO M1 cells with DHT in the cell culture medium, and effects were measured as decreased activity compared to the DHT-treated control (Figure 4). Twelve out of 23 FCM extracts induced an antiandrogenic response in a dose-related manner. Hydroxyflutamide was used as a standard for antiandrogenic effects (Figure SI-11). The BEQ values ranged from 0.1 to 3.7 µM hydroxyflutamide equivalents in 10 mg FCM/mL (Table SI-2). The antiandrogenic responses may be explained by chemicals like phenols, phthalates or organotins; the latter is used as a fungicide in paper and pulp (Muncke 2010). Phenolic compounds found in coatings and plastic food packaging have been reported to induce antagonistic response of the androgen receptor in the AR CALUX (Krüger et al. 2008).

Tests of antiandrogenic effects have been carried out by Mertl et al. (2014) with yeast androgen screen (YAS) and human cell-based AR CALUX bioassays. Two out of three paperboard samples showed antagonistic effects in the YAS reporter gene assay. However, the response was not detected in the antagonistic AR CALUX assay, except in one sample that showed a positive response in both assays. The difference in responses between the two models are not known, but it may be explained by underlying cytotoxicity of the FCM extracts causing false-positive results in the yeast screen assay. Conversely, Rosenmai et al. (2017) also tested antiandrogenic activity, in which nine out of twenty paper and board samples had antiandrogenic activity. It was speculated by the authors that the effect may be explained by the resin acids abietic (AA) and dehydroabietic (DHA) used in paper products, as seen in a study by Rostkowski et al. (2011). Rosenmai et al. (2017) reported antiandrogenic effects for cake tray, baking mould and paper wraps, which is similar to observations in this study. Besides this, other studies have focused on studying chemicals on food packaging materials like inks. Peijnenburg et al. (2010) studied the commonly used ink component photoinitiator 2 isopropylthioxanthone (2-ITX), which was found to have antioestrogenic and antiandrogenic effects in two yeast-based assays.

Aryl hydrocarbon receptor activity

The AhR assay utilises DR-EcoScreen cells, which are mouse hepatoma cells stably transfected with an aryl hydrocarbon responsive element (AhRE) that regulates the expression of the luciferase gene (Anezaki et al. 2009).

A dose-related increase in AhR activity was observed for nearly all of the samples (20/23) (Figure 5). The strongest effects were observed for pizza slice trays, cake/pastry boxes/mats, pizza boxes and bag of cookies.

Strong activity was already observed at the lowest concentrations for boxes for porridge and flour mixes, boxes of cereals and pizza boxes. At the highest concentrations, the boxes for infant formula/skimmed milk and boxes for cookies (from the supermarket) showed a slight reduction in AhR activity compared to the lower concentration of 3 mg/mL, indicating cytotoxicity that was not detected in the MTS assay. TCDD was used as a standard (Figure SI-12). The BEQ values, at non-cytotoxic concentrations, ranged from 0.2 to 13 ρM TCDD equivalents (Table SI-2).

The positive response in AhR is supported by previous data on both paper and cardboard using the stably transfected rat hepatoma CALUX-assay

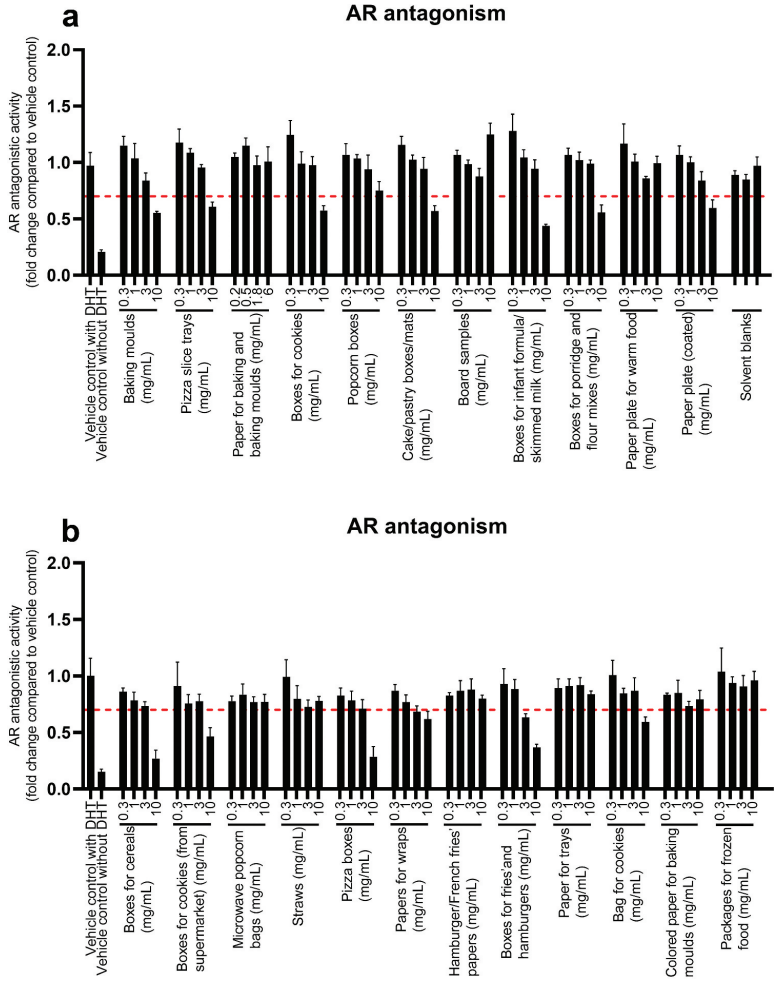

Figure 4. Antiandrogenic response in AR-EcoScreen GR-KO M1 cells after 24 h of exposure to FCM extracts (a,b). Unspiked medium with MeOH/MQ water was used as the control for the assay (1%). The panel shows mean ± SD of quadruplicates (n = 4) from one representative experiment. The dotted line illustrates the cut-off of 0.7. Samples with an activity below the cut-off was defined as bioactive.

(Binderup et al. 2002; Bengtström et al. 2014; Rosenmai et al. 2017). In a recent study by Rosenmai et al. (2017), all samples induced AhR, with the pizza box, tomato punnet, sausage tray and paperboard with offset print showing pronounced inductions of the AhR. The strong response was suggested to be due to additive response caused by the presence of contaminants and/or natural components within the paper and board that have the ability to function as AhR ligands. Furthermore, the photoinitiator 2-ITX in ink have shown to have AhR agonistic activity in the DR CALUX assay, as well as induce the AhR responsive enzyme cytochrome P450 1A1 activity in the EROD assay using the rat hepatoma H4IIE cell line (Peijnenburg et al. 2010). Further studies are needed to understand FCMs impact on AhR, as it has vital functions in biotransformation of xenobiotic substances,

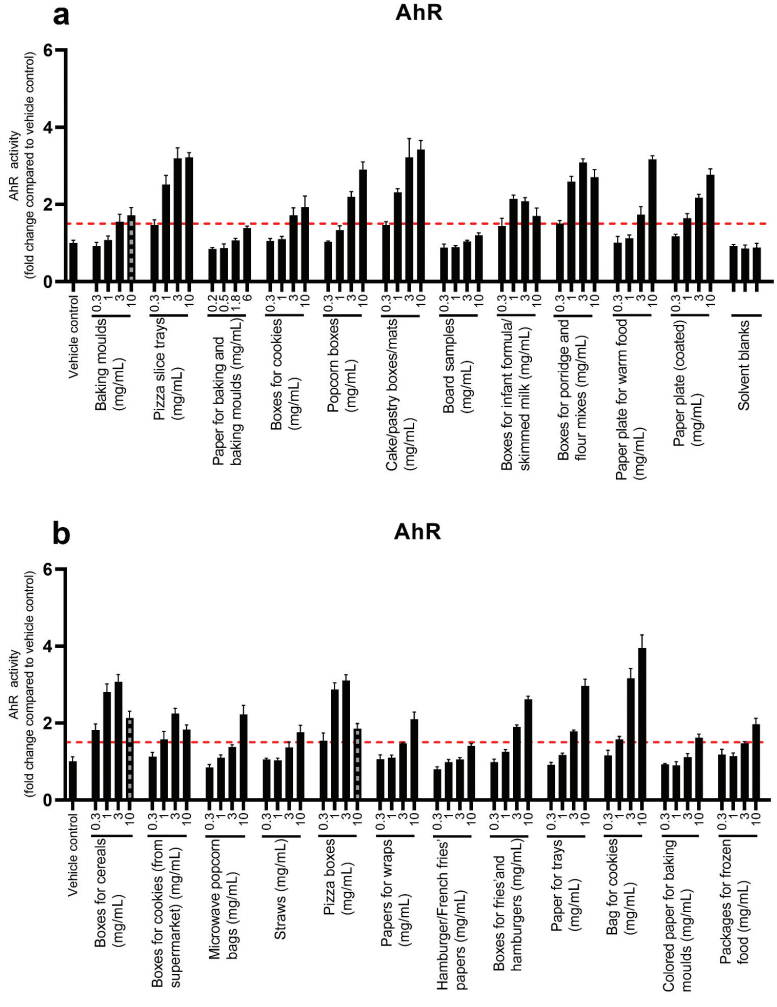

Figure 5. AhR activity in DR-EcoScreen cells after 24 h of exposure to FCM extracts (a,b). The graphs illustrates mean ± SD of quadruplicates (n = 4) from one representative experiment. The dotted line shows the cut-off limit of 1.5. The hashed grey black bars represent concentrations that were cytotoxic.

reproduction, development and intestinal immunological response (Gutiérrez-Vázquez and Quintana 2018; Bock 2019).

NFκβ activity

NFκβ activity was tested in HepG2-NFκβ cells, which is a stably transfected cell line with a NFκβ responsive element controlling the luciferase gene (Figure SI-8). The transcription factor NFκβ has vital functions in the immune system, and dysfunction has been related to cancer, autoimmune diseases and viral infections (Brasier 2006). None of the FCM extracts caused an increased activity in NFκβ, which had the cut-off at 1.5.

The lack of response in the NFκβ reporter gene assay could be explained by several factors, that the extracts did not induce an immune response, potential immunosuppressive effects or lack of cell communication that is critical for proper immunological.

Kejlová et al. (2019) also investigated the inflammatory response and observed the induction of cytokine IL-8 in heavy-printing FCM samples in the sophisticated 3D human intestine model EpiIntestinal FT, which suggest that FCMs may affect important functions of leukocytes. Still, few studies have investigated the inflammatory response from FCM and future studies should focus on the potential immunotoxic effects in the gut, since it is the main route of exposure of FCMs.

Future perspective

We have observed activation of oxidative stress, genotoxicity, xenobiotic metabolism and antagonistic effects on the oestrogen as well as androgen receptors. Packages that are of potential concern includes cake/pastry boxes/mats, boxes for infant formula/skimmed milk, pizza boxes, pizza slice trays and bag of cookies. Two materials that were particularly noticeable were cake/pastry boxes/mats and boxes for infant formula/skimmed milk, which suggests that these materials seem to be the most problematic, potentially due to the heavy colouration from the printing inks. These findings are of importance given that substances that causes these effects could migrate into food and thus constitute a health hazard for humans.

One important aspect of the present study is whether the extraction method is representative of realistic migrations from the FCMs to food and subsequently in relation to human exposure. The methanol extraction with microwave treatment at high temperature may exaggerate the migration of water-soluble compounds, although conversely more-lipid-soluble contaminants may not be extracted. Additionally, the extraction procedure was done on the food packaging as a whole product, and single sided extraction of materials having a secondary packaging like infant formula/skimmed milk could have resulted in different results. Nevertheless, the resin acids dehydroabietic (DHA) and abietic (AA) in paper products have shown to migrate under mild extraction procedures, and have been speculated to cause antiandrogenic effects in food package materials (Ozaki et al. 2006; Rosenmai et al. 2017). In addition, worst-case scenario extractions can be relevant for certain materials that are exposed to high temperatures in their normal use, for example, microwave popcorn bags, and as a screening method to identify potential problematic substances/FCMs.

It is important to keep in mind that volatile substances may seep through cardboard and plastic bag materials, such that dry foods can still be contaminated with chemicals from inks or recycled fibres, particularly after longer storage conditions (Lorenzini et al. 2010). Furthermore, it is essential to ensure that observed effects are not from substances present in the food itself.

Several challenges exist when studying food package materials and one of these are NIAS, which currently are not possible to identify and quantitatively determine in targeted chemical analysis. As toxicity can arise from both unknown and known compounds individually and as components in mixtures, it is necessary to base the hazard identification and risk assessment on the material as a whole and not the single known chemicals. An effect-based strategy enables hazard identification; however, there is a need to standardise bioassays in future studies to ensure high-quality performance, reporting, sensitivity, specificity and consistency between laboratories (Groh and Muncke 2017).

