Downloaded from orbit.dtu.dk on: Dec 20, 2017

Technical University of Denmark

Compounds in food packaging materials - toxicological profiling of knowns and unknowns

Rosenmai, Anna Kjerstine; Vinggaard, Anne Marie; Taxvig, Camilla

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Rosenmai, A. K., Vinggaard, A. M., & Taxvig, C. (2014). Compounds in food packaging materials - toxicological profiling of knowns and unknowns. Søborg: National Food Institute, Technical University of Denmark.

DTU Library
Technical Information Center of Denmark

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from orbit.dtu.dk on: Sep 27, 2016

Technical University of Denmark

DTU

Compounds in food packaging materials

toxicological profiling of knowns and unknowns

Rosenmai, Anna Kjerstine; Vinggaard, Anne Marie; Taxvig, Camilla

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Link to publication

Citation (APA):
Rosenmai, A. K., Vinggaard, A. M., & Taxvig, C. (2014). Compounds in food packaging materials: toxicological profiling of knowns and unknowns. Søborg: National Food Institute, Technical University of Denmark.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

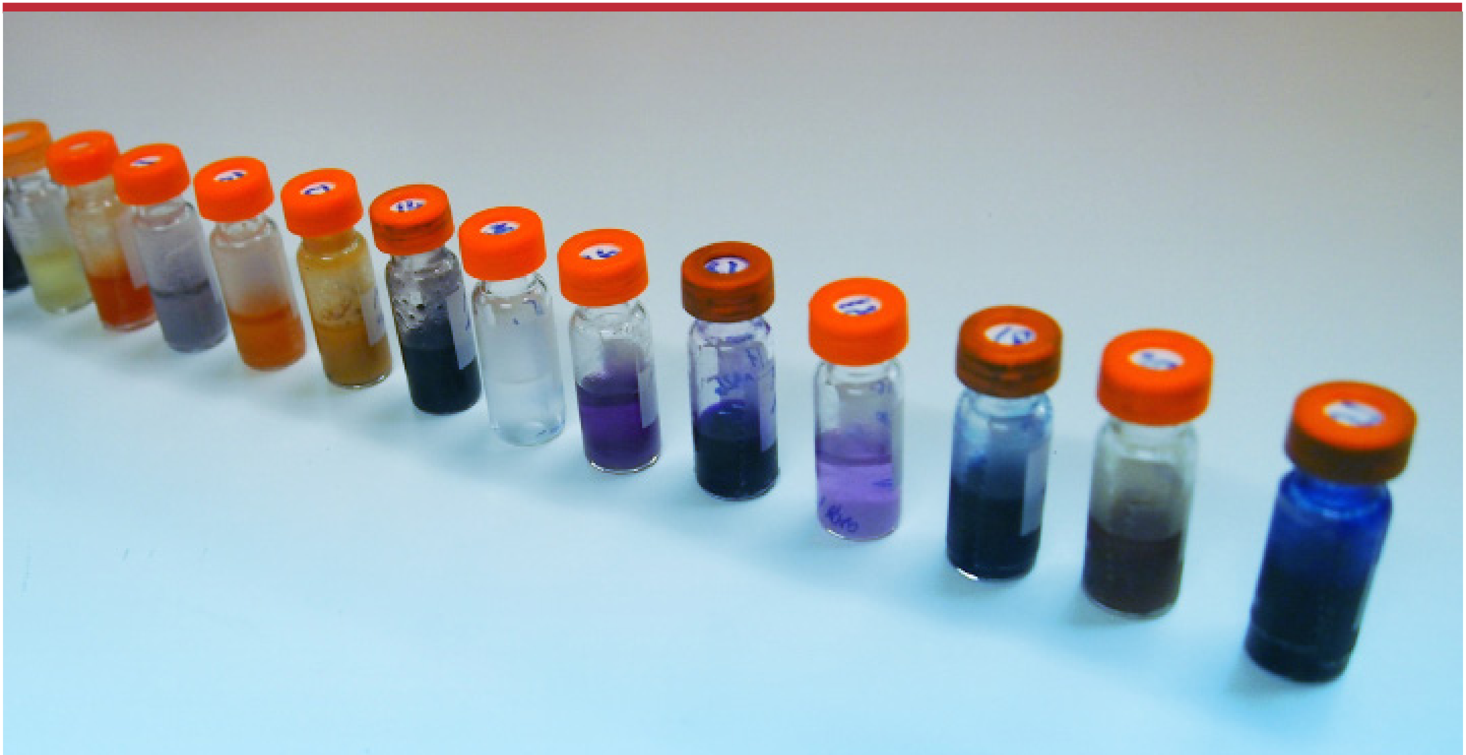
- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

DTU

Compounds in food packaging materials

- toxicological profiling of knowns and unknowns



Anna Kjerstine Rosenmai
PhD Thesis
2014

DTU Food
National Food Institute

Compounds in food packaging materials

- *toxicological profiling of knowns and unknowns*

PhD thesis

Anna Kjerstine Rosenmai

Division of Toxicology and Risk Assessment
National Food Institute
Technical University of Denmark

October 2014

Thesis title
Compounds in food packaging materials - toxicological profiling of knowns and unknowns

Author and place of employment
Anna Kjerstine Rosenmai
Division of Toxicology and Risk Assessment
National Food Institute
Technical University of Denmark

Supervisors
Professor Anne Marie Vinggaard
Division of Toxicology and Risk Assessment
National Food Institute
Technical University of Denmark

Scientist Camilla Taxvig
Division of Toxicology and Risk Assessment
National Food Institute
Technical University of Denmark

Evaluation committee
Christopher Lau, Lead Research Biologist, US EPA, USA
Helle Ravn Andersen, Associate Professor, SDU, Denmark
Julie Boberg, Senior Scientist, DTU FOOD, Denmark

Cover photo
Extracts from food contact materials, photographer Anna Kjerstine Rosenmai

Copyright
Reproduction of this publication in whole or in part must include the customary bibliographic citation, including author attribution, report title, etc.

Published by
Technical University of Denmark, Ledelse og Administration, Anker Engelunds Vej 1, Bygning 101 A, 2800 Kgs. Lyngby, 2014

Funding
The Ministry of Food, Agriculture, and Fisheries of Denmark and the Danish Veterinary and Food Administration

ISBN
978-87-93109-29-2

Preface

The practical work for this PhD project was conducted at The National Food Institute, Technical University of Denmark, in the laboratories of Professor Bernard Jegou at Rennes University (France), and at BioDetection Systems (BDS) in The Netherlands.

Acknowledgements

I would like to thank a lot of people who in one way or another have been directly involved in this project or offered me support on a personal level. Firstly, thanks to my supervisors, Anne Marie Vinggaard and Camilla Taxvig, for your guidance throughout the project, for believing in my abilities, and being supportive. Thanks to the laboratory technicians, Birgitte Plesning Møller, Lene Sofie Dahl Svensson, Heidi Letting, Dorte Lykkegaard Korsbech, and Kitt Lademann for being brilliant in the laboratory and always being helpful. As part of the project I had the opportunity to work in the laboratory of Bernard Jegou for which I am grateful. The people there were very welcoming and introduced me to new techniques. All the PhD students in the ULF network have been part of creating a space for relaxation, outlet for frustration, and laughter. The lunch team and everybody in the G-section of our Division have been my partners in crime in drinking far too much coffee and having lunch breaks with great discussions and conversations. Thanks to Terje Svingen for being a super feedback person and pushing me to becoming better at communicating science. Thank you Xenia Trier, my scientific inspiration, for always pushing me to do better, padding my shoulder when needed and lending a shoulder during hard times. Last, but not the least, thanks to friends and family, for always listening, keeping me grounded and being up for a good time. A special thanks to Katrine Scheibye, Hanna Johansson, Kristine Rasmussen, Astrid Kristensen, Lillian Rosenmai, and Helge Nielsen.

Copenhagen, October 2014

Anna Kjerstine Rosenmai
PhD student

Summary

Food contact materials (FCMs) are sources of food contamination and human chemical exposure. Some chemicals in these materials are known to cause adverse effects, but many are poorly characterized for their potential toxicological hazards making risk assessment a challenge.

The aim of the project was to obtain knowledge on the potential hazards posed by chemicals present in FCMs through examining compounds with known usages or suspected of being used in these materials, namely bisphenol A (BPA), BPA analogues and fluorinated substances. Furthermore, we developed a strategy to identify problematic compounds present in these materials. Specific focus was placed on *in vitro* endpoints assessing endocrine activity.

BPA, five BPA analogues, and 19 fluorinated substances including fluorochemical containing technical mixtures (TMs) were investigated. The *in vitro* assays included the androgen receptor (AR), estrogen receptor (ER), aryl hydrocarbon receptor, retinoic acid receptor, glucocorticoid receptor, p53, and nuclear factor (erythroid-derived 2)-like 2 reporter gene assays, and the H295R steroidogenesis assay. The FCM strategy was a step-by-step procedure in which extracts from FCMs of paper and board were tested *in vitro*, active extracts were fractionated and tested *in vitro*, tentative identification was performed in active fractions, and tentatively identified compounds were tested *in vitro* and quantified in the extract.

BPA analogues generally led to similar estrogenic and antiandrogenic effects *in vitro* compared to BPA. However, the BPA analogue BPS caused less marked effects on most of these endpoints, but led to a more pronounced effects on progestagen levels compared to BPA. Likewise, the effects on corticoid levels in the H295R steroidogenesis assay differed between the six compounds. These data suggest that the tested analogues overall have similar effects on the parameters investigated, though some differed. Therefore based on the presented data, the BPA analogues may not be suitable BPA alternatives.

The fluorinated chemicals exhibited endocrine activities distinct from one another, but subgroups of fluorinated chemicals had similar profiles. Polyfluoroalkyl phosphate ester surfactants (PAPs) generally decreased progestagen and androgen levels, fluorotelomer alcohols (FTOHs) generally increased ER activity, and long-chained perfluorinated alkyl carboxylic acids (PFCAs) generally increased 17β-estradiol levels. Two TMs caused estrogenicity *in vitro*, whereas none of the short-chained PFCAs caused effects. It is recommended to conduct further studies on polyfluorinated chemicals as well as TMs to obtain more information on the implications of these differences in effect.

The developed FCM strategy proved useful for identifying potential problematic compounds in FCMs of paper and board. All extracts from FCMs led to effects in at least one of the applied *in vitro* assays and we successfully identified five causative agents in two FCMs when applying the full strategy. It is recommended to test more FCMs of paper and board with the strategy to obtain information on other potentially problematic compounds present in these materials.

The presented data overall suggest that some compounds present in FCMs or suspected of being used can exert endocrine activities *in vitro*, though the implications of these findings with respect to effects in humans and exposure to humans remain largely unknown. Nevertheless, it is of concern that so many of the materials and compounds led to effects and calls for further studies to be conducted. The data obtained in this PhD can be used as a prioritization tool for these purposes.

Resumé (summary in Danish)

Fødevarekontaktmaterialer (FKM’er) er kilder til forurening af fødevarer og menneskelig kemisk eksponering. Nogle af de kemikalier, der er tilstede i disse materialer, har kendte toksikologiske effekter, men der findes dog stadig mange stoffer, som er dårligt karakteriseret for deres potentielle toksikologiske effekter, hvilket gør risikovurdering til en udfordring.

Formålet med projektet var at få viden om de potentielle toksikologiske effekter forbundet med kemikalier i FKM'er ved at undersøge kemikalier, hvis anvendelse er kendt eller som er mistænkt for at blive anvendt i disse materialer, såsom bisphenol A (BPA), BPA-analoger og fluorstoffer. Endvidere blev der udviklet en strategi til at identificere problematiske kemiske stoffer i disse materialer. Fokus blev i særlig grad lagt på *in vitro* karakterisering af endokrin aktivitet.

BPA, fem BPA analoger, og 19 fluorerede stoffer, herunder fluorholdige tekniske blandinger (TB’er), blev undersøgt *in vitro*. De anvendte *in vitro* assays omfattede androgen receptor (AR), østrogen receptor (ER), aryl hydrocarbon receptor, retinoic acid receptor, glucocorticoid receptor, p53, and nuclear factor (erythroid-derived 2)-like 2 reporter gene assays, og H295R steroidogenese assayet. FKM-strategien bestod af en trin-for-trin procedure, hvor ekstrakter fra FKM'er af papir og pap blev testet *in vitro*, aktive ekstrakter blev fraktioneret og testet *in vitro*, aktive fraktioner blev tentativt identificeret, og tentativt identificerede stoffer blev derefter testet *in vitro* og kvantificeret i ekstraktet.

Analogerne til BPA udviste generelt lignende østrogene og antiandrogene effekter *in vitro* sammenlignet med BPA. Dog forårsagede BPA analogen, BPS, mindre markante effekter på de fleste af disse effektmål, men førte til en mere udtalt effekt på niveauet af progestagener i forhold til BPA. Effekterne på niveauerne af kortikosteroiderne, i H295R assayet, var forskellig for de seks stoffer. Overordnet tyder data dog på, at de testede analoger samlet set har effekter som lignede BPAs. Baseret på de konkrete undersøgelser ser analogerne derfor ikke ud til at være bedre alternativer til BPA.

De fluorerede kemikalier udviste forskellige endokrine aktiviteter, omend undergrupper af fluorstoffer havde lignende profiler. De polyfluorerede alkyl phosphate ester surfaktanter (PAPs) medførte generelt et fald i progestagen og androgen niveauer, hvor fluortelomeralkoholer (FTOHs) generelt medførte forøget ER aktivitet og de langkædede perfluoralkyl carboxylsyrer (PFCA'er) forøgede 17β-østradiol niveauer. To TB’er udviste østrogenicitet *in vitro*, mens ingen af de kortkædede PFCA'er forårsagede effekter. Det anbefales at foretage yderligere undersøgelser af polyfluorerede kemikalier samt TB’er for at få mere information om konsekvenserne af disse forskelle i effekt.

Den udviklede FKM strategi viste sig nyttig til at identificere potentielle problematiske stoffer i FKM'er af papir og pap. Alle ekstrakter fra FKM'er udviste effekt i mindst et af de anvendte assays og ved anvendelse af strategien var det muligt at identificere fem stoffer i to FKM’er, som kunne forklare de observerede effekter.

Det anbefales at bruge den udviklede strategi til at teste flere FKM'er af papir og pap for bedre at forstå omfanget, såvel som identiteten, af potentielt problematiske forbindelser.

De præsenterede data tyder samlet set på, at stoffer der anvendes eller mistænkes anvendt i FKM'er, kan have endokrine aktiviteter *in vitro*. Selvom konsekvenserne af disse resultater med hensyn til effekter på mennesker, og eksponering af mennesker i nogle tilfælde er ukendte, er det bekymrende, at så mange af de testede materialer og kemikalier medførte effekter. Derfor opfordres der til, at der gennemføres yderligere undersøgelser af denne type materialer, hvor data fra dette PhD projekt kan bruges som en prioriteringsredskab til dette formål.

List of abbreviations

AA	-	Abietic acid
AEQ	-	Androgen equivalence factor
AhR	-	Aryl hydrocarbon receptor
AR	-	Androgen receptor
BBP	-	Butyl-benzyl phthalate
BPA	-	Bisphenol A
BPB	-	Bisphenol B
BPE	-	Bisphenol E
BPF	-	Bisphenol F
BPS	-	Bisphenol S
DBP	-	Di-butyl phthalate
DHAA	-	Dehydroabietic acid
DiBP	-	Di-isobutyl phthalate
EC_{50}	-	Values for concentrations causing 50% of the maximum response
ED	-	Endocrine disruptor
EEQ	-	Estrogen equivalence factor
EQ	-	Equivalence factor
ER	-	Estrogen receptor
FCM	-	Food contact material
FTOH	-	Fluorotelomer alcohol
GR	-	Glucocorticoid receptor
HPLC-MS/MS	-	High pressure liquid chromatography-tandem mass spectrometry
HPP	-	4-cumylphenol
Nrf2	-	Nuclear factor (erythroid-derived 2)-like 2
PAPs	-	Polyfluoroalkyl phosphate ester surfactant
PFAS	-	Polyfluoroalkyl substances
PFBA	-	Perfluorobutanoic acid
PFCA	-	Perfluorinated alkyl carboxylic acid
PFDA	-	Perfluorodecanoic acid
PFdoDA	-	Perfluorododecanoic acid
PFHpA	-	Perfluoroheptanoic acid
PFHxA	-	Perfluorohexanoic acid
PFNA	-	Perfluorononanoic acid
PFOA	-	Perfluorooctanoic acid
PFPA	-	Perfluoropentanoic acid
PFunDA	-	Perfluoroundecanoic acid
PPAR	-	Peroxisome proliferator-activated receptor
QSAR	-	Quantitative Structure-Activity Relationship
RAR	-	Retinoic acid receptor
TM	-	Technical mixture

List of publications derived from the PhD

Manuscript 1

Rosenmai, A.K., Nielsen, F.K., Pedersen, M., Hadrup, N., Trier, X., Christensen, J.H., and Vinggaard, A.M., 2013. Fluorochemicals used in food packaging materials inhibit male sex hormone synthesis. Toxicol. Appl. Pharmacol. 266, 132-142.

Manuscript 2

Rosenmai, A.K., Dybdahl, M., Pedersen, M., van Vugt-Lussenburg, B.M.A., Wedebye, E.B., Taxvig, C., and Vinggaard, A.M., 2014. Are structural analogues to bisphenol A safe alternatives? Tox Sci. 139, 35-47.

Manuscript 3

Rosenmai, A.K., Trier, X., Taxvig, C., van Vugt-Lussenburg, B.M.A., and Vinggaard, A.M. (2014). Fluorinated compounds and technical mixtures for use in food contact materials have estrogenic activity in an *in vitro* screening. Manuscript in preparation.

Manuscript 4

Rosenmai, A.K., Bengtström, L., Taxvig, C., Trier, X., Petersen, J.H., Granby, K., and Vinggaard, A.M. (2014). A strategy to identify problematic chemicals in food contact materials of paper and board. Manuscript in preparation.

Additional publications

Manuscript 5

Taxvig, C., **Rosenmai**, A.K., and Vinggaard, A.M., (2014). Polyfluorinated alkyl phosphate ester surfactants (PAPs) – Current knowledge and knowledge gaps. Basic Clin Pharmacol Toxicol. 115, 41-44.

Manuscript 6

Bengtström, L., Trier, X., Granby, K., **Rosenmai**, A.K., and Petersen, J.H., 2014. Fractionation of extracts from paper and board food contact materials for *in vitro* screening of toxicity. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 12, 1-10.

Content

1. INTRODUCTION **1**

1.1 Study objectives, hypotheses, and methodology **2**

1.2 Contribution to publications **3**

2. BACKGROUND **4**

2.1 Endocrine disruption **4**

2.1.1 The endocrine system and mechanisms of endocrine disruption 4

2.2 Bisphenol A and its structural analogues **7**

2.2.1 Usage, exposure sources and human biomonitoring 7

2.2.2 Toxicokinetics 9

2.2.3 Toxicological effects 9

2.2.4 Endocrine activity *in vitro* 9

2.3 Fluorochemicals **10**

2.3.1 Exposure sources and human biomonitoring 11

2.3.2 Toxicokinetics 11

2.3.3 Toxicological effects 13

2.3.4 Endocrine disruptive potential 13

2.4 Strategies to address toxicity of food contact materials **15**

3. EXPERIMENTAL DESIGN **17**

3.1 The bottom-up approach **17**

3.2 The top-down approach **18**

4. RESULTS **20**

4.1 Bisphenol A and structural analogues **20**

4.2 Fluorinated chemicals **20**

4.3 Food contact materials **22**

5. DISCUSSION **23**

5.1 The tested hypotheses **23**

5.1.1 Bisphenol A analogues 23

5.1.2 Fluorinated chemicals 24

5.1.3 Strategy to assess complex mixtures of unknown composition 25

5.2 Interpretation of *in vitro* results **26**

5.3 Sources and exposure to compounds in food contact materials **27**
5.3.1 Human biomonitoring and sources 27
5.3.2 Migration of compounds from food contact materials 28

6. CONCLUSIONS **30**

7. FUTURE PERSPECTIVES **32**

8. REFERENCES **34**

9. APPENDICES **49**

1. Introduction

Ideally, foods for human consumption are free from chemical contamination. In the modern world this has become practically impossible and the efforts are instead put towards minimizing human exposure as much as possible to problematic substances that can cause adverse health effects. However, potential sources of food contamination are many and include environmental toxicants, pesticide residues, packaging materials and many more (Borchers et al. 2010). Thus, continued monitoring and testing to assure food safety is both a challenge and an obligation.

Food contact materials (FCMs), which include packaging and any material the foods come into contact with during production, are potential sources of chemical exposure (Borchers et al. 2010; Grob et al. 2006). Many chemicals are used for FCMs and to date more than 3000 indirect food additives, including chemicals used in FCMs, have been catalogued (Neltner et al. 2013). Notably, these catalogues do not differentiate between substances presently in use in the production of FCMs with those that are not (Geueke et al. 2014). Furthermore, it is estimated that more than 70% of the catalogued substances are not tested for toxicological effects to an extent which allows for appropriate risk assessment (Neltner et al. 2013).

Paper and board FCMs are of particular interest, as no specific regulation exists within the EU for these materials. A general framework directive (*Article 3*) for FCMs exist, which states that substances in FCMs should not transfer to foods in amounts that could endanger human health (The European Commission 2004). However, in practical terms such guidelines are empty promises if the potential toxicity of the substances is unknown. Thus, there is a clear need for an approach that gives empirical data on the compounds used in FCMs, especially those of paper and board, as well as a strategy on how to prioritize a toxicological investigation of these compounds.

Bisphenol A (BPA) and fluorinated compounds are examples of chemicals or chemical groups used in FCMs (EFSA 2006; Geens et al. 2011; Kissa 2001) and that also have been measured in humans. For instance, BPA has been measured in human urine from the general US population (Calafat et al. 2008), and also in human amniotic fluid (Edlow et al. 2012; Yamada et al. 2002), umbilical cord blood (Chou et al. 2011; Lee et al. 2008), and in the placenta (Cao et al. 2012; Jimenez-Diaz et al. 2010). Similarly, fluorinated compounds have been measured in human sera (D'eon et al. 2009; EFSA 2008; Houde et al. 2006), umbilical cord blood (Kim et al. 2011; Monroy et al. 2008), and breast milk (So et al. 2006). These studies clearly show that the general population, including unborn babies, is exposed to these compounds. Foetal exposure is of particular concern, as endocrine disruptors (EDs) have been implicated in impaired foetal development, which can manifest as disease or developmental disorders at birth or later in life

(UNEP/WHO 2013) and the endocrine disruptive potential of these compounds have been demonstrated by numerous studies (Biegel et al. 1995; Biegel et al. 2001; Bookstaff et al. 1990; Cook et al. 1992; Feng et al. 2009; Gould et al. 1998; Grignard et al. 2012; Kitamura et al. 2005; Krishnan et al. 1993; Lee et al. 2003; Liu et al. 2009; Liu et al. 2010a; Liu et al. 2010b; Paris et al. 2002; Zhang et al. 2011a).

In recent years the public awareness of BPA and its potential effects has increased and several BPA-free products are on the market. BPA has been prohibited in the manufacture of polycarbonate baby feeding bottles within the EU (The European Commission 2011) and in Canada the import, sale and advertisement of polycarbonate baby bottles containing BPA has been prohibited (Government of Canada 2010). This inevitably will lead to the introduction of alternative compounds to replace the use of BPA. A growing concern is that structural analogues could be used as substitutions and that these may exert similar effects as BPA.

The toxicology of some fluorinated chemicals is fairly well established, however a range of fluorinated alternatives are being produced (Wang et al. 2013) for which little information on the structures, usages, and toxicology is publically available (Scheringer et al. 2014). There is some evidence that longer-chained perfluorinated alkyl carboxylic acids (PFCAs) have endocrine disruptive activities both *in vivo* and *in vitro*, itself warranting further investigations. Furthermore, many of the alternatives are shorter-chained (Scheringer et al. 2014), which potentially break-down to short-chained PFCAs, however almost nothing is currently known about their potential endocrine activities. Therefore, it is imperative to conduct further studies on fluorinated alternatives and their degradation products to ascertain that these do not exhibit similar activities as the long-chained PFCAs.

1.1 Study objectives, hypotheses, and methodology

Objectives

The overall aim was to understand the potential toxicology of chemicals present in FCMs by,

1. expanding on toxicological knowledge of BPA analogues to understand if these would be good alternatives to BPA.
2. characterizing the endocrine activity of fluorinated compounds for which little is known, as well as compare the endocrine disruptive potential across groups of fluorinated compounds.
3. developing a strategy to identify problematic compounds used in FCMs of paper and board.

Hypotheses

The following hypotheses were tested to fulfil the aim of the project,

1. The endocrine mechanisms-of-action of structural analogues to BPA are comparable to that of BPA.
2. Alternative fluorinated chemicals, including short-chained fluorinated compounds, harbour endocrine activity *in vitro* through similar mechanism-of-action as long-chained PFCAs.
3. Biodirected analysis in combination with analytical chemical tools can be used to identify problematic compounds present in food packaging materials of paper and board.

Study methodology

A panel of *in vitro* assays was applied. The data presented in this PhD mainly focus on the endocrine endpoints covered in this panel including estrogen receptor (ER) and androgen receptor (AR) activities, and interferences with steroid hormone synthesis.

Two approaches were applied,

- *The bottom-up approach* was used to test for effects of single compounds to elucidate the toxicological potential of single chemicals known to be present or suspected of being present in food packaging. We investigated BPA and five analogues, as well as nineteen fluorinated substances by this approach.

- *The top-down approach* was used to test extracts from FCMs of paper and board of unknown composition to characterize the hazard posed by these as well as identify potential causative agents. We investigated 20 extracts from paper and board food packaging materials.

1.2 Contribution to publications

My expertise is within *in vitro* assays for endocrine activity and knowledge on fluorinated chemicals, BPA and BPA analogues. Collaborative partners have been responsible for high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis of steroid hormones, Quantitative Structure-Activity Relationship (QSAR) modelling, *in vitro* activity in the retinoic acid receptor (RAR), nuclear factor (erythroid-derived 2)-like 2 (nrf2), p53, and glucocorticoid receptor (GR) CALUX reporter gene assays, and the extraction, fractionation, identification, and quantification in FCMs of paper and board. Thus these methods are not my areas of expertise.

2. Background

The experimental work of this PhD project centred around three areas; BPA analogues, fluorinated substances, and complex mixtures extracted from FCMs. This chapter will give a brief introduction to the field of endocrine disruption, as well as give insight into the two known groups of compounds used or suspected of being used in FCMs; fluorinated substances, BPA, and BPA analogues. It will also outline some methods previously applied to obtain information on the potential toxicology of chemicals used in FCMs.

2.1 Endocrine disruption

Endocrine disruptors (EDs) are compounds that, by affecting the hormone system, lead to adverse health effects in the exposed individual or its offspring and is defined in the *Global assessment of the state-of-the-science of endocrine disruptors* report (IPCS 2002) as,

> *"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations."*

Endocrine related diseases in humans have increased such as genital malformations, endocrine related cancers, neurodevelopmental disorders, and obesity (UNEP/WHO 2013). These increases occur at a rate that exclude genetic changes as the sole origin of disease, and thus it has been proposed that other factors might play a role, including chemical exposures. Associations between some of these diseases and chemical exposure have been shown in epidemiological studies (UNEP/WHO 2013). Similarly, some of these effects have been induced *in vivo* with exposure to compounds (Diamanti-Kandarakis et al. 2009), such as changes in mammary gland development with exposure to a known estrogenic compound (Mandrup et al. 2012) and malformations of male sex organs with exposure to known antiandrogenic compounds (Kim et al. 2010; McIntyre et al. 2001).

2.1.1 The endocrine system and mechanisms of endocrine disruption

Hormones control processes such as organ formation and cell differentiation during development, but also maintain adult organ and tissue function. Due to this critical role during development, it is believed that the foetus and neonate are particularly sensitive to exposure to EDs, as it may lead to permanent damage manifesting at birth or later in life (UNEP/WHO 2013). In order to understand how EDs can exert their effect on the endocrine system a brief introduction to the system will be given here.

The endocrine system consists of tissues and glands, such as the gonads, the thyroid gland, the adrenal gland, and the pituitary gland, that communicate with one another and other parts of the body by producing and secreting hormones, which are transported throughout the body through the vasculature. Many different hormones exist, such as insulin, androgens, estrogens, leptin, and thyroid hormones, however as this thesis examines effects on the estrogen and androgen receptors as well as steroidogenesis, steroid hormones will be the main focus of this chapter.

Steroidogenesis is the process by which steroid hormones are synthesized. The synthesis mainly takes place in the adrenal gland and the gonads; albeit the major hormone products differ between these tissues. The adrenal gland synthesises aldosterone, cortisol, corticosterone, as well as adrenal androgens. The testes synthesise androgens, foremost testosterone, whereas the ovaries synthesise estrogens (UNEP/WHO 2013). Figure 1 gives an overview of steroidogenesis.