The results presented here prompt future studies on the presence of hazardous chemicals in paper and board FCMs. Specifically, studies should focus on using more relevant extraction methods and investigate potential alterations in toxicity during passage through the intestinal epithelium in combination with reporter gene bioassays. Finally, the use of effect-based approaches to evaluate the potential effects of such chemicals in food packages should be emphasised, since it cannot be ruled out that the chemicals causing activity in the FCM extracts could migrate and contaminate the food.

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Declaration of interest

The authors declare no competing financial interest.

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RESEARCH

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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

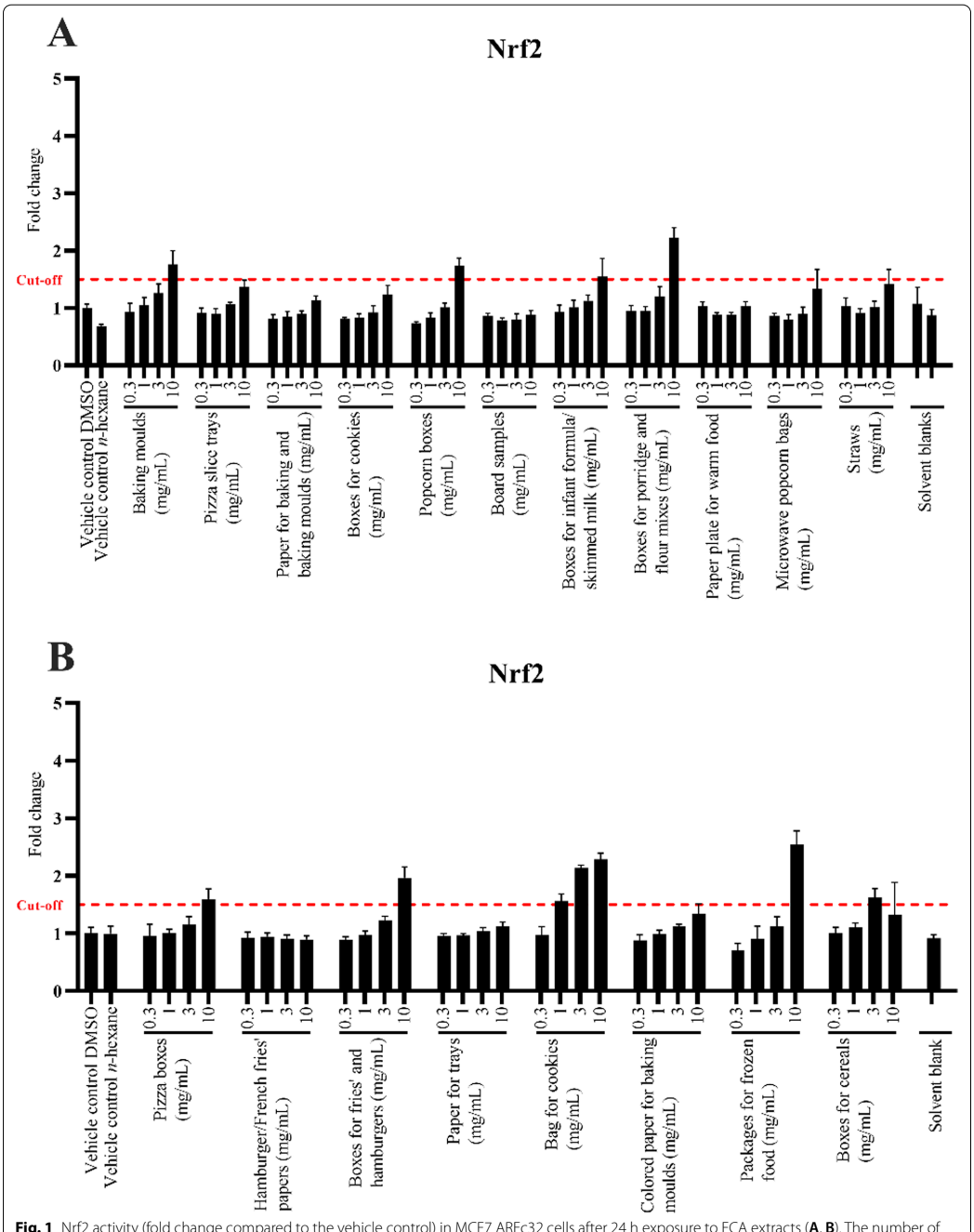

Fig. 1 Nrf2 activity (fold change compared to the vehicle control) in MCF7 AREc32 cells after 24 h exposure to FCA extracts (**A**, **B**). The number of technical repeats (*n*) was 8 for the vehicle controls and 4 for the samples (mean ± SD)

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

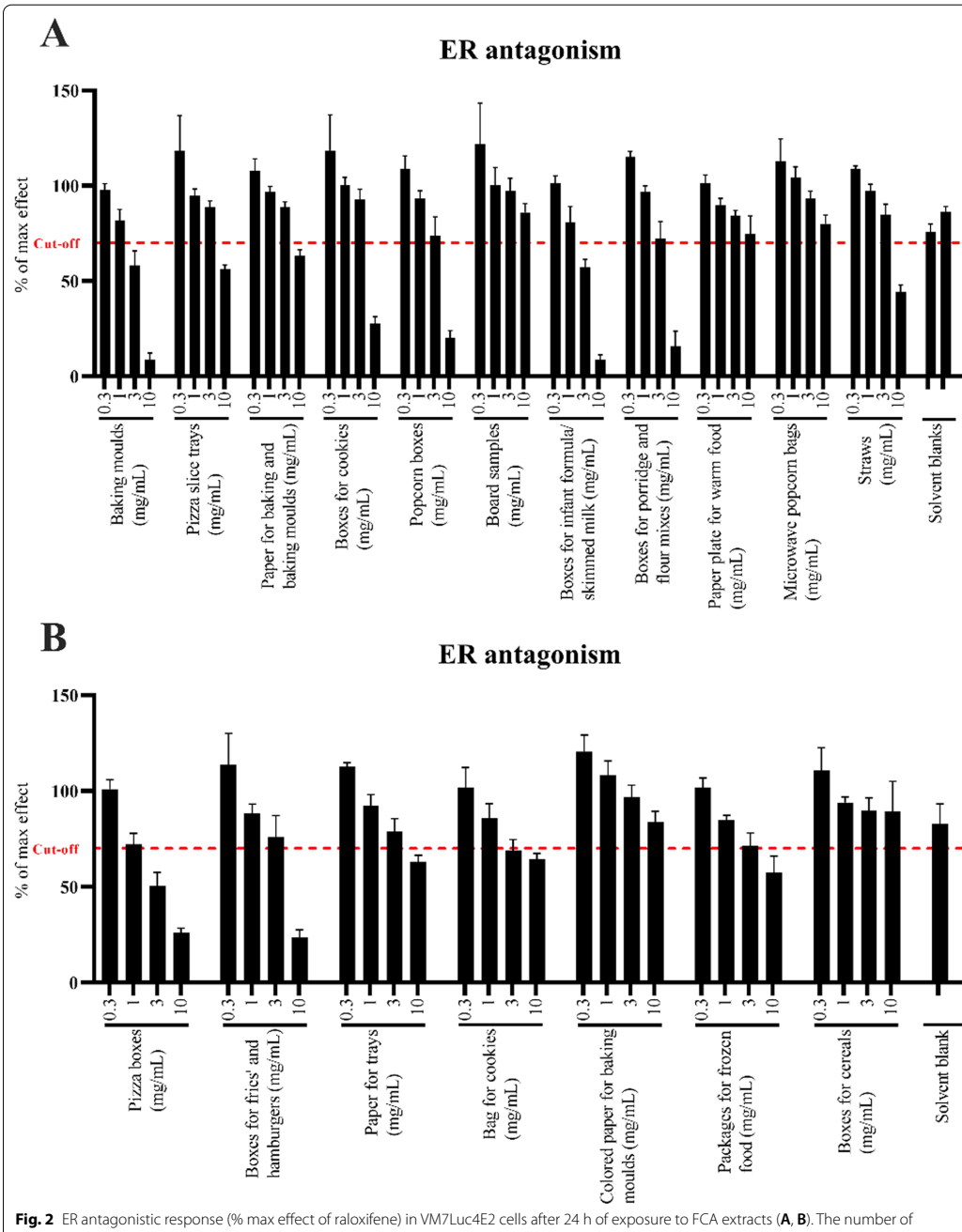

Fig. 2 ER antagonistic response (% max effect of raloxifene) in VM7Luc4E2 cells after 24 h of exposure to FCA extracts (**A**, **B**). The number of technical repeats (*n*) was 8–12 for the vehicle controls and 4 for the samples (mean ± SD). Samples with an activity below the cut-off limit were defined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

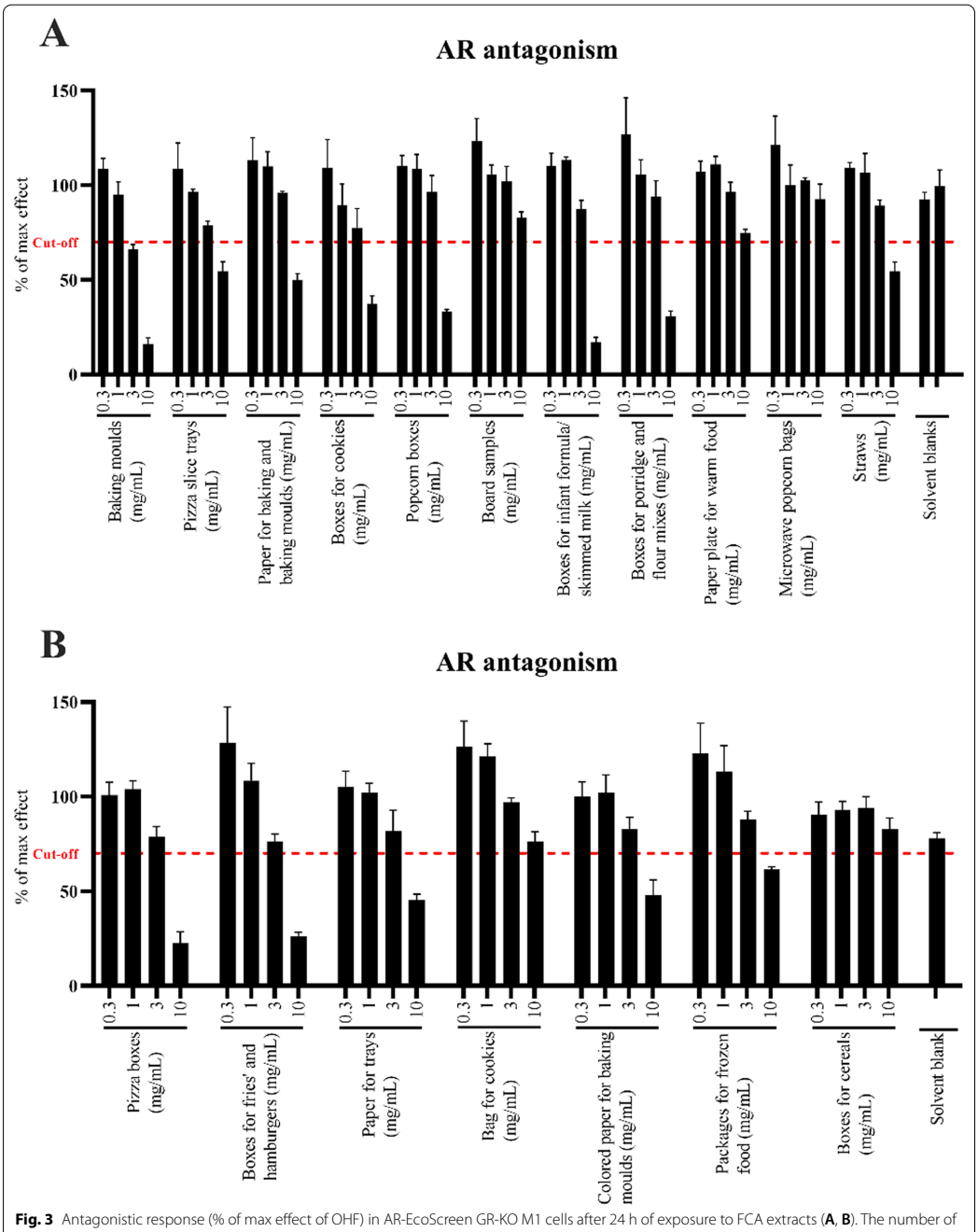

Fig. 3 Antagonistic response (% of max effect of OHF) in AR-EcoScreen GR-KO M1 cells after 24 h of exposure to FCA extracts (**A**, **B**). The number of technical repeats (*n*) was 8–12 for the vehicle controls and 4 for the samples (mean ± SD). The cut-off limit is represented by the red dotted line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

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00666-4.

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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IN VITRO SYSTEMS

An in vitro-based hazard assessment of liquid smoke food flavourings

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Abstract
Liquid smoke products are widely used as a food additive to create a desired smoke flavour. These products may contain hazardous chemicals generated during the wood-burning process. However, the toxic effects of these types of hazardous chemicals constituting in the commercially available products are largely unknown. Therefore, a test battery of cell-based in vitro methods, covering different modes of actions of high relevance to human health, was applied to study liquid smoke products. Ten liquid smoke flavourings were tested as non-extracted and extracted. To assess the potential drivers of toxicity, we used two different solvents. The battery of in vitro methods covered estrogenicity, androgenicity, oxidative stress, aryl hydrocarbon receptor activity and genotoxicity. The non-extracted samples were tested at concentrations 0.002 to 1 µL liquid smoke flavouring/mL culture medium, while extracted samples were tested from 0.003 to 200 µL/mL. Genotoxicity was observed for nearly all non-extracted and all hexane-extracted samples, in which the former had higher potency. No genotoxicity was observed for ethyl acetate-extracted samples. Oxidative stress was activated by almost all extracted and non-extracted samples, while approximately half of the samples had aryl hydrocarbon receptor and estrogen receptor activities. This study used effect-based methods to evaluate the complex mixtures of liquid smoke flavourings. The increased bioactivities seen upon extractions indicate that non-polar chemicals are driving the genotoxicity, while polar substances are increasing oxidative stress and cytotoxic responses. The differences in responses indicate that non-extracted products contain chemicals that are able to antagonize toxic effects, and upon extraction, the protective substances are lost.

Keywords Smoke flavouring · Commercial liquid smoke flavouring · Food additives · Bioassays · Bioanalytical tool · Effect-based methods

Introduction

While smoking of foods traditionally has been performed mainly as a mean of preservation, it is today also used to create foods with a desired flavour of smoke. This has resulted in the development of smoke flavouring products, which are adding smoke flavour to food without actual smoking of the food item. Smoke flavourings are produced by thermal treatment of wood in the absence of oxygen (pyrolysis), followed by condensation of the vapours and fractionation of the liquid products, resulting in a complex mixture of compounds (EFSA FAF Panel 2021; Sikorski 2004). It is well known that this process also produces hazardous compounds that could pose a risk to public health, e.g., polycyclic aromatic hydrocarbons (PAHs) like benzo[a]pyrene (BaP) (Šimko 2018; Yabiku et al. 1993). Smoke flavourings are specifically regulated according to Regulation (EC) No 2065/2003, which focuses on the usage of smoke flavourings on or in food items (European Parliament, Council of the European Union 2003). There are currently ten primary smoke flavourings authorized to be used in or on food items (Council of the European Union 2013). Primary products are the primary smoke condensates and primary tar fractions, which are further processed to produce the smoke flavourings applied in food. European Food Safety Authority (EFSA) recently issued an updated guidance document for application on smoke flavouring primary products (EFSA FAF Panel 2021). The initial toxicity studies needed focus on potential genotoxic properties of the smoke flavours, and a tiered approach is applied by combining in silico, in vitro and in vivo evaluations of genotoxic properties. In addition, tier I safety data for developmental and reproductive toxicity

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are required for new authorizations (EFSA FAF Panel 2021, Appendix E). In 2010–2012, EFSA published a number of safety assessments of smoke flavouring primary products where they concluded that there were safety concerns for the proposed uses and levels for several products, whereas others were of no safety concern (EFSA Panel on Food Contact Materials 2011a, b, 2012).

The smoke flavouring primary products, evaluated by EFSA, are mainly used in the food industry. However, there are also smoke flavouring products commercially available directly to consumers. The products available on the market and the primary products are supposedly produced in a similar manner by pyrolysis, but from different sorts of woods. To differentiate the products, we have tested in this study from the smoke flavouring primary products evaluated by EFSA, the tested products will hereafter be called: liquid smoke flavourings.

Liquid smoke flavourings are characterized by having a high variability and complex chemical composition with limited information on toxicity of individual chemical constituents, a large number of unidentified chemicals, and potential interaction between chemicals in the mixture (EFSA FAF Panel 2021). Thus, alternative methods for toxicity testing would be useful (Montazeri et al. 2013). Effect-based methods, often based on cultured mammalian cells that have been modified to respond to key molecular events early in toxicity pathways, have proven to be valuable to evaluate highly complex mixtures (Escher et al. 2021; Rosenmai et al. 2017; Selin et al. 2021).

In this study, we used a panel of in vitro bioassays to evaluate effects of hazardous chemicals in ten commercially available liquid smoke flavourings. The liquid smoke flavourings were tested as non-extracted and extracted, and potential drivers of toxicity were tested by using two different solvents for the extraction. Endpoints covered were estrogenicity, androgenicity, oxidative stress, aryl hydrocarbon receptor activity (AhR), and genotoxicity.

Materials and methods

Liquid smoke flavourings

Ten liquid smoke flavourings marketed to consumers were purchased from different online stores, and were produced from apple, hickory, mesquite, oak, and pecan wood (Table 1). All samples were used within their expiration date. Information of the ingredients and recommended doses are provided in Table SI-2. No information on the flavouring ingredient other than the wood was given on the product itself.

For the non-extracted liquid smoke flavouring, 2 mL was filtered using a 0.22 µm syringe. Thereafter, the samples were transferred into Eppendorf tubes and stored at + 4 °C until analysis.

Table 1 Sample ID analysed as non-extracted and extracted liquid smoke flavourings

Smoke flavouring	Extraction method			
	Non-extracted	SPE (Hex)	LLE (Hex)	LLE (EA)
Apple	A1	A1 SPE		
Hickory	H1	H1 SPE		
	H2	H2 SPE		
	H3	H3 SPE		
	H4	H4 SPE	H4 Hex	H4 EA
	H5	H5 SPE	H5 Hex	H5 EA
Mesquite	M1	M1 SPE		
	M2	M2 SPE		
Oak	O1	O1 SPE		
Pecan	P1	P1 SPE		

Hex hexane, *EA* ethyl acetate

The liquid smoke flavourings were also extracted by two different solvents, namely hexane (Hex, log $K_{ow} = 3.8$) and ethyl acetate (EA, log $K_{ow} = 0.7$), to investigate to what extent polar or non-polar substances are driving the toxic effects.

Samples were extracted with either solid-phase extraction (SPE) or liquid–liquid extraction (LLE), the latter using both hexane and ethyl acetate. We wanted to compare the simpler and more traditionally used extraction method LLE against the more automated SPE method. SPE was performed with Oasis HLB 20 cc cartridges that are able to extract a wide range of compounds with pH ranging from 0 to 14. The extraction procedures are described in the Supplementary Information (SI-1, Sect. 1). In short, samples were either extracted by SPE with hexane or LLE using either hexane or ethyl acetate (Table 1). After extraction, samples were evaporated to dryness using nitrogen and resuspended in 0.5 mL of DMSO before being transferred into Eppendorf tubes for bioanalysis. Hickory samples 1, 2, and 5 were not dissolved in DMSO due to their oily composition and were diluted in cell culture media instead of DMSO. Only two samples were successfully extracted through LLE, namely hickory sample 4 and 5, since a clear separable solvent phase was not obtained for the rest of the samples. The concentrations of extracted samples are given as µL liquid smoke flavouring used for the extraction per mL cell culture medium, to enable a comparison of effect concentrations between non-extracted and extracted samples. The extracted samples were stored at – 20 °C.

Effect-based in vitro methods

Effect-based tests that covered reactive, non-specific, and specific modes of actions were applied (Escher et al. 2021). The methods assessed activation of AhR, androgenicity (AR), estrogenicity (ER), oxidative stress (Nrf2), and genotoxicity (micronucleus test) (Table 2). Detailed information of the methods is presented in the Supplementary Information (Table SI-1).

For all assays, a specific reference compound was used as a standard to validate each run. The vehicle controls consisted of cell medium for the non-extracted smoke flavourings and hickory samples 1, 2, as well as 5, and DMSO was used for the remaining extracted samples.

Cytotoxicity was evaluated by MTS and ATPase assay, as described in the Supplementary Information (SI, Sect. 1.5), and by ethidium monoazide stain (EMA) in the genotoxicity (micronucleus) assay.

The non-extracted smoke flavouring samples were tested at concentrations 0.002–1 µL liquid smoke flavouring/mL cell culture medium and the extracted samples were tested at concentrations from 0.003 to 200 µL liquid smoke flavouring/mL cell culture media. The concentrations used in the bioassays were decided from the effects on cytotoxicity to ensure that bioactivity was assessed at non-cytotoxic concentrations. Samples were analysed in either twofold or fivefold dilutions.

Data evaluation

Cell viability results were normalized to the vehicle control, which was set as 100%. Samples causing more than 20% reduction were considered cytotoxic, except for the micronucleus test where the cytotoxicity limit was defined as fourfold increase in %EMA-positive events compared to the vehicle control.

For nuclear receptor agonistic response, the activity was first normalized to the vehicle control, and then normalized to the maximum (max) effect of the standard. The antagonistic receptor activities of samples were normalized to vehicle controls without DHT, followed by normalization to the max effect of the vehicle control exposed to DHT. Standard curves for the nuclear receptors were created in GraphPad Prism 9 Software (San Diego, California, USA) using non-linear (log logistic) sigmoidal curve fit. For oxidative stress response, the activity was normalized to the vehicle control, since no max effect can be reached (Escher et al. 2018). The response was therefore fitted to a linear regression dose–response curve.

The limit of detection (LOD) was calculated as three times the standard deviation of the vehicle control. The cut-off limit was based on the LOD and used to define a sample as bioactive. The cut-off was set as the even number above the LOD for agonistic activity and below the LOD for antagonistic activity (Escher et al. 2018, Table SI-1).

The cut-off was set at 70% for antagonistic samples; thus, samples with activities at or below 70% were considered bioactive. The effect concentration 20% (EC_{20}) or inhibitory concentration 30% (IC_{30}) was calculated for agonistic and antagonistic activity for all bioactive samples, respectively.

Effect concentration induction ratio 1.5 ($EC_{IR1.5}$) was calculated for samples in the oxidative stress Nrf2 assay. Nrf2 activity was presented as fold change compared to the vehicle controls.

For genotoxicity, the micronucleus formation data were analysed in GraphPad Prism 9 using one-way ANOVA with Dunnett's multiple comparison test. Samples were defined as bioactive if the responses were statistically significant compared to the vehicle control value (p value < 0.05).

The bioanalytical equivalent concentration (BEQ) was calculated, to relate the effect of the sample to a known reference compound, according to the following formula (Escher et al. 2015):

$$\mathrm{BEQ} = \frac{\mathrm{EC_{20}orEC_{IR1.5}orIC_{30}(referencecompound)}}{\mathrm{EC_{20}orEC_{IR1.5}orIC_{30}(sample)}}.$$

The BEQ was then multiplied with the recommended serving size from the manufacturers to retrieve the estimated exposure in bioequivalents of reference compound

Table 2 Summary of the effect-based in vitro methods

In vitro method	Cell line	Reference compound	Concentration (µM)
Cytotoxicity	All cell lines mentioned below	N/A	N/A
Aryl hydrocarbon receptor activity	DR-EcoScreen	2,3,7,8-Tetrachlorodibenzodioxin (TCDD)	1×10^{-8} to 3×10^{-4}
Androgen receptor agonistic activity	AR-EcoScreen GR-KO M1	Dihydrotestosterone (DHT)	1×10^{-9} to 1×10^{-3}
Androgen receptor antagonistic activity	AR-EcoScreen GR-KO M1	Hydroxyflutamide (OHF)	1×10^{-5} to 1×10^{1}
Estrogen receptor agonistic activity	VM7Luc4E2	Estradiol (E2)	4×10^{-7} to 4×10^{-4}
Oxidative stress response	MCF7 AREc32	Tert-Butylhydroquinone (tBHQ)	8×10^{-1} to 2.5×10^{1}
Micronucleus test	TK6	Mitomycin C* (MMC)	1×10^{-1} and 2×10^{-1}

*MMC was used as a positive control

per serving size/portion. If the recommended serving size was not stated, it was assumed to be 5 mL (Table SI-3). For the manufacture that stated that the recommended serving size was a few drops, we estimated that one drop was 0.05 mL and that three drops would be representative as the serving dosage.

Results

Cytotoxicity

Cell viability was investigated after 24 h exposure of MCF7 AREc32, DR-Ecoscreen, VM7Luc4E2, and AR-EcoScreen with glucocorticoid receptor knockout mutant 1 (GR-KO MI) cells to liquid smoke flavourings (Figs. SI-1, SI-3, SI-5, SI-7). Treatments that reduced the viability with more than 20% were considered cytotoxic and were excluded from further testing.

Non-extracted samples exhibited higher cytotoxicity in comparison to all extracted samples. For the majority of the non-extracted samples, cytotoxicity was observed at the highest concentration tested (Figs. 2A, SI-1, SI-3, SI-5, SI-7). Non-extracted hickory samples 2, 4, and 5 retrieved the highest potency of all tested samples in MCF7 AREc32, DR-EcoScreen GR-KO M1, VM7Luc4E2, and AR-EcoScreen cell lines (Figs. SI-1, SI-3, SI-5, SI-7).