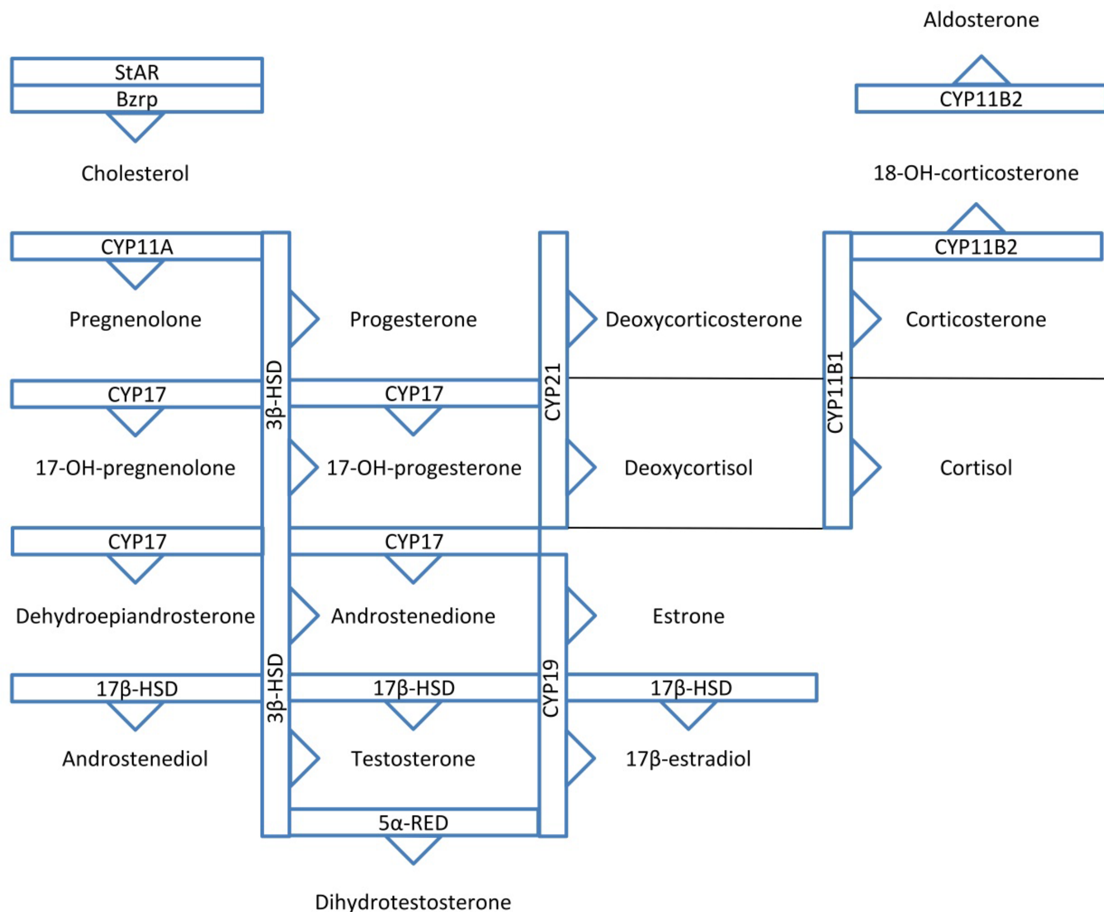


Figure 1: Steroidogenesis. Boxes = proteins involved in conversion or transport.

The step-by-step synthesis of steroid hormones starts with cholesterol, which is transported to the inner mitochondrial membrane by steroidogenic acute regulatory (StAR) protein and peripheral-type benzodiazepine receptor (Bzrp). Here cholesterol side-chain cleavage enzyme (CYP11A) converts cholesterol into pregnenolone, which diffuse to the smooth endoplasmatic reticulum where the conversion into androgens and estrogens takes place. Several enzymes are involved in this process including CYP17, 3β-hydroxysteroid dehydrogenase (3β-HSD2), 17β-HSD, and aromatase (CYP19A1). In the adrenal gland the synthesis of the corticoids is catalysed by CYP21A2 and CYP11B1. The conversion of testosterone into its more potent form dihydrotestostesterone mainly takes place in the target tissue catalysed by 5α-reductase (Maglich et al. 2014; Scott et al. 2009). Steroidogenesis is regulated by the hypothalamus and the anterior pituitary, and the hormones produced in the adrenal gland and testes exert negative feedback on these glands (IPCS 2002).

After synthesis, steroid hormones are secreted into the bloodstream where they are transported bound to proteins to their respective target tissues, where non-bound hormone diffuse across the cell membrane and act by binding to their respective receptors, Figure 2. The androgen and estrogen receptors are located in the cytosol of cells. Upon ligand binding, the receptor translocate to the nucleus where it binds to specific DNA sequences to regulate transcription of target genes, which ultimately lead to changes in cell function (Porcher et al. 2009).

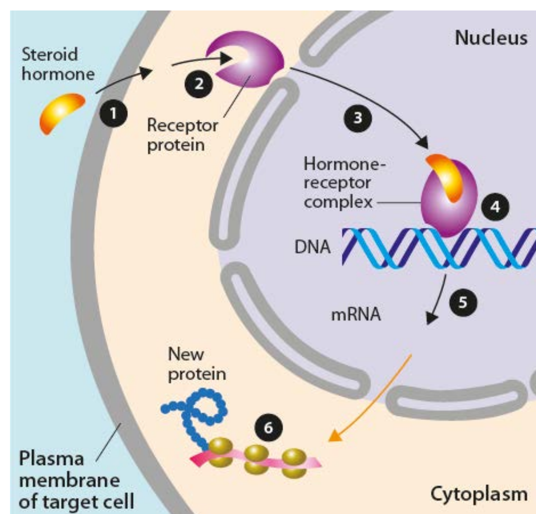


Figure 2: Steroid hormone receptor activation (from UNEP/WHO 2013).

Endocrine disruption can occur through interference with any of the above processes, Figure 3, and EDs can interfere with several of these mechanisms simultaneously. Interferences can occur through receptor-mediated and non-receptor-mediated mechanisms, of which the latter cover mechanisms such as interferences with synthesis, protein bound transport, or causing increased/decreased metabolism of endogenous hormones. At the receptor level EDs can exert agonistic or antagonistic effects leading to induced or inhibited receptor activity (Porcher et al. 2009). Many xenobiotics have shown the ability to decrease the activity of the AR and increase the activity of the ERs (Kojima et al. 2004; Vinggaard et al. 2008).

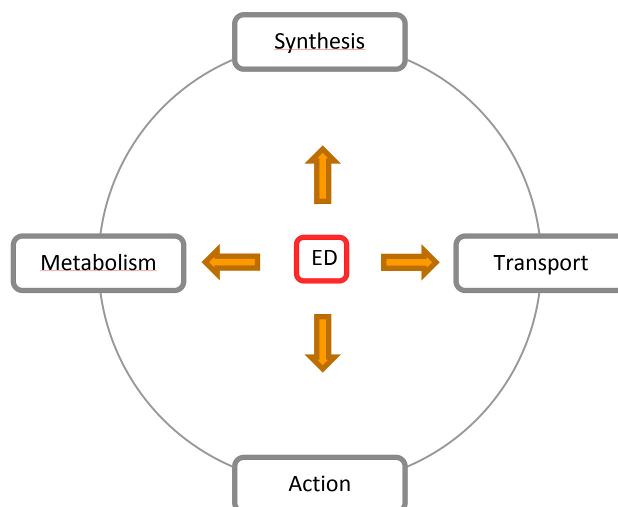


Figure 3: Mechanisms of an endocrine disruptor (ED) (Adapted from Porcher et al., 2009).

2.2 Bisphenol A and its structural analogues

Bisphenol A is widely investigated for its endocrine disruptive potential and the literature on the use, exposure, toxicokinetics and effects of this compound is extensive. BPA analogues are much less studied, and thus this chapter will give an introduction to BPA and the analogues based on currently available information.

The term bisphenol is used for compounds containing two hydroxyphenyls attached to one another by a bridge. In BPA the hydroxyl groups are placed in the para position and the bridge consists of one carbon to which two methyl groups are attached. Many other bisphenols exists, in which the bridge can be more or less polar and which vary with respect to the placement of the hydroxyl groups. BPA and the analogues included in this PhD project are shown in Figure 4. In this thesis, the term BPA analogues also covers compounds not containing two hydroxyphenyl groups, but rather refers to compounds that have structural similarities to BPA.

2.2.1 Usage, exposure sources and human biomonitoring

The primary application of BPA is as a starting material in the production of polymers such as polycarbonate and epoxy resins, which are used for multiple applications. In FCMs, polycarbonate is used for food and beverage containers, whereas epoxy resins are used as coating for food and beverage cans (Geens et al. 2011). Besides these applications, BPA have been reported in recycled paper products (Ozaki et al. 2004; Vinggaard et al. 2000), presumably originating from thermal paper containing BPA (Geens et al. 2011). It has been estimated that diet is the main source of human BPA exposure in the general population followed by exposure through contact with thermal paper (EFSA 2013).

BPB, BPF, and BPS have been detected in foods and drinks on several accounts (Cunha et al. 2011; Gallart-Ayala et al. 2011; Grumetto et al. 2008; Liao and Kannan 2014; Vinas et al. 2010). In addition, BPS has been reported in thermal paper (Becerra and Odermatt 2012; Liao et al. 2012c) and BPF as well as BPS in dust (Liao et al. 2012b). These data suggests that humans could be exposed to the BPA analogues through foods, drinks, dust and thermal paper.

The majority of the human population in developed countries is exposed to BPA. Urine concentrations of free BPA plus conjugated BPA was measured in more than 90% of samples (n = 2517) in the general US population from 6 years of age and upwards in the period 2003-2004 (Calafat et al. 2008). Similarly, total BPA was measured in human urine collected from seven Asian countries from 2006-2010 (Zhang et al. 2011b), as well as in Danish children and adolescents (Frederiksen et al. 2013). BPA in its free or conjugated form have also been measured in amniotic fluid (Edlow et al. 2012; Yamada et al. 2002), umbilical cord blood (Chou et al. 2011; Lee et al. 2008) and in the placenta (Cao et al. 2012; Jimenez-Diaz et al. 2010), indicating that the foetus is exposed during pregnancy. Since the foetus is suspected of being particularly sensitive to ED exposure, this could be an issue of concern.

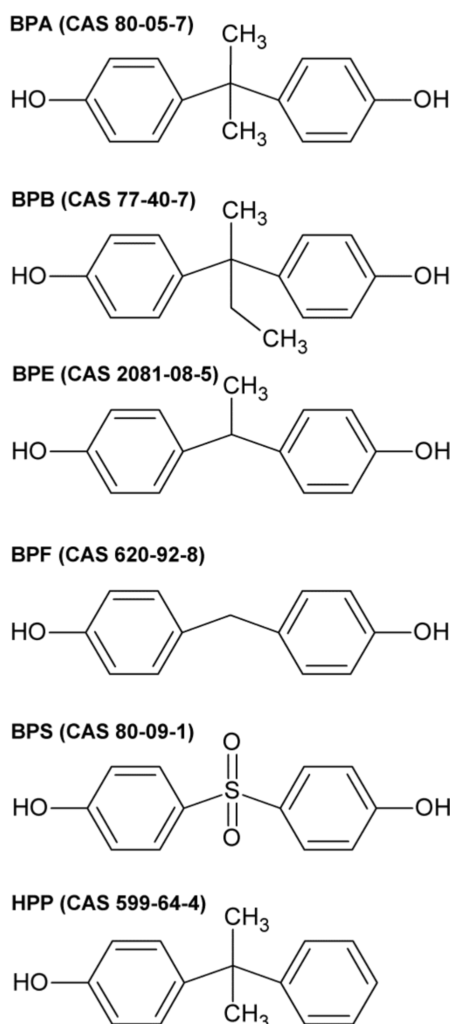


Figure 4: Chemical structures and CAS numbers of BPA and BPA analogues investigated in this PhD project.

The occurrence of BPA analogues in humans is only described in a few studies and the studies are not based on large cohorts as that for BPA. Of the BPA analogues tested in this study three has currently been measured in humans. Liao and co-workers (2012a) found BPS both in its free and conjugated form in the general US population and in seven Asian countries (n = 315) (Liao et al. 2012a) and BPB has been reported in human blood from endometriotic women from Italy in a small cohort (n = 58) (Cobellis et al. 2009). Finally, BPF as well as BFS was detected in urine samples (n=94) collected from residents living near a bisphenol AF manufacturing plant, though BPF was only detected in a small subset of the samples, and BPB was not detected in any of the samples (Yang et al. 2014).

2.2.2 Toxicokinetics

In humans, BPA is readily absorbed across the gastrointestinal tract and subsequent elimination is rapid (Völkel et al. 2002). Human urine elimination half-life has been reported to be between 4-5.4 hrs after oral administration of labelled BPA in two small study populations (Völkel et al. 2002; Völkel et al. 2005). In human urine BPA is mainly measured in its conjugated form, BPA-glucuronide or BPA-sulfate, of which the former is the major metabolite (Vandenberg et al. 2007).

Only few studies have reported on the metabolism of BPA analogues and thus I here refer to data obtained from sea and river water, well knowing that these may not be representative of metabolism in humans or animals. In river water the degradation of BPA, BPB, BPE, BPF, and BPS was examined, and it was found that BPF degraded faster than BPA, whereas the remaining BPA analogues degraded at a slower rate (Ike et al. 2006). In accordance with the findings in river water, BPF showed slightly better degradation efficiencies than BPA in seawater, whereas BPS showed no degradation during the time course of the experiments (Danzl et al. 2009). These data suggest that the degradation at least in water systems may vary between these structurally similar compounds. If a similar degradation pattern takes place in humans, this could mean varying half-lives of the different analogues, which might affect their potential to exert effects *in vivo*.

2.2.3 Toxicological effects

In recent years, a debate on the potential low-dose effects of BPA has been ongoing as some *in vivo* studies have shown low-dose effects on endpoints such as changes in normal behavioural development (Xu et al. 2010), changes in obesity associated parameters (Miyawaki et al. 2007), decreased anogenital distance (Christiansen et al. 2014), and disturbed mammary gland development (Moral et al. 2008) with prenatal followed in some cases by postnatal exposure. The recently proposed tolerable daily intake of BPA of 5µg/kg/d is however based on general toxicity on the kidney in male mice in a two-generational toxicity study (EFSA 2014).

2.2.4 Endocrine activity *in vitro*

BPA is widely investigated for affecting several endocrine targets *in vitro*. The estrogenic potential of BPA has been reported in numerous studies, in which the values for concentrations causing 50% of the maximum response (EC_{50}) ranged from 0.2-2.32 µM (Grignard et al. 2012; Kitamura et al. 2005; Kuruto-Niwa et al. 2005; Paris et al. 2002). In these studies the potency of 17β-estradiol was >10,000 times more potent than BPA. Besides, antiandrogenic potential of BPA has been reported on the AR (Kitamura et al. 2005; Lee et al. 2003; Paris et al. 2002), as well as the ability to decrease androgen levels in the H295R steroidogenesis assay (Zhang et

al. 2011a). This decrease in androgen levels were accompanied by increased estrogen levels (Zhang et al. 2011a).

The effects of BPA analogues are not as thoroughly investigated for endocrine disruptive potential as BPA itself. However some studies suggest that structurally similar chemicals cause effects on the same endpoints *in vitro*. Amongst the compounds investigated were BPA, BPB, BPE, BPF, BPS, and 4-cumylphenol (HPP). These compounds all showed the potential to be estrogenic and antiandrogenic (Kitamura et al. 2005), however the potency differed between the compounds and also between the studies (Grignard et al. 2012; Kitamura et al. 2005).

A few studies have examined a broad range of structural analogues to BPA for estrogenicity and antiandrogenicity and found some structural traits which affected the effect on these endpoints (Kitamura et al. 2005; Paris et al. 2002; Rivas et al. 2002). Overall the results from these studies suggest that estrogenicity as well as antiandrogenicity increase when the hydroxyl groups are placed in the para position compared to the meta and the ortho position. For estrogenicity at least one phenyl group is needed for effect to occur and the effect increase when two phenyl groups are present, whereas for antiandrogenic effects only one phenyl group is needed and the response does not change when a second phenyl group is introduced. Finally, for both estrogenicity and antiandrogenicity it seems that the introduction of a polar group in/on the central carbon reduces the effects (Kitamura et al. 2005; Paris et al. 2002; Rivas et al. 2002).

2.3 Fluorochemicals

Fluorinated chemicals cover a wide range of structurally diverse compounds, which are used for multiple applications. In FCMs of paper and board fluorinated chemicals impart repellency properties to the materials (Kissa 2001). Within the last couple of decades, a shift in fluorochemical production methods has taken place, as a consequence of voluntary phase-outs by the industry as well as implementation of regulations for some fluorochemicals (OECD 2013; Stockholm Convention 2009; US EPA 2000). These actions have in some cases led to a shift in the production towards short-chained chemistry (Scheringer et al. 2014). The following chapter will focus on the compounds investigated in this study including compounds used in coatings for FCMs of paper and board, polyfluoroalkyl phosphate ester surfactants (PAPs), and their metabolic products, fluorotelomer alcohols (FTOHs) and perfluorinated alkyl carboxylic acids (PFCAs).

The term fluorochemicals or fluorinated chemicals are here defined as an aliphatic substance containing at least one fully fluorinated carbon atom, also called polyfluoroalkyl substances (PFAS). These compounds contain a fluorinated moiety and a functional head group such as an alcohol or carboxylic acid (Buck et al. 2011; Trier et al. 2011) and the fluorinated moiety can

vary in chain lengths from typically 4-15 carbons (Prevedouros et al. 2006). The degree of fluorination classifies the compounds as either a polyfluoroalkyl substances, defined as above, or perfluoroalkyl substances in which all carbons except that in the functional head group are fully fluorinated (Buck et al. 2011). PAPs, FTOHs, and PFCAs tested in this PhD project thus belong to the polyfluoroalkyl substances, whereas only the PFCAs are perfluoroalkyl substances.

2.3.1 Exposure sources and human biomonitoring

Human exposure sources to perfluorooctanoic acid (PFOA) have been estimated to be through intake of food and drinking water (Trudel et al. 2008; Vestergren et al. 2008), however fluorinated chemicals have also been measured in indoor dust (D'Hollander et al. 2010), suggesting this as an alternative source of human exposure. Furthermore, D'eon et al. (2011b) have suggested that additional sources to PFOA exposure may exist, such as exposure to fluorinated chemicals, for instance in FCMs of paper and board, which can be converted into PFCAs (D'eon and Mabury 2011b). In support of these findings, PAPs, a precursor of PFCAs, can migrate from FCMs of paper and board into food and food simulants (Begley et al. 2005; Begley et al. 2008).

The serum half-life of PFOA in humans is long, $T_{½}$ of approximately 3.8 years (Olsen et al. 2007) and in animals the $T_{½}$ generally decrease with PFCA chain length (Lau 2012). PFOA in serum/plasma/whole blood has been reported ranging from 3 to 213 nM in the general population worldwide (EFSA 2008). Furthermore, several other PFCAs have been measured in human serum/plasma (Calafat et al. 2007; Olsen et al. 2012), as well as homologues of diPAPs in a total concentration of 4.5 µg/L in one study (D'eon et al. 2009).

Fluorinated compounds have also been measured in human body compartments of relevance to foetal and neonatal exposure. PFOA has in two studies been reported in 100% of umbilical cord blood samples (Apelberg et al. 2007; Monroy et al. 2008) and correlations between maternal and umbilical serum levels of PFOA and longer-chained PFCAs was found in women from Seoul (Kim et al. 2011). Transfer to breast milk of PFOA and diPAPs has also been reported (Kubwabo et al. 2013; So et al. 2006; Völkel et al. 2008). These human biomonitoring data suggests that not only is the general population exposed to fluorinated compounds, but the unborn or newborn child may also be exposed through the placenta or breast milk.

2.3.2 Toxicokinetics

Based on human biomonitoring data, some fluorinated compounds can pass biological barriers and enter the human body, as the compounds are measured in blood and other human body

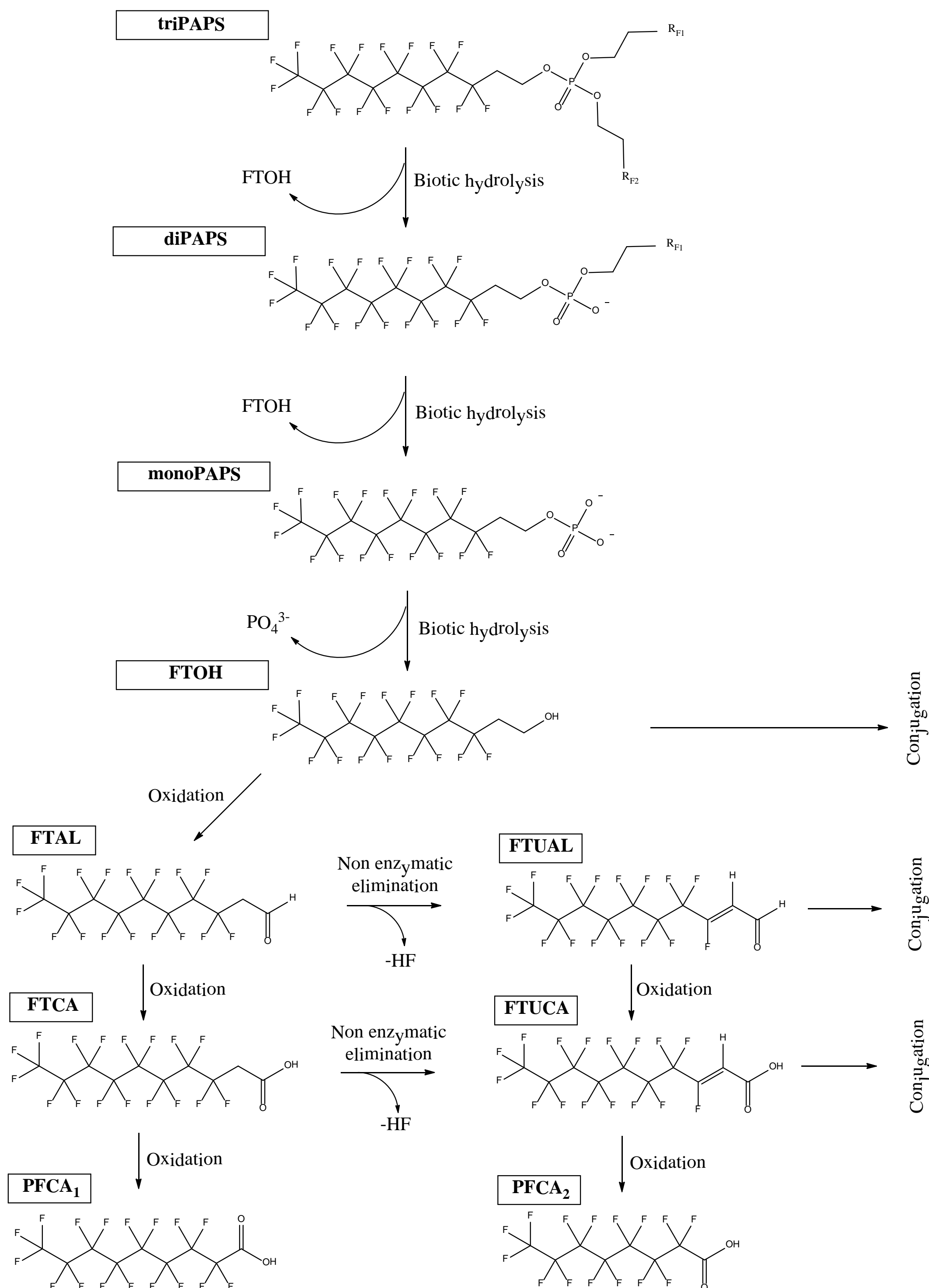


Figure 5: Metabolic pathway of trialkylated, dialkylated, and monoalkylated polyfluoroalkyl phosphate ester surfactants (PAPs) into perfluorinated alkyl carboxylic acids (PFCAs) through the intermediate metabolite fluorotelomer alcohol (FTOH). R_F = fully fluorinated carbon chains. Abbreviations: fluorotelomer aldehyde (FTAL), fluorotelomer carboxylic acid (FTCA), fluorotelomer unsaturated aldehyde (FTUAL), and fluorotelomer unsaturated carboxylic acid (FTUCA). (D'eon and Mabury 2007; D'eon and Mabury 2011a; Fasano et al. 2006; Kudo et al. 2005; Martin et al. 2005).

compartments. These findings have also been corroborated by animal studies where animals subjected to oral exposure of PFOA are found to absorb this fluorinated compound into the circulation (Kennedy et al. 2004). The absorption of the precursor compounds, PAPs, were examined only in a few studies; however they illustrated that diPAPs homologues can be measured in blood after a single oral dose (D'eon and Mabury 2007; D'eon and Mabury 2011a). 8:2 monoPAPs has also displayed an ability to absorb across the gastrointestinal tract (D'eon and Mabury 2007). After absorption, fluorinated chemicals are typically found at the highest concentrations in the liver, kidney and serum (Lau et al. 2007).

Some polyfluoroalkyl substances have the potential to metabolize into the highly persistent PFCAs of which the latter most likely does not undergo further metabolism or conjugation (Martin et al. 2005). 8:2 diPAPs was administered in rats and successively monoPAPs, PFOA, and other intermediate metabolites were measured, suggesting that diPAPs was dephosphorylated in several steps to FTOH, which in turn was metabolized into PFCAs in the rat (Figure 5) (D'eon and Mabury 2007). The metabolism of FTOH into PFCAs involves the formation of several intermediate metabolites, several of which form conjugates including FTOH itself (Fasano et al. 2006; Kudo et al. 2005; Martin et al. 2005).

2.3.3 Toxicological effects

In animal studies, exposure to PFCAs of varying chain lengths led to enlarged liver mainly through hepatocytic hypertrophy and effects such as hepatocellular vacuolation, degeneration, necrosis, and lipid droplet accumulation has also been observed with exposure to some fluorochemicals. Tumour induction in the rat liver, pancreas, and testis has been reported with chronic PFOA exposure. Furthermore, developmental effects with prenatal PFOA exposure in mice include neonatal mortality, growth deficits, as well as developmental delays. Finally, PFOA exposure has been associated with immunotoxicity (Lau 2012).

2.3.4 Endocrine disruptive potential

Fluorinated chemicals have been reported to exert endocrine disruptive effects by interfering with the thyroid hormone system or the sex hormone system (Lau et al. 2007). The latter, which is the focus of this thesis, has been shown to be affected by several long-chained PFCAs, including PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluorododecanoic acid (PFdoDA). However, only limited to no data exists for FTOHs, short-chained PFCAs, and PAPs.

Several studies have reported effects on steroid hormones levels following PFOA exposure. *In vivo* studies generally report on an increase in serum 17β-estradiol levels (Biegel et al. 1995; Biegel et al. 2001; Cook et al. 1992), but show variable effects on testosterone levels; no

change (Biegel et al. 1995; Biegel et al. 2001) or decreasing trend (Cook et al. 1992) following exposure. An increase in testosterone levels *in vivo* within the testicular interstitium have also been reported (Biegel et al. 1995). *In vitro*, PFOA led to decreased testosterone synthesis in isolated Leydig cells (Biegel et al. 1995; Zhao et al. 2010). At the receptor level, PFOA can increase ER and inhibit AR mediated transactivation *in vitro* (Kjeldsen and Bonefeld-Jorgensen 2013), though the latter was not observed in a previous study (Vinggaard et al. 2008). In essence, these data suggest that PFOA have estrogenic potential, however the ability to affect testosterone synthesis and AR activity of PFOA remain unclear.

Similar to PFOA, *in vivo* exposure to PFNA can lead to increased serum 17β-estradiol levels and decreased testosterone levels at 5 mg/kg/day. At lower concentrations, testosterone levels were elevated (Feng et al. 2009), however as body weight measurements were not reported, it is difficult to exclude general toxicity as accounting for these effects. *In vitro* studies have found similar effects on these hormones (Kraugerud et al. 2011), and also report decreased activity of the AR and no change in ER activity (Kjeldsen and Bonefeld-Jorgensen 2013). These data indicate that PFNA, at least *in vitro*, have the potential to exert antiandrogenic effects at the levels of androgen synthesis and receptor activity and estrogenic effects through increasing 17β-estradiol levels. It remains unclear if the similar observed effects *in vivo* are caused by general toxicity or through similar mechanisms.

PFDA and PFdoDA have been reported to decrease plasma/serum androgen levels (Bookstaff et al. 1990; Shi et al. 2009a). These changes were also observed *in vitro* following PFDA exposure and challenge with human chorionic gonadotropin (Boujrad et al. 2000). In addition, 17β-estradiol levels was either not affected or decreased with exposure to PFdoDA in male and female rats respectively, though the decrease in females occurred only when body weight was reduced (Shi et al. 2009a; Shi et al. 2009b). PFDA, but not PFdoDA, was reported to decrease AR mediated transactivation *in vitro*, but neither compound affected ER activity (Kjeldsen and Bonefeld-Jorgensen 2013). This suggests that these longer-chained PFCAs exhibit antiandrogenic potential *in vivo* and/or *in vitro* and further, that PFDA and PFdoDA are not estrogenic. However, considering the estrogenicity of PFNA and PFOA, this should be investigated further.

In accordance with the observed increased in 17β-estradiol levels for long-chained PFCAs, both 6:2 and 8:2 FTOH led to similar increases in adult zebrafish (Liu et al. 2009; Liu et al. 2010a). 6:2 FTOH led to increased testosterone in both sexes (Liu et al. 2009), whereas the effects of 8:2 FTOH were sex specific, causing increased levels in females and decreased levels in males (Liu et al. 2010a). Decreased testosterone levels were also shown in the H295R steroidogenesis assay following exposure to 8:2 FTOH (Liu et al. 2010b). *In vitro* studies also

suggest that both compounds are estrogenic by increasing proliferation in the MCF-7 proliferation assay (Maras et al. 2006).

Albeit somewhat inconsistent, data on FTOHs and long-chained PFCAs point towards these compounds being both estrogenic and antiandrogenic. Therefore, it can be speculated that any of these compounds, alone or in combination, can affect sex hormone synthesis and action, ultimately impacting on reproductive health. In support of this, foetal as well as neonatal exposure to PFOA has led to changes in mammary gland development (White et al. 2011), an effect which has previously been reported with exposure to synthetic estrogens (Mandrup et al. 2012), suggesting that PFOA may lead to this effect through exhibiting estrogenic potential *in vivo*. Therefore based on all these data, any putative endocrine disruptive potential of fluorinated chemicals should be investigated further.

2.4 Strategies to address toxicity of food contact materials

Part of this PhD project revolved around developing a strategy to address toxicities of chemical in FCMs of paper and board as well as identifying causative agents arising from FCM by use of bioassay guided analysis and analytical chemical tools. The following chapter will briefly introduce some *in vitro* endpoints which have been used previously for investigating effects of paper and board materials. These studies included endpoints such as genotoxicity, cytotoxicity, estrogenicity, and effects on the aryl hydrocarbon receptor (AhR) activity (Binderup et al. 2002; Lopez-Espinosa et al. 2007; Ozaki et al. 2004; Ozaki et al. 2005; Vinggaard et al. 2000; Weber et al. 2006).