A similar trend of cytotoxicity was seen for ethyl acetate LLE samples, where higher potencies were obtained for ethyl acetate-extracted samples than for hexane-extracted samples (Figs. 2A, SI-1C, SI-3C SI-5C, SI-7C).

SPE samples exhibited varying cytotoxicity, although to a considerable lower degree compared to the non-extracted samples (Figs. 2A, SI-1, SI-3, SI-5, SI-7).

Oxidative stress (Nrf2)

Oxidative stress, measured as Nrf2 activity, was induced by all non-extracted hickory-smoke product samples 1–5 in a dose-related manner (Fig. 1A). Highest potency was obtained for H2, H4 and H5. H2 was the most potent of all non-extracted samples tested, causing a 25-fold induction at a concentration of 0.04 µL/mL. This specific sample was even bioactive at the lowest concentration of 0.003 µL/mL (Fig. 1A).

Nearly all SPE samples (8/10) were bioactive at the highest concentration tested, however, at considerable higher concentrations (12.5–200 µL/mL) than in the non-extracted samples. Mesquite sample 2 caused oxidative stress to the highest degree, reaching a 31-fold induction at 200 µL/mL (Fig. 1E). LLE samples extracted with ethyl acetate induced Nrf2 with a higher potency than SPE extracted (Fig. 1C). Hickory sample 5, extracted with ethyl acetate, showed the highest activity and was bioactive at all concentrations tested, ranging from 0.1 to 6.3 µL/mL (Fig. 1C). In contrast, samples extracted with hexane showed a very low induction of Nrf2 (Fig. 1C). Hickory sample 4 exhibited a similar Nrf2 efficacy when tested non-extracted, extracted through SPE and LLE with ethyl acetate, however, at different concentrations, 0.04, 100, and 6.3 µL/mL, respectively (Fig. 1A–C). A similar oxidative stress response was seen for non-extracted and SPE mesquite sample 1 at the highest concentration tested, although the former being 1000 times less concentrated (Fig. 1D, E).

The linear dose–response of the standard tBHQ is presented in the Supplementary Information (Fig. SI-2). For all non-extracted bioactive samples, defined by the cut-off limit of 1.5-fold change, BEQ values were calculated as mg tBHQ equivalent concentrations (eq) per serving size/dose. The BEQ values ranged from 0.9 to 452 mg tBHQeq per 5 mL sample (Table SI-3). Hickory sample 2, 4, and 5 retrieved the highest BEQ values of 452, 384, and 345 mg tBHQeq/5 mL sample, respectively (Table SI-3).

Genotoxicity

Samples inducing oxidative stress to a high degree were investigated in the micronucleus test (MN) (Fig. 2). Liquid smoke samples detected as cytotoxic, as indicated by a fourfold increase in % EMA events compared to the control, were excluded for MN assessment (Fig. 2A).

The non-extracted tested hickory sample 2, 5 and mesquite sample 2 showed a statistically significant increase in the micronuclei formation (Fig. 2B). In agreement with the oxidative stress assay, hickory sample 4 and 5 extracted with SPE also caused a statistically significant increase in the genotoxic response (Fig. 2B). Extraction with ethyl acetate did not affect the micronuclei formation. Genotoxicity was observed for both non-extracted and SPE mesquite sample 2, but the potency was higher in the non-extracted sample (Fig. 2B).

The positive control mitomycin C caused an elevated genotoxic response in a dose-dependent manner, in which the highest concentration of 200 nM caused the highest MN formation.

AhR activity

Activation of AhR, defined by the cut-off limit of 15% of max effect, was observed for both non-extracted and SPE extracted samples. The sample being the most potent and having the highest efficacy was hickory sample 2 of all non-extracted samples, while hickory sample 4 was the most potent of all extracted samples (Fig. 3A, B). The highest concentration of SPE extracted hickory sample 2 had 23% of max effect and 40% with hickory sample number 4 (Fig. 3B).

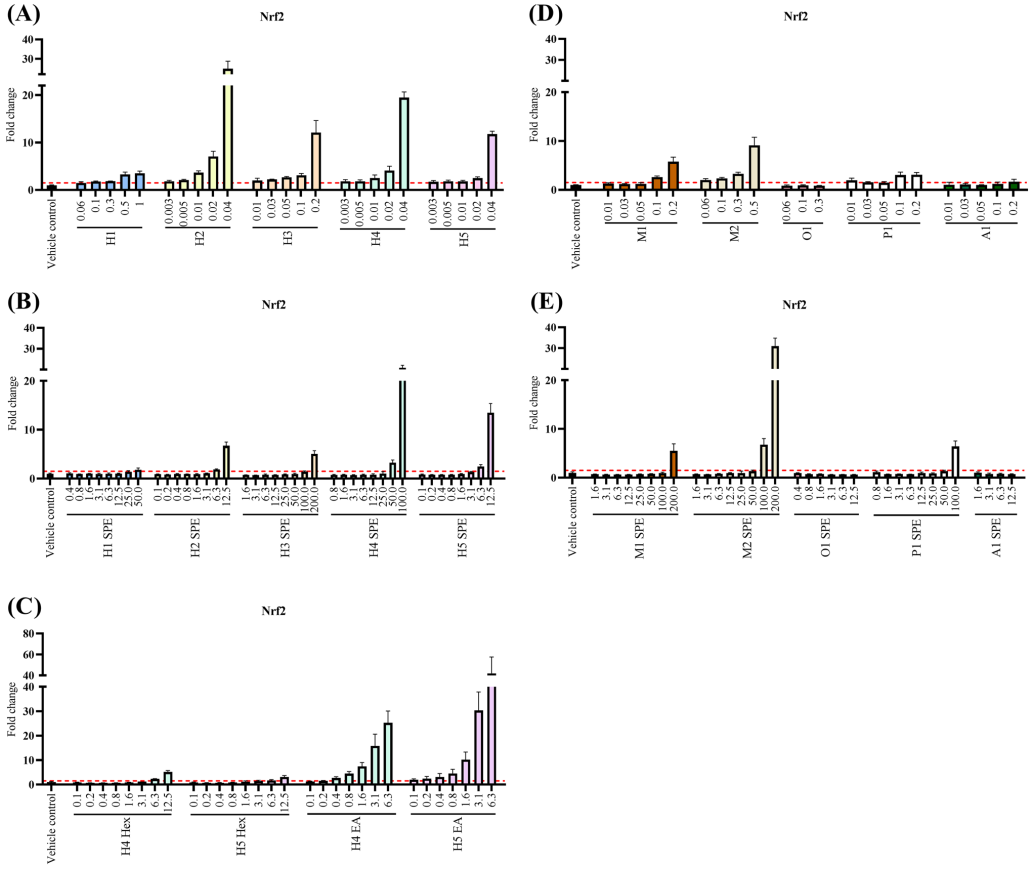

Fig. 1 Nrf2 response (fold change compared to control) upon 24 h exposure of MCF7 AREc32 cells to liquid smoke flavourings: non-extracted (**A**, **D**), SPE extracted (**B**, **E**), and LLE extracted (**C**). Concentrations on the *x*-axis are expressed as µL liquid smoke flavouring/mL cell culture medium. Data illustrate mean ± SD ($n = 4$), and the dotted line represents the induction ratio of 1.5-fold change, defined as the cut-off limit of bioactivity

Non-extracted and SPE extracted mesquite sample 1 and 2 induced AhR activity even at the lower tested concentrations (Fig. 3D, E). The AhR response drastically increased upon SPE extraction, whereas it remained inactive when tested non-extracted or extracted with hexane and ethyl acetate through liquid–liquid (Fig. 3A–C). Still, worth mentioning is that the effect for the majority of the samples was only visible at the highest non-cytotoxic concentration. The only exceptions were for SPE mesquite sample 1 and 2 (Fig. 3E). Hickory sample 1, 5 and apple sample 1 remained inactive when tested non-extracted and extracted (Fig. 3A–E). Pecan sample 1 evoked a higher AhR response upon SPE extraction, likely due to the higher concentration used and the activity reached a max effect of 30% (Fig. 3E).

TCDD was used as standard and the non-linear dose response is shown in the Supplementary Information (Fig. SI-4). The BEQ values ranged from 14,000 to 300,000 pg TCDDeq per 5 mL sample for the non-extracted samples, in which hickory sample 2 obtained the highest BEQ value (Table SI-3).

Estrogenicity

We observed estrogenic activity in three out of ten non-extracted samples (Figs. SI-6A, 6D). The activity was only seen at the highest concentration tested for hickory sample 1 and 2, as well as mesquite sample 2.

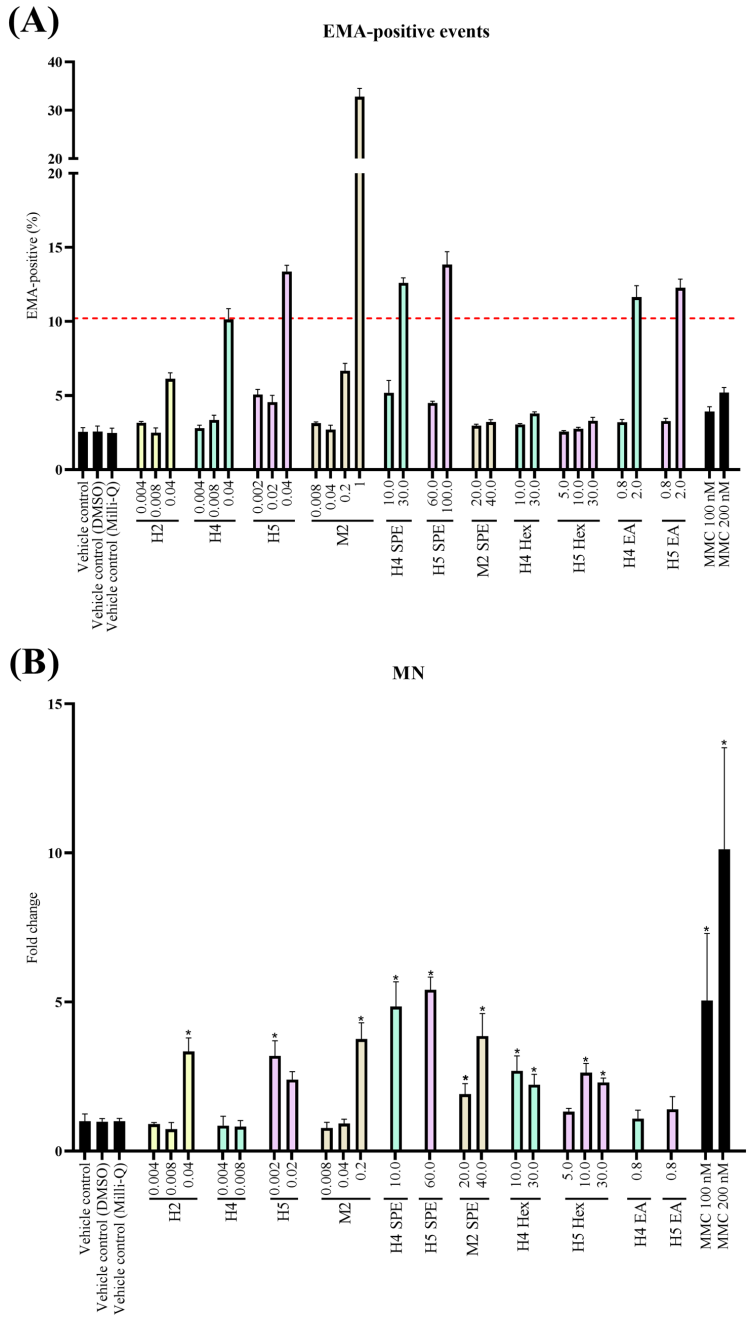

Fig. 2 Cytotoxic and genotoxic response (fold change of micronuclei events compared to control) upon 24 h exposure of TK6 cells to liquid smoke flavourings: cytotoxicity (**A**) and micronuclei events (**B**). Concentrations on the *x*-axis are expressed as µL liquid smoke flavouring/mL cell culture medium. The graph demonstrates mean ± SD, $n = 12$ for controls and $n = 4$ for samples. Mitomycin C (MMC) was used as a positive control at concentrations 100 and 200 nM. Samples that were statistically significantly different from the control are indicated with an asterisks (*p value < 0.05)

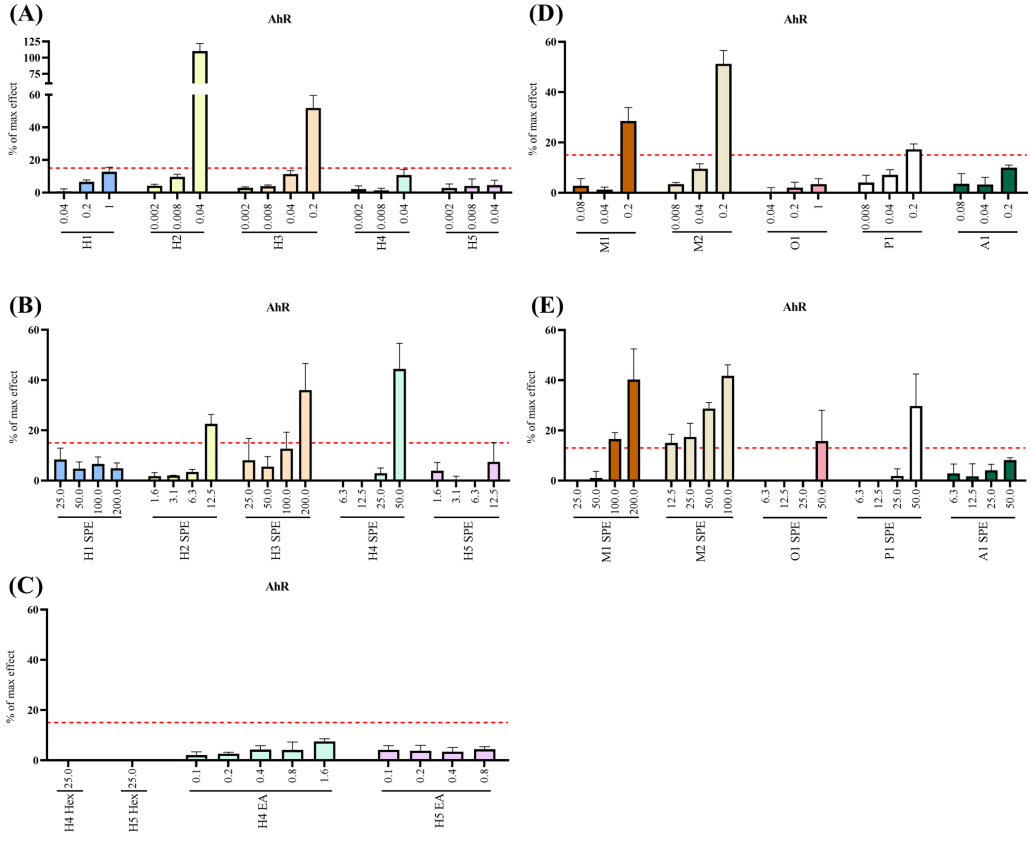

Fig. 3 AhR activity (% of max effect) after 24 h exposure of DR-EcoScreen cells to liquid smoke flavourings: non-extracted (**A**, **D**), SPE extracted (**B**, **E**) and LLE extracted (**C**). Concentrations on the x-axis are expressed as µL liquid smoke flavouring/mL cell culture medium. Data illustrate mean ± SD ($n = 4$) and the dotted line represents the % max effect of 15, defined as the cut-off limit of bioactivity

No ER activity was observed for extracted hickory sample 1 and 2 (Fig. SI-6B). On the contrary, mesquite sample 2 had a drastically increased activity, between 60 and 108% of the max effect, after SPE extraction (Figs. SI-6B, 6E).

ER activity was highly increased in several of the extracted products, this was especially true for SPE extracted samples (Figs. SI-6B, 6E). When comparing the extraction techniques, samples extracted with SPE elicited higher estrogenic response for hickory sample 4 compared to LLE hexane samples (Figs. SI-6B, SI-6C).

SPE caused a higher induction, likely explained by being more concentrated (Figs. SI-6B, SI-6C). Hickory sample 4 extracted with ethyl acetate did not induce estrogenicity, in comparison to the hexane extractions which showed a strong estrogenic response. On the other hand, hickory sample 5 exerted no response as non-extracted or SPE extracted, while LLE extraction by hexane and ethyl acetate caused an elevated response of the estrogen receptor (Figs. SI-6C).

E2 was used as a standard and the non-linear dose–response curve is found in the Supplementary Information (Fig. SI-6F). Only one BEQ value was obtained for the non-extracted samples, which was 1.6 ng E2eq/5 mL for mesquite sample 2, as the remaining samples either remained inactive or were below the detection limit (Table SI-3).

Androgenicity

The non-extracted and SPE hickory samples did not activate the androgen receptor, defined by the cut-off limit of 4% of max effect (Figs. SI-8A, SI-8B). The lack of response could be explained by the usage of low concentrations, as higher

concentrations exerted cytotoxicity. Nevertheless, upon SPE extraction, the oak sample 1 and pecan sample 1 elicited agonistic response of the androgen receptor (Figs. SI-8D, SI-8E). Furthermore, the agonistic response of hickory sample 5 drastically increased in a dose-related manner after LLE with hexane, but it remained inactive when extracted with ethyl acetate (Fig. SI-8C).

No antagonistic mode of action on the androgen receptor was observed when cells were exposed to the non-extracted liquid smoke flavourings (Figs. SI-9A, SI-9D). A few samples exhibited antagonistic effects, but the sudden drop in activity suggests that these effects more likely can be explained by undetected cytotoxicity and should therefore be interpreted with caution (Figs. SI-9B, SI-9E). A similar profile of antagonistic effect was seen for hickory sample 4 that was liquid–liquid extracted with ethyl acetate (Fig. SI-9C).

The non-linear dose–response curves of DHT and OHF are available in the Supplementary Information (Figs. SI-8F, 9F). No BEQ values were obtained for agonistic and antagonistic androgen receptor response (Table SI-3).

Discussion

In this study, we used a panel of effect-based methods to retrieve information on potential toxicity of the mixture of chemicals that defines liquid smoke flavourings. The specific endpoints studied were oxidative stress, genotoxicity, aryl hydrocarbon, estrogen, and androgen receptor activities in addition to general cytotoxicity.

A high cytotoxicity was observed in all but two of the non-extracted samples. For several of the samples, cytotoxicity was already seen at 1 µL liquid smoke flavouring per 1 mL cell culture medium, and for some even at 0.2 µL/mL. Cytotoxicity was considerably reduced after SPE and LLE with hexane, thus allowing higher concentrations to be tested, and therefore, higher effects were seen in comparison to non-extracted products. The results indicate that cytotoxicity mainly originates from polar substances. This is further supported by the higher cytotoxicity in samples after LLE with ethyl acetate, where cytotoxicity was almost as high as in the non-extracted samples. The results emphasize the impact of extraction procedure in bioanalysis (Abbas et al. 2019).

Exposure of cells to liquid smoke flavourings induced oxidative stress response, determined as Nrf2 activity. All hickory and the two mesquite samples induced oxidative stress especially in non-extracted but also in extracted samples. Extraction procedure had a main impact on the oxidative stress response, and induction of Nrf2 activity was pronounced upon extraction with ethyl acetate, supporting the suggestion that polar substances are main drivers of oxidative stress, as discussed above for cytotoxicity. As oxidative stress may be associated with genotoxicity, four of the samples which induced Nrf2 activity were tested for genotoxic potential by a micronucleus test. Non-extracted samples had a higher potency compared to hexane-extracted samples, except for one non-extracted sample (H4), which was not genotoxic at non-cytotoxic concentrations. Interestingly, ethyl acetate-extracted samples did not increase micronuclei formation, which may be explained by the low concentrations used as higher concentration caused toxicity, or by the fact that polar substances do not drive genotoxicity.

Previous studies have shown increased DNA single-strand breaks (Ohshima et al. 1989), altered pyloric glands in rats after oral exposure to hickory-smoke condensate (Shichino et al. 1992) and mutation in human lymphocytes in vitro after exposure to aqueous wood smoke flavourings (Braun et al. 1987). A more recent study confirmed the genotoxic potential of commercially available liquid smoke flavourings in a human p53 reporter gene cell line, and reported higher p53 response in hickory than mesquite samples (Hossain et al. 2013). Additionally, increased γ-H2AX, p21 and p53 protein levels were detected. However, other studies failed to detect genotoxicity or obtained inconclusive results in the Ames test (Braun et al. 1987; Putnam et al. 1999). The lack of effect in the Ames test may be explained by the low sensitivity of the test and/or usage of different smoke flavoured products (Kirkland et al. 2014). PAHs are generated during the smoke formation and are thought to covalently bind to protein and nucleic acids, forming DNA adducts that may be carcinogenic (Luo et al. 2008; Oz 2020; Šimko 2011).

The formation of PAHs is of human health concern and has to be analysed for authorization of liquid smoke flavourings (Commission Regulation EC No 627/2006 2006). Metabolic activation is needed for PAHs to exert DNA damaging effects. In this study, we did not include a metabolic activation system and the results therefore suggest that the genotoxicity observed is mediated through other chemicals. Furthermore, the specificity of TK6 cells to distinguish between clastogen and aneugen modes of action seems to be lower in comparison to when other cell lines are used (Bryce et al. 2011; Smart et al. 2020). The results in this study together with previous studies show that a variety of commercially available liquid smoke flavourings may have genotoxic properties in vitro, which needs to be further investigated.

AhR activity was induced in five of the ten non-extracted samples and in seven of the SPE samples, although at much higher concentrations. This was most obvious for hickory sample 2, where the non-extracted sample had the highest efficacy of all tested liquid smoke samples, and the activity was drastically reduced after SPE extraction. AhR activity can be induced by numerous chemicals, for example by PAHs (Boonen et al. 2020).

Apart from a single positive sample and two at the cut-off level, no ER activity was observed in the non-extracted samples. However, higher concentrations could be tested than of the non-extracted samples due to cytotoxicity, and seven of the SPE samples exhibited estrogenic activities. Boonen et al. (2020) reported ER activity by BaP in bioassays. No AR activities, agonistic or antagonistic, were detected in the non-extracted samples, while two of the SPE samples were active in the highest concentration. PAHs have been shown to induce antagonistic androgen receptor activity in water samples (Xu et al. 2019).

Bioactivities varied widely between the various products. Some products exhibited no or a low activity in all assays (H1, O1), while others had a high activity in several of the assays (H2, M2). The bioactivities depend on the concentrations of the individual bioactive compounds and interactions between the compounds in the mixture, which are unknown factors. Wood type, burning conditions, purification, pH-, total acid, chemical, and water content are factors influencing the chemical composition of smoke flavourings (Budaraga et al. 2016; Sikorski 2004; Šimko 2005). The commercially available liquid smoke flavourings investigated in the present study had limited information on manufacturing and identity, compared to the registered smoke flavourings (Council of the European Union 2013). However, it is supposed that the smoke flavourings in general should be regarded as safer than smoke products generated directly from the traditional smoking procedure, as toxic chemicals can be removed during the filtration and purification processes (European Parliament, Council of the European Union 2003).

The bioequivalent concentrations corresponding to the observed bioactivity for the non-extracted products in each assay was calculated and expressed as bioequivalents of the reference compound per serving size, to allow a comparison to the estimated intake via food or drinking water. For estrogenic activity, the only sample with a BEQ value was M2, corresponding to 1.6 ng E2eq per serving. This can be compared to the WHO benchmark value for drinking water of 1 ng E2/L (WHO 2017). The daily consumption of drinking water is estimated to 2 L, which means that the exposure of E2eq from one serving size of M2 is below the benchmark value of E2 in drinking water.

For oxidative stress, the BEQ values ranged from 0.9 to 452.0 mg tBHQeq/serving. This value can be compared to the acceptable daily intake (ADI) of tBHQ provided by EFSA, which is 0.7 mg/kg bw/day, corresponding to 49 mg/day at a body weight of 70 kg (EFSA 2004). Six of the ten liquid smoke flavourings resulted in intakes above the ADI for one serving size, of which hickory samples 2, 4, and 5 had the highest BEQ values.

The calculated guidance value for AhR activity was in this case not appropriate, as the liquid smoke products obviously is not induced by dioxins or planar PCBs, but rather by other chemicals with different toxicokinetics and toxicodynamics. The calculated TCDD equivalents from the liquid smoke flavourings ranged from 14,000 to 300,000 pg per serving, and greatly exceeded the tolerable weekly intake (TWI) established by EFSA of 2 pg/kg body weight, corresponding to 140 pg/person/week (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al. 2018).

Information on potential toxic effects of the commercially available liquid smoke flavourings is scarce. In contrast, toxicity of smoke products in E-cigarettes has attracted more attention. Smoke flavourings as food additives and in E-cigarettes are both based on the same concept, namely to remove the most toxic substances produced from natural combustion, while retaining flavouring compounds. Cell-based bioassays have been used for hazard evaluation of cigarette smoke constituents, but we are not aware of a similar approach for hazard evaluation of liquid smoke flavourings (Barhdadi et al. 2021; Moore et al. 2020; Rudd et al. 2020; Stabbert et al. 2017). Even if the route of exposure differs between E-cigarettes and liquid smoke flavourings, both will reach the systemic circulation after absorption. Rudd et al. (2020) reported that E-cigarettes should be considered as a safer option to cigarette smoke, which likely can be explained by the fact that the flavour and nicotine are received through aerosolization of E-cigarettes, compared to burning of tobacco in cigarette smoke, allowing fewer toxicants to be formed. It was concluded that less cytotoxicity in the neutral red uptake (NRU) assay and no mutagenicity (Ames test) or genotoxicity (MN test) was seen for E-cigarettes, compared to the reference cigarette. However, it is not agreed within the research field that e-cigarettes should be considered as safer, as these liquids may contain genotoxicants (Barhdadi et al. 2021).