In a project called the BIOSAFEPAPER project endpoints covering cytotoxicity and genotoxicity were used. When testing extracts from FCMs of paper and board several led to cytotoxicity, whereas only few led to effects on genotoxicity (Weber et al. 2006). However, other studies have shown genotoxic potential of several FCM samples (Ozaki et al. 2004; Ozaki et al. 2005). Estrogenicity of paper and board has been investigated in at least two studies, where several of the samples caused effects (Lopez-Espinosa et al. 2007; Vinggaard et al. 2000). Finally, the AhR reporter gene assay showed positive response in all extracts in one study, though the samples size was small (Binderup et al. 2002).

Furthermore, one study have implemented a fractionation step as part of the process, when wanting to identify causative agents in FCMs. Ozaki and co-workers tested FCMs of paper and board for their potential to cause DNA damage (Ozaki et al. 2004) and found several of these to cause effect. By fractionating the extracts and subsequently testing the fractions *in vitro*, they successfully identified two potential causative agents (Ozaki et al. 2005).

The studies above all illustrate the usefulness of applying a bioassay-guided approach to obtain knowledge on potential hazards of compounds in FCMs. Further, they show that including genotoxicity, cytotoxicity, estrogenic endpoints, the AhR reporter gene assay, as well as a fractionation step to assess toxicities of FCMs and identifying causative agents in these, may be a promising starting point.

3. Experimental design

Two approaches were applied to test the potential toxicology of compounds present or suspected of being used in FCMs *in vitro*, the bottom-up and the top-down approach. Many of the same assays were applied in these approaches and thus a brief introduction to the assays in which endocrine activity was assessed is given here. Other assays were also included in the Manuscripts covering endpoints such as genotoxicity and oxidative stress. A detailed description of the experimental methods is given in Manuscript 1-4.

The AR reporter gene assay is conducted in the Chinese hamster ovary cell-line transfected with the human AR expression vector as well as the mouse mammary tumour virus (MMTV)-luciferase vector (Vinggaard et al. 1999). Upon activation of the receptor the receptor-ligand complex is translocated to the nucleus leading to transcription of luciferase. Successively luciferin is added and is converted by luciferase into a product, which can be measured as luminescence. The ER reporter gene assay is based on a similar principle. The human ovarian adenocarcinoma cell-line is used, which is stably transfected with the reporter gene and endogenously express both ERα and ERβ (OECD 2012). In the H295R steroidogenesis assay, the human adreno-carcinoma cell-line is used. The cells have characteristics of zonally undifferentiated human foetal adrenal cells and contain all the proteins necessary to synthesize corticosteroids and sex hormones (OECD 2011). Steroid hormones can be measured in the supernatant after assay completion. In this PhD up to 10 steroid hormones were measured, and gene expression of several genes coding for central proteins involved in steroidogenesis were examined for some compounds.

These assays cover some of the proposed mechanisms-of-action, which may facilitate endocrine disruptive effects, which are illustrated in figure 3. Thus, data obtained from the assays provide information on whether the compounds can target several mechanisms simultaneously leading to an overall greater endocrine activity.

3.1 The bottom-up approach

The bottom-up approach covers the classical toxicological approach in which effects of single compounds are tested. BPA, five BPA analogues, and nineteen fluorinated substances were investigated in 4-9 different *in vitro* assays, Table 1. The BPA analogues were chosen based on the usage of some of these; however the main objective was to test if slight structural changes in the compounds could lead to altered potency, efficacy, and mechanism-of-action compared to BPA. The selected fluorinated substances represent active ingredients in some technical mixtures (TMs) intended for coating of FCMs (PAPs), intermediate metabolites of PAPs (FTOHs), and final metabolites of PAPs (PFCAs). Finally, three TMs intended for use in FCMs

of paper and board were included. The TMs contained fluorochemicals for which the specific structures, concentrations and levels of residuals are largely unknown.

Table 1: Substances tested in vitro in the bottom-up approach. In vitro assays included the androgen receptor (AR), estrogen receptor (ER), aryl hydrocarbon receptor (AhR) reporter gene assays and the H295R steroidogenesis assay (H295R). Further, the retinoic acid receptor (RAR), the glucocorticoid receptor (GR), the nrf2, and the p53 CALUX reporter gene assays were used. For abbreviations of compounds refer to the Abbreviation list. Color code: Grey = tested, white = not tested.

	BPA and analogues						**Fluorinated substances**																		
	BPA	BPB	BPE	BPF	BPS	HPP	PFBA	PFPA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFdoDA	4:2 FTOH	6:2 FTOH	8: FTOH	8:2/8:2/8:2 triPAPs	10:2/10:2 diPAPs	8:2/8:2 diPAPs	8:2 monoPAPs	TM1	TM2	TM3
AR	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
ER	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
H295R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
AhR	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
RAR	■	■	■	■	■	■					■	■	■	■	■										
GR											■	■	■	■	■										
nrf2	■	■	■	■	■	■					■	■	■	■	■										
p53	■	■	■	■	■	■					■	■	■	■	■										

3.2 The top-down approach

The top-down approach was devised to assess potential toxicity of chemicals present in FCMs of paper and board for which the chemical composition is unknown. The FCM samples are presented in Manuscript 4 and represent a broad range of intended applications, storage times, and materials. At present only data from the AR, ER, and AhR reporter gene assays are included in Manuscript 4, however it is intended that the final strategy will contain several other assays including the nrf2 CALUX, p53 CALUX, RAR CALUX, GR CALUX, peroxisome proliferator-activated receptor alpha and gamma (PPARα, PPARγ) reporter gene assays as well as the Comet assay and the Ames test.

The strategy consists of several steps, as illustrated in Figure 6, which are described in detail in Manuscript 4 and include 1) production of extracts from FCMs of paper and board, 2) testing of extracts *in vitro*, 3) fractionation of selected active extracts, 4) testing of fractions *in vitro*, 5) tentative identification of compounds in active fractions, 6) selection of final list of tentatively identified compounds for testing based on read-across, biophores, and commercial availability, 7) *in vitro* testing of selected tentatively identified compounds, 8) confirmation of tentatively identified compounds and quantification of identified compounds, and 9) calculation of equivalence factors (EQs) based on concentrations of identified compounds as well as dose-response parameters for the identified compounds as well the extract.

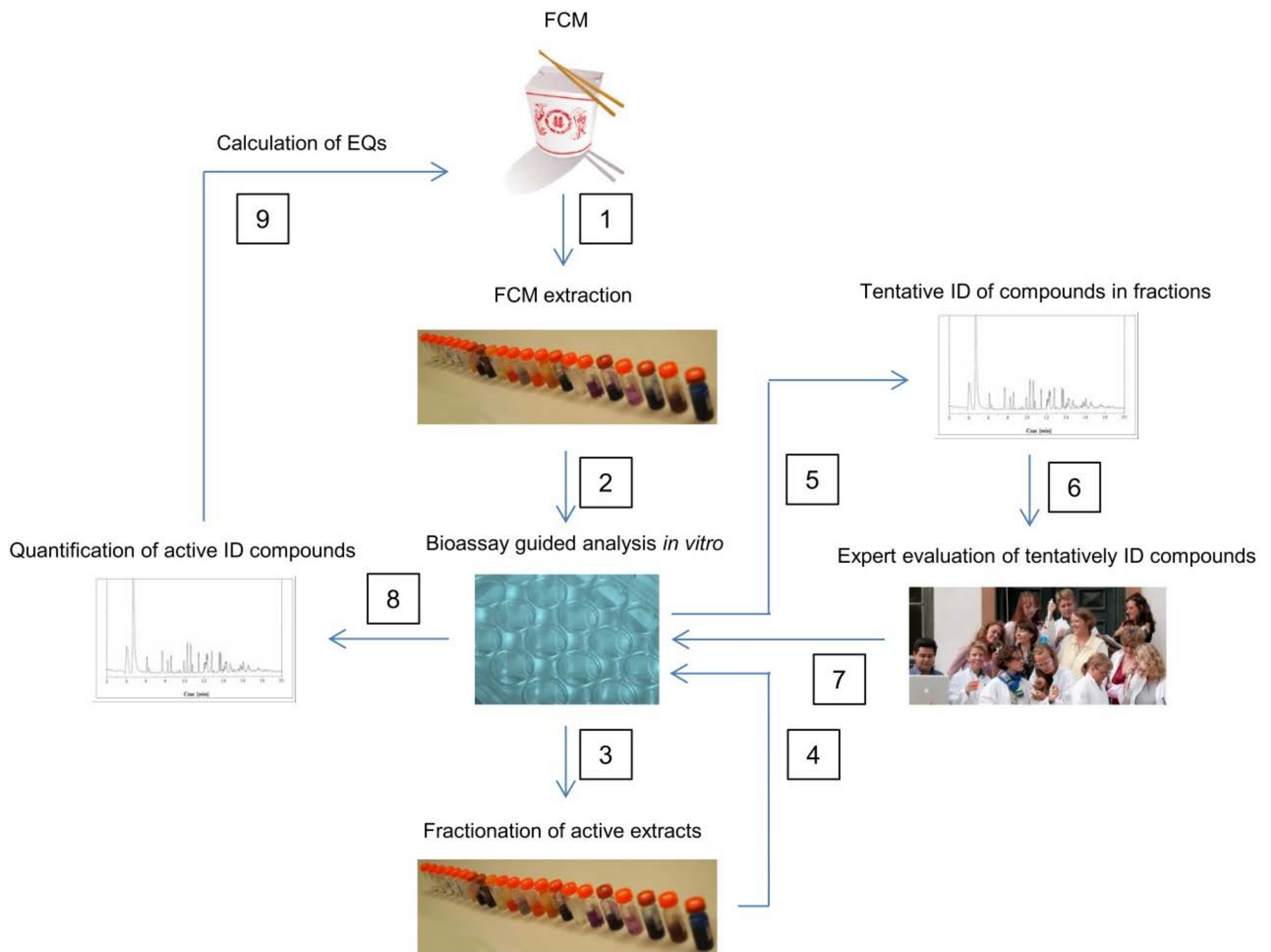


Figure 6: Step-by-step workflow for the FCM strategy. Numbers indicate, 1) production of extracts from FCMs of paper and board, 2) in vitro testing of extracts, 3) fractionation of extracts exhibiting in vitro activity, 4) in vitro testing of fractions from active extracts, 5) tentative identification of compounds based on in vitro activity of fractions, 6) selection of tentatively identified compounds by expert judgment for further in vitro examination, 7) testing of selected tentatively identified compounds in vitro, 8) quantification of selected tentatively identified compounds causing in vitro activity, 9) calculation of equivalence factors (EQs). Abbreviations: ID = identification, FCM = food contact material.

4. Results

A brief summary of major findings presented in Manuscript 1-4 are listed below.

4.1 Bisphenol A and structural analogues

Manuscript 2

Are structural analogues to bisphenol A safe alternatives?
Rosenmai, A.K., Dybdahl, M., Pedersen, M., van Vugt-Lussenburg, B.M.A., Wedebye, E.B., Taxvig, C., and Vinggaard, A.M., 2014. Tox Sci. 139, 35-47.

The aim of this study was to compare the toxicological effects of BPA to that of its analogues BPB, BPE, BPF, BPS, and HPP. Together with BPA, these five analogues were investigated across a battery of *in vitro* assays and QSAR models. The endpoints examined covered endocrine disruption such as ER and AR activities as well as effects on steroidogenesis, but also endpoints related to metabolism, teratogenicity, genotoxicity, and carcinogenicity.

The observed effects on AR and ER activity were similar for BPA and the five analogues, and the compounds generally exhibited similar potencies. One exception was BPS, which was less potent on ER activity compared to the remaining test compounds and showed only a decreasing trend on AR activity. Overall, compound exposure in the steroidogenesis assays led to decreased androgen levels and increased estrogen and progestagen levels. An exception is BPS, which did not affect estrogen levels and showed a marked effect on progestagen levels compared to BPA, which did not affect progesterone and led to a decreasing trend on 17α-OH progesterone in this assay. Corticosteroid levels differed qualitatively between the test compounds.

4.2 Fluorinated chemicals

Manuscript 1

Fluorochemicals used in food packaging materials inhibit male sex hormone synthesis
Rosenmai, A.K., Nielsen, F.K., Pedersen, M., Hadrup, N., Trier, X., Christensen, J.H., and Vinggaard, A.M., 2013. Toxicol. Appl. Pharmacol.. 266, 132-142.

The aim of the investigation was to obtain information on the potential endocrine activities on AR and steroidogenesis of six fluorinated compounds. To achieve this, we examined four PAPs,

as well as 8:2 FTOH and PFOA *in vitro* in the AR reporter gene assay and the H295R steroidogenesis assay.

Three of the test compounds, namely 8:2 diPAPs, 8:2 monoPAPs, and 8:2 FTOH, generally led to decreases in androgen and progestagen levels, and led to increased levels of at least one of the measured estrogens. CYP19 mRNA levels were increased with exposure to 8:2 diPAPs, 8:2 monoPAPs, and 8:2 FTOH, whereof the two latter also led to decreased Bzrp mRNA levels. None of the remaining genes were affected with exposure to any of the six fluorinated chemicals. 8:2 triPAPS, 10:2 diPAPs, and PFOA also led to decreases in some of androgens measured and caused an increase in at least one of the measured estrogens. No effect was observed on AR activity of any of the compounds.

Manuscript 3

Fluorinated compounds and technical mixtures for use in food contact materials have estrogenic activity in an in vitro screening
Rosenmai, A.K., Trier, X., Taxvig, C., van Vugt-Lussenburg, B.M.A. and Vinggaard, A.M.
Manuscript in preparation.

In this study, we examined sixteen fluorinated chemicals and three fluorine-containing TMs for the ability to interfere with the endocrine system *in vitro* as well as compare the activities on different endpoints between the compounds. Endpoints such as genotoxicity and oxidative stress were also included in the panel of *in vitro* assays. The compounds included were four PAPs, their intermediate metabolites (FTOH), as well as their final metabolites (PFCAs ranging from perfluorobutanoic acid (PFBA) to PFdoDA). Furthermore, we included three fluorine-containing TMs, TM1-TM3, of unknown composition intended for coating of FCMs of paper and board.

The main effects observed for several of the tested compounds were estrogenic potential either through increasing ER activity or increasing estrogen levels. TM2, PFDA, perfluoroundecanoic acid (PFunDA), and PFdoDA led to increases in 17β-estradiol levels, whereas TM2, TM3, 8:2 monoPAPs, 8:2 FTOH 6:2 FTOH, 4:2 FTOH, PFNA, and PFOA increased ER activity. Only 8:2 triPAPs and 8:2 monoPAPs led to increased AhR activity. The short-chained PFCAs did not cause effect in any of the assays, and none of the compounds led to effects on AR activity.

4.3 Food contact materials

Manuscript 4

A strategy to identify problematic chemicals in food contact materials of paper and board
Rosenmai, A.K., Bengtström, L., Taxvig, C., Trier, X., Petersen, J.H., Granby, K., and Vinggaard, A.M. Manuscript in preparation.

In this study we wanted to obtain information on the potential hazards of chemicals present in FCMs of paper and board by developing a step-by step FCM strategy applying both analytical chemistry tools as well as bioassay guided analysis. Only data from the AR, ER, and AhR reporter gene assays are included in the Manuscript at present, though the final FCM strategy is intended to include 11 assays covering genotoxicity, oxidative stress and many more.

In the initial screening of the 20 extracts from paper and board, we identified that all extracts led to effects in the AhR reporter gene assay, whereas only some led to changes in ER and AR activity. Two extracts underwent further investigation by use of the full strategy developed, extract S4 and extract S8.

Extract S8 exhibited ER activity. The extract was fractionated and fractions were tested in the ER reporter gene assay. Tentative identification was conducted in active fractions, and BPA, di-isobutyl phthalate (DiBP), Di-butyl phthalate (DBP), and butyl-benzyl phthalate (BBP) were selected for further testing. BPA, DBP, and BBP led to effects individually in the ER reporter gene assay and their identity was confirmed in the extract and fractions. Based on quantification of these tree compounds in the extracts, we calculated estrogen equivalence factors (EEQs) for BPA, DBP, and BBP as well as the extract. The sum of EEQs calculated for identified compounds exceeded that of the extract. Extract S4 exhibited AR activity. The step-by-step procedure described above was also applied for this extract. Based on the tentative identification in fractions abietic acid (AA), dehydroabietic acid (DHAA), 4-oxo-retinoic acid, isorhamnetin, and rhamnetin was selected for testing *in vitro*. All led to decreased AR activity when tested individually, except isorhamnetin, however only the identity of DHAA and AA was confirmed. The sum of androgen equivalence factors (AEQs) for DHAA and AA exceeded that of the extract.

5. Discussion

Food packaging materials constitute a potential source of human exposure to chemicals, as compounds may migrate into foods from the packaging. Many compounds are catalogued as food contact substances, including some intended for food packaging materials, but many of these are likely not sufficiently assessed for toxicological effects to allow for a complete risk assessment. This is of concern since it implies that humans are exposed to compounds through foods, for which limited data on the toxicology is available.

In this PhD project, the aim was to investigate if compounds known, or suspected to be used in FCM could have the potential to cause adverse effects, with specific focus on endocrine activities. Two approaches were applied; the bottom-up and the top-down approach, in which BPA analogues, fluorinated chemicals, and extracts from FCMs of paper and board were examined.

5.1 The tested hypotheses

5.1.1 Bisphenol A analogues

The aim was to gain knowledge about the toxicology of BPA analogues to ultimately be able to allow for the assessment of putative toxicological effects caused by these compounds. I hypothesized that the analogues would exhibit similar endocrine mechanisms-of-action as BPA *in vitro*. Overall, I found this to be the case as described in Manuscript 2. Here, a comparable qualitative effect on ER and AR activity, as well as effect on androgen and estrogen levels, was evident. However, there were also differences in effects of the analogues for some of the endpoints investigated, as the qualitative effects on corticoids differed and some compounds led to marked effects on progestagens compared to BPA. From Manuscript 2, I established that the BPA analogues display effects for most endocrine mechanisms-of-action that are comparable to BPA, but for some endocrine endpoints they do not. This similarity in effects suggests that neither of the analogues are good alternatives to BPA based on the parameters investigated here.

In many of the applied assays, BPS had a profile that was distinct from BPA. BPS only led to decreasing trends on AR activity, exhibited a less potent effect on ER activity, and no effect on estrogen levels. However, BPS led to a marked increase in progestagen levels compared to BPA. The differences in ER and AR activities between BPS and BPA was expected based on previous findings (see chapter 2.2.4), as a polar bridge is suggested to decrease the activity for both these endpoints (Kitamura et al. 2005; Paris et al. 2002; Rivas et al. 2002). These data on

estrogenicity and antiandrogenicity indicates that BPS is a good alternative to BPA. However, the toxicokinetics of BPS should also be considered, as there are indications that degradation might be slower (Danzl et al. 2009; Ike et al. 2006), potentially leading to a longer half-life. Furthermore, the potential effects on progestagen levels need to be further examined, as these effects could lead to a distinct toxicology of BPS compared to BPA *in vivo*.

As evidenced by these experiments, simply substituting a known endocrine disruptive chemical, BPA in this case, with structurally similar compounds is not straight forward, as they can have both similar and different toxicological effects. BPB, BPE, BPF, and HPP generally led to very similar effects compared to BPA on estrogen and androgen levels, as well as activity of ER and AR. As BPA *in vivo* caused effects associated with exposure to estrogenic and antiandrogenic compounds (Christiansen et al. 2014; Moral et al. 2008), it may be inferred that the substitutes we tested will also exert such effects, which is supported by the reported estrogenic potential of BPF and BPS *in vivo* (Stroheker et al. 2003; Yamasaki et al. 2004). To my knowledge, no publicly available *in vivo* data exists on any potential endocrine disruptive activities of BPB, BPE, and HPP. Thus, it can only be speculated whether the estrogenicity and antiandrogenicity observed *in vitro* in this study will lead to similar effects *in vivo*.

In my opinion, we need to sincerely consider results from the *in vitro* measurements, as they strongly suggest that BPA analogues have similar mechanisms-of-action as BPA. We need to use this knowledge to design further studies to better understand the toxicology of the chemicals, with the ultimate goal of assuring that humans are not exposed to harmful compounds.

5.1.2 Fluorinated chemicals

Since knowledge about endocrine activity of some fluorinated chemicals is presently very limited, another aim was to gain further insight into this. I hypothesized that fluorochemicals with unknown endocrine disruptive potential would exhibit comparable effects to longer-chained PFCAs through similar mechanisms-of-action and performed experiments to test this. The results are described and discussed in Manuscript 1 and 3. These studies showed that the fluorinated substances targeted different mechanisms within the experimental setup, and thus the hypothesis can be rejected. However, some sub-groups of fluorinated compounds seemingly exhibit similar effects: PAPs inhibited steroidogenesis leading to decreased androgen and/or progestagen levels, FTOH activated the ER, and the long-chained PFCAs increased 17β-estradiol levels. Furthermore, we observed no discernible effect of short-chained PFCAs, which suggest these as good alternatives to long-chained PFCAs based on the parameters investigated here. Finally, two of three tested TMs exhibited estrogenic potential, which

warrants further studies into characterizing the specific chemical structures of the fluorinated compounds present in these TMs.

Previous studies have mainly focused on the ability of long-chained PFCAs to exhibit sex hormone-related endocrine disruptive activities (Biegel et al. 1995; Biegel et al. 2001; Bookstaff et al. 1990; Cook et al. 1992; Kjeldsen and Bonefeld-Jorgensen 2013; Kraugerud et al. 2011; Shi et al. 2009a), which makes sense as these constitute the final metabolites of many fluorinated chemicals (D'eon and Mabury 2007; Fasano et al. 2006; Kudo et al. 2005; Martin et al. 2005) and PFOA has a long half-life in humans (Olsen et al. 2007). However, as several of the PAPs, TMs, and FTOHs also exhibit endocrine activity and some show markedly different profiles to long-chained PFCAs, our data emphasize that the contribution of these substances should not be neglected, or at the very least be characterized further. This is particularly important since they may cause similar effects *in vivo* as long-chained PFCAs, or lead to different effects due to for instance the antiandrogenic potential of PAPs.

Though long-chained PFCAs have been reported to affect hormone levels as well as sex hormone receptor activity, only few studies have to my knowledge reported on the implications of these changes on reproductive parameters sensitive to endocrine disruption. PFdoDA exposure led to no changes on age of vaginal opening, age of first estrous cycle, and ovarian histology in pre-pubertal female rats (Shi et al. 2009b). However, PFOA exposure *in utero* or neonatally has been shown to cause permanent changes on mammary gland development in female offspring, potentially by interfering with ovarian steroids (White et al. 2011). Based on these studies, it can be speculated that other fluorinated chemicals affecting 17β-estradiol levels, including TM2 and other long-chained PFCAs investigated in this study, might lead to changes in mammary gland development *in vivo*.

5.1.3 Strategy to assess complex mixtures of unknown composition

In collaboration with others I developed a strategy to screen FCMs of paper and board for *in vitro* effects and to identify potentially problematic compounds used in such materials from a toxicological perspective. I hypothesized that using biodirected analysis in combination with analytical chemical tools would be a useful strategy for these purposes. Results are described in Manuscript 4. Overall, the hypothesis was supported, as all the *in vitro* assays applied were responsive to some or all of the extracts. Furthermore, it was also possible to identify five agents, DHAA, AA, BPA, BBP, and DBP, which had the ability individually to either affect AR activity or ER activity. This is to our knowledge the first report of the antiandrogenic effect of DHAA. Based on parameters obtained from testing these individually and quantification of these in the extract we calculated EQs, which were greater than that observed for the extract, suggesting that the main causative agents have been identified. As BPA was more potent than

both DBP and BBP in the ER reporter gene assay, this compound mainly contributed to the effects observed in the extract, despite the lower concentration of BPA compared to BBP and DBP. AA mainly contributed to the effect in the AR active extract, as this compound was present at high concentrations.

Our data show that FCMs of paper and board contain compounds that have the potential to cause effects *in vitro*. Of the 20 extracts we produced, it remains unknown what the causative agents are in 18 of them, as we did not investigate these further. Nevertheless, it is of concern that so many FCMs led to effects at all and the worry is that these 20 samples represent only a subset of the FCMs on the market, which can exhibit such activities. This notion is supported by several other studies, which have shown that FCMs of paper and board can exhibit both estrogenic, genotoxic, cytotoxic potential as well as lead to increased AhR activities (Binderup et al. 2002; Lopez-Espinosa et al. 2007; Ozaki et al. 2004; Ozaki et al. 2005; Weber et al. 2006). Though much data on other parameters related to human exposure and relevance of the above activities in humans are still needed, these findings points towards FCMs of paper and board as a source of exposure with many potentially problematic compounds.

Based on our findings as well as previous studies, I recommend testing a much broader spectrum of the FCMs of paper and board presently on the market. This could likely give information on whether the samples included in this study are representative of the market or whether they by chance represent a subset of samples that exhibit a high degree of activity. Furthermore, it is recommended that actions should be taken to identify causative agents in more FCMs, to determine if these are common components in such materials.

5.2 Interpretation of *in vitro* results

Both the top-down and bottom-up approaches are based on a battery of *in vitro* assays that all consider biochemical effects within isolated systems. Extrapolation from these multifaceted systems is not straight forward, and in many cases impossible. In an *in vivo* environment, absorption, distribution, metabolism and elimination may greatly affect the fate of the compound within the organism and thus the potential effect, as the compounds might not enter the body, might be metabolised to active/inactive compounds, might not be distributed to the relevant tissues, and/or might be quickly eliminated from the body. Furthermore, *in vivo* experiments take into account the complex interactions of not only multicellular organs and tissues, but also cross-talk between organs, which are of particular importance in endocrine regulation.

Besides these shortcomings of *in vitro* methods, there is however studies in which known *in vitro* endocrine disruptive compounds have led to effects *in vivo*. For example, *in vivo* exposure to ethinyl estradiol, a known ER agonist *in vitro* (Mattison et al. 2014), led to changes in mammary gland development (Mandrup et al. 2012) and in utero exposure to flutamide, a

known AR antagonist *in vitro* (Vinggaard et al. 1999), caused decreased anogenital distance, increased nipple retention, cryptorchidism and hypospadias in male offspring (McIntyre et al. 2001). Furthermore, studies comparing *in vitro* predictivity of *in vivo* effects, suggests that *in vitro* results can be used as predictive. As an example of this, Maglich et al. (2014) tested compounds with known reproductive toxicity as well as compounds not causing reproductive toxicity in the H295R steroidogenesis assay, and found results from this assay as good predictors of reproductive toxicity (Maglich et al. 2014). Furthermore, compiling information from chemicals tested in 13 *in vitro* assays related to ER signalling including ER agonism, antagonism, binding, and cell growth responses, showed that these collectively could predict *in vivo* effects in the uterotrophic assay (Rotroff et al. 2014); an assay sensitive to estrogenic compounds (Stroheker et al. 2003; Yamasaki et al. 2004).

Collectively, these data illustrate that though many *in vivo* parameters cannot be accounted for *in vitro*, *in vitro* assays can be predictive of at least some *in vivo* effects. As many of the investigated compounds led to effects in both the ER and AR reporter gene assay as well as the H295R steroidogenesis assay, some of which are suggested from the above to be predictive endpoints, these mechanisms could potentially contribute to effects *in vivo*.

5.3 Sources and exposure to compounds in food contact materials

The top-down approach provides information on both the use and effect of compounds in FCMs. However, the identification of a problematic compound in one FCM does not necessarily equate to it being used generally in such materials and, more importantly, does not necessarily mean that humans are exposed. The following chapter will discuss information dealing with human exposure to identified compounds, as this can significantly affect the proposed actions based on the results obtained from the test strategy.

5.3.1 Human biomonitoring and sources

An approach to assess the likely exposure to a compound is to consult previous measurements from human cohorts. However, caution should be made regarding ‘non-detected’ compounds as it may reflect that they have not been examined, they are quickly eliminated, or are measured in the wrong body compartment. On the other hand, if the compound is measurable in humans, it suggests that humans are exposed. For example, BPA, identified as the driver behind the ER activity of the extract in the FCM strategy (Manuscript 4), has been detected in the general population in a large human cohort (Calafat et al. 2008), suggesting human exposure is widespread. For BPA, diet is estimated as the main contributor to human exposure (EFSA 2013). However, biomonitoring data as the above does not in itself reveal the source of exposure, except in cases where only very specific applications exists for the compound of

interest. Thus, obtaining information on the sources in general is important before inferring FCMs as the origin, since other sources can add to the overall human exposure load.

Alternatively, information about origin of compounds in FCM of paper and board can be used to understand if these are ubiquitously present in these materials, and thus whether it can be expected that FCMs in general contribute to human exposure. For instance, are they added intentionally, are they non-intentionally added residuals, are they carry-overs from recycled materials, or are they natural constituents of paper and board. In the case of AA, identified as the driver of AR activity in the extract (Manuscript 4), the compound is a natural constituent of resin acids in wood (Roberts 1996; Sjöström and Alen 1998). Thus, if wood rich in resins is utilized in the paper, it can be expected that levels of AA will be present in the final paper product. AA have been found in extracts from FCMs of paper and board on several accounts (Ozaki et al. 2006; Weber et al. 2006) and in foods (Mitani et al. 2007), supporting this notion, though the compound is also used during the actual paper making process (Leach and Pierce 1993; Roberts 1996). Overall, in cases where little data on human levels exist, information as the above can be useful, as it illustrates that the compound, in this case AA, can be expected to be present in many FCMs of paper and board dependent on the starting material and the production process, that it is actually measured in these materials, and that it is present in food packed in paper and board. All these findings suggest that human exposure through food consumption may occur.