The same controversy can be said for liquid smoke flavourings in comparison to the traditional smoking of food. A similar approach to use and generate in vitro data of E-cigarettes would be recommended to be applied to liquid smoke flavourings (Moore et al. 2020).

In this study, we have tested ten commonly used liquid smoke flavourings and used two different solvents to investigate if polar or non-polar substances are driving the toxic effects. The increased bioactivities upon extraction indicate that non-polar substances are driving the genotoxicity, whereas polar substances are driving the oxidative stress and cytotoxicity. The usage of effect-based methods allowed testing of the complex whole mixture, enabling us to study interactive effects of the product. Findings in this study indicate that liquid smoke flavourings contain compounds with hazardous properties and to ensure that these widely used products are safe further studies should be carried out.

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Availability of data and materials All data are included in the manuscript or in the Supplementary Material. Further data or information will be supplied upon request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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Evaluation of *in vitro* bioassays as a screening tool to monitor chemical hazards in cow's milk

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ABSTRACT

Studies on cow's milk have mainly focused on analyzing specific chemical groups and natural components. Therefore, in this study, we evaluated if effect-based *in vitro* methods could be used as a screening tool to monitor chemical hazards in milk. In total, 32 milk samples were collected from a Swedish dairy company throughout one year. These samples included conventional and organic semi-skimmed as well as raw milk. The milk samples were tested in five *in vitro* methods covering eight endpoints. These endpoints included cytotoxicity, endocrine disruption (estrogen/androgen induction/inhibition), aryl hydrocarbon receptor activity, oxidative stress and DNA damage. Estrogen and androgen receptor inhibition, in addition to aryl hydrocarbon receptor activity, were the most responsive endpoints, where 10 to 13 out of the 32 milk samples were bioactive. Organic and conventional milk showed no major differences. Overall, no or only low activities were observed in milk samples in the remaining *in vitro* assays, which is a promising result with regard to applying effect-based methods as a screening tool. Concerning the most responsive assays, more research is needed to understand the normal background variations before they can be used as a screening tool for chemical hazards in milk.

1. Introduction

Chemical pollutants have been extensively studied in matrices like surface, drinking and wastewater (Escher et al., 2013; König et al., 2017; Lundqvist et al., 2021; Oskarsson et al., 2021). Multiple studies have shown that the most often analyzed and/or well-known pollutants only explain a small fraction of the toxicity observed within the *in vitro* methods (Escher et al., 2013; König et al., 2017; Oskarsson et al., 2021). Thus, relying solely on chemical analysis of a limited number of individual substances provides an inadequate picture of the hazards posed by chemical pollutants. Since milk and milk products are food groups that are consumed by numerous people, it is important to have a good control system in place to ensure that these products are not contaminated. The main causes of contamination are via feed and water (Schulz et al., 2005).

Most research efforts in milk monitoring have focused on quantifying specific chemical groups, natural compounds and the composition of the milk (Foroutan et al., 2019; Di Bella et al., 2020; Hasan et al., 2022; Róin et al., 2023), but there is scarce information on the overall biological effects of the total milk chemical exposome that potentially can be related to adverse health effects, and how these effects may vary throughout the year. This underlines the necessity to adopt a holistic approach, where the effects of known, unknown and mixtures of biologically active chemicals are efficiently evaluated. *In vitro methods*, also referred to as effect-based methods, yield information about the modes of action of chemicals and indicate if there are chemicals of concern in a sample. These methods can be used early in the hazard assessment (Escher et al., 2021b). Consequently, we wanted to apply a similar approach with the aim to evaluate if *in vitro* bioassay methods could be used as a screening tool to monitor chemical hazards in cow's milk.

The present study, therefore, used a panel of five *in vitro* methods, all closely linked to toxicity pathways of high relevance to human health. We wanted to investigate the background levels of bioactive compounds in Swedish milk samples and see if any differences between organic and conventional raw and semi-skimmed milk could be quantified. Additionally, we also wanted to explore if there were any seasonal variations in cow's milk. We hypothesized that higher activities in the cow's milk may be observed in the mandatory grazing period of the cow (i.e. outdoor period), as the consumption of grass and unintentional ingestion of soil increases, which can contain chemicals like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (McLachlan, 1993; Hasan et al., 2022). These chemical groups are known to

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increase the activity of AhR. Furthermore, we also hypothesized that the estrogen receptor activities potentially could be altered during the consumption of clover when grazing, due to the phytoestrogen content (Róin et al., 2023), that is if the phytoestrogens are broken down during the conservation of the silage. The endpoints focused on were cytotoxicity, endocrine disruption (estrogen/androgen receptor induction/inhibition), xenobiotic metabolism (aryl hydrocarbon receptor activity), oxidative stress (in the form of Nrf2 activity) and DNA damage (micronucleus test).

2. Materials and methods

2.1. Chemicals and solvents

The solvents acetonitrile (75-05-8, ≥99.9%) and formic acid (64-18-6, ≥98%) were purchased from Sigma-Aldrich. Methanol (67-56-1, ≥99.8%) was supplied from VWR. Ultrapure water (Milli-Q®) was sourced from a Millipore® facility system using a 0.22 μm filter.

Dimethyl sulfoxide (DMSO, CAS 67-68-5 >99.9%), 5α-androstan-17β-ol-3-one (DHT, CAS 521-18-6, ≥97.5%), β-estradiol (CAS 50-28-2, ≥98%), hydroxyflutamide (OHF, CAS 52806-53-8, ≥98%), methoxychlor (CAS 72-43-5, 98.7%), raloxifene hydrochloride (Ral, CAS 82640-04-8), tamoxifen (CAS 10540-29-1, ≥99%), 2,3,7,8-tetrachlorodibenzo-p-dioxin solution (TCDD, CAS 1746-01-6), tert-butylhydroquinone (tBHQ, CAS 1948-33-0, 97%) and mitomycin C (MMC, CAS 50-07-7) were acquired from Sigma-Aldrich.

2.2. Sample preparation and extraction

Representative milk samples were collected monthly from the largest dairy company in Sweden between June 2020 to May 2021 (weeks 26, 2020 to 21, 2021) and consisted of in total 32 samples; organic semi-skimmed milk (n = 4), conventional semi-skimmed milk (n = 12), organic raw milk (n = 4) and conventional raw milk (n = 12). Organic milk was collected every third week, starting from week 26 2020 and ending at week 12 2021. The raw milk was pooled from multiple farms around the dairy plant and was collected before any processing occurred. Normally, raw milk has a fat content of 4.3%. The raw milk arrived in sterile polyethylene terephthalate (PET) sampling bottles with blue polypropylene (PP) caps (VWR®, #3310269). Semi-skimmed milk, on the other hand, had a fat content of 1.5% and was homogenized as well as pasteurized. These were delivered in commercially available coated paperboard cartons. Directly after packaging, milk samples were frozen (−20 °C) until the sample preparation started.

A similar method for milk sample preparation and extraction was applied as the one developed by Waters (Huang et al., 2015). Each milk sample was mixed by inversion a few times prior to opening. For 50 mL of milk, 200 mL of 0.2% formic acid in acetonitrile was added and mixed to precipitate proteins. The samples were then centrifuged for 30 min at 5000 rpm and supernatants were collected for solid-phase extraction (SPE). The 3 cc Oasis PRiME HLB Cartridge (Oasis, #186008056) was conditioned with 0.2% formic acid in acetonitrile. Thereafter, the supernatants were loaded onto the cartridge and collected. The cartridge allowed matrix interferences like phospholipids and fats to efficiently be removed from the milk. The fast and effective modified SPE method allows acidic, basic and neutral compounds to be retrieved with high recoveries (Huang et al., 2015). After collection, the samples were filtered using a 0.22 μm filter. Evaporation to dryness occurred on the TurboVap II Evaporation System (Biotage) and samples were dissolved in 0.5 mL of 5% methanol in Milli-Q® water (5% MeOH/H_20).

The concentrations of the milk samples were expressed as the relative enrichment factor (REF). Milk samples were 100× enriched during the extraction procedure and 100× diluted in the *in vitro* assays, resulting in the highest concentration of 1. The final plate concentrations ranged from REF 1.00 to 0.02. REF <1 represents diluted samples. The final concentration of the milk samples depended on the cell viability results.

Three solvents blanks treated in the same way as the samples, but without any milk, were also prepared and tested. All samples were stored at −20 °C until analysis.

2.3. In vitro test methods

A panel of *in vitro* methods, all closely linked to toxicity pathways of high relevance to human health, was applied to the milk samples. These covered specific action modes (estrogen/androgen receptor induction/inhibition, aryl hydrocarbon receptor induction), non-specific (cytotoxicity) and reactive toxicity (micronucleus formation; MN, oxidative stress). Additional details of the *in vitro* methods and cell maintenance are found in the Supplementary Information (SI 1–3).

Each run was validated by using an assay-specific reference compound to generate a dose-response curve. Further information on the standards as well as yielded effect concentration (EC) and inhibitory concentrations (IC) can be found in the Supplementary Information (Table S1).

The vehicle controls consisted of 5% methanol in Milli-Q® water for the milk samples and DMSO for each standard. The standards tested were tBHQ, TCDD, DHT and β-estradiol, for oxidative stress, AhR response, induction of androgen as well as estrogen receptors, respectively. Hydroxyflutamide was used for the inhibitory response of the androgen receptor, while raloxifene was used for the inhibitory estrogen receptor activity. The positive controls included tamoxifen (ER inhibitory response), methoxychlor (ER induction response) and mitomycin C (genotoxic response).

2.4. Data analysis

Cell viability data were expressed as fold change compared to the vehicle controls, which was set as 100% for all *in vitro* tests apart from the micronucleus test. A reduction of more than 25% was defined as cytotoxic. For the micronucleus test, cytotoxicity was evaluated by staining the cells with ethidium monoazide stain (EMA), and a 4-fold increase of %EMA-positive events to the vehicle control was considered cytotoxic (Bryce et al., 2013; Laboratories, 2018).

The limit of detection (LOD) of all the *in vitro* endpoints was calculated to identify the concentration of the reference compound that induces three times the standard deviation (SD) of the normalized vehicle control, except for oxidative stress (Escher et al., 2021c). For oxidative stress, LOD was instead calculated as one plus three times the SD of the normalized vehicle control (Escher, Neale and Leusch, 2021c). Based on the LOD the cut-off was set to express the sample as bioactive.

The cut-off for oxidative stress, induction mode of the hormonal receptors and AhR response was set as the even number above the LOD. For the inhibitory mode of the hormonal receptors, the cut-off was set as the even number below the LOD (Table S1).

The data generated from the AhR and induction of hormonal receptors were normalized to the vehicle control, followed by being normalized to the maximum (max) effect of the corresponding standard. The inhibitory mode of the receptors was, on the other hand, first normalized to the unspiked vehicle control and then normalized to the max effect of the spiked vehicle control.

The bioanalytical equivalent concentrations (BEQ) were only calculated for bioactive samples to relate the effect of a known standard to the effect of a milk sample. It was derived by dividing the ratio of the effect concentration (EC) of the reference compound by the EC value of the sample, per the following equation (Escher et al., 2021a):

$$BEQ_{bioassay} = \frac{EC_{IR1.7}, EC_x \text{ or } IC_{30}\ (reference\ compound)}{EC_{IR1.7}, EC_x \text{ or } IC_{30}(sample)}$$

$x = 10, 20$

The standard error (SE) for BEQ for oxidative stress was calculated

according to the formulas (Escher, Neale and Leusch, 2021a):

$$EC_{IR1.7} = \frac{0.7}{slope}$$

$$SE(EC_{IR1.7}) = \frac{0.7}{slope^2} \times SE(slope)$$

For the remaining assays, where the effect was linear up to 30% of the max effect, the SE was derived by the equations (Escher et al., 2021a):

$$y = slope \times concentration$$

$$EC_x = \frac{y}{slope}$$

$$SE(EC_x) = \frac{y}{slope^2} \times SE(slope)$$

$$SE\left(BEQ_{bioassay}\right) = \sqrt{\frac{1}{EC_x(sample)^2} \times SE(EC_x(reference\ compound))^2 + \frac{EC_x(reference\ compound)^2}{EC_x(sample)^4} \times SE(EC_x(sample))^2}$$

Linear regression analysis was applied in GraphPad Prism version 9.5.0 (San Diego, California, USA), after normalizing the data to the vehicle control (fold change) to fit the oxidative stress data, as no max response exists (Escher et al., 2014). Nonlinear regression (log-logistic) analysis was used for the remaining standards with a four-parameter sigmoidal curve fit in GraphPad Prism (Table S1).