5.3.2 Migration of compounds from food contact materials

Another approach to delineate the potential exposure to humans from FCMs of paper and board is to obtain information on migration of compounds of interest into food. Migration studies are often conducted in food simulants, which is intended to represent different types of foods (Arvanitoyannis and Kotsanopoulos 2014). In the top-down approach, the applied extraction method is rather crude, involving double-sided ethanol extraction at high temperatures for two hours, and thus no consideration to the intended use of the FCMs was taken. The amounts extracted will thus most likely overestimate the levels in foods under normal storage conditions and in this sense represent a “worst-case scenario”. However, DHAA and AA have shown the ability to migrate under milder conditions, including water and 20% ethanol for shorter periods and lower temperatures (Ozaki et al. 2006), suggesting that although the extraction method was crude, at least some of the compounds present in the extracts would also be present under milder conditions, which may be more relevant with respect to human exposure.

Applying classical food simulants to estimate chemical migration can in some cases underestimate the transfer depending on the intended food type for which the FCM is to be used. This is the case with some groups of fluorinated chemicals, which will migrate to a higher

degree when an emulsifier is present, such as in butter, than they will migrate into classical simulants such as ethanol, water, and oil (Begley et al. 2008). However, in the BIOSAFEPAPER project in which softer extraction method were applied followed by a filtration step (Weber et al. 2006), they did tentatively identify several fluorinated chemicals in one extract (Bradley et al. 2008). Therefore, it is plausible that the compounds would be extracted by our methods if present in the FCMs. Nevertheless, these limitations in extraction of fluorinated compounds, lend support to them being tested individually, which was done in the bottom-up approach.

As the applied extraction method used here are crude, in my opinion conducting migration tests would be a natural continuum when a causative agent has been identified in FCMs by use of our strategy, as it would give insight into the transfer into foods, and thus indirectly human exposure.

6. Conclusions

In this project the overall aim was to obtain more knowledge about the toxicology of compounds present or suspected of being present in FCMs.

We investigated two groups of compounds, namely BPA analogues and fluorinated chemicals for potential to exhibit endocrine activity *in vitro*. Overall the tested BPA analogues exhibited similar estrogenic and antiandrogenic activities, but caused qualitative different effects on corticoids. BPS showed a markedly different profile on the above endpoint, exhibiting less potent and efficacious effects or no effect at all; however BPS showed marked effects on progestagens compared to BPA. Overall, these finding indicate that structurally similar compounds in most cases cause similar effects, at least *in vitro*. Based on the presented data, it is not recommended to substitute BPA with these analogues, before more information is available.

The fluorochemicals exhibited different mechanisms-of-action on the endocrine system, but subgroups of fluorinated chemicals exhibit similar effects. Overall, PAPs decreased androgen and progestagen levels, FTOHs activated the ER, and long-chained PFCAs increased 17β-estradiol levels. Furthermore two TMs were estrogenic and none of the short-chained PFCAs caused effects in the applied assays. These data emphasise the need to consider effects posed by polyfluorinated substances and not just those by the PFCAs, as the PAPs and FTOHs could cause similar or different effects than long-chained PFCAs. Furthermore, the data points towards examining more TMs available on the market, as two of three tested, were estrogenic and finally that short-chained PFCAs as are better alternatives than the long-chained PFCAs based on the parameters examined here.

All the tested FCMs led to effects in at least one of the applied *in vitro* assays, and further when applying the full strategy to two FCMs we successfully identified DHAA and AA as causing antiandrogenic effects on the AR activity, and BPA, DBP, and BBP as causing estrogenic effects on the ER activity. The FCM strategy using bioassay guided analysis in combination with analytical chemical tools thus proved a useful tool to screen for hazards of FCMs and to identify potentially problematic compounds in FCMs.

The aforementioned findings all point towards compounds in FCMs or compounds suspected of being used in FCMs as having the potential to exert endocrine activities. However, large data gaps related to *in vivo* and human relevance of these findings remains to be fully elucidated. Previous findings however support that *in vitro* data can be predictive of *in vivo* effects, and thus the data obtained from the present study can be used to prioritize future studies to be conducted. Furthermore, data gaps exists with respect to potential human exposure, which

could be obtained by understanding the sources of the compounds in FCMs, utilizing human biomonitoring data, or to obtain information on the potential of these compounds to migrate into foods.

The data from this PhD project shows that many of the investigated compounds and FCMs exhibited *in vitro* endocrine activities. This is of great concern and the data obtained should be used to prioritize further studies on the hazards posed by chemicals in these materials.

7. Future perspectives

Based on the work performed in this PhD, we have created a platform to characterize potential hazards from chemicals in FCMs, which can be used to prioritize further studies. Much work still remains with respect to expanding the knowledge on FCMs already investigated, but also with respect to understanding the relevance of the results from a human effect and exposure perspective.

In the top-down approach we investigated 20 FCM extracts of which only two were investigated in the full step-by-step strategy. Several other extracts led to effects in the AR, ER, and AhR reporter gene assays. In future we should examine these extracts by the full strategy to elucidate whether the same compounds cause the effect across all extracts or whether other compounds could explain the responses. Furthermore, additional FCMs should be tested by the strategy to understand if the selected samples are representative of the market.

In a strategy developed previously, the BIOSAFEPAPER project, 20 FCM extracts were tested and several were found cytotoxic, but only one extract genotoxic. The endpoints included in the strategy presented here, AR, ER, and AhR activity, were not covered by the BIOSAFEPAPER strategy. As we observe effects of many of the extracts, it would be interesting to test the same FCMs in both strategies to shed light on whether the differences in findings are caused by the effect range covered by the two strategies, the FCMs tested, or other differences such as the extraction methods used.

With respect to the compound classes tested in the bottom-up approach, first and foremost we need to characterize the TMs tested in this project to obtain knowledge on the specific chemical structures of the fluorinated constituents in the mixture. Further, only three TMs were tested of which two showed estrogenic potential, and thus in future studies more TMs should be tested in supplement with identification work.

The 8:2 PAPs tested in this study overall led to antiandrogenic effects. Recently short-chained PAPs, which could potentially break down to short-chained PFCAs, have become available on the market (Chiron, Norway). Neither of the short-chained PFCAs tested here caused effects in the assay applied. Thus, it would be interesting to test the short-chained PAPs, to understand if these will lead to similar effects as the short-chained PFCAs or whether they will exhibit similar antiandrogenic effects as the long-chained PAPs.

Finally, five BPA analogues were tested *in vitro* and the compounds generally led to similar qualitative effects as BPA. As a proof of concept, it would be interesting to investigate these five analogues *in vivo* to see if the same is evident in a full organism. If similar toxicological profiles

are observed *in vivo*, such data would illustrate that *in vitro* data can in some cases be used as predictive of *in vivo* effects.

8. References

Apelberg BJ, Goldman LR, Calafat AM, Herbstman JB, Kuklenyik Z, Heidler J et al. 2007. Determinants of fetal exposure to polyfluoroalkyl compounds in Baltimore, Maryland. Environ Sci Technol 41(11):3891-3897; doi: 10.1021/es0700911.

Arvanitoyannis IS, Kotsanopoulos KV. 2014. Migration phenomenon in food packaging. Food-package interactions, mechanisms, types of migrants, testing and relative legislation - A review. Food and Bioprocess Technology 7(1):21-36; doi: 10.1007/s11947-013-1106-8.

Becerra V, Odermatt J. 2012. Detection and quantification of traces of bisphenol A and bisphenol S in paper samples using analytical pyrolysis-GC/MS. Analyst 137(9):2250-2259; doi: 10.1039/c2an15961a.

Begley TH, Hsu W, Noonan G, Diachenko G. 2008. Migration of fluorochemical paper additives from food-contact paper into foods and food simulants. Food Addit Contam 25(3):384-390; doi: 10.1080/02652030701513784 ER.

Begley TH, White K, Honigfort P, Twaroski ML, Neches R, Walker RA. 2005. Perfluorochemicals: Potential sources of and migration from food packaging. Food Addit Contam 22(10):1023-1031; doi: 10.1080/02652030500183474.

Biegel LB, Liu RCM, Hurt ME, Cook JC. 1995. Effects of ammonium perfluorooctanoate on leydig-cell function - in-vitro, in-vivo, and ex-vivo studies. Toxicol Appl Pharmacol 134(1):18-25; doi: 10.1006/taap.1995.1164.

Biegel L, Hurtt M, Frame S, O'Connor J, Cook J. 2001. Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicological Sciences 60(1):44-55; doi: 10.1093/toxsci/60.1.44.

Binderup M, Pedersen G, Vinggaard A, Rasmussen E, Rosenquist H, Cederberg T. 2002. Toxicity testing and chemical analyses of recycled fibre-based paper for food contact. Food Addit Contam 19:13-28; doi: 10.1080/02652030110089878.

Bookstaff RC, Moore RW, Ingall GB, Peterson RE. 1990. Androgenic deficiency in male-rats treated with perfluorodecanoic acid. Toxicol Appl Pharmacol 104(2):322-333.

Borchers A, Teuber SS, Keen CL, Gershwin ME. 2010. Food safety. Clin Rev Allergy Immunol 39(2):95-141; doi: 10.1007/s12016-009-8176-4.

Boujrad N, Vidic B, Gazouli M, Culty M, Papadopoulos V. 2000. The peroxisome proliferator perfluorodecanoic acid inhibits the peripheral-type benzodiazepine receptor (PBR) expression and hormone-stimulated mitochondrial cholesterol transport and steroid formation in leydig cells. Endocrinology 141(9):3137-3148.

Bradley EL, Honkalampi-Hamalainen U, Weber A, Andersson MA, Bertaud F, Castle L et al. 2008. The BIOSAFEPAPER project for in vitro toxicity assessments: Preparation, detailed chemical characterisation and testing of extracts from paper and board samples. Food and Chemical Toxicology 46(7):2498-2509; doi: 10.1016/j.fct.2008.04.017.

Buck RC, Franklin J, Berger U, Conder JM, Cousin IT, de Voogt P et al. 2011. Perfluroalkyl and polyfluoroalkyl substances in the environment: Terminology, classification, and origins. Integrated Environment Assessment and Management 7(4):513-541.

Calafat AM, Wong L, Kuklenyik Z, Reidy JA, Needham LL. 2007. Polyfluoroalkyl chemicals in the US population: Data from the national health and nutrition examination survey (NHANES) 2003-2004 and comparisons with NHANES 1999-2000. Environ Health Perspect 115(11):1596-1602; doi: 10.1289/ehp.10598.

Calafat AM, Ye X, Wong L, Reidy JA, Needham LL. 2008. Exposure of the US population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect 116(1):39-44; doi: 10.1289/ehp.10753.

Cao X, Zhang J, Goodyer CG, Hayward S, Cooke GM, Curran IHA. 2012. Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998-2008. Chemosphere 89(5):505-511; doi: 10.1016/j.chemosphere.2012.05.003.

Chou W, Chen J, Lin C, Chen Y, Shih F, Chuang C. 2011. Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: A birth cohort study in Taiwan. Environ Health 10:94; doi: 10.1186/1476-069X-10-94.

Christiansen S, Axelstad M, Boberg J, Vinggaard AM, Pedersen GA, Hass U. 2014. Low-dose effects of bisphenol A on early sexual development in male and female rats. Reproduction 147(4):477-487; doi: 10.1530/REP-13-0377.

Cobellis L, Colacurci N, Trabucco E, Carpentiero C, Grumetto L. 2009. Measurement of bisphenol A and bisphenol B levels in human blood sera from endometriotic women. Biomed Chromatogr 23(11):1186-1190; doi: 10.1002/bmc.1241.

Cook JC, Murray SM, Frame SR, Hurtt ME. 1992. Induction of leydig-cell adenomas by ammonium perfluorooctanoate - a possible endocrine-related mechanism. Toxicol Appl Pharmacol 113(2):209-217; doi: 10.1016/0041-008X(92)90116-A.

Cunha SC, Almeida C, Mendes E, Fernandes JO. 2011. Simultaneous determination of bisphenol A and bisphenol B in beverages and powdered infant formula by dispersive liquid-liquid micro-extraction and heart-cutting multidimensional gas chromatography-mass spectrometry. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28(4):513-526; doi: 10.1080/19440049.2010.542551.

Danzl E, Sei K, Soda S, Ike M, Fujita M. 2009. Biodegradation of bisphenol A, bisphenol F and bisphenol S in seawater. Int J Environ Res Public Health 6(4):1472-1484; doi: 10.3390/ijerph6041472.

D'eon JC, Crozier PW, Furdui VI, Reiner EJ, Libelo EL, Mabury SA. 2009. Observation of a commercial fluorinated material, the polyfluoroalkyl phosphoric acid diesters, in human sera, wastewater treatment plant sludge, and paper fibers. Environ Sci Technol 43(12):4589-4594; doi: 10.1021/es900100d.

D'eon JC, Mabury SA. 2007. Production of perfluorinated carboxylic acids (PFCAs) from the biotransformation of polyfluoroalkyl phosphate surfactants (PAPS): Exploring routes of human contamination. Environ Sci Technol 41(13):4799-4805; doi: 10.1021/es070126x.

D'eon JC, Mabury SA. 2011a. Exploring indirect sources of human exposure to perfluoroalkyl carboxylates (PFCAs): Evaluating uptake, elimination, and biotransformation of polyfluoroalkyl phosphate esters (PAPs) in the rat. Environ Health Perspect 119(3):344-350; doi: 10.1289/ehp.1002409.

D'eon JC, Mabury SA. 2011b. Is indirect exposure a significant contributor to the burden of perfluorinated acids observed in humans? Environ Sci Technol 45(19):7974-7984; doi: 10.1021/es200171y.

D'Hollander W, de Voogt P, De Coen W, Bervoets L. 2010. Perfluorinated substances in human food and other sources of human exposure. Reviews of Environmental Contamination and Toxicology, Vol 208: Perfluorinated Alkylated Substances 208:179-215; doi: 10.1007/978-1-4419-6880-7_4.

Diamanti-Kandarakis E, Bourguignon J, Giudice LC, Hauser R, Prins GS, Soto AM et al. 2009. Endocrine-disrupting chemicals: An endocrine society scientific statement. Endocr Rev 30(4):293-342; doi: 10.1210/er.2009-0002.

Edlow AG, Chen M, Smith NA, Lu C, McElrath TF. 2012. Fetal bisphenol A exposure: Concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters. Reprod Toxicol 34(1):1-7; doi: 10.1016/j.reprotox.2012.03.009.

EFSA. 2006. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission related to 2,2-BIS(4-HYDROXYPHENYL)PROPANE (bisphenol A). 428:The EFSA Journal.

EFSA. 2008. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts - scientific opinion of the panel on contaminants in the food chain. 653.

EFSA. 2013. Draft scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs - part: Exposure assessment. EFSA-Q-2012-00423:EFSA Journal 2013.

EFSA. 2014. Draft Scientific Opinion on the Risks to Public Health Related to the Presence of Bisphenol A (BPA) in Foodstuffs. http://www.efsa.europa.eu/en/consultations/call/140117.pdf ed. .

Fasano WJ, Carpenter SC, Gannon SA, Snow TA, Stadler JC, Kennedy GL et al. 2006. Absorption, distribution, metabolism, and elimination of 8-2 fluorotelomer alcohol in the rat. Toxicological Sciences 91(2):341-355; doi: 10.1093/toxsci/kfj160.

Feng Y, Shi Z, Fang X, Xu M, Dai J. 2009. Perfluorononanoic acid induces apoptosis involving the fas death receptor signaling pathway in rat testis. Toxicol Lett 190(2):224-230; doi: 10.1016/j.toxlet.2009.07.020.

Frederiksen H, Aksglaede L, Sorensen K, Nielsen O, Main KM, Skakkebaek NE et al. 2013. Bisphenol A and other phenols in urine from Danish children and adolescents analyzed by isotope diluted TurboFlow-LC-MS/MS. Int J Hyg Environ Health 216(6):710; doi: 10.1016/j.ijheh.2013.01.007.

Gallart-Ayala H, Moyano E, Galceran MT. 2011. Analysis of bisphenols in soft drinks by on-line solid phase extraction fast liquid chromatography tandem mass spectrometry. Anal Chim Acta 683(2):227-233; doi: 10.1016/j.aca.2010.10.034.

Geens T, Goeyens L, Covaci A. 2011. Are potential sources for human exposure to bisphenol-A overlooked? Int J Hyg Environ Health 214(5):339-347; doi: 10.1016/j.ijheh.2011.04.005.

Geueke B, Wagner CC, Muncke J. 2014. Food contact substances and chemicals of concern: A comparison of inventories. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment 31(8):1438-1450; doi: 10.1080/19440049.2014.931600.

Gould J, Leonard L, Maness S, Wagner B, Conner K, Zacharewski T et al. 1998. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142(1-2):203-214; doi: 10.1016/S0303-7207(98)00084-7.

Government of Canada. 2010. Order Amending Schedule I to the Hazardous Products Act (Bisphenol A), Part II, Vol. 144, no. 7. http://www.chemicalsubstanceschimiques.gc.ca/challenge-defi/batch-lot-2/bisphenol-a/bpa-risk_hazard-eng.php ed. :Canada Gazette.

Grignard E, Lapenna S, Bremer S. 2012. Weak estrogenic transcriptional activities of bisphenol A and bisphenol S. Toxicol In Vitro 26(5):727-731; doi: 10.1016/j.tiv.2012.03.013.

Grob K, Biedermann M, Scherbaum E, Roth M, Rieger K. 2006. Food contamination with organic materials in perspective: Packaging materials as the largest and least controlled source? A view focusing on the european situation. Crit Rev Food Sci Nutr 46(7):529-535; doi: 10.1080/10408390500295490.

Grumetto L, Montesano D, Seccia S, Albrizio S, Barbato F. 2008. Determination of bisphenol A and bisphenol B residues in canned peeled tomatoes by reversed-phase liquid chromatography. J Agric Food Chem 56(22):10633-10637; doi: 10.1021/jf802297z.

Houde M, Martin JW, Letcher RJ, Solomon KR, Muir DCG. 2006. Biological monitoring of polyfluoroalkyl substances: A review. Environ Sci Technol 40(11):3463-3473; doi: 10.1021/es052580b ER.

Ike M, Chen M, Danzl E, Sei K, Fujita M. 2006. Biodegradation of a variety of bisphenols under aerobic and anaerobic conditions. Water Sci Technol 53(6):153-159; doi: 10.2166/wst.2006.189.

IPCS. 2002. Global assessment of the state-of-the-science of endocrine disruptors. Geneva, Switzerland:International Programme on Chemical Safety, World Health Organization.

Jimenez-Diaz I, Zafra-Gomez A, Ballesteros O, Navea N, Navalon A, Fernandez MF et al. 2010. Determination of bisphenol A and its chlorinated derivatives in placental tissue samples by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 878(32):3363-3369; doi: 10.1016/j.jchromb.2010.10.021.

Kennedy GL, Butenhoff JL, Olsen GW, O'Connor JC, Seacat AM, Perkins RG et al. 2004. The toxicology of perfluorooctanoate. Crit Rev Toxicol 34(4):351-384; doi: 10.1080/10408440490464705.

Kim S, Lee KT, Kang CS, Tao L, Kannan K, Kim K et al. 2011. Distribution of perfluorochemicals between sera and milk from the same mothers and implications for prenatal and postnatal exposures. Environmental Pollution 159(1):169-174; doi: 10.1016/j.envpol.2010.09.008.

Kim TS, Jung KK, Kim SS, Kang IH, Baek JH, Nam H et al. 2010. Effects of in utero exposure to di(n-butyl) phthalate on development of male reproductive tracts in sprague-dawley rats. Journal of Toxicology and Environmental Health-Part A-Current Issues 73(21-22):1544-1559; doi: 10.1080/15287394.2010.511579.

Kissa E. 2001. Fluorinated Surfactants and Repellents. 2nd ed. New York:Marcel Dekker.

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K et al. 2005. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84(2):249-259; doi: 10.1093/toxsci/kfi074.

Kjeldsen LS, Bonefeld-Jorgensen EC. 2013. Perfluorinated compounds affect the function of sex hormone receptors. Environmental Science and Pollution Research 20(11):8031-8044; doi: 10.1007/s11356-013-1753-3.

Kojima H, Katsura E, Takeuchi S, Niiyama K, Kobayashi K. 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using chinese hamster ovary cells. Environ Health Perspect 112(5):524-531.

Kraugerud M, Zimmer KE, Ropstad E, Verhaegen S. 2011. Perfluorinated compounds differentially affect steroidogenesis and viability in the human adrenocortical carcinoma (H295R) in vitro cell assay. Toxicol Lett 205(1):62-68; doi: 10.1016/j.toxlet.2011.05.230.

Krishnan A, Stathis P, Permuth S, Tokes L, Feldman D. 1993. Bisphenol-a - an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132(6):2279-2286; doi: 10.1210/en.132.6.2279.

Kubwabo C, Kosarac I, Lalonde K. 2013. Determination of selected perfluorinated compounds and polyfluoroalkyl phosphate surfactants in human milk. Chemosphere 91(6):771-777; doi: 10.1016/j.chemosphere.2013.02.011.

Kudo N, Iwase Y, Okayachi H, Yamakawa Y, Kawashima Y. 2005. Induction of hepatic peroxisome proliferation by 8-2 telomer alcohol feeding in mice: Formation of perfluorooctanoic acid in the liver. Toxicological Sciences 86(2):231-238; doi: 10.1093/toxsci/kfi191.

Kuruto-Niwa R, Nozawa R, Miyakoshi T, Shiozawa T, Terao Y. 2005. Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. Environ Toxicol Pharmacol 19(1):121-130; doi: 10.1016/j.etap.2004.05.009.

Lau C. 2012. Perfluorinated compounds. In: Molecular, Clinical and Environmental Toxicology, Volume 3: Environmental Toxicology (Luch A, ed), 47.

Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J. 2007. Perfluoroalkyl acids: A review of monitoring and toxicological findings. Toxicol Sci 99(2):366-394; doi: 10.1093/toxsci/kfm128.

Leach RH, Pierce RJ. 1993. The Printing Ink Manual. 5th ed. Dordrecht, The Netherlands:Kluwer Academic Publishers.

Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, Lee K. 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol Sci 75(1):40-46; doi: 10.1093/toxsci/kfg150.

Lee YJ, Ryu H, Kim H, Min CS, Lee JH, Kim E et al. 2008. Maternal and fetal exposure to bisphenol A in Korea. Reprod Toxicol 25(4):413-419; doi: 10.1016/j.reprotox.2008.05.058.

Liao C, Kannan K. 2014. A survey of bisphenol A and other bisphenol analogues in foodstuffs from nine cities in China. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment 31(2):319-329; doi: 10.1080/19440049.2013.868611.

Liao C, Liu F, Alomirah H, Vu Duc Loi, Mohd MA, Moon H et al. 2012a. Bisphenol S in urine from the United States and seven Asian countries: Occurrence and human exposures. Environ Sci Technol 46(12):6860-6866; doi: 10.1021/es301334j.

Liao C, Liu F, Guo Y, Moon H, Nakata H, Wu Q et al. 2012b. Occurrence of eight bisphenol analogues in indoor dust from the United States and several Asian countries: Implications for human exposure. Environ Sci Technol 46(16):9138-9145; doi: 10.1021/es302004w.

Liao C, Liu F, Kannan K. 2012c. Bisphenol S, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol A residues. Environ Sci Technol 46(12):6515-6522; doi: 10.1021/es300876n.

Liu C, Deng J, Yu L, Ramesh M, Zhou B. 2010a. Endocrine disruption and reproductive impairment in zebrafish by exposure to 8:2 fluorotelomer alcohol. Aquatic Toxicology 96(1):70-76; doi: 10.1016/j.aquatox.2009.09.012.

Liu C, Yu L, Deng J, Lam PKS, Wu RSS, Zhou B. 2009. Waterborne exposure to fluorotelomer alcohol 6:2 FTOH alters plasma sex hormone and gene transcription in the hypothalamic-

pituitary-gonadal (HPG) axis of zebrafish. Aquatic Toxicology 93(2-3):131-137; doi: 10.1016/j.aquatox.2009.04.005.

Liu C, Zhang X, Chang H, Jones P, Wiseman S, Naile J et al. 2010b. Effects of fluorotelomer alcohol 8/2 FTOH on steroidogenesis in H295R cells: Targeting the cAMP signalling cascade. Toxicol Appl Pharmacol 247(3):222-228; doi: 10.1016/j.taap.2010.06.016.

Lopez-Espinosa M-, Granada A, Araque P, Molina-Molina J-, Puertollano M-, Rivas A et al. 2007. Oestrogenicity of paper and cardboard extracts used as food containers. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment 24(1):95-102; doi: 10.1080/02652030600936375.

Maglich JM, Kuhn M, Chapin RE, Pletcher MT. 2014. More than just hormones: H295R cells as predictors of reproductive toxicity. Reproductive Toxicology 45:77-86; doi: 10.1016/j.reprotox.2013.12.009.

Mandrup KR, Hass U, Christiansen S, Boberg J. 2012. Perinatal ethinyl oestradiol alters mammary gland development in male and female wistar rats. Int J Androl 35(3):385-396; doi: 10.1111/j.1365-2605.2012.01258.x.

Maras M, Vanparys C, Muylle F, Robbens J, Berger U, Barber J et al. 2006. Estrogen-like properties of fluorotelomer alcohols as revealed by MCF-7 breast cancer cell proliferation. Environ Health Perspect 114(1):100-105; doi: 10.1289/ehp.8149.

Martin JW, Mabury SA, O'Brien PJ. 2005. Metabolic products and pathways of fluorotelomer alcohols in isolated rat hepatocytes. Chem Biol Interact 155(3):165-180; doi: 10.1016/j.cbi.2005.06.007.

Mattison DR, Karyakina N, Goodman M, LaKind JS. 2014. Pharmaco- and toxicokinetics of selected exogenous and endogenous estrogens: A review of the data and identification of knowledge gaps. Crit Rev Toxicol 44(8):696-724; doi: 10.3109/10408444.2014.930813.

McIntyre B, Barlow N, Foster P. 2001. Androgen-mediated development in male rat offspring exposed to flutamide in utero: Permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicological Sciences 62(2):236-249; doi: 10.1093/toxsci/62.2.236.

Mitani K, Fujioka M, Uchida A, Kataoka H. 2007. Analysis of abietic acid and dehydroabietic acid in food samples by in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. Journal of Chromatography a 1146(1):61-66; doi: 10.1016/j.chroma.2007.01.118.

Miyawaki J, Sakayama K, Kato H, Yamamoto H, Masuno H. 2007. Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice. J Atheroscler Thromb 14(5):245-252.

Monroy R, Morrison K, Teo K, Atkinson S, Kubwabo C, Stewart B et al. 2008. Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. Environ Res 108(1):56-62; doi: 10.1016/j.envres.2008.06.001.

Moral R, Wang R, Russo IH, Lamartiniere CA, Pereira J, Russo J. 2008. Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. J Endocrinol 196(1):101-112; doi: 10.1677/JOE-07-0056.

Neltner TG, Alger HM, Leonard JE, Maffini MV. 2013. Data gaps in toxicity testing of chemicals allowed in food in the United States. Reproductive Toxicology 42:85-94; doi: 10.1016/j.reprotox.2013.07.023.

OECD. 2011. OECD Guideline for the Testing of Chemicals, H295R Steroidogenesis Assay, Test no. 456. http://www.oecd-ilibrary.org/environment/test-no-456-h295r-steroidogenesis-assay_9789264122642-en ed. :OECD.

OECD. 2012. Test no. 457: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists. http://www.oecd-ilibrary.org/environment/test-no-457-bg1luc-estrogen-receptor-transactivation-test-method-for-identifying-estrogen-receptor-agonists-and-antagonists_9789264185395-en ed. :OECD.

OECD. 2013. Synthesis paper on per- and polyfluorinated chemicals:OECD/UNEP Global PFC Group.

Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL et al. 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and

perfluorooctanoate in retired fluorochemical production workers. Environ Health Perspect 115(9):1298-1305; doi: 10.1289/ehp.10009.

Olsen GW, Lange CC, Ellefson ME, Mair DC, Church TR, Goldberg CL et al. 2012. Temporal trends of perfluoroalkyl concentrations in american red cross adult blood donors, 2000-2010. Environ Sci Technol 46(11):6330-6338; doi: 10.1021/es300604p.

Ozaki A, Ooshima T, Mori Y. 2006. Migration of dehydroabietic and abietic acids from paper and paperboard food packaging into food-simulating solvents and tenax TA. Food Addit Contam 23(8):854-860; doi: 10.1080/02652030600743813.

Ozaki A, Yamaguchi Y, Fujita T, Kuroda K, Endo G. 2004. Chemical analysis and genotoxicological safety assessment of paper and paperboard used for food packaging. Food and Chemical Toxicology 42(8):1323-1337; doi: 10.1016/j.fct.2004.03.010.

Ozaki A, Yamaguchi Y, Fujita T, Kuroda K, Endo G. 2005. Safety assessment of paper and board food packaging: Chemical analysis and genotoxicity of possible contaminants in packaging. Food Addit Contam 22(10):1053-1060; doi: 10.1080/02652030500090885.

Paris F, Balaguer P, Terouanne B, Servant N, Lacoste C, Cravedi J et al. 2002. Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines. Mol Cell Endocrinol 193(1-2):43-49; doi: 10.1016/S0303-7207(02)00094-1.

Porcher JM, Devillers J, Marchand-Geneste N. 2009. Mechanisms of endocrine disruption - A tentative review. In: Endocrine Disruption Modeling (Devillers J, ed). Boca Raton, FL, USA:CRC Press, 11.

Prevedouros K, Cousins I, Buck R, Korzeniowski S. 2006. Sources, fate and transport of perfluorocarboxylates. Environ Sci Technol 40(1):32-44; doi: 10.1021/es0512475.

Rivas A, Lacroix M, Olea-Serrano F, Laios I, Leclercq G, Olea N. 2002. Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 82(1):45-53; doi: 10.1016/S0960-0760(02)00146-2.

Roberts JC. 1996. The Chemistry of Paper . 1st ed. Cambridge, England:Royal Society of Chemistry Paperbacks.

Rotroff DM, Martin MT, Dix DJ, Filer DL, Houck KA, Knudsen TB et al. 2014. Predictive endocrine testing in the 21st century using in vitro assays of estrogen receptor signaling responses. Environ Sci Technol 48(15):8706-8716; doi: 10.1021/es502676e.

Scheringer M, Trier X, Cousins IT, de Voogt P, Fletcher T, Wang Z et al. 2014. Helsingør statement on poly- and perfluorinated alkyl substances (PFASs). Chemosphere 114:337-339; doi: 10.1016/j.chemosphere.2014.05.044.

Scott HM, Mason JI, Sharpe RM. 2009. Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. Endocr Rev 30(7):883-925; doi: 10.1210/er.2009-0016.

Shi Z, Ding L, Zhang H, Feng Y, Xu M, Dai J. 2009a. Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. Toxicol Lett 188(3):192-200; doi: 10.1016/j.toxlet.2009.04.014.

Shi Z, Zhang H, Ding L, Feng Y, Xu M, Dai J. 2009b. The effect of perfluorododecanonic acid on endocrine status, sex hormones and expression of steroidogenic genes in pubertal female rats. Reproductive Toxicology 27(3-4):352-359; doi: 10.1016/j.reprotox.2009.02.008.

Sjöström E, Alen R. 1998. Analytical Methods in Wood Chemistry, Pulping, and Papermaking. Berlin, Heidelberg:Springer-Verlag.

So MK, Yamashita N, Taniyasu S, Jiang QT, Giesy JP, Chen K et al. 2006. Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. Environ Sci Technol 40(9):2924-2929; doi: 10.1021/es060031f.

Stockholm Convention. 2009. Listing of POPs in the Stockholm Convention, Annex B. http://chm.pops.int/TheConvention/ThePOPs/ListingofPOPs/tabid/2509/Default.aspx ed. .

Stroheker T, Chagnon MC, Pinnert MF, Berges R, Canivenc-Lavier MC. 2003. Estrogenic effects of food wrap packaging xenoestrogens and flavonoids in female wistar rats: A comparative study. Reproductive Toxicology 17(4):421-432; doi: 10.1016/S0890-6238(03)00044-3.

The European Commission. 2004. REGULATION (EC) no 1935/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 October 2004 on Materials and Articles

Intended to Come into Contact with Food and Repealing Directives 80/590/EEC and 89/109/EEC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:338:0004:0017:en:PDF ed. .

The European Commission. 2011. Commission Directive 2011/8/EU of 28 January 2011 Amending Directive 2002/72/EC as Regards the Restriction of use of Bisphenol A in Plastic Infant Feeding Bottles. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:026:0011:0014:EN:PDF.

Trier X, Granby K, Christensen JH. 2011. Polyfluorinated surfactants (PFS) in paper and board coatings for food packaging. Environmental Science and Pollution Research; doi: 10.1007/s11356-010-0439-3.

Trudel D, Horowitz L, Wormuth M, Scheringer M, Cousins IT, Hungerbuhler K. 2008. Estimating consumer exposure to PFOS and PFOA. Risk Analysis 28(2):251-269; doi: 10.1111/j.1539-6924.2008.01017.x.

UNEP/WHO. 2013. State of the science of endocrine disruptive chemicals - 2012. Geneva, Switzerland:WHO Press.

US EPA. 2000. EPA and 3M Announce Phase Out of PFOS. http://yosemite.epa.gov/opa/admpress.nsf/d0cf6618525a9efb85257359003fb69d/33aa946e6cb11f35852568e1005246b4!OpenDocument&Highlight=2,risk ed. .

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol A (BPA). Reproductive Toxicology 24(2):139-177; doi: 10.1016/j.reprotox.2007.07.010.

Vestergren R, Cousins IT, Trudel D, Wormuth M, Scheringer M. 2008. Estimating the contribution of precursor compounds in consumer exposure to PFOS and PFOA. Chemosphere 73(10):1617-1624; doi: 10.1016/j.chemosphere.2008.08.011.

Vinas P, Campillo N, Martinez-Castillo N, Hernandez-Cordoba M. 2010. Comparison of two derivatization-based methods for solid-phase microextraction-gas chromatography-mass spectrometric determination of bisphenol A, bisphenol S and biphenol migrated from food cans. Anal Bioanal Chem 397(1):115-125; doi: 10.1007/s00216-010-3464-7.

Vinggaard A, Joergensen E, Larsen J. 1999. Rapid and sensitive reporter gene assays for detection of antiandrogenic and estrogenic effects of environmental chemicals. Toxicol Appl Pharmacol 155(2):150-160; doi: 10.1006/taap.1998.8598.

Vinggaard A, Korner W, Lund K, Bolz U, Petersen J. 2000. Identification and quantification of estrogenic compounds in recycled and virgin paper for household use as determined by an in vitro yeast estrogen screen and chemical analysis. Chem Res Toxicol 13(12):1214-1222; doi: 10.1021/tx000146b.

Vinggaard AM, Niemela J, Wedebye EB, Jensen GE. 2008. Screening of 397 chemicals and development of a quantitative structure-activity relationship model for androgen receptor antagonism. Chem Res Toxicol 21(4):813-823; doi: 10.1021/tx7002382.

Völkel W, Bittner N, Dekant W. 2005. Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. Drug Metab Disposition 33(11):1748-1757; doi: 10.1124/dmd.105.005454.

Völkel W, Colnot T, Csanady G, Filser J, Dekant W. 2002. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem Res Toxicol 15(10):1281-1287; doi: 10.1021/tx025548t.

Völkel W, Genzel-Boroviczeny O, Demmelmair H, Gebauer C, Koletzko B, Twardella D et al. 2008. Perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA) in human breast milk: Results of a pilot study. Int J Hyg Environ Health 211(3-4):440-446; doi: 10.1016/j.ijheh.2007.07.024.

Wang Z, Cousins IT, Scheringer M, Hungerbuehler K. 2013. Fluorinated alternatives to long-chain perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs) and their potential precursors. Environ Int 60:242-248; doi: 10.1016/j.envint.2013.08.021.

Weber A, von Wright A, Honkalampi-Hämäläinen U, Järvinen M, Lhuguenet JC, Severin I et al. 2006. Biosafepaper - application of bioassays for safety assessment of paper and board for food contact. QLK1-CT-2001-00930.

White SS, Fenton SE, Hines EP. 2011. Endocrine disrupting properties of perfluorooctanoic acid. J Steroid Biochem Mol Biol 127(1-2):16-26; doi: 10.1016/j.jsbmb.2011.03.011.

Xu X, Zhang J, Wang Y, Ye Y, Luo Q. 2010. Perinatal exposure to bisphenol-A impairs learning-memory by concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice. Horm Behav 58(2):326-333; doi: 10.1016/j.yhbeh.2010.02.012.

Yamada H, Furuta I, Kato EH, Kataoka S, Usuki Y, Kobashi G et al. 2002. Maternal serum and amniotic fluid bisphenol A concentrations in the early second trimester. Reprod Toxicol 16(6):735-739; doi: 10.1016/S0890-6238(02)00051-5.

Yamasaki K, Noda S, Imatanaka N, Yakabe Y. 2004. Comparative study of the uterotrophic potency of 14 chemicals in a uterotrophic assay and their receptor-binding affinity. Toxicol Lett 146(2):111-120; doi: 10.1016/j.toxlet.2003.07.003.

Yang Y, Guan J, Yin J, Shao B, Li H. 2014. Urinary levels of bisphenol analogues in residents living near a manufacturing plant in south China. Chemosphere 112:481-486; doi: 10.1016/j.chemosphere.2014.05.004.

Zhang X, Chang H, Wiseman S, He Y, Higley E, Jones P et al. 2011a. Bisphenol A disrupts steroidogenesis in human H295R cells. Toxicol Sci 121(2):320-327; doi: 10.1093/toxsci/kfr061.

Zhang Z, Alomirah H, Cho H, Li Y, Liao C, Tu Binh Minh et al. 2011b. Urinary bisphenol A concentrations and their implications for human exposure in several Asian countries. Environ Sci Technol 45(16):7044-7050; doi: 10.1021/es200976k.

Zhao B, Hu G, Chu Y, Jin X, Gong S, Akingbemi BT et al. 2010. Inhibition of human and rat 3 beta-hydroxysteroid dehydrogenase and 17 beta-hydroxysteroid dehydrogenase 3 activities by perfluoroalkylated substances. Chem Biol Interact 188(1):38-43; doi: 10.1016/j.cbi.2010.07.001.

9. Appendices

9.1 Manuscript 1

Rosenmai, A.K., Nielsen, F.K., Pedersen, M., Hadrup, N., Trier, X., Christensen, J.H., and Vinggaard, A.M., 2013. Fluorochemicals used in food packaging materials inhibit male sex hormone synthesis. Toxicol. Appl. Pharmacol. 266, 132-142.

9.2 Manuscript 2

Rosenmai, A.K., Dybdahl, M., Pedersen, M., van Vugt-Lussenburg, B.M.A., Wedebye, E.B., Taxvig, C., and Vinggaard, A.M., 2014. Are structural analogues to bisphenol A safe alternatives? Tox Sci. 139, 35-47.

9.3 Manuscript 3

Rosenmai, A.K., Trier, X., Taxvig, C., van Vugt-Lussenburg, B.M.A., and Vinggaard, A.M. (2014). Fluorinated compounds and technical mixtures for use in food contact materials have estrogenic activity in an *in vitro* screening. Manuscript in preparation.

9.4 Manuscript 4

Rosenmai, A.K., Bengtström, L., Taxvig, C., Trier, X., Petersen, J.H., Granby, K. and Vinggaard, A.M. (2014). A strategy to identify problematic chemicals in food contact materials of paper and board. Manuscript in preparation.

9.1 Manuscript 1

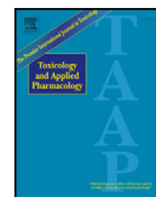
Rosenmai, A.K., Nielsen, F.K., Pedersen, M., Hadrup, N., Trier, X., Christensen, J.H., and Vinggaard, A.M., 2013. Fluorochemicals used in food packaging materials inhibit male sex hormone synthesis. Toxicol. Appl. Pharmacol. 266, 132-142.

Toxicology and Applied Pharmacology 266 (2013) 132–142

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap



Fluorochemicals used in food packaging inhibit male sex hormone synthesis

A.K. Rosenmai [a,*], F.K. Nielsen [b], M. Pedersen [c], N. Hadrup [a], X. Trier [c], J.H. Christensen [d], A.M. Vinggaard [a]

[a] Division of Toxicology & Risk Assessment, National Food Institute, Technical University of Denmark, DK-2860 Søborg, Denmark
[b] Section of Toxicology, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2100 Copenhagen, Denmark
[c] Division of Food Chemistry, National Food Institute, Technical University of Denmark, DK-2860 Søborg, Denmark
[d] Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg C., Denmark

ARTICLE INFO

Article history:
Received 23 July 2012
Revised 15 October 2012
Accepted 17 October 2012
Available online 7 November 2012

Keywords:
H295R steroidogenesis assay
Androgen receptor reporter gene assay
Polyfluorinated chemicals
Fluorotelomer alcohol
Perfluorooctanoic acid
Polyfluoroalkyl phosphate surfactants

ABSTRACT

Polyfluoroalkyl phosphate surfactants (PAPS) are widely used in food contact materials (FCMs) of paper and board and have recently been detected in 57% of investigated materials. Human exposure occurs as PAPS have been measured in blood; however knowledge is lacking on the toxicology of PAPS. The aim of this study was to elucidate the effects of six fluorochemicals on sex hormone synthesis and androgen receptor (AR) activation *in vitro*. Four PAPS and two metabolites, perfluorooctanoic acid (PFOA) and 8:2 fluorotelomer alcohol (8:2 FTOH) were tested.

Hormone profiles, including eight steroid hormones, generally showed that 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH led to decreases in androgens (testosterone, dehydroepiandrosterone, and androstenedione) in the H295R steroidogenesis assay. Decreases were observed for progesterone and 17-OH-progesterone as well. These observations indicated that a step prior to progestagen and androgen synthesis had been affected. Gene expression analysis of StAR, Bzrp, CYP11A, CYP17, CYP21 and CYP19 mRNA showed a decrease in Bzrp mRNA levels for 8:2 monoPAPS and 8:2 FTOH indicating interference with cholesterol transport to the inner mitochondria. Cortisol, estrone and 17β-estradiol levels were in several cases increased with exposure. In accordance with these data CYP19 gene expression increased with 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH exposures indicating that this is a contributing factor to the decreased androgen and the increased estrogen levels.

Overall, these results demonstrate that fluorochemicals present in food packaging materials and their metabolites can affect steroidogenesis through decreased Bzrp and increased CYP19 gene expression leading to lower androgen and higher estrogen levels.

© 2012 Elsevier Inc. All rights reserved.

Introduction

Fluorochemicals are a group of manmade compounds that possess the unique ability to repel water and oil simultaneously (Kissa, 2001). This makes them ideal for multiple purposes including in coatings for paper and board (Begley et al., 2008). In a recent study 61% of selected food contact materials (FCMs) made of paper and board were found to contain fluorochemicals and of these 57% contained polyfluoroalkyl phosphate surfactants (PAPS) (Trier, 2011) (Fig. 1). The technical mixtures of paper coatings contain dialkylated (diPAPS), monoalkylated (monoPAPS) and trialkylated (triPAPS) homologues (Trier et al., 2011). Among these diPAPS has been reported to migrate from FCMs into foods (Begley et al., 2008). Despite these findings there are no specific EU legislation for the use of PAPS in paper and board FCMs (Trier et al., 2011).

Reports of diPAPS in human sera collected in the United States suggested that not only are these chemicals used, but humans are exposed and can absorb these across the gastrointestinal tract. The reported total concentration of diPAPS homologues (4:2, 6:2, 8:2 and 10:2 diPAPS) was 4.5 μg/L (D'eon et al., 2009). Metabolism of PAPS can lead to the formation of highly persistent perfluorinated carboxylic acids (PFCAs) (Fig. 1), such as perfluorooctanoic acid (PFOA), which has a half-life in humans of 3.8 years (Olsen et al., 2007). In male rats PAPS metabolize into PFCAs possibly through the intermediate fluorotelomer alcohol (FTOH) (D'eon and Mabury, 2007, 2011). FTOH oxidize into PFCAs in isolated rat hepatocytes (Martin et al., 2005). Several body compartments have been found to contain PFCAs, including human blood/serum (Houde et al., 2006), umbilical cord blood (Kim et al., 2011; Monroy et al., 2008) and breast milk (So et al., 2006). PFOA has been measured at mean serum/plasma/whole blood concentrations ranging from 3 to 213 nM

* Corresponding author at: Division of Toxicology & Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, Building G, DK-2860 Søborg, Denmark.
E-mail address: akjro@food.dtu.dk (A.K. Rosenmai).

0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2012.10.022

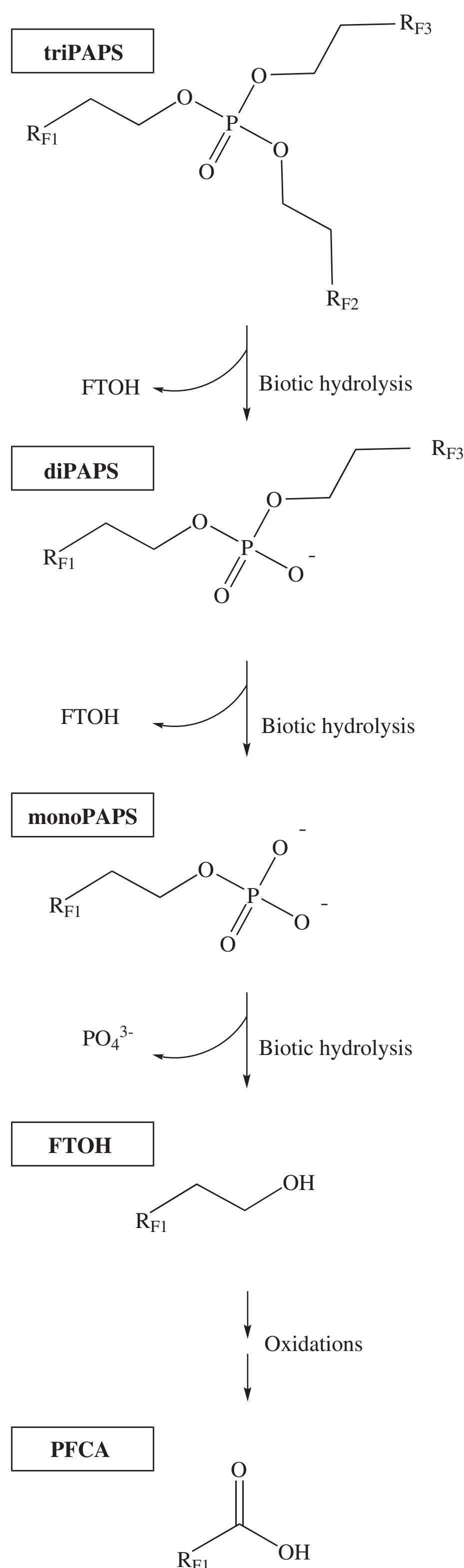


Fig. 1. Metabolic pathway of polyfluoroalkyl phosphate surfactants (PAPS) in rat, mice and isolated rat hepatocytes into fluorotelomer alcohol (FTOH) and perfluorinated carboxylic acids (PFCAs) (D'eon and Mabury, 2007, 2011; Fasano et al., 2006; Kudo et al., 2005; Martin et al., 2005). R_{F1}, R_{F2} and R_{F3} represent fully fluorinated carbon chains of varying lengths.

in the general population worldwide (EFSA, 2008). This indicates that not only are the general population exposed to PFCAs, but also that fetuses and infants may be exposed through placental passing and/or breast feeding. Based on the above studies it seems likely that PAPS also in humans is a precursor of PFCAs and as PAPS is present in human blood, it is relevant to establish if PAPS in itself have the potential to cause adverse effects in humans.

To our knowledge no data exists on the toxicology of PAPS, whereas PFOA and perfluorooctane sulfonic acid (PFOS) have been widely reviewed (Andersen et al., 2008; Kennedy et al., 2004; Lau et al., 2007; Steenland et al., 2010). Studies conducted in male rats have shown that exposure to PFCAs of varying chain lengths, which are all potentially final metabolites of PAPS, could decrease testosterone levels *in vivo* (Feng et al., 2009; Shi et al., 2007, 2009). Furthermore decreased gene expression of key enzymes and transporters involved in steroidogenesis was observed as a result of perfluorododecanoic acid (PFdoDA) exposure in male rats (Shi et al., 2007, 2009). For PFOA a study showed a decrease in testosterone levels in isolated rat Leydig cells with exposure (Zhao et al., 2010). Finally 8:2 FTOH, a metabolite of PAPS, also showed the capacity to decrease testosterone levels in the human adrenal cortico-carcinoma cell line (Liu et al., 2010). The above mentioned studies suggest that PFCAs and 8:2 FTOH have testosterone-inhibiting potential, and that the mechanism of action is interference with sex hormone synthesis. In accordance with these data an epidemiological study investigated associations between high PFOA and PFOS exposure, semen variables and hormone levels. A statistically significant association was found between high levels of PFOA and PFOS exposure and a decreased number of normal sperms as the only parameter (Joensen et al., 2009). As PAPS resemble PFOA and 8:2 FTOH by containing fluorinated chains and being surfactant molecules, it is therefore relevant to investigate if PAPS also have endocrine disrupting potential.

The aim of this study was to examine the potential of four PAPS (8:2 monoPAPS, 8:2 diPAPS, 10:2 diPAPS and 8:2 triPAPS) and metabolic products of these, PFOA and 8:2 FTOH, to interfere with steroidogenesis and androgen receptor activation using two *in vitro* assays, the H295R steroidogenesis assay and the androgen receptor (AR) reporter gene assay, respectively. The levels of eight steroid hormones and gene expression levels of six proteins involved in the steroidogenic pathway were end-points in the H295R steroidogenesis assay.

Materials and methods

Chemicals

Test chemicals 8:2 triPAPS (65.1%), 10:2 diPAPS (94.6%), 8:2 diPAPS (97.4%), and 8:2 monoPAPS (88.7%) were synthesized by Chiran (Trondheim, Norway). 8:2 FTOH (97%) was purchased from Fluorochem Ltd. (Derbyshire, UK) and PFOA (95%) was purchased from VWR (Herlev, Denmark). The notations 8:2 and 10:2 refer to the chain length of the fluorinated chains and the CH_2 group within the molecule e.g. the notation 10:2 diPAPS reflects that the two chains contain ten carbon atoms that are fully fluorinated and two carbon atoms to which hydrogen atoms are attached (Fig. 1). Stock solutions of 20 mM were prepared in DMSO (Sigma-Aldrich, Copenhagen, Denmark).

The calibration standard solution for high pressure liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) analysis contained dihydrotestosterone, dehydroepiandrosterone (DHEA), progesterone, estrone, testosterone and 17β-estradiol, all purchased from Sigma-Aldrich, (Copenhagen, Denmark), as well as 17-OH-progesterone, cortisol and androstenedione purchased from Steraloids (Rhode Island, USA), Riedel-de Haën (Seelze, Germany) and Cerilliant (Round Rock, USA), respectively. Internal standard solution containing testosterone-d_2, 17β-estradiol-d_3 and methyltestosterone-d_3 were purchased from RIKILT (Wageningen, Nederlands).

R1881 which is used in the AR reporter gene assay was purchased from PerkinElmer (Skovlunde, Denmark).

H295R steroidogenesis assay

The H295R human adrenal cortico-carcinoma cell line was obtained from the American Type Culture Collection (LGC standards, Boras, Sweden). Cells were cultured in DMEM/F12 + glutamine medium with HEPES buffer (Invitrogen, Taastrup, Denmark) containing 1% Insulin–Transferrin–Sodium selenite plus premix (VWR, Herlev, Denmark) and 2.5% Nu-Serum (BD Bioscience, Brøndby, Denmark) at 37 °C in a 5% CO_2 atmosphere. $3*10^5$ cells/well were seeded in 24-well plates (VWR, Herlev, Denmark) and cultured for 24 h. Subsequently, cells were exposed to 1.6, 3.1, 6.3, 12.5, 25.0, and 50.0 μM of test chemicals for 48 h. Each exposure concentration was tested in triplicates within each experiment. DMSO concentrations were 0.25% in all exposure groups—a non-cytotoxic concentration (see Supplementary materials 1). After 48 h exposure the supernatant was sampled for hormone analysis.

To test whether cell viability was compromised as a result of chemical exposure 500 μL media and 50 μL MTT stock solution (Sigma-Aldrich, Copenhagen, Denmark) of 5 mg/mL were added to wells and incubated for 1–2 h. The medium was removed and 500 μL isopropanol was added. Absorption was measured at 570 nm using a spectrophotometer (Victor[2], 1420 Multilabel Counter, PerkinElmer). Concentration–response relationships for cell viability testing were performed in all experiments except for those involving gene expression analysis and hormone analysis by HPLC–MS/MS. If cell viability decreased by more than 20% in any exposure group compared to control viability, these hormone data were removed from the data set according to the OECD test guideline procedure (OECD, 2011).

Hormone analysis by immunoassay

Three H295R steroidogenesis assay experiments were conducted for hormone measurements by immunoassay.

Hormones were extracted from cell supernatants using a C_{18} solid phase extraction (SPE) cartridge (200 mg, 3 mL) (Biotage, Uppsala, Sweden) as previously described (Vinggaard et al., 2002). Time-resolved fluoroimmunoassays were purchased from PerkinElmer (Skovlunde, Denmark) and were used for quantification of 17β-estradiol, testosterone, and progesterone. The experimental procedure was performed according to the protocol supplied by the manufacturer. A sample from each well was tested in duplicate in the immunoassay and the mean of these measurements was used for further calculation. Data points obtained from each well were normalized against the mean of the controls from the same cell plate, and data from the three experiments were pooled.

Test compounds were tested for autofluorescence and underwent a chemical-immunoassay interference test in the 17β-estradiol assay as HPLC–MS/MS measurements of this hormone were below the limit of quantification and thus comparison of the HPLC–MS/MS and immunoassay results for this hormone was not possible. No chemical interference in the immunoassay was observed.

Hormone analysis by high pressure liquid chromatography–tandem mass spectrometry (HPLC–MS/MS)

Hormone measurements by HPLC–MS/MS were conducted for one H295R steroidogenesis experiment including exposure concentrations of 3.1, 12.5 and 50.0 μM for each test compound.

Hormones were extracted according to a method published previously (Mortensen and Pedersen, 2007). Supernatant was extracted using a C_{18} endcapped SPE cartridge (500 mg, 3 mL) (Merck, Darmstadt, Germany) after addition of an internal standard solution of testosterone-d_2, 17β-estradiol-d_3 and methyltestosterone-d_3. Impurities were removed with demineralized water followed by elution of steroid hormones from the cartridge with MeOH. The extract was then evaporated to dryness using nitrogen, and resuspended in 40% solution of MeOH in demineralized water.

Nine hormones, progesterone, 17-OH-progesterone, dehydroepiandrosterone, testosterone, androstenedione, dihydrotestosterone, cortisol, estrone and 17β-estradiol, were separated, detected, and quantified using the HPLC–MS/MS method described elsewhere (Mortensen and Pedersen, 2007). Minor modifications were made to accommodate a smaller sample size and to include more hormones. The LC system (Agilent 1100) was equipped with an Atlantis C_{18} column (2.1 × 150 mm, 3 μm) (Waters Corp., Milford, MA, USA) maintained at 40 °C. The sample injection volume was 50 μL. 17β-estradiol and estrone were measured in ESI^- mode using 65% MeOH and 0.01% ammonia for mobile phase (0.15 mL/min, isocratic flow rate). The remaining steroid hormones were measured in ESI^+ mode using 65% MeOH and 0.1% acetic acid for mobile phase (0.2 mL/min, isocratic flow rate). The mass spectrometer was a Quattro Ultima triple quadropole instrument (Waters Corp., Milford, MA, USA). Calibration standards were run before and after sample analysis at levels of 0.25, 1.25, 2.5, 5.0, and 10.0 ng/mL.

The absolute recoveries of the hormones in the cell extracts were estimated to be 70–87%, based on the absolute recoveries of the three internal standards in 90 experiments. Thus the sensitivity of the method is comparable to the analysis in blood (Mortensen and Pedersen, 2007).

The limit of quantification (LOQ) of the cell extracts was estimated as 6 times signal-to-noise at a low concentration level, and was <0.1 ng/mL for all hormones except for DHEA which was <0.8 ng/mL. The dihydrotestosterone and 17β-estradiol levels in extracts were $<$LOQ and will thus not be further discussed.

Each obtained data point was normalized against the mean of plate control.

mRNA isolation and cDNA synthesis

The H295R steroidogenesis assay was conducted as described above with the exception that $0.6*10^5$ cells/well were seeded in 96-well plates. Gene expression analysis was conducted for three H295R steroidogenesis experiments including exposure concentrations of 3.1, 12.5 and 50.0 μM.

After supernatant removal, mRNA isolation from cells and conversion of mRNA to cDNA were done by using the TaqMan® Fast Cells-to-CT™ Kit (Applied Biosystems, Nærum, Denmark) according to product protocol. During mRNA isolation DNase was added to the lysis solution (1:100) to remove genomic DNA. cDNA was synthesized by reverse transcription on a thermal cycler (PTC-200, MJ Research). mRNA isolate and cDNA were stored at −20 °C.

Table 1
Names, abbreviations and catalogue numbers on Taqman gene expression assay.

Protein/enzyme name	Abbreviation	Catalogue number
Steroidogenic acute regulatory protein	StAR	Hs00264912_m1
Benzodiazepine receptor	Bzrp	Hs00559362_m1
Cholesterol side-chain cleavage enzyme	CYP11A	Hs00167984_m1
17α-hydroxylase/17,20 lyase/17,20 desmolase	CYP17A1	Hs01124136_m1
21-hydroxylase	CYP21A2	Hs00365734_g1
Aromatase	CYP19A1	Hs00903413_m1
β-actin		Hs99999903_m1

Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was analyzed on a 7900 HT Fast Real-time PCR system using TaqMan® Gene Expression Assays (Applied Biosystems, Nærum, Denmark) containing primers and probes for genes coding for six proteins involved in steroidogenesis (Table 1). β-actin was used as housekeeping gene. 384-well plates (Applied Biosystems, Nærum Denmark) were used for qRT-PCR analysis. In each well TaqMan® Gene Expression Assay (0.5 μL), TaqMan® Gene Expression Master Mix (5.0 μL), nuclease free water (2.5 μL) and cDNA template (2.0 μL) were added. All genes were analyzed on a single plate in duplicates. The thermal cycling program was initiated with 95 °C for 20 s followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s.

C_t values were determined for each target. Relative quantification of mRNA was calculated by first normalizing each data point to the housekeeping gene. No consistent significant effect on the housekeeping gene was observed. Successively data was normalized to the average of the non-exposed group ($\Delta\Delta C_t = (C_{t(target\ gene)} - C_{t(housekeeping\ gene)}) - C_{t(average\ control)}$) within each of the three experiments. Finally fold change values ($2^{-\Delta\Delta Ct}$) were calculated, the three experiments were pooled and these data were plotted.

Androgen receptor reporter gene assay

Two experiments in triplicates for each exposure concentration were conducted. The assay was performed as previously described by Vinggaard et al. (2002). The Chinese hamster ovary (CHO) cell line was obtained from the American Type Culture Collection (LGC standards, Boras, Sweden). Cells were transfected with pSVAR0 and MMTV-LUC for testing of agonistic and antagonistic effects on the activation of the AR, and with pSVAR13, encoding for a mutated AR, and MMTV-LUC for testing of cell viability. All plasmids were generous gifts from Dr. Albert Brinkmann (Erasmus University, Rotterdam). A known androgen receptor agonist, R1881 was added to all wells on the antagonism plates, at a constant concentration of 0.1 nM. Test chemicals were added leading to final exposure concentrations of 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, and 50.0 μM on agonism, antagonism and toxicity plates. Luciferase activity was measured on a luminometer (BioOrbit, Galaxy) by the addition of luciferin. Within an experiment each data point was normalized against the mean of control from the same cell plate and successively the data from the two experiments were pooled.