Data generated from the micronucleus test was first assessed in FCS Express 7 Flow Research Edition, then in GraphPad Prism version 9.5.0, where bioactivity was statistically evaluated by one-way ANOVA with Dunnett's Multiple Comparison test. Bioactive samples were defined by being statistically significant to the normalized vehicle control (p-value below 0.05).

3. Results and discussion

3.1. Bioactivities

3.1.1. Cytotoxicity testing

Generally, no cytotoxicity was observed in MCF7 AREc32, VM7Luc4E2 and TK6 cells in the concentration range tested (Figs. S1, S3 and S5). The only exception was raw milk at week 2 in VM7Luc4E2 cells, which exerted a high cytotoxic effect at REF 1 (Fig. S3). On the contrary, 22 out of the 32 milk samples were cytotoxic at the highest REF of 1 in the DR-EcoScreen cell line (Fig. S4), while 26 out of 32 milk samples were cytotoxic in the AR-EcoScreen GR-KO M1 cell line at REF 1 (Fig. S2). Two of these samples (W35 - raw milk, W2 – semi-skimmed milk) were cytotoxic down to REF 0.25.

The solvent blanks did not affect the viability (*data not shown*). Based

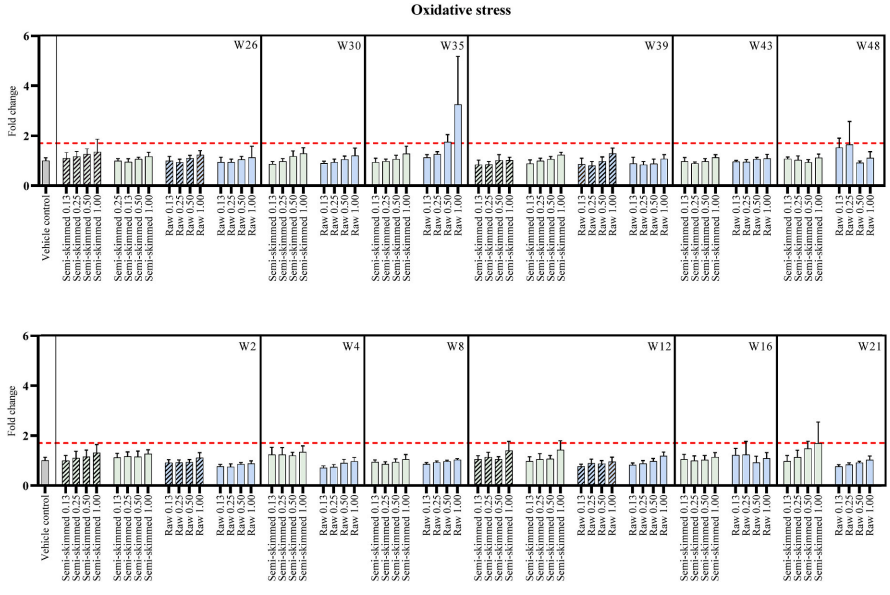

Fig. 1. Bioactivity of milk samples after exposure for 24 h in the oxidative stress *in vitro* assay. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations are expressed as REF. Data are shown as mean ± SD from two independent experiments (n = 4 for milk samples/run, n = 8 for vehicle control/run). The red dotted lines mark the cut-off level of 1.7-fold change. "W" denotes the week the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

on these findings, non-cytotoxic concentrations were used for the remaining *in vitro* test methods.

3.1.2. Oxidative stress

Only one sample, raw milk at week 35, was bioactive in the oxidative stress assay (Fig. 1). Raw milk, at week 35, was bioactive at the two highest concentrations and reached a 3.3-fold change increase compared to the vehicle control (Fig. 1). The BEQ for the raw milk sample at week 35 was 4.92×10^{-6} M tBHQ equivalents (eq) (Table S2).

There are many differences between raw and semi-skimmed milk at week 35, besides the difference in fat content, the raw milk itself is not processed (i.e. pasteurized or homogenized). Also, as milk is pooled, the raw and semi-skimmed milk may not come from the same batch of milk. Additionally, they were also stored in different containers. Before the extraction, the semi-skimmed milk was stored in the commercially available coated paperboard carton while raw milk was stored in PET bottles. However, the reason why only one specific sample in our study deviated from the remaining PET-stored samples and/or semi-skimmed milk samples is not known. It may be possible that it was exposed to more light before being delivered, resulting in the degradation of light-sensitive protective molecules with antioxidant properties like vitamin A. Another hypothesis is that the fat-soluble substances are driving the oxidative stress and these exist at higher concentrations in raw milk.

One previous study has emphasized the importance when selecting the storage bottle, where for example higher lipid oxidation of homogenized whole milk (3.5% fat) was seen in PET bottles with increasing time (0–7 days of storage) compared to pigmented high-density polyethylene (HDPE) and coated paperboard cartons (Zygoura et al., 2004). Furthermore, the degradation of vitamin A was most pronounced for the clear PET bottles (51% loss), followed by pigmented PET (30% loss) and control samples consisting of coated paperboard carton (14%). These results illustrate that there can be changes in the composition of the milk samples depending on the type of storage bottle that is chosen (Zygoura et al., 2004). Regardless, we have earlier stored Milli-Q® water and tap water in the same type of PET bottles, as used in this study, for longer times and they did not show any activity of the assays tested (Lundqvist et al., 2021).

Mojica and Bisso (2021) reported increased total antioxidant capacity in commercially available non-fat chocolate milk using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, while all the other commercially available milk samples (whole, non-fat and reduced fat) showed a similar antioxidant response.

The high antioxidant response in non-fat chocolate milk was hypothesized to be due to the increased content of polyphenols in cocoa powder (Mojica and Bisso, 2021). Nonetheless, it is important to highlight that the study mentioned above did not extract the milk samples, which we did in our study to remove the milk matrix that could interfere with the assay.

Generally, we did not observe oxidative stress in the samples, which is of great benefit if the method is to be used as a screening tool. Oxidative stress was observed in one case, which also is good from a methodological standpoint because it demonstrates that we can capture oxidative stress-causing substances, if present in the milk.

3.1.3. Genotoxicity

As oxidative stress is one of the multiple mechanisms that can cause genotoxic effects, raw milk at week 35 was hypothesized to potentially be genotoxic. Even though only one sample was recognized to be bioactive in the oxidative stress assay, four conventional raw milk samples, close to the cut-off limit of the oxidative stress assay, were tested at two concentrations (REF 1.00 and 0.50) in the micronucleus test to evaluate their genotoxic potential. However, none of the tested milk samples proved to be genotoxic (Table 1).

Anthropogenic pollutants like PCB congeners, polybrominated diphenyl ethers (PBDEs) and/or PAHs have been detected in raw as well as commercially available whole and fat-free milk samples (Chen et al., 2017; Di Bella et al., 2020; Hasan et al., 2022). The presence of these pollutants within milk was hypothesized to be related to the feedstuff as they may consume contaminated feed through grass, maize and soil. Both PCBs and PAHs are classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC), where several of the chemicals need metabolic activation to cause their DNA-damaging effects (IARC, 2010, 2016). Since no metabolic components were added in this study, future studies need to investigate the potential genotoxic effect of metabolically active chemicals in milk. Still, the non-existing background activity of genotoxicity observed in milk samples is promising in regard to the idea of using effect-based methods as screening tools.

Table 1
Summary of micronucleus test results from two independent runs (n = 4 for samples/run, n = 3–4 for vehicle controls/run). Concentrations of the milk samples and vehicle controls are expressed as REF.

Milk sample	Week (year)	REF	MN formation	
			Average %MN ± SD	Average MN fold change ± SD
Raw milk	35 (2020)	1.00	0.16 ± 0.04	0.68 ± 0.16
		0.50	0.14 ± 0.04	0.62 ± 0.16
	48 (2020)	1.00	0.22 ± 0.07	0.94 ± 0.29
		0.50	0.24 ± 0.04	1.05 ± 0.17
	12 (2021)	1.00	0.16 ± 0.05	0.68 ± 0.21
		0.50	0.14 ± 0.03	0.60 ± 0.14
	21 (2021)	1.00	0.11 ± 0.04	0.47 ± 0.17
		0.50	0.15 ± 0.02	0.64 ± 0.09
Vehicle control				
5% MeOH/ H_20	N/A	1.00	0.23 ± 0.07	1.00 ± 0.28
Milli-Q®		1.00	0.21 ± 0.05	1.00 ± 0.26
Positive control				
MMC	N/A	200 nM	1.08 ± 0.37[a]	5.17 ± 1.75[a]
		100 nM	0.65 ± 0.18[a]	3.10 ± 0.88[a]

[a] Samples that were statistically significant from its vehicle control are marked with an asterisk (p-value <0.0001).

3.1.4. AhR

In total, 12 out of 32 milk samples were bioactive in the AhR assay, of which four samples were semi-skimmed milk and the remaining eight were raw milk (Fig. 2). All bioactive samples demonstrated activity at their highest REF in a dose-related manner, except for raw milk at week 4 that was bioactive from REF 0.50. The highest REF of this particular sample was below the cut-off limit and it is likely related to undetected cytotoxicity and it was thus omitted from the BEQ calculation.

Raw milk at week 16 obtained the highest efficacy, reaching 19% of the max effect and was bioactive down to REF 0.13 (Fig. 2). Organic raw milk was slightly more bioactive than organic semi-skimmed milk, as seen at weeks 26 and 12, which potentially could be explained by the fact that raw milk has higher fat content than semi-skimmed milk, and dioxin as well as dioxin-like compounds are known to associate with fat. The organic semi-skimmed milk was only found to be active at week 2 (Fig. 2). The BEQs ranged from 4.99×10^{-13}–2.60×10^{-12} M TCDD eq (Table S2). The BEQ values were recalculated into TCDD eq per gram fat in the milk, this corresponds to 3.74 to 19.47 ρg TCDD/g fat for raw milk and 14.80 to 24.27 ρg TCDD/g fat for semi-skimmed milk.

The European Union (EU) has defined a max level for the sum of dioxins, which is 2.0 ρg World Health Organisation (WHO)-polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/PCDFs)-TEQ/g fat (European Commission, Directorate-General for Health and Food Safety, 2023). It should, however, be noted that the BEQ values measured in our AhR assay are not directly comparable to the WHO-TEQ, even though they both are expressed as TCDD

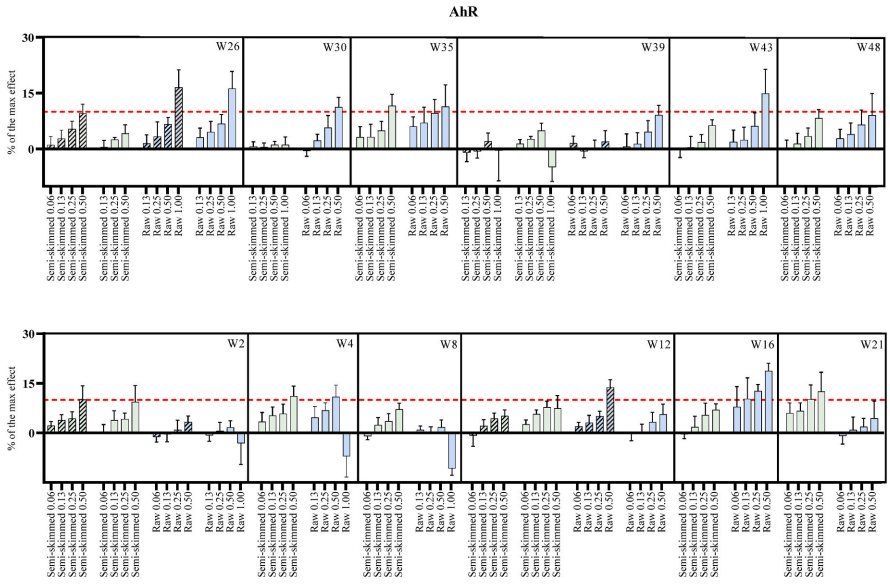

Fig. 2. Induction of the aryl hydrocarbon receptor (% of the max effect of the reference compound) after exposure for 24 h to milk samples. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations of the milk samples are expressed as REF. Data represent mean ± SD from two independent experiments (n = 2–4 for samples/run, n = 8 for vehicle control/run). Samples with activity above the cut-off limit, represented by the red-dotted line, were defined as bioactive. "W" denotes the week the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

equivalents. The WHO-TEQ system is based on a principle to potency-scale a set of predefined individual chemicals for which the concentrations in a sample have been measured, and then calculate the potency-scaled sum concentrations, expressed as TCDD equivalents. The BEQ values measured in our AhR bioassay are the sum biological effects of both the dioxin and dioxin-like compounds covered by the WHO-TEQ system, and the thousands of other chemicals that have been reported to activate AhR. Hence, it could be expected that the BEQ value of a sample is higher than the WHO-TEQ value. To elucidate the fraction of the BEQ value that is constituted of the WHO-TEQ substances in a sample, parallel chemical analysis for the compounds covered by the WHO-TEQ system would be needed.