Statistical analysis

One-way analysis of variance (ANOVA) was performed on hormone, gene expression, AR reporter gene and cell viability data with a successive Dunnett's post-test to examine differences between exposed groups and controls. The criterion for statistical significance was $p<0.05$, $p<0.01$, and $p<0.001$ leading to the marking *, **, and ***, respectively. $\Delta\Delta C_t$ values were used for statistical analysis of gene expression data (Zeuthen et al., 2010). Data was assessed for normal distribution and homogeneity of variance.

All statistical analysis was conducted in GraphPad Prism 5. All information obtained from the data was taken into account. Thus immunoassay and gene expression data represent three experiments in triplicates, HPLC–MS/MS data represent triplicates within one experiment and AR reporter gene assay represents two experiments in triplicates.

Results

The H295R steroidogenesis assay was conducted to gain information on the ability of the test compounds to affect the synthesis of sex hormones. Measurements of steroid hormones, including progesterone, 17-OH-progesterone, dehydroepiandrosterone, testosterone, androstenedione, dihydrotestosterone, cortisol, estrone and 17β-estradiol, and expression of central genes involved in steroidogenesis allow assessment of the potential of the compounds to exert such effects as well as obtaining information on the mechanism of an effect.

Figs. 2–4 show an overview of the steroid hormone profiles after exposure to increasing concentrations of 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH, respectively, with concentration–effect relationships for each measured hormone and bar windows illustrating effects on gene expression of proteins involved in steroidogenesis. An overview of the qualitative effects, lowest observable effect concentrations (LOECs), and the tentative maximum efficacy (E_{max}) values for all hormones after 8:2 triPAPS, 10:2 diPAPS, 8:2 diPAPS, 8:2 monoPAPS, 8:2 FTOH, and PFOA exposure is shown in Table 2. For further reference to LOEC values it should be noted that significant effects observed at one low concentration, which was not replicated at higher concentrations, have not been considered as representative LOECs. Such data is reported in Table 2, but indicated with the marking [b]. Data for 8:2 triPAPS, 10:2 diPAPS, and PFOA will only be described briefly.

Effects on hormones

8:2 diPAPS exposure led to a statistically significant decrease in the androgens, testosterone, androstenedione, and DHEA with LOECs of 25.0, 12.5, and 50.0 μM, respectively (Fig. 2 and Table 2). Furthermore a decrease in the progestagens, progesterone and 17-OH-progesterone was observed after exposure to 8:2 diPAPS with LOECs of 3.1 and 12.5 μM, respectively. Estrone increased significantly as a result of exposure to 8:2 diPAPS at the highest test concentration of 50.0 μM, whereas a non-significant increasing trend was observed for 17β-estradiol (Fig. 2). Finally, cortisol levels also increased significantly with a LOEC of 50.0 μM (Fig. 2).

Like for 8:2 diPAPS, exposure to 8:2 monoPAPS resulted in decreases in the level of all measured androgens and progestagens. 8:2 monoPAPS also caused an increase in estrone whereas the 17β-estradiol level was unaffected (Fig. 3 and Table 2). Where cortisol level was increased with exposure to 8:2 diPAPS this remained unaffected after 8:2 monoPAPS exposure. The LOECs for 8:2 monoPAPS on testosterone, androstenedione and DHEA were 25.0, 12.5, and 12.5 μM, respectively. Progesterone and 17-OH-progesterone LOECs were 50.0 and 3.1 μM, respectively. Finally, estrone had a LOEC of 50.0 μM. Generally the potencies and tentative efficacies of 8:2 monoPAPS were greater than that of 8:2 diPAPS for the affected hormones, except for progesterone for which a greater potency and efficacy was found after 8:2 diPAPS exposure (Table 2).

The effect of 8:2 FTOH on the measured hormones was similar to that of 8:2 diPAPS and 8:2 monoPAPS, showing decreased androgen and progestagen levels (Fig. 4 and Table 2). LOECs for testosterone, androstenedione, progesterone and 17-OH-progesterone were 3.1 μM. As for 8:2 monoPAPS the cortisol level was unaffected. 17β-estradiol increased significantly with a LOEC of 12.5 μM, and for estrone all exposure groups were increased compared to the control. 8:2 FTOH showed greater potency for progesterone, 17-OH-progesterone, androstenedione and testosterone than 8:2 diPAPS and 8:2 monoPAPS (Table 2).

8:2 triPAPS exposure (Table 2) did not lead to changes in levels of DHEA, androstenedione, 17β-estradiol and cortisol. A decrease was observed in some exposure groups in progestagens, but these data did not seem to be concentration-dependent. An increase in estrone and a decrease in testosterone levels were observed with LOECs of 12.5 and 50.0 μM, respectively. Exposure to PFOA caused no change in androgens, progestagens, and cortisol, whereas 17β-estradiol decreased significantly at low concentrations and increased significantly at higher concentrations and estrone increased with a LOEC of 12.5 μM (Table 2). 10:2 diPAPS led to significant decreases in

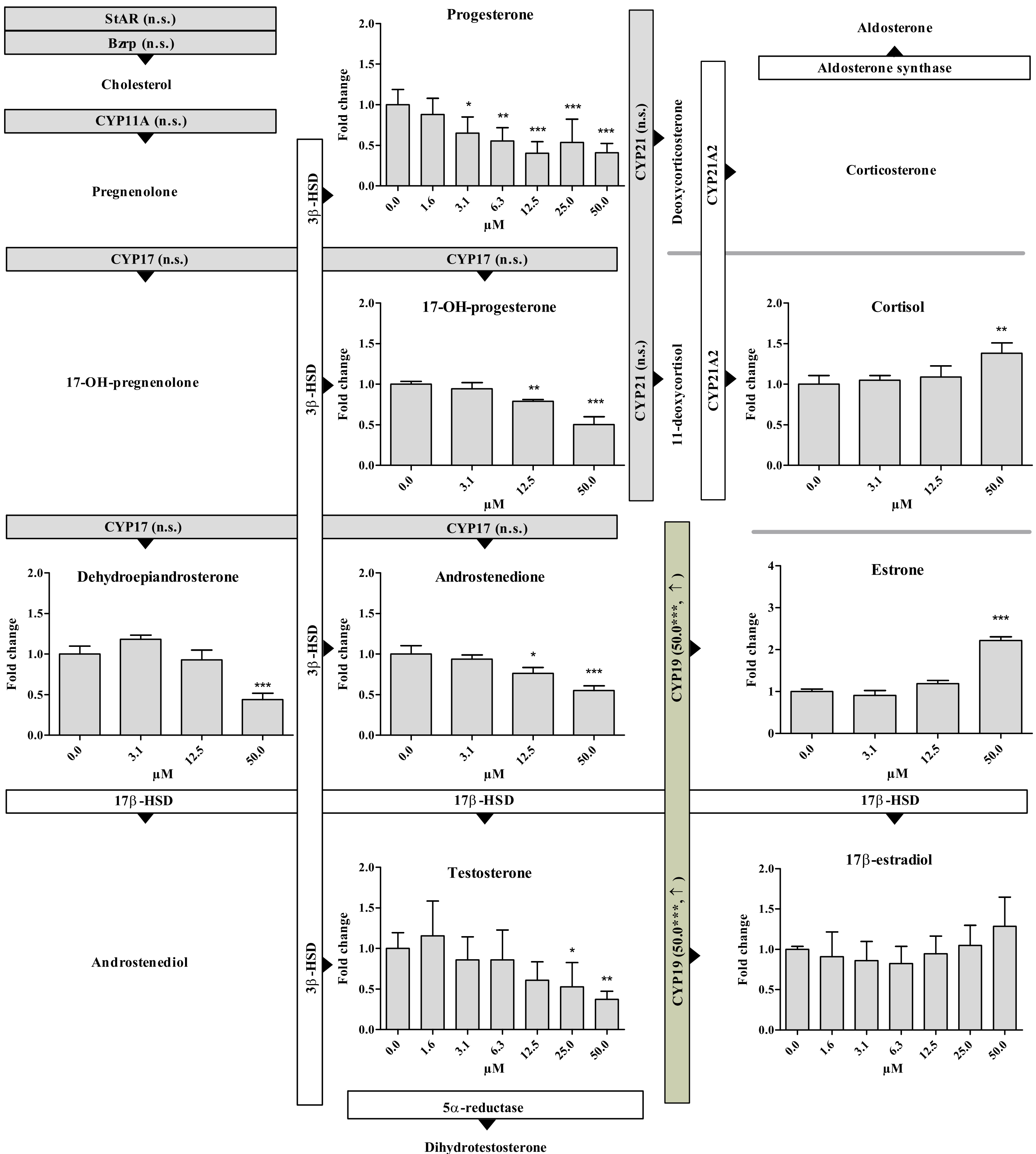


Fig. 2. Steroid hormone profile caused by 8:2 diPAPS. Concentrations are given in µM and response in fold change. Concentration–effect relationships for progesterone, testosterone and 17β-estradiol are based on immunoassay data from three independent experiments of three replicates in each experiment. Each data point was normalized to the mean of the experiment controls and subsequently the three experiments were pooled. The concentration–effect relationships for the remaining hormones are based on HPLC–MS/MS data from one experiment performed in triplicates. The bars represent proteins involved in steroidogenesis of which StAR, Bzrp, CYP11A, CYP17, CYP21, and CYP19 gene expression levels were measured in three experiments in triplicates. Arrow up (↑) indicates an increase in the relative level of mRNA, arrow down (↓) represents a decrease in the relative level of mRNA and n.s. indicates that no statistically significant change was observed. Markings of *, **, and *** represent a statistically significant level of $p<0.05$, <0.01 and <0.001, respectively.

progesterone and 17-OH-progesterone with LOECs of 50.0 µM and 3.1 µM, respectively. DHEA and androstenedione levels also decreased with LOECs of 3.1 and 12.5 µM, respectively; however the level of testosterone, cortisol and 17β-estradiol was not affected by exposure. Estrone level increased significantly at an exposure concentration of 50 µM of 10:2 diPAPS (Table 2).

Quality control of the hormone measurements was done by comparing HPLC–MS/MS and immunoassay measurements, conducting

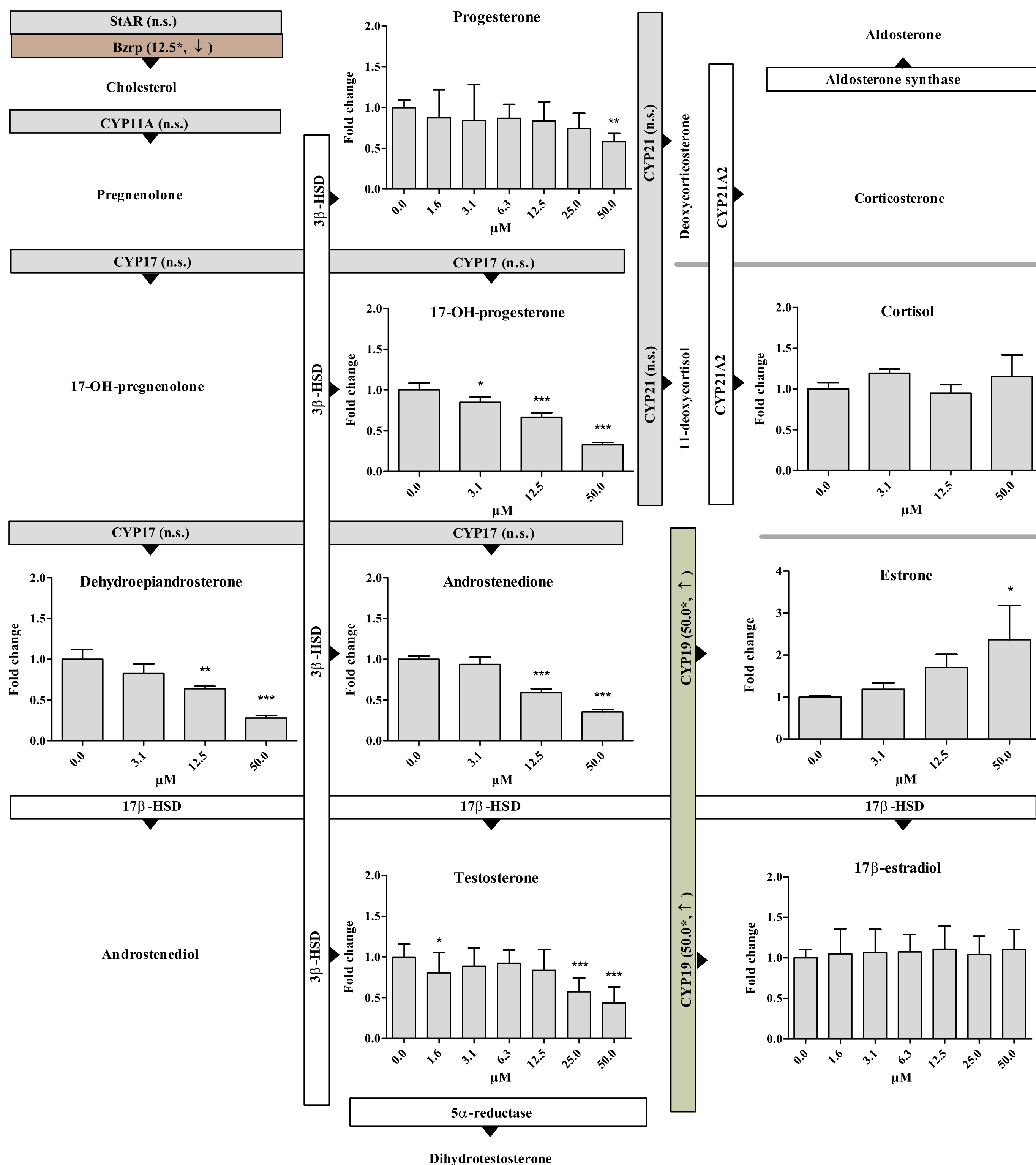


Fig. 3. Steroid hormone profile caused by 8:2 monoPAPS. Concentrations are given in μM and response in fold change. Concentration–effect relationships for progesterone, testosterone and 17β-estradiol are based on immunoassay data from three independent experiments of three replicates in each experiment. Each data point was normalized to the mean of the experiment controls and subsequently the three experiments were pooled. The concentration–effect relationships for the remaining hormones are based on HPLC–MS/MS data from one experiment performed in triplicates. The bars represent proteins involved in steroidogenesis of which StAR, Bzrp, CYP11A, CYP17, CYP21, and CYP19 gene expression levels were measured in three experiments in triplicates. Arrow up (↑) indicates an increase in the relative level of mRNA, arrow down (↓) represents a decrease in the relative level of mRNA and n.s. indicates that no statistically significant change was observed. Markings of *, **, and *** represent a statistically significant level of $p<0.05$, <0.01 and <0.001, respectively.

chemical-immunoassay interference test and securing that cell viability was not compromised. Comparing progesterone and testosterone hormone data obtained from the HPLC–MS/MS and immunoassay methods, these generally showed similar concentration–effect relationships as a result of exposure to the six fluorochemicals. It was not possible to compare 17β-estradiol data between the two methods, as the levels were

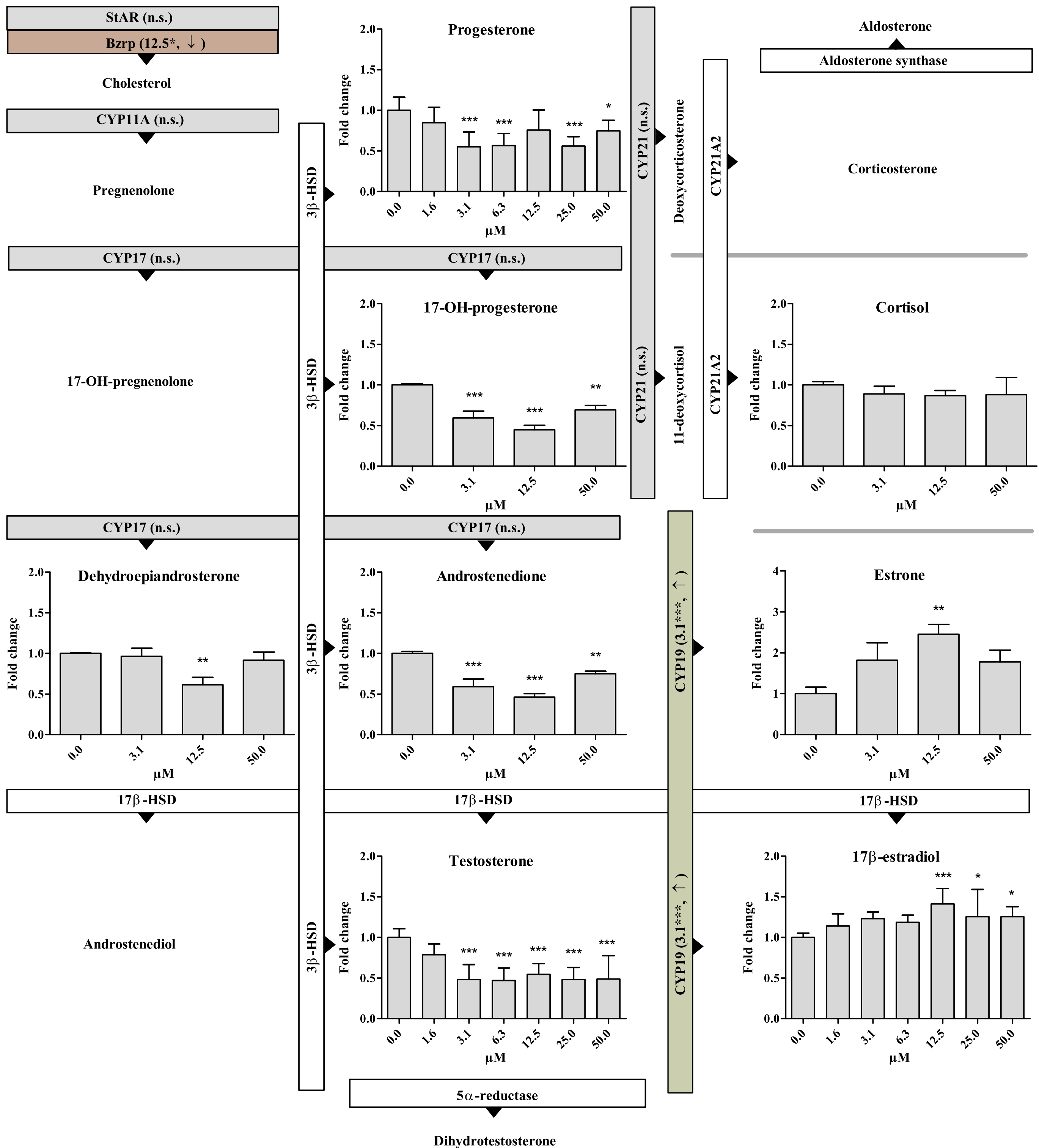


Fig. 4. Steroid hormone profile caused by 8:2 FTOH. Concentrations are given in μM and response in fold change. Concentration–effect relationships for progesterone, testosterone and 17β-estradiol are based on immunoassay data from three independent experiments of three replicates in each experiment. Each data point was normalized to the mean of the experiment controls and subsequently the three experiments were pooled. The concentration–effect relationships for the remaining hormones are based on HPLC–MS/MS data from one experiment performed in triplicates. The bars represent proteins involved in steroidogenesis of which StAR, Bzrp, CYP11A, CYP17, CYP21, and CYP19 gene expression levels were measured in three experiments in triplicates. Arrow up (↑) indicates an increase in the relative level of mRNA, arrow down (↓) represents a decrease in the relative level of mRNA and n.s. indicates that no statistically significant change was observed. Markings of *, **, and *** represent a statistically significant level of $p<0.05$, <0.01 and <0.001, respectively.

below the limit of quantification when measured by HPLC–MS/MS. No consistent concentration-dependent effect on cell viability was observed for the six tested fluorochemicals in cells used for immunoassay hormone analysis (see Supplementary materials 1) and thus it was assumed that compromised cell viability was generally not an issue for any of the data obtained in the H295R steroidogenesis assay.

Table 2
The effect of six fluorochemicals on steroidogenesis in the H295R steroidogenesis assay. The qualitative effect (QE) (↓ or ↑), the lowest observable effect concentration (LOEC) and the tentative efficacy (E_{max}) given in % change are shown. Immunoassay values are based on three experiments performed in triplicates and the HPLC–MS/MS values are based on one experiment performed in triplicates. All values have a significance level of $p<0.05$.

		8:2 triPAPS	10:2 diPAPS	8:2 diPAPS	8:2 monoPAPS	8:2 FTOH	PFOA
Progesterone[a]	QE	↓	↓	↓	↓	↓	n.s.
	LOEC (μM)	12. 5[b]	50	3.1	50	3.1	n.s.
	E_{max} (%)	26 ± 10	22 ± 16	60 ± 14	42 ± 10	45 ± 18	n.s.
17-OH-Progesterone[c]	QE	↓	↓	↓	↓	↓	n.s.
	LOEC (μM)	12.5[b]	3.1	12.5	3.1	3.1	n.s.
	E_{max} (%)	14 ± 6	28 ± 3	50 ± 10	67 ± 3	55 ± 6	n.s.
Cortisol[c]	QE	n.s.	↓	↑	n.s.	n.s.	n.s.
	LOEC (μM)	n.s.	3.1[b]	50	n.s	n.s .	n.s.
	E_{max} (%)	n.s.	25 ± 15	38 ± 13	n.s.	n.s.	n.s.
DHEA[c]	QE	n.s.	↓	↓	↓	↓	n.s.
	LOEC (μM)	n.s.	3.1	50	12.5	12.5[b]	n.s.
	E_{max} (%)	n.s.	29 ± 3	56 ± 8	72 ± 3	39 ± 9	n.s.
Androstenedione[c]	QE	n.s.	↓	↓	↓	↓	↓
	LOEC (μM)	n.s.	12.5	12.5	12.5	3.1	12.5[b]
	E_{max} (%)	n.s.	17 ± 3	45 ± 6	65 ± 3	54 ± 4	19 ± 9
Testosterone[a]	QE	↓	n.s.	↓	↓	↓	n.s.
	LOEC (μM)	50	n.s.	25	25	3.1	n.s.
	E_{max} (%)	34 ± 21	n.s.	63 ± 10	56 ± 20	53 ± 16	n.s.
Estrone[c]	QE	↑	↑	↑	↑	↑	↑
	LOEC (μM)	12.5	50	50	50	12.5[b]	12.5
	E_{max} (%)	34 ± 8	30 ± 11	121 ± 9	137 ± 82	146 ± 24	75 ± 9
17β-estradiol[a]	QE	n.s.	n.s.	n.s.	n.s.	↑	↓/↑
	LOEC (μM)	n.s.	n.s.	n.s.	n.s.	12.5	1.6/50
	E_{max} (%)	n.s.	n.s.	n.s.	n.s.	41 ± 19	24 ± 19/17 ± 22

n.s. = not statistically significant.
The light red shade indicates a decrease in the respective hormone level; the light green shade indicates an increase in hormone level; the gray shade indicates that both a decrease and an increase in hormone level were observed.
[a] Immunoassay data.
[b] The LOEC is based on a single significant finding which is not reflected in higher concentrations.
[c] HPLC–MS/MS data.

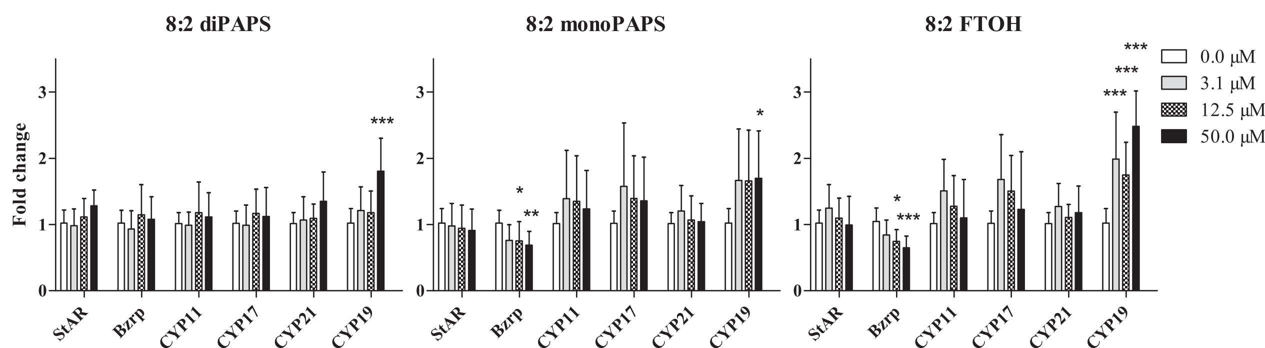


Fig. 5. Gene expression levels following 8:2 diPAPS, 8:2 monoPAPS, and 8:2 FTOH exposure are shown as fold change values ($2^{-\Delta\Delta Ct}$) of mRNA for steroidogenic acute regulatory protein (StAR), benzodiazepine receptor (Bzrp), cholesterol side-chain cleavage enzyme (CYP11), 17α-hydroxylase/17,20 lyase/17,20 desmolase enzyme (CYP17), CYP21 and aromatase (CYP19). Concentration–effect relationships are based on data from three experiments in triplicates. All data are corrected for housekeeping gene β-actin, each data point was normalized against the mean of experiment controls and the three experiments were pooled. Markings of *, **, and *** represent a statistically significant level of $p<0.05$, <0.01 and <0.001, respectively.

Effects on gene expression

8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH led to increased expression of CYP19 mRNA with LOECs of 50.0, 50.0 and 3.1 μM, respectively (Fig. 5). Both 8:2 monoPAPS and 8:2 FTOH led to decreased levels of Bzrp mRNA with LOECs of 12.5 μM.

Neither StAR, CYP11A, CYP17, nor CYP21 showed significant changes as a result of exposure to 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH. Likewise none of the examined target genes showed changes in gene expression after exposure to 8:2 triPAPS, 10:2 diPAPS and PFOA (data not shown).

Effects on AR transcriptional activation

All six fluorochemicals were tested for their ability to agonize or antagonize the activation of the AR by using a reporter gene assay. The test chemicals showed no agonistic or antagonistic effect in the applied assay, at test concentrations ≤50 μM (see Supplementary materials 2).

Discussion

Three of the tested chemicals, namely 8:2 diPAPS, 8:2 monoPAPS, and 8:2 FTOH, showed significant effects on androgen levels in the H295R steroidogenesis assay in a concentration-dependent manner. Testosterone and androstenedione decreased with exposure to all three chemicals, and DHEA decreased after exposure to 8:2 diPAPS and 8:2 monoPAPS (Figs. 2–4). Neither 8:2 diPAPS, 8:2 monoPAPS nor 8:2 FTOH appeared to be AR agonists or antagonists, and thus among the two endpoints, inhibition of androgen production through interference with steroidogenesis appeared to be the main target.

8:2 triPAPS, 10:2 diPAPS and PFOA showed a less marked effect on androgens in the H295R steroidogenesis assay, and none of these test compounds caused agonistic or antagonistic effects in the AR reporter gene assay. Results for 8:2 triPAPS and 10:2 diPAPS will not be discussed further. It should be mentioned however that results obtained with exposure to 8:2 triPAPS may be a result of exposure to a mixture of chemicals as the purity of the 8:2 triPAPS product only was 65.1%. Impurities in the 8:2 triPAPS product are mainly 8:2 diPAPS and 8:2 monoPAPS (personal communication, Chiron, Norway) and thus the cells in the 8:2 triPAPS experiment are exposed to a mixture of these three compounds. It is believed that the observed changes in hormones, estrone and testosterone (Table 2) with exposure to 8:2 triPAPS are a result of 8:2 diPAPS and 8:2 monoPAPS exposure.

To understand the mechanism underlying the decreases in androgens in the steroidogenic pathway caused by 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH, the hormone profiles were examined in detail (Figs. 2–4). An inhibition was also observed for progesterone and 17-OH-progesterone with exposure to these compounds, indicating a target located prior to both progestagen and androgen synthesis. A disturbance at any level prior to these in steroidogenesis could potentially lead to the observed decreases in both of these steroid hormone classes. Gene expression data was obtained for three genes involved prior to androgen and progestagen formation in steroidogenesis. StAR and Bzrp which facilitate the transport of cholesterol to the inner mitochondrial membrane and constitute the rate-limiting step in steroidogenesis, and CYP11A which cleaves the side chain of cholesterol after its arrival to the inner mitochondria, and constitute the enzymatically rate-limiting step. Among these three genes, Bzrp gene expression was inhibited following exposure to 8:2 monoPAPS and 8:2 FTOH indicating a down-regulation of this gene, leading to a decreased transport of cholesterol into the inner mitochondria. Another mechanism that could contribute to the observed decreases in androgens is through pushing of the equilibrium from androgens to estrogens leading to increased formation of 17β-estradiol and estrone. The levels of estrogens generally increased after exposure to 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH (Table 2), despite the decrease in substrates, androstenedione and testosterone. This suggests that the equilibrium between the two groups of sex hormones was affected. This was supported by an observed increase in CYP19 gene expression levels following 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH exposure which potentially can lead to the observed decreases in androgens and increases in estrogens.

Besides the above suggested mechanisms of action the decreased androgen levels could be caused by interferences with translation of mRNA into proteins and activity of important proteins in steroidogenesis. As the test compounds are surface active they may also affect the fluidity of cell membranes as described previously for PFOS (Hu et al., 2003; Xie et al., 2010), though our results do not seem to support this hypothesis, but rather suggests that the mechanism is specific, as described above.

Liu et al. (2010) have previously studied the effects of 8:2 FTOH in the H295R steroidogenesis assay at an exposure period of 24 h. Despite the differences in exposure time, 24 h versus 48 h, many of the measured hormones showed similar trends in the two studies, including a decrease in 17-OH-progesterone, androstenedione and testosterone. However Liu et al. (2010) also found a decrease in cortisol levels and no change in progesterone levels. They hypothesized that 8:2 FTOH caused a general down-regulation of steroidogenesis through decreased cAMP levels (Liu et al., 2010). In the present study cortisol levels stayed unaffected and progesterone levels decreased with 8:2 FTOH exposure. The absence of a cortisol decrease and the increase in 17β-estradiol indicate that a site specific mechanism of action is likely to occur in the present study, which is

supported by gene expression data for Bzrp and CYP19. Differences in hormone profiles in the two studies may be a result of different incubation periods.

PFOA showed a significant increase in estrogen levels at higher concentrations in the H295R steroidogenesis assay. However, no effect was observed on androgen levels in the H295R steroidogenesis assay and in the AR reporter gene assay. Previously PFOA has been tested for AR antagonism *in vitro* (Vinggaard et al., 2008) showing results in accordance with those in the present study. The hormone data are in agreement with previous *in vivo* studies where no convincing downward change in testosterone and an increase in 17β-estradiol serum levels were found with exposure to PFOA (Biegel et al., 2001; Cook et al., 1992). In contrast, studies conducted on isolated rat Leydig cells have shown that PFOA has the potential to decrease testosterone levels *in vitro* (Zhao et al., 2010). The fact that PFOA showed no effect on AR activation or androgen levels in the present *in vitro* studies does not exclude however, that PFOA could interfere with the androgen pathway *in vivo* for instance by disturbance of metabolism of steroid hormones. This has been suggested previously, as expression of genes associated with steroid metabolism was induced in fetal mouse liver after exposure to PFOA (Rosen et al., 2007). The discrepancy between *in vitro* effects on testosterone synthesis in the present study and the study by Zhao et al. (2010) could be explained by species differences, human versus rat, or by qualitative differences in the steroidogenic pathway in the adrenal and testis. Overall the present study does not supply additional evidence of PFOA interfering with androgen synthesis, but our study supports the hypothesis that PFOA can cause increased estrogen levels.

The technical mixtures used for coating of food packaging materials mainly contain diPAPS homologues, but also triPAPS and monoPAPS (Trier et al., 2011). These can undergo dephosphorylation and oxidation into FTOH and PFCA homologues in rats (D'eon and Mabury, 2007, 2011). The present study included an assessment of individual metabolites in a shared metabolic pathway. The OECD guideline states that the metabolic capacity of H295R cells is currently unknown, but that it is probably limited (OECD, 2011). Moreover, Liu et al. (2010) found no conjugates of the perfluorinated acids or the acids themselves indicating that no biotransformation of 8:2 FTOH into PFCAs occurred (Liu et al., 2010). When comparing the effect on the hormone levels of 8:2 FTOH and PFOA in the present study, the lack of uniformity between the two profiles indicates that a full biotransformation of 8:2 FTOH into PFOA has not occurred, as qualitatively similar effects otherwise would have been expected for the two compounds. It can thus be assumed that the data obtained for 8:2 FTOH is a result of exposure to 8:2 FTOH itself and not the metabolite PFOA. The potential of H295R cells to dephosphorylate PAPS into FTOH can indirectly be elucidated through comparison of hormone profiles. As described previously it is believed that the effects on testosterone and estrone with 8:2 triPAPS exposure are a result of impurities. The lack of qualitative uniformity between hormone profiles of 8:2 triPAPS compared to 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH indicates that no or minor metabolism of 8:2 triPAPS has occurred. The apparent lack of metabolism of PAPS and FTOH in H295R cells suggests that the individual metabolites exert an effect in this system.

As pointed out the technical mixture used for FCMs consists of PAPS of various degrees of alkylation, but also varying lengths of the fluorinated chain. In the present study compounds with a fluorinated chain length of eight or ten carbon atoms were included, however contributions from shorter chained or longer chained PAPS should be examined in future studies. Furthermore, longer chained PAPS would be metabolized *in vivo* into longer chained PFCAs, which may contribute to the overall effect on the androgen pathway as described previously (Feng et al., 2009; Shi et al., 2007, 2009).

Human serum levels of diPAPS homologues with chain lengths 4:2, 6:2, 8:2 and 10:2 were measured to be 4.5 μg/L (D'eon et al., 2009). This approximately equals to 5 nM if all homologues contribute equally. LOEC for 8:2 diPAPS on testosterone was 25.0 μM, which is far above the blood level. However, the short exposure period of 48 h applied in the present study does not reflect a life-long human exposure. In addition, the contribution of diPAPS homologues in combination with metabolic products of these (monoPAPS and FTOH), as well as the general cocktail of chemicals that humans are exposed to, may together cause sex hormone disturbances *in vivo* and adverse effects on human health.

In summary 8:2 diPAPS, 8:2 monoPAPS, and 8:2 FTOH all gave rise to decreases in levels of all measured androgens in the steroidogenic pathway. The mechanism underlying this effect is suggested to be specific and to have two targets, inhibition of Bzrp and induction of CYP19 gene expression, thereby giving rise to the characteristic hormone profiles for these three chemicals. This study is a first contribution in gaining knowledge on the toxicology of PAPS. There is a need for further toxicological studies to be conducted on these chemicals, as very little is known within this field, the chemicals are widely used, humans are exposed and no specific EU legislation to control the use of PAPS in FCMs exists.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2012.10.022.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors wish to thank Camilla Taxvig for assistance with handling of data, proof reading and general support. Furthermore Heidi Letting, Dorte Lykkegaard Korsbech, Birgitte Møller Plesning, and Lis Abildgaard Andersen are thanked for technical assistance. Finally Katrine Scheibyes' help with conducting the initial testing of PAPS in the AR reporter gene assay is appreciated.

References

Andersen, M.E., Butenhoff, J.L., Chang, S., Farrar, D.G., Kennedy Jr., G.L., Lau, C., Olsen, G.W., Seed, J., Wallacekj, K.B., 2008. Perfluoroalkyl acids and related chemistries—toxicokinetics and modes of action. Toxicol. Sci. 102, 3–14.
Begley, T.H., Hsu, W., Noonan, G., Diachenko, G., 2008. Migration of fluorochemical paper additives from food-contact paper into foods and food stimulants. Food Addit. Contam. 25, 384–390.
Biegel, L., Hurtt, M., Frame, S., O'Connor, J., Cook, J., 2001. Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicol. Sci. 60, 44–55.
Cook, J.C., Murray, S.M., Frame, S.R., Hurtt, M.E., 1992. Induction of Leydig-cell adenomas by ammonium perfluorooctanoate—a possible endocrine-related mechanism. Toxicol. Appl. Pharmacol. 113, 209–217.
D'eon, J.C., Mabury, S.A., 2007. Production of perfluorinated carboxylic acids (PFCAs) from the biotransformation of polyfluoroalkyl phosphate surfictants (PAPS): exploring routes of human contamination. Environ. Sci. Technol. 41, 4799–4805.
D'eon, J.C., Mabury, S.A., 2011. Exploring indirect sources of human exposure to perfluoroalkyl carboxylates (PFCAs): evaluating uptake, elimination, and biotransformation of polyfluoroalkyl phosphate esters (PAPs) in the rat. Environ. Health Perspect. 119, 344–350.
D'eon, J.C., Crozier, P.W., Furdui, V.I., Reiner, E.J., Libelo, E.L., Mabury, S.A., 2009. Observation of a commercial fluorinated material, the polyfluoroalkyl phosphoric acid diesters, in human sera, wastewater treatment plant sludge, and paper fibers. Environ. Sci. Technol. 43, 4589–4594.
European Food Safety Authority (EFSA), 2008. Opinion of the Scientific Panel on Contaminants in the Food chain on Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. EFSA J. 653, 1–131.
Fasano, W.J., Carpenter, S.C., Gannon, S.A., Snow, T.A., Stadler, J.C., Kennedy, G.L., Buck, R.C., Korzeniowski, S.H., Hinderliter, P.M., Kemper, R.A., 2006. Absorption, distribution, metabolism, and elimination of 8–2 fluorotelomer alcohol in the rat. Toxicol. Sci. 91, 341–355.
Feng, Y., Shi, Z., Fang, X., Xu, M., Dai, J., 2009. Perfluorononanoic acid induces apoptosis involving the Fas death receptor signaling pathway in rat testis. Toxicol. Lett. 190, 224–230.
Houde, M., Martin, J.W., Letcher, R.J., Solomon, K.R., Muir, D.C.G., 2006. Biological monitoring of polyfluoroalkyl substances: a review. Environ. Sci. Technol. 40, 3463–3473.
Hu, W.Y., Jones, P.D., DeCoen, W., King, L., Fraker, P., Newsted, J., Giesy, J.P., 2003. Alterations in cell membrane properties caused by perfluorinated compounds. Comp. Biochem. Physiol. C 135, 77–88.

Joensen, U.N., Bossi, R., Leffers, H., Jensen, A.A., Skakkebaek, N.E., Jorgensen, N., 2009. Do perfluoroalkyl compounds impair human semen quality? Environ. Health Perspect. 117, 923–927.

Kennedy, G.L., Butenhoff, J.L., Olsen, G.W., O'Connor, J.C., Seacat, A.M., Perkins, R.G., Biegel, L.B., Murphy, S.R., Farrar, D.G., 2004. The toxicology of perfluorooctanoate. Crit. Rev. Toxicol. 34, 351–384.

Kim, S., Lee, K.T.L., Kang, C.S., Tao, L., Kannan, K., Kim, K., Kim, C., Lee, J.S., Park, P.S., Yoo, Y.W., Ha, J.Y., Shin, Y., Lee, J., 2011. Distribution of perfluorochemicals between sera and milk from the same mothers and implications for prenatal and postnatal exposures. Environ. Pollut. 159, 169–174.

Kissa, E., 2001. Fluorinated Surfactants and Repellents, 2nd ed. Marcel Dekker, New York.

Kudo, N., Iwase, Y., Okayachi, H., Yamakawa, Y., Kawashima, Y., 2005. Induction of hepatic peroxisome proliferation by 8–2 telomer alcohol feeding in mice: formation of perfluorooctanoic acid in the liver. Toxicol. Sci. 86, 231–238.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J., 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99, 366–394.

Liu, C., Zhang, X., Chang, H., Jones, P., Wiseman, S., Naile, J., Hecker, M., Giesy, J.P., Zhou, B., 2010. Effects of fluorotelomer alcohol 8/2 FTOH on steroidogenesis in H295R cells: targeting the cAMP signalling cascade. Toxicol. Appl. Pharmacol. 247, 222–228.

Martin, J.W., Mabury, S.A., O'Brien, P.J., 2005. Metabolic products and pathways of fluorotelomer alcohols in isolated rat hepatocytes. Chem. Biol. Interact. 155, 165–180.

Monroy, R., Morrison, K., Teo, K., Atkinson, S., Kubwabo, C., Stewart, B., Foster, W.G., 2008. Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. Environ. Res. 108, 56–62.

Mortensen, S.K., Pedersen, M., 2007. Confirmatory analysis of acetylgestagens in plasma using liquid chromatography–tandem mass spectrometry. Anal. Chim. Acta 586, 217–222.

OECD, 2011. OECD Guideline for the Testing of Chemicals - The H295R Steroidogenesis Assay (internet) OECD Publishing. ([230512]. Last revised 2011. Available at: <www.oecd-ilibrary.org/docserver/download/fulltext/9745601e.pdf?expires=1337758392&id=id&accname=freeContent&checksum=86FA5AD706D7FAF9CB836567248E636).

Olsen, G.W., Burris, J.M., Ehresman, D.J., Froehlich, J.W., Seacat, A.M., Butenhoff, J.L., Zobel, L.R., 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ. Health Perspect. 115, 1298–1305.

Rosen, M.B., Thibodeaux, J.R., Wood, C.R., Zehr, R.D., Schmid, J.E., Lau, C., 2007. Gene expression profiling in the lung and liver of PFOA-exposed mouse fetuses. Toxicology 239, 15–33.

Shi, Z., Zhang, H., Liu, Y., Xu, M., Dai, J., 2007. Alterations in gene expression and testosterone synthesis in the testes of male rats exposed to perfluorododecanoic acid. Toxicol. Sci. 98, 206–215.

Shi, Z., Ding, L., Zhang, H., Feng, Y., Xu, M., Dai, J., 2009. Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. Toxicol. Lett. 188, 192–200.

So, M.K., Yamashita, N., Taniyasu, S., Jiang, Q., Giesy, J.P., Chen, K., Lam, P.K.S., 2006. Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. Environ. Sci. Technol. 40, 2924–2929.

Steenland, K., Fletcher, T., Savitz, D.A., 2010. Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environ. Health Perspect. 118, 1100–1108.

Trier, X., 2011. Polyfluorinated surfactants in food packaging of paper and board. Ph.D. thesis. Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Denmark.

Trier, X., Granby, K., Christensen, J.H., 2011. Polyfluorinated surfactants (PFS) in paper and board coatings for food packaging. Environ. Sci. Pollut. Res. 18, 1108–1120.

Vinggaard, A., Nellemann, C., Dalgaard, M., Jorgensen, E., Andersen, H., 2002. Antiandrogenic effects *in vitro* and *in vivo* of the fungicide prochloraz. Toxicol. Sci. 69, 344–353.

Vinggaard, A.M., Niemela, J., Wedebye, E.B., Jensen, G.E., 2008. Screening of 397 chemicals and development of a quantitative structure-activity relationship model for androgen receptor antagonism. Chem. Res. Toxicol. 21, 813–823.

Xie, W., Ludewig, G., Wang, K., Lehmler, H., 2010. Model and cell membrane partitioning of perfluorooctanesulfonate is independent of the lipid chain length. Colloids Surf. B: Biointerfaces 76, 128–136.

Zeuthen, L.H., Fink, L.N., Metzdorff, S.B., Kristensen, M.B., Licht, T.R., Nellemann, C., Frokiaer, H., 2010. Lactobacillus acidophilus induces a slow but more sustained chemokine and cytokine response in naive foetal enterocytes compared to commensal *Escherichia coli* RID F-8344-2010. BMC Immunol. 11, 2.

Zhao, B., Chu, Y., Hardy, D.O., Li, X., Ge, R., 2010. Inhibition of 3 beta- and 17 beta-hydroxysteroid dehydrogenase activities in rat Leydig cells by perfluorooctane acid. J. Steroid Biochem. Mol. Biol. 118, 13–17.

9.2 Manuscript 2

Rosenmai, A.K., Dybdahl, M., Pedersen, M., van Vugt-Lussenburg, B.M.A., Wedebye, E.B., Taxvig, C., and Vinggaard, A.M., 2014. Are structural analogues to bisphenol A safe alternatives? Tox Sci. 139, 35-47.

TOXICOLOGICAL SCIENCES **139(1)**, 35–47 2014
doi: 10.1093/toxsci/kfu030
Advance Access publication February 22, 2014

Are Structural Analogues to Bisphenol A Safe Alternatives?

Anna Kjerstine Rosenmai,* Marianne Dybdahl,* Mikael Pedersen,† Barbara Medea Alice van Vugt-Lussenburg,‡ Eva Bay Wedebye,* Camilla Taxvig,* and Anne Marie Vinggaard*,[1]

*Division of Toxicology & Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark; †Division of Food Chemistry, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark; and ‡BioDetection Systems b.v., Science Park 406, 1098 XH Amsterdam, The Netherlands

[1]To whom correspondence should be addressed at Division of Toxicology & Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark. E-mail: annv@food.dtu.dk.

Received December 17, 2013; accepted February 6, 2014

Background: **Bisphenol A (BPA) is a chemical with widespread human exposure suspected of causing low-dose effects. Thus, a need for developing alternatives to BPA exists. Structural analogues of BPA have already been detected in foods and humans. Due to the structural analogy of the alternatives, there is a risk of effects similar to BPA.**

Objectives: **The aim was to elucidate and compare the hazards of bisphenol B (BPB), bisphenol E (BPE), bisphenol F (BPF), bisphenol S (BPS) and 4-cumylphenol (HPP) to BPA.**

Methods: In vitro **studies on steroidogenesis, receptor activity, and biomarkers of effect, as well as Quantitative Structure-Activity Relationship (QSAR) modeling.**

Results: **All test compounds caused the same qualitative effects on estrogen receptor and androgen receptor activities, and most of the alternatives exhibited potencies within the same range as BPA. Hormone profiles for the compounds indicated a specific mechanism of action on steroidogenesis which generally lead to decreased androgen, and increased estrogen and progestagen levels. Differential effects on corticosteroid synthesis were observed suggesting a compound-specific mechanism. Overall, BPS was less estrogenic and antiandrogenic than BPA, but BPS showed the largest efficacy on 17α-hydroxyprogesterone (17α-OH progesterone). Finally, there were indications of DNA damage, carcinogenicity, oxidative stress, effects on metabolism, and skin sensitization of one or more of the test compounds.**

Conclusions: **Interference with the endocrine system was the predominant effect of the test compounds. A substitution of BPA with these structural analogues should be carried out with caution.**

Key words: **bisphenol A; BPA analogues; *in vitro*; QSAR; steroidogenesis.**

Bisphenol A (BPA) is a chemical used in many applications including production of polymer products such as polycarbonate plastics and epoxy resins which are used in various food contact materials (EFSA, 2006). BPA can furthermore be used as an additive (Geens *et al.*, 2011), e.g., in thermal papers for receipts (Liao and Kannan, 2011; Mendum *et al.*, 2011) and have been found in recycled paper (Geens *et al.*, 2011; Vinggaard *et al.*, 2000).

Total BPA levels (conjugated + free) have been measured in more than 90% of urine samples from the U.S. population ($n = 2517$; Calafat *et al.*, 2008) and the population of seven Asian countries ($n = 296$; Zhang *et al.*, 2011b) between 2003–2004 and 2006–2010, respectively, suggesting that the vast majority of the population is exposed. Furthermore, conjugated and/or unconjugated BPA has been detected in human amniotic fluid (Edlow *et al.*, 2012; Yamada *et al.*, 2002), umbilical cord blood (Chou *et al.*, 2011; Lee *et al.*, 2008), and placenta (Cao *et al.*, 2012; Jimenez-Diaz *et al.*, 2010) suggesting that the fetus is exposed. Diet is estimated the main source of human exposure followed by thermal paper (EFSA, 2013).

BPA has been widely investigated with respect to its toxicological hazard and has shown low-dose effects including disturbed mammary gland development (Moral *et al.*, 2008), changes in normal behavioral development (Xu *et al.*, 2010), and changes in obesity associated parameters (Miyawaki *et al.*, 2007) in rodents. BPA is well known for its estrogenic activity (Gould *et al.*, 1998; Grignard *et al.*, 2012; Kitamura *et al.*, 2005; Krishnan *et al.*, 1993; Paris *et al.*, 2002), but other mechanisms of action have also been reported such as human pregnane X receptor (PXR) agonism (Sui *et al.*, 2012), effects on steroid hormone synthesis (Zhang *et al.*, 2011a), and androgen receptor (AR) antagonism (Kitamura *et al.*, 2005; Lee *et al.*, 2003a; Paris *et al.*, 2002; Vinggaard *et al.*, 2008), all of which contribute to the overall potential of BPA to interfere with hormone systems.

In 2010, the Canadian Government prohibited the import, sale, and advertisement of polycarbonate baby bottles containing BPA (Government of Canada, 2010), and in 2011, the European Union prohibited the use of BPA in the manufacture of polycarbonate feeding bottles for infants (The European Commission, 2011). Thus, an incentive for developing alternative substances exists. Structural analogues such as bisphenol S (BPS) have already been found in canned soft drinks, canned foods (Gallart-Ayala *et al.*, 2011; Vinas *et al.*, 2010), and thermal receipt papers (Becerra and Odermatt, 2012; Liao *et al.*, 2012b). Bisphenol B (BPB) has been found in canned tomatoes (Grumetto *et al.*, 2008), canned soft drinks, and canned beers

© The Author 2014. Published by Oxford University Press on behalf of the Society of Toxicology.
All rights reserved. For permissions, please email: journals.permissions@oup.com

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

(Cunha *et al.*, 2011). In addition, BPS (free and conjugated) has been detected in 81% of urine samples from the general population collected from USA and seven Asian countries ($n = 315$; Liao *et al.*, 2012a), and BPB has been found in 28% of blood sera from Italian endometriotic women ($n = 58$; Cobellis *et al.*, 2009).

Generally limited information on the hazards of these BPA analogues is available. However, as a consequence of the structural similarity of these alternatives to BPA, there is a risk that they could lead to similar adverse effects. Thus, an urgent need for investigating the hazards of the alternatives to BPA exists. In the present study, the overall aim was to compare the hazard of BPB, bisphenol E (BPE), bisphenol F (BPF), BPS, and 4-cumylphenol (HPP) to BPA by characterizing their toxicological profiles *in silico* and *in vitro*. The toxicological profiling included acute and local effects, endocrine disruption, teratogenicity, genotoxicity, carcinogenicity, and effects on metabolism.

METHODS

In Silico *Profiling*

Two QSAR modeling tools, MultiCASE (version 2.4.1.4) and Leadscope (version 3.04-10) were applied. The individual QSAR models are described in Supplementary materials 1 and in Dybdahl *et al.* (2012), Jensen *et al.* (2008, 2011), Jonsdottir *et al.* (2012), and Vinggaard *et al.* (2008). Experimental data for compounds included in the training set of QSAR models for a given endpoint are indicated in Table 1.

MultiCASE is a statistical model system that aims to discover fragment combinations, called biophores/biophobes for active/inactive molecules, relevant for an observed effect. Further MultiCASE identifies modulators, such as physiochemical properties, which may affect the probability of a fragment being a biophore/biophobe. Warnings are given in predictions if a fragment is not represented in the training set, or if a contradictory modulator is present in a prediction. Warnings were considered an indication that the molecule was outside the model applicability domain. MultiCASE predictions are reported as positive or negative.

Leadscope uses a library of 27,000 structural features typically found in small drug molecules and eight calculated molecular descriptors for QSAR modeling. The best correlated features are selected when building a predictive model. In this study, a compound is in domain if there is ≥30% structural similarity with a training set compound and if the compound contains at least one structural feature from the model. Predictions are provided as probabilities (p) for the presence of a given effect. In this study, $p \geq 0.7$ is considered a positive prediction and $p \leq 0.3$ a negative prediction.

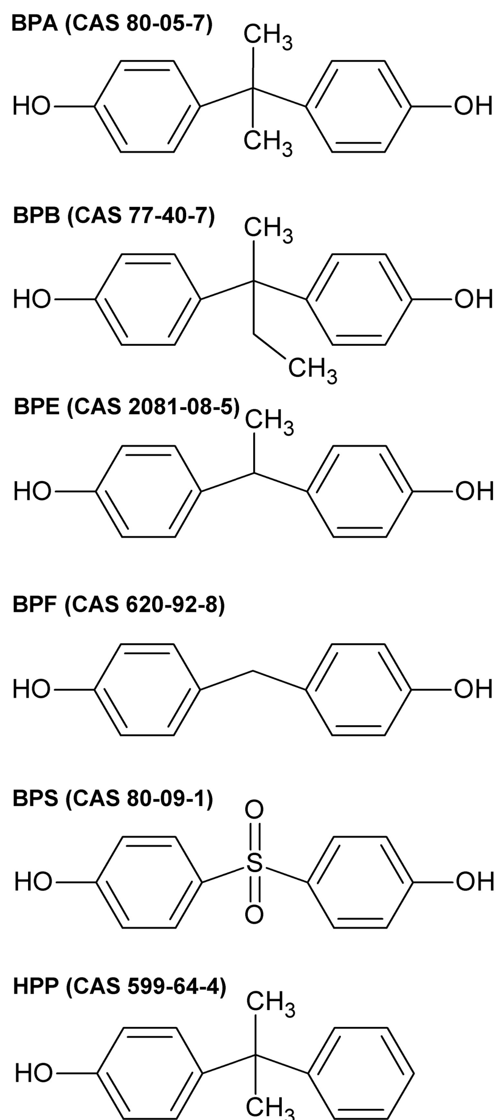


FIG. 1. Chemical structures and CAS numbers for BPA, BPB, BPE, BPF, BPS, and HPP.

In Vitro *Profiling*

Test chemicals. BPA, BPB, BPE, BPF, BPS, and HPP stock solutions of 40mM were prepared in dimethyl sulfoxide (DMSO). BPA, BPF, BPS, and HPP were purchased from Sigma-Aldrich (Copenhagen, Denmark) and BPB and BPE were purchased from VWR (Herlev, Denmark). For chemical structures and CAS numbers, see Figure 1.

H295R steroidogenesis assay. The H295R steroidogenesis assay (H295R assay) was performed to assess test compound potential to affect steroid hormone synthesis. The assay was conducted as described previously (Rosenmai *et al.*, 2013) using human adrenal cortico-carcinoma cells (ATCC, LGC standards,

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

Boras, Sweden). Prochloraz (Ehrenstorfer, Augsburg, Germany) and forskolin (Sigma-Aldrich, Brøndby, Denmark) were included as a negative and positive control, respectively. Test compounds were added in seven 2-fold dilutions in triplicates ranging from 0.8 to 50μM with a constant vehicle concentration in all wells. Cell viability was assessed in the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann, 1983) as described previously (Rosenmai *et al.*, 2013). For information on criteria for cytotoxicity and exclusion of data due to cytotoxicity for all assays see Supplementary materials 2.

Hormones were extracted from cell supernatants by solid phase extraction (SPE) as previously described (Mortensen and Pedersen, 2007; Vinggaard *et al.*, 2002). Time-resolved fluoroimmunoassays (PerkinElmer, Skovlunde, Denmark) were used for quantification of 17β-estradiol, testosterone, and progesterone in two to three H295R steroidogenesis experiments. Additionally 10 hormones were analyzed by high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)including progesterone, 17α-OH progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, dihydrotestosterone, corticosterone, cortisol, 17β-estradiol, and estrone in one experiment. Hormones were separated, identified, and quantified as previously described (Mortensen and Pedersen, 2007) with a few modifications (Rosenmai *et al.*, 2013). The limit of quantification (LOQ) was estimated as described in Rosenmai *et al.* (2013), and was 1000 pg/ml for DHEA, 500 pg/ml for dihydrotestosterone, 100 pg/ml for androstenedione and 17β-estradiol, and 50 pg/ml for the remaining hormones measured. Both methods were used to quantify testosterone and progesterone, and the results were generally in accordance. DHEA and corticosterone levels at 50μM HPP exposure as well as all dihydrotestosterone and 17β-estradiol measurements by HPLC-MS/MS were below LOQ.

Estrogen receptor (ER) reporter gene assay. Activation of ERs by test compounds were tested in a stably transfected human ovarian adenocarcinoma cell line ($BG1Luc4E_2$) which was provided by Michael Denison (University of California, USA). Experiments were generally conducted according to the Organisation for Economic Co-operation and Development (OECD) test guideline (OECD, 2012). Approximately 72 h before experiment set up, cells were transferred to estrogen-stripped medium. Successively cells were seeded (4×10^4 cells/well) ~48 h before exposure. After ~22 h exposure, luciferase activity was measured. Positive controls, 17β-estradiol (Sigma-Aldrich), and methoxychlor (ICN biomedicals, Aurora, Ohio, USA) were included in experiments. Two experiments of three to four replicates were conducted with seven 10-fold dilutions of test compounds ranging from 0.0001 to 100μM. The DMSO concentration was 0.25% in all treatment groups and 0.2% in controls. Cell viability was scored by visual evaluation.

Androgen receptor (AR) reporter gene assay. The potential of the chemicals to interfere with the activation of the AR was tested in an AR reporter gene assay as described by Vinggaard *et al.* (2002). Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection (ATCC, LGC standards, Boras, Sweden) and plasmids for receptors and reporter gene were gifts from Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). A positive and negative control, R1881 (PerkinElmer) and hydroxyflutamide (OHF) (Toronto Research Chemicals, Toronto, Canada), respectively, was included in experiments. R1881 was added at a concentration of 0.1nM on antagonism plates. Three experiments in triplicates were conducted with eight 2-fold dilutions of test compound between 0.4 and 100μM. Vehicle concentrations were constant in all wells.

Aryl hydrocarbon receptor (AhR) reporter gene assay. AhR activation leads to transcription of metabolizing enzymes (Ma, 2008). Stably transfected rat hepatoma (H4IIE-CALUX) cells provided by Dr Michael Denison (University of California, USA) were used. The assay was conducted as previously described (Laier *et al.*, 2003) with a few modifications. Cells were incubated for ~22 h in minimum essential medium (MEM) α [1% foetal bovine serum (FBS), 1% penicillin/streptomycin/fungizone (PSF)], chemical exposure was performed for 24 h, and successively luminescence was measured. Cell viability was tested as previously (Laier *et al.*, 2003), but cells were seeded at a concentration of 1.1×10^4 cells/well. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was used as a positive control. Two experiments in triplicates were conducted with nine 2-fold dilutions of test compounds ranging from 0.4 to 100μM with a constant vehicle concentration in all wells.

Nrf2, retinoic acid receptor (RAR), and p53 reporter gene assays. The Nrf2, p53, and RAR are involved in responses to oxidative stress (Motohashi and Yamamoto, 2004), responses to DNA damage (Horn and Vousden, 2007), and play a central role during embryonic development (Mark *et al.*, 2009), respectively. The BDS-CALUX reporter gene assays, developed and carried out by BioDetection Systems b.v. (Amsterdam, The Netherlands), are based on the human U2OS osteosarcoma cell line which is stably transfected with a reporter gene and a receptor, RAR assay (Van Vugt-Lussenburg et al., unpublished data) or solely a reporter gene, p53-CALUX (Van der Linden *et al.*, 2014) and Nrf2-CALUX (Van Vugt-Lussenburg et al., unpublished data).

The U2OS-CALUX cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Breda, Netherlands) with 7.5% fetal calf serum (Invitrogen), 1× nonessential amino acids (Invitrogen) and 10 U/ml penicillin and 10 μg/ml streptomycin (Invitrogen). The assays were conducted in DMEM without phenol-red (Invitrogen) supplemented with 5% dextran coated charcoal (DCC) stripped fetal calf serum,

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

and amino acids and antibiotics as above. Positive controls, actinomycin D, all-*trans*-retinoic acid, and curcumin (Sigma-Aldrich, Zwijndrecht, Netherlands) for the p53, RAR, and Nrf2 assays, respectively were included in experiments. Two independent experiments of three to six replicates were conducted with eight 2-fold dilutions of test compounds in concentrations ranging from 0.8 to 100μM with a constant vehicle concentration in all wells. 3×10^3 cells/well were seeded in 384-well plates and incubated for 24 h. After incubation, cells were exposed to test compounds for 24 h. Successively the luciferase signal was measured on a luminometer (Berthold Centro, Bad Wildbad, Germany).