Both Mayilsamy et al. (2022) and Chou et al. (2008) evaluated PCDD and PCDFs in bovine milk, where the latter study showed levels below the earlier set threshold limit of 3 ρg WHO-TEQ/g fat (European Commission, Directorate-General for Health and Food Safety, 2006). The former study, on the other hand, retrieved total dioxin values of 0.03–7.33 ρg TEQ/g fat (Mayilsamy et al., 2022). Higher concentrations of dioxin-like compounds were thought to be attributed to the greatly populated areas and industrialized districts.

Contamination of dioxin in the soil, as well as grass, is well known (Schulz et al., 2005) and the replacement of feed at contaminated sites, especially hay, has reduced the contamination levels in milk (Bertocchi et al., 2015). Noteworthy, the sample preparations and extraction procedures used between the above-discussed studies and this study are dissimilar, which unquestionably will impact which chemicals that are captured and their effect(s).

We observed AhR activity in almost half of the samples, further studies are needed to understand whether the background variation of bioactive substances is because of natural compounds or anthropogenic contaminants.

3.1.5. Androgen receptor

All samples were below the cut-off limit in the androgen induction assay, meaning that none of the samples were bioactive (Fig. S6). Courant et al. (2007) reported overall lower concentrations of free androgens (dehydroepiandrosterone, α-testosterone and 4-androstenedione) in commercially available skimmed and half-skimmed milk compared to whole milk, where quantification of α-testosterone on average was 31.8 ng l^{-1} in skimmed milk and 51.3 ng l^{-1} in half-skimmed milk, while whole milk contained up to 78.1 ng l^{-1} on average. However, questions regarding analyses of phytoestrogens have been raised for extraction procedures using hydrolytic enzymes originating from *Helix pomatia* (Bláhová et al., 2016). It was seen that the use of this hydrolytic enzyme overestimated phytoestrogen content in milk, due to potential enzyme contamination, which also could be of importance for phytoandrogens.

Androgen receptor inhibition was observed in a dose-related manner for several of the milk samples (Fig. 3). Semi-skimmed milk at weeks 35, 12, 16 and 21, in addition to raw milk at weeks 39, 2, 4, 12, 16 and 21, were bioactive (Fig. 3). Semi-skimmed milk at week 16 obtained the highest BEQ value of 1.42×10^{-7} M OHF eq (Table S2). Studies on the androgen receptor-inhibitory activities in milk are very limited, highlighting that more research is needed to understand if the background levels are due to pollutants or naturally occurring chemicals.

The lack of response in the androgen induction assay is a promising finding, as it increases the chances of detecting contaminants that activate this parameter, compared to the situation with the inhibition of the androgen receptor where the milk itself was bioactive throughout the year and could mask the effects from the contaminants. Thus, more research is needed for the inhibitory mode of the androgen receptor.

3.1.6. Estrogen receptor

None of the milk samples induced the estrogen receptor after 24 h of

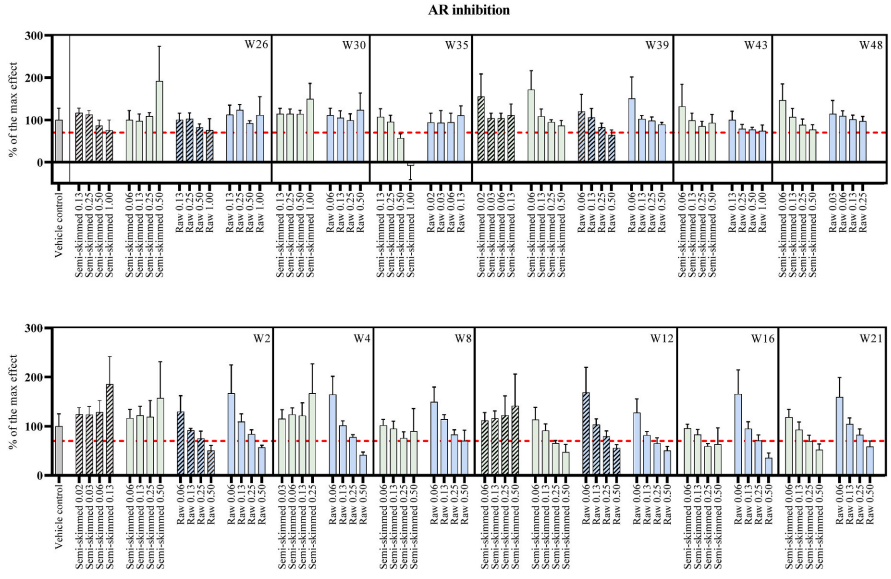

Fig. 3. Inhibition of the androgen receptor (% of the max effect of the reference compound) after exposure for 24 h to milk samples. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations of the milk samples are expressed as REF. Data represent mean ± SD from two independent experiments (n = 2–4 for samples/run, n = 6–8 for vehicle control/run). Samples with activity below the cut-off limit, represented by the red-dotted line, were defined as bioactive. "W" denotes the week the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

exposure (Fig. S7). Dose-related inhibition of the estrogen receptor, on the other hand, was detected for a few semi-skimmed and raw milk samples between weeks 4–21 (Fig. 4). The inhibitory activity was most pronounced for semi-skimmed milk at week 21, reaching 44% effect at the highest REF, followed by 68% at REF 0.50 (Fig. 4). The organic milk lacked activity at all weeks, apart from week 12 where semi-skimmed milk was bioactive at REF 1. The BEQ ranged from 1.78×10^{-9}–3.20×10^{-9} M Ral eq (Table S2).

There is great potential to use effect-based methods as a screening tool, in regards to induction of the estrogen receptor, as low background activity was observed.

In agreement with the androgen inhibitory assay, the inhibitory effects of the estrogen receptor are not currently well reported in other studies. The studies existing mainly focus on the identification of specific phytoestrogens (Antignac et al., 2003; Steinshamn et al., 2008; Mustonen et al., 2009; Njåstad et al., 2014), the transfer of phytoestrogens into the milk (Mustonen et al., 2009) or seasonal variations of phytoestrogen in milk (Róin et al., 2023). However, it should be noted that, as previously stated, several studies may have overestimated the phytoestrogen concentration in the milk due to enzyme contamination (Bláhová et al., 2016). Regardless, the variation of phytoestrogenic compounds in milk between different areas in the world can be explained by the different plant species grazed by the cow, farm management systems (conventional, biodynamic and/or organic) and seasonal variations (Róin et al., 2023). Nevertheless, additional research needs to be conducted to understand the impact of the background levels of the inhibitory estrogen assay.

3.2. Monitoring using in vitro assays

This work utilized five *in vitro* methods covering eight toxicological endpoints to detect bioactivities in Swedish milk over one year. In general, no or low bioactivities were displayed for induction hormonal activities (ER/AR), genotoxicity and oxidative stress, while the activities of AhR and inhibition of the hormonal receptors were found to be more commonly occurring in the milk samples. None of the solvent blanks showed an effect in the eight endpoints tested (*data not shown*).

Interestingly, inhibitory estrogen activities were not observed until week 4 and the inhibition continued until week 21. Nearly all samples (10/32) between these weeks were bioactive only at the highest REF with relatively similar activities between the semi-skimmed and raw milk samples. It therefore appears to be seasonal differences in the presence of antiestrogens in the milk and it indicates that repeated sampling is of importance to understand the variations. It would be beneficial in the future to possibly perform effect-directed analysis to identify if the driving chemicals are of natural origin or pollutants (Brack et al., 2016), in a similar way that has been conducted by Hashmi et al. (2018) on wastewater. With this being said, AhR activity and inhibitory modes of action on the androgen receptor, appear to be even the whole year. These results confirm the value to conduct a follow-up of the present study and further develop *in vitro* methods to detect potentially hazardous chemicals within cow's milk. Such a follow-up study could further be enhanced by the inclusion of recovery experiments with the reference compound for each assay before and after the extraction. Additionally, the inclusion of an additional clean-up step with silica would be beneficial, in order to see how much of the AhR activity is driven by persistent chemicals.

The assays where activity was seen in a few samples would be the best candidates to follow-up because the background level of these substances is low. This means that we more easily could detect any contaminants since the signal would not be disturbed by a high background activity. The endpoints with higher occurring activities are more challenging to use as a screening tool, as we currently do not know if the background levels are due to pollutants (like dioxins), foreign

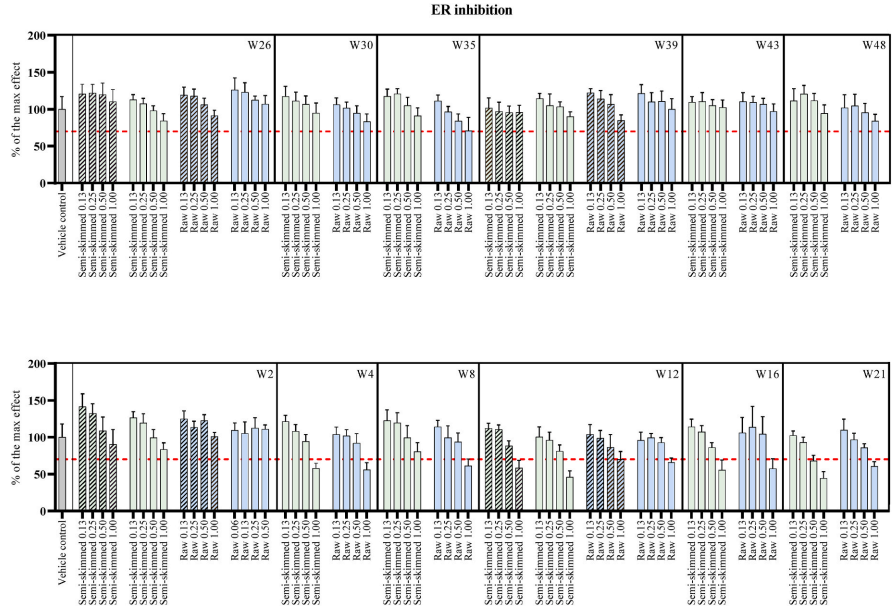

Fig. 4. Inhibition of the estrogen receptor (% of the max effect of the reference compound) after exposure for 24 h to milk samples. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations of the milk samples are expressed as REF. Data represent mean ± SD from two independent experiments (n = 4 for samples/run, n = 16 for vehicle control/run). Samples with activity below the cut-off limit, represented by the red-dotted line, were defined as bioactive. "W" denotes the week when the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substances (such as antibiotics) or naturally occurring chemicals (estrogens, androgens, etc.). One has to analyze a larger number of samples in the future to draw any definitive conclusion about which biological activities in the assays that are caused by endogenous compounds and which activities that are caused by chemical pollutants. Applying *in vitro* methods could act as an early warning system to detect potentially hazardous chemicals within milk and thereby ensure the safety of milk that is needed to determine recommended actions, in a similar fashion that has been done for water samples (Lundqvist et al., 2019; Oskarsson et al., 2021). However, these methods may not be used daily, as milk has a high turnover on the market and results need to be delivered rapidly. Thus, the methods could rather be used seasonally or in a monthly fashion to investigate changes in trends.

4. Conclusions

An *in vitro*-based approach consisting of testing hormonal activities (estrogen and androgen receptors), DNA damage, oxidative stress and xenobiotic metabolism (AhR) was used to detect bioactive compounds in cow's milk. Generally, the study showed that the milk did not appear to contain detectable amounts of bioactive substances in the oxidative stress, genotoxicity and induction of estrogen/androgen receptor assays, as shown by the lack or minor response. The inhibitory mode of action on the hormonal receptor as well as AhR exerted more activity, where approximately 10–13 samples out of 32 were bioactive. Overall, no cytotoxicity was detected in three cell lines (MCF7 AREc32, TK6 and VM7Luc4E2), while nearly all milk samples at the highest REF exerted cytotoxicity in the AR-EcoScreen GR-KO M1 and the majority in DR-EcoScreen cells. The use of *in vitro* methods as a screening tool to monitor chemical hazards in milk shows great promise.

CRediT authorship contribution statement

Erica Selin: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Geeta Mandava:** Formal analysis, Investigation, Writing – review & editing. **Maria Karlsson:** Writing – review & editing, Supervision. **Johan Lundqvist:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2023.114025.

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Food toxicology heavily relies on the chemical quantification of a limited number of chemicals. This approach is challenged when thousands of anthropogenic chemicals exist, and it is impossible to monitor them all. This thesis focused on applying an alternative approach of using *in vitro* bioassays to evaluate food packages, liquid smoke flavourings and milk. *In vitro* bioassays proved to be a valuable tool when assessing these products' complex mixtures, as these can provide mechanistic information on health-relevant endpoints.

Erica Selin received her doctoral education at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences. Her bachelor's and master's degrees were obtained at Karolinska Institutet.

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