Data Handling and Statistical Analysis

In vitro data were normalized to the mean of the control from the same cell plate within each experiment. Normalized residuals to means were assessed for normal distribution (D'Agostino & Pearsons omnibus test) and variance homogenicity (one-way ANOVA). All variances were homogenous. A one-way ANOVA (Dunnett's post hoc test) was conducted if all data for a specific endpoint were normally distributed. In other cases a Kruskal-Wallis test (Dunn's post hoc test) was used to assess differences between means. If the test showed significant differences between means and the same qualitative dose-response relationship in the majority of the experiments, the independent experiments and the pooled means of the experiments was fitted to a sigmoidal dose-response fit. Constraints in the curve fits were set at 1 for controls and at the maximum/minimum response for the upper/lower limit of the model for the specific compound (Hadrup *et al.*, 2013). The exposure concentration leading to 50% of the maximum response (EC_{50}) and the tentative maximum efficacy (E_{max}) values, which describe the observed maximum change in response compared with control, were predicted based on curve fits from the independent experiments. Means of these or single values are presented on tabular form dependent on the number of experiments. All data analysis was conducted in GraphPad Prism 5.

RESULTS

A broad toxicological characterization of BPA, BPB, BPE, BPF, BPS, and HPP was conducted using seven *in vitro* assays as well as predictions performed in an array of QSAR models. In Table 1, predictions based on QSAR modeling are shown. In Tables 2 and 3, predicted E_{max} and EC_{50} values are shown for *in vitro* experiments.

Endocrine Interference and Teratogenicity

Steroidogenesis was affected by all test compounds in the human adrenal cortico-carcinoma cells (Fig. 3, Table 3).

Overall, progestagen levels increased with exposure to BPB, BPE, BPF, BPS, and HPP; however, BPA and BPB did not increase progesterone levels significantly in the majority of the experiments. BPF and BPS led to the greatest E_{max} values on progesterone and 17α-OH progesterone levels, respectively.

Testosterone, androstenedione, and DHEA levels generally decreased with exposure to the test compounds. However, DHEA did not change with HPP exposure and increased in level with BPB exposure. BPA and BPE showed the most potent effect on androgens levels compared with the remaining test compounds. The EC_{50} and E_{max} values for decreased androgens ranged from 0.3 to 28.0μM and 44 to 89%, respectively (Table 3).

Generally 17β-estradiol and estrone levels were increased by test compounds though BPS did not cause an effect. BPB and HPP increased estrone levels significantly with efficacies of 1592 and 845%, respectively. EC_{50} values for both estrogens differed by less than a factor of ~3 between the test compounds.

BPA, BPB, BPS, and HPP decreased cortisol levels with E_{max} values in the same range, 72–78%, and EC_{50} values ranging by a factor ~3.5. BPF led to increased cortisol levels. BPB, BPS, and HPP led to decreased corticosterone levels; whereas BPE and BPF led to increased levels (Table 3). BPA exposure did not affect corticosterone levels.

ER activity was increased by all six compounds (Fig. 2). BPB and HPP were equally potent to BPA ($EC_{50} \sim 0.1\mu M$) (Table 2). BPE, BPF, and BPS were less potent; however, the EC_{50} values were all within a factor of ~15 to BPA. BPS was the least potent of the six compounds. E_{max} values ranged from 156 to 386%. In the QSAR models for estrogenic effects, all six compounds, except BPB, have been experimentally tested positive for ER binding and activation and were part of the training sets. BPB was predicted positive for ER binding and out of domain for ER activation.

All test compounds decreased the activity of the AR (Fig. 2). BPS only led to a decreasing trend and was not modeled. BPA, BPB, and BPF had similar EC_{50} values, whereas BPE had higher and HPP lower potency (Table 2). The EC_{50} values differed by less than a factor of ~2.7 and E_{max} values ranged from 72 to 92%. Of the tested compounds, only BPE led to an agonistic response in the AR assay with an EC_{50} of 16.0μM (SD = 2.0) and E_{max} of 169% (SD = 37; data not shown). The QSAR model for AR antagonism included BPA and BPF in the training set, which has previously shown AR antagonism experimentally. BPB and BPE were predicted positive for antiandrogenicity, while BPS and HPP were predicted negative. The most relevant biophores in the QSAR model require either substitutions on both benzene rings or one substituted benzene ring combined with an unsubstituted carbon atom in the methylene bridge, which could explain the difference in QSAR prediction and *in vitro* result for HPP.

Finally, the test compounds were predicted negative in a QSAR model for human teratogenicity (Table 1). This is in accordance with experimental data on RAR activation showing that all test compounds, except BPF, had no effect on this endpoint (data not shown).

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

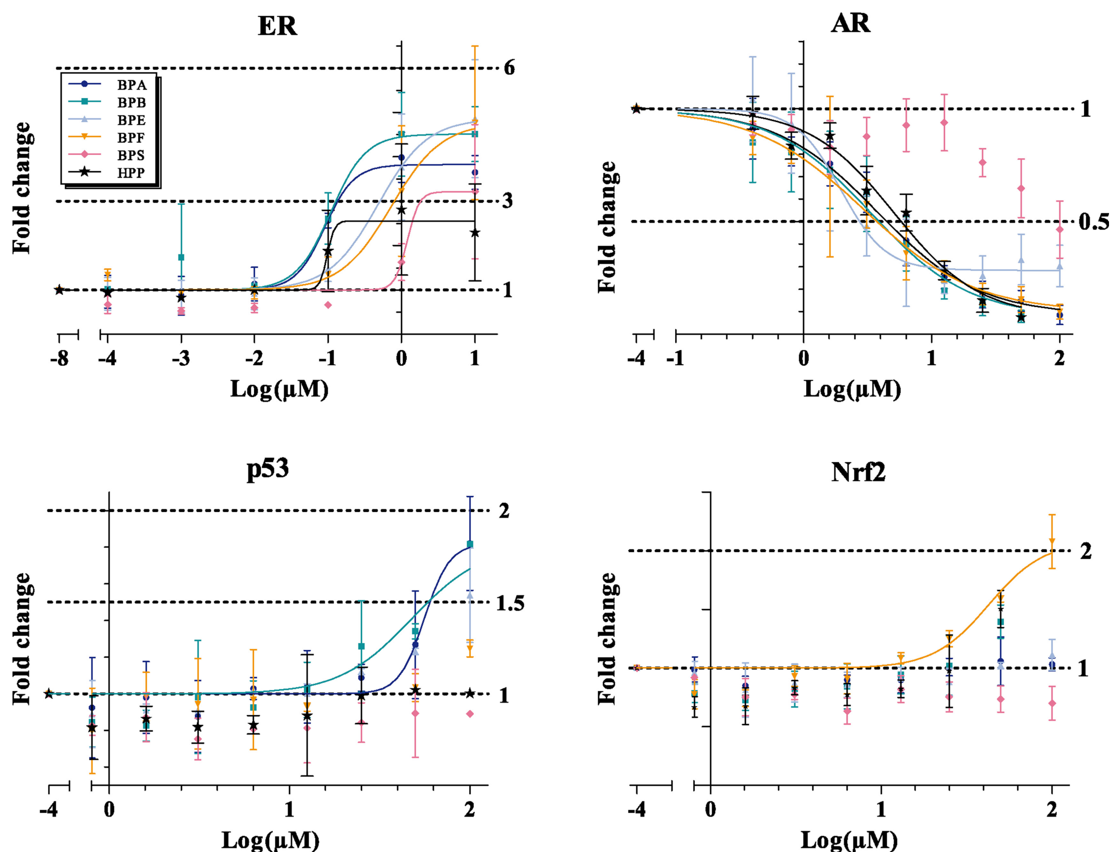


FIG. 2. ER agonism, AR antagonism, p53 agonism, and Nrf2 agonism data for BPA (dark blue), BPB (green), BPE (light blue), BPF (orange), BPS (pink), and HPP (black) shown as fold change compared with control (±SD) as a function of the logarithm to the concentration (log(μM)). Curve fits and data points are based on means of replicates from independent experiments.

Oxidative Stress, DNA Damage, and Cancer

BPF and HPP led to reporter gene activation in the Nrf2-CALUX assay (Fig. 2, Table 2), whereas the remaining compounds did not lead to changes in response.

BPA and BPB caused reporter gene activation in the p53-CALUX assay (Fig. 2, Table 2). BPE showed an increasing trend; however, this was not modeled as it was not significant in both experiments. In the QSAR models, BPA had been tested negative for several genotoxicity endpoints and was part of the training sets for these models. BPA was either predicted negative or was out of domain of the genotoxicity models which did not include BPA in the training set. The remaining compounds gave either negative predictions for genotoxicity or were out of domain of the models.

BPA has been tested experimentally and was part of the training sets for the QSAR models for rodent cancer (AF1–AF4) (Table 1). BPA was positive for cancer in male rats, marginally positive in male mice, and negative for female rats and mice. BPB and BPF were predicted positive in male mice and female mice, respectively. However, the prediction for BPB was based on only five chemicals containing the biophore of which two where tested marginally positive including BPA. The overall Research Collaboration Agreement (RCA) QSAR cancer call were negative for all compounds, except BPB which was out of domain. An estimate of the carcinogenic potency in rodents (TD_{50}) indicated very low potency (>1000 mg/kg/day) for all compounds (data not shown).

Metabolism

BPA, BPE, and BPF activated the AhR *in vitro* showing potencies ranging from 48.8 to 54.8μM. BPA led to a lower efficacy than BPE and BPF. BPB, BPS, and HPP had no effect on AhR activity (Table 2).

All compounds were predicted positive in at least one of the applied models for cytochrome P450 (CYP) substrate/inhibition (Table 1). Only robust predictions ($p \geq 0.7$ or $p \leq 0.3$) are mentioned in the following. HPP and BPB were predicted CYP2D6 substrate and inhibitor, respectively. All compounds except BPS were predicted CYP3A4 substrates, and BPS and HPP were predicted CYP2C9 substrates. All compounds except BPB were predicted negative for CYP2C9 inhibition and BPS was furthermore predicted negative for CYP2D6 inhibition. Robust predictions for PXR binding was only obtained for BPB and HPP which were predicted positive.

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

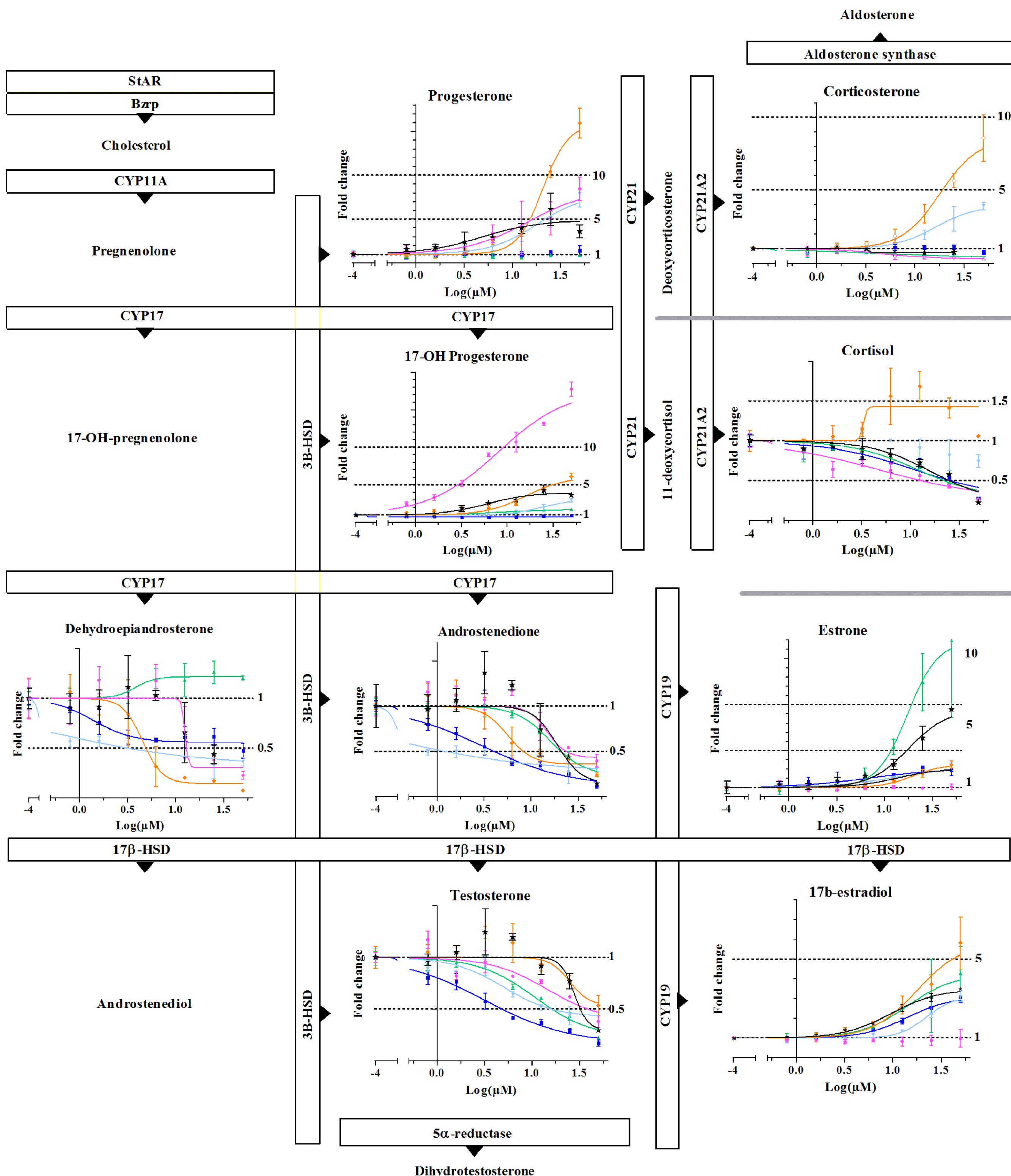


FIG. 3. Hormone profiles for BPA (dark blue), BPB (green), BPE (light blue), BPF (orange), BPS (pink), and HPP (black) from the H295R steroidogenesis assay. Data shown as fold change compared with control (±SD) as a function of the logarithm to the concentration (log(μM)). Curve fits and data points are based on means of replicates from independent experiments for 17β-estradiol analyzed by immunoassay and one experiment in triplicates for the remaining hormones analyzed by HPLC-MS/MS. Basal production of progesterone, 17α-OH progesterone, corticosterone, cortisol, DHEA, androstenedione, testosterone, and estrone were 494 ± 136, 5732 ± 1009, 185 ± 46, 3202 ± 931, 10,122 ± 4932, 45,347 ± 13,088, 1686 ± 298, and 247 ± 154 pg/ml, respectively, given as means of controls ± SD from six cell plates ($n = 18$) measured by HPLC-MS/MS.

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

TABLE 1
QSAR Predictions for Test Co

			BPA	BPB	BPE	BPF	BPS	HPP
Metabolism[a]	CYP2D6	Substrates	0.69	0.69	0.69	0.69	0.34	0.74
		Inhibitors	0.64	0.73	0.64	0.62	0.16	0.67
	CYP3A4	Substrates	0.82	0.83	0.80	0.80	0.36	0.84
		Inhibitors	0.59	0.62	0.58	0.57	0.37	0.55
	CYP2C9	Substrates	0.64	0.65	0.64	0.62	0.73	0.70
		Inhibitors	0.27	0.33	0.26	0.25	0.29	0.26
	PXR binding[b]		0.64	0.75	0.60	0.53		0.74
Endocrine disruption	ER binding[b]		*		*	*	*	*
	Estrogenicity reporter gene[b]		*		*	*	*	*
	Antiandrogen[b]		*			*		
Reprotoxicity	Teratogenicity FDA TERIS							
Genotoxicity	Ashby structural alerts for DNA reactivity		*					
	Reverse, mutation test, Ames[b]		*				*	
	Chromosomal aberrations in CHO[b]		*					
	Chromosomal aberration in CHL[b]							
	Mouse lymphoma[b]		*					
	HGPRT/CHO[b]							
	UDS Rat hepatocytes[b]							
	SHE cell transformation[b]		*					
	Rodent dominant lethal[c]							
	Drosophila melanogaster SLRL[c]		*					
	SCE Mouse[c]							
	Mouse micronucleus[c]		*					
	COMET assay[c]							
Cancer	Carcinogenicity	Male rats (AF1)	*					
		Female rats (AF2)	*					
		Male mice (AF3)	*					
		Female mice (AF4)	*					
	RCA overall QSAR call (AF1-4)							
Sensitization	Skin		*					
	Respiratory							
Irritation	Skin		*				*	

Notes. Color code: red, positive; green, negative; white, out of domain. (*) Included in the training set of the model and the experimental result is indicated.
[a]Leadscope model, $p \geq 0.7$ and $p \leq 0.3$ is a positive and negative prediction, respectively.
[b]*In vitro*.
[c]*In vivo*.

Sensitization and Skin Irritation

All six compounds were either predicted or tested positive for skin sensitization. All compounds gave negative predictions for respiratory sensitization. Training set compounds, BPA and BPS were tested negative for skin irritation, and BPB, BPF, and HPP were predicted negative.

DISCUSSION

Endocrine Interference

BPA and the five analogues showed a clear effect on AR and ER activity as well as on steroid hormone synthesis in the present study, suggesting that these compounds may interfere with the endocrine system through several modes of action. In general, we found similar qualitative effects of the test compounds on endocrine activity.

Estrogenic effects of the test compounds were observed both for ER activation and for estrogen synthesis. In the ER reporter gene assay BPA showed a similar potency as BPB and HPP. BPE, BPF, and BPS were slightly less potent than BPA. BPS showed both low potency and efficacy. Furthermore, BPS was the only compound not leading to an effect on estrogen levels in the H295R assay, suggesting BPS as the overall least estrogenic compound. BPB and HPP showed a higher efficacy on estrone levels than the remaining compounds indicating a greater overall estrogenic potential of these compounds compared with BPA.

Antiandrogenic effects were observed for the six test compounds on AR activity and androgen synthesis. BPS only showed a decreasing trend at higher concentrations on AR activity, indicating a less antiandrogenic potential of BPS compared with the remaining test compounds. Androgen levels in the H295R assay were generally decreased by all test compounds with BPA and BPE being the most potent.

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

TABLE 2

Tentative Maxi Efficacy (E_{max}) and Values for Concentrations Causing 50% of the Maximum Response (EC_{50}) for Test Compounds in Reporter Gene Assays

		BPA	BPB	BPE	BPF	BPS	HPP
ER	E_{max} (%)	**286**	**357**	**386**	**377**	**222**	**156**
	SD (%)	42	70	133	173	151	128
	EC_{50} (μM)	**0.08**	**0.12**	**0.47**	**0.82**	**1.17**	**0.10**
	SD (μM)	0.02	0.03	0.08	0.36	0.04	0.01
AR	E_{max} (%)	**91**	**92**	**72**	**89**		**92**
	SD (%)	5	3	11	6		2
	EC_{50} (μM)	**3.8**	**3.4**	**1.9**	**3.0**		**5.1**
	SD (μM)	1.3	1.8	0.9	1.5		0.7
AhR	E_{max} (%)	**47**[a]		**83**[a]	**86**		
	SD (%)				34		
	CI (%)	—		—			
	EC_{50} (μM)	**54.8**[a]		**53.0**[a]	**48.8**		
	SD (μM)				10.2		
	CI (μM)	VW		VW			
Nrf2	E_{max} (%)				**108**		**62**[a]
	SD (%)				23		
	CI (%)						—
	EC_{50} (μM)				**42.2**		**25.5**[a]
	SD (μM)				4.9		
	CI (μM)						VW
P53	E_{max} (%)	**82**	**61**				
	SD (%)	26	30				
	EC_{50} (μM)	**50.9**	**33.6**				
	SD (μM)	14.2	29.1				

Notes. E_{max} and EC_{50} values (bold) based on means of predicted values from dose-response curve fits for independent experiments. Standard deviations (SD) are shown if more than one experiment was modeled and 95% confidence intervals (CI) if one experiment was modeled. Color code: green, activation; red, deactivation; white, no significant change. VW: very wide; —: the constraints of the model were reached and thus no confidence interval could be predicted.

[a]Value based on one experiment, as other significant experiment could not be fitted to the nonlinear regression model applied.

The estrogenic and antiandrogenic potential of BPA and the remaining test compounds have been reported previously (Cabaton *et al.*, 2009; Chen *et al.*, 2002; Grignard *et al.*, 2012; Kitamura *et al.*, 2005; Kuruto-Niwa *et al.*, 2005; Rivas *et al.*, 2002; Xu *et al.*, 2005). Exposure to estrogenic and antiandrogenic chemicals have been associated with breast cancer development (Yue *et al.*, 2013) and effects on the male reproductive system (Sharpe and Skakkebaek, 2008; Skakkebaek *et al.*, 2001), respectively. Potent effects were observed on ER activity ($EC_{50} = 0.08–1.17 \mu M$), AR activity ($EC_{50} = 1.9–5.1 \mu M$) and steroid hormone levels ($EC_{50} = 0.3–28.0 \mu M$) in the present study, which are in the range of high-end urine concentrations of BPA (conjugated and free) in Danish children of ∼100nM (Frederiksen *et al.*, 2013). As the test compounds all exhibited estrogenic and antiandrogenic potential they may contribute to these types of effects.

The effects on progestagen levels differed between BPA and the remaining test compounds as BPA had little effect on these hormones whereas the remaining compounds generally led to increased progestagen levels. BPS as well as BPF led to the greatest changes in efficacy on 17α-OH progesterone and progesterone levels, respectively. Administration of a synthetic progestagen *in utero* has been associated with virilization of female mice and feminization in male mice (Willingham *et al.*, 2006). Thus, these data indicate that the five analogues, especially BPS and BPF, may have effects which are not prominent for BPA on this endpoint.

Finally, the effect on the corticosteroids differed between the six compounds. Generally BPE and BPF led to increased corticosteroid levels, whereas the remaining test compounds led to decreased levels, suggesting a compound-specific mechanism of effect which could be caused by dissimilar upstream or downstream effects. The increased corticosteroid levels observed for BPE and BPF may be of concern as such increases *in utero* have been associated with development of effects involved in the metabolic syndrome, as well as changes in behavior later in life (Drake *et al.*, 2007), and thus these compounds may be of specific concern for these endpoints.

In summary, the six test compounds generally led to increased progestagen and estrogen levels, and decreased androgen levels in the H295R assay (Fig. 3, Table 3). This suggests that the observed effects were caused by specific interactions, and were

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

TABLE 3
Te Maximum Efficacy (E_{max}) and Values for Concentrations Causing 50% of the Maximum Response (EC_{50}) for Test Compounds in the H295R Steroidogenesis Assay

		BPA	BPB	BPE	BPF	BPS	HPP
Progesterone	E_{max} (%)			**689**	**1493**	**744**	**387**
	CI (%)			—	—	—	240–513
	EC_{50} (μM)			**18.2**	**20.8**	**14.7**	**4.8**
	CI (μM)			8.8–37.9	18.4–23.5	3.4–63.5	1.9–12.0
17α-OH progesterone	E_{max} (%)	**22**	**74**	**198**	**510**	**1676**	**298**
	CI (%)	17–28	—	—	—	—	249–329
	EC_{50} (μM)	**0.004**	**8.2**	**23.0**	**16.0**	**8.0**	**6.1**
	CI (μM)	VW	4.6–14.4	14.9–35.7	10.8–23.9	5.2–12.3	4.3–8.7
Cortisol	E_{max} (%)	**73**	**72**		**43**	**74**	**78**
	CI (%)	—	—		27–59	—	—
	EC_{50} (μM)	**11.0**	**11.8**		**3.3**	**4.8**	**16.3**
	CI (μM)	2.2–55.3	5.4–25.5		VW	1.0–22.6	6.5–41.1
Corticosterone	E_{max} (%)		**66**	**292**	**757**	**70**	**30**
	CI (%)		—	—	—	—	19–32
	EC_{50} (μM)		**4.5**	**16.1**	**18.1**	**4.7**	**3.4**
	CI (μM)		0.1–145.0	10.6–24.6	13.0–25.2	2.4–9.1	VW
Dehydroandrosterone	E_{max} (%)	**44**	**22**	**68**	**86**	**70**	
	CI (%)	38–50	16–26	—	74–93	52–77	
	EC_{50} (μM)	**1.4**	**3.8**	**0.5**	**4.6**	**12.5**	
	CI (μM)	1.0–2.0	2.2–6.7	0.2–1.6	3.5–5.9	VW	
Androstenedione	E_{max} (%)	**89**	**77**	**73**	**64**	**57**	**86**
	CI (%)	—	—	—	53–75	39–61	—
	EC_{50} (μM)	**3.1**	**16.0**	**0.3**	**5.6**	**14.9**	**19.0**
	CI (μM)	2.1–4.7	12.4–20.7	0.1–0.9	4.1–7.7	9.7–22.8	11.0–32.9
Testosterone	E_{max} (%)	**83**	**78**	**58**	**47**	**62**	**70**
	CI (%)	—	—	—	—	—	—
	EC_{50} (μM)	**3.2**	**10.8**	**5.0**	**24.9**	**14.5**	**28.0**
	CI (μM)	2.1–4.8	7.2–16.2	3.4–7.3	15.0–41.5	4.5–47.0	7.9–98.8
Estrone	E_{max} (%)	**205**[a]	**1592**	**226**	**248**		**845**
	CI (%)	—	—	—	—		—
	EC_{50} (μM)	**7.2**[a]	**17.4**	**13.6**	**19.7**		**17.9**
	CI (μM)	1.7–31.3	11.0–27.5	6.2–29.7	14.0–27.7		13.0–24.6
17β-Estradiol	E_{max} (%)	**209**	**326**	**212**	**481**		**248**
	SD (%)	31	138	29	132		7
	EC_{50} (μM)	**14.0**	**13.6**	**22.2**	**17.6**		**9.7**
	SD (μM)	3.2	2.1	0.7	1.3		1.7

Notes. E_{max} and EC_{50} values (bold) based on means of predicted values from dose-response curve fits for independent experiments. Standard deviations (SD) are shown if more than one experiment was modeled and 95% confidence intervals (CI) if one experiment was modeled. Color code: green, activation; red, deactivation; white, no significant change. VW: very wide; —: the constraints of the model was reached and thus no confidence interval could be predicted.
[a]Normalized to means of controls from all cell plates within experiment.

not a result of a general down- or upregulation of steroidogenesis. The specific interactions within steroidogenesis have previously been investigated for BPA in the H295R assay (Zhang *et al.*, 2011a). BPA exposure was suggested to cause an increase in progesterone and decrease in androgen levels through inhibition of the CYP17 lyase reaction and to increase estrogen levels through inhibition of metabolism of estrogens (Zhang *et al.*, 2011a). Overall, the present study is in accordance with these findings (Table 3), suggesting that one or both of the specific interactions of BPA suggested by Zhang *et al.* (2011a) may be

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

evident for the test compounds. Differences in hormone profiles for BPA between the two studies may reflect temporal or compensatory effects as a result of differential exposure times, 24 h versus 48 h.

Oxidative Stress, DNA Damage, and Cancer

BPA and BPB increased p53 activity and BPE caused an increasing trend indicating potential to cause DNA damage. BPF and HPP led to increased Nrf2 activity indicating potential for oxidative stress (Table 2). Furthermore, BPB and BPF had positive predictions in one of the QSAR models for cancer (Table 1) in which BPA was tested experimentally positive.

In previous studies BPA has been reported to have genotoxic potential (Atkinson and Roy, 1995; Iso *et al.*, 2006; Tsutsui *et al.*, 1998), but other studies have reported negative findings for genotoxicity (Ibuki *et al.*, 2008; Lee *et al.*, 2003b). Furthermore, BPF has shown genotoxic potential in the HepG2 cells using the Comet assay (Cabaton *et al.*, 2009) and when assessing the ability to cause histone H2AX phosphorylation (Audebert *et al.*, 2011), but BPF showed no effect in the micronucleus assay (Cabaton *et al.*, 2009). These contradicting data for BPA and BPF from previous studies are in accordance with the data from the present study. To our knowledge, no data exists on the potential of the remaining test compounds to cause DNA damage or cancer. The *in vitro* assays were conducted without a metabolizing system and thus bioactivated metabolites are not assessed in this study. The authors suggest that further investigations of these compounds into their genotoxic and carcinogenic potential as well as potential to cause oxidative stress should be performed.

Metabolism

Interactions with several CYP enzymes as well as two receptors, AhR and PXR, associated with metabolism were investigated either *in silico* or *in vitro*. In the AhR reporter gene assay effects were observed at high concentrations for BPA, BPE, and BPF. All compounds were predicted positive in at least one of the QSAR models for CYP substrate recognition and inhibition, and BPB and HPP were predicted PXR binders (Table 1).

All the test compounds have previously been reported to induce activation of the human PXR receptor *in vitro* (Sui *et al.*, 2012). Thus, these data indicate that at least BPB and HPP may lead to PXR activation though binding directly to the receptor. PXR as well as AhR activation induce expression of enzymes involved not only in the metabolism of xenobiotics but also of endogenous hormones (Arlotto *et al.*, 1991; Ma, 2008; You, 2004). PXR activation has been associated with decreased androgen levels (Zhang *et al.*, 2010) and increased corticosterone and aldosterone levels (Zhai *et al.*, 2007). Thus, activation of these receptors by the test compounds may add to the overall endocrine potential by increasing or decreasing the removal of endogenous hormones *in vivo* causing disruption of homeostasis.

Metabolism of the bisphenol analogues have to our knowledge not been investigated *in vivo* previously. However, several BPA analogues were studied in river water (Ike *et al.*, 2006), pond sediments (Ike *et al.*, 2006), and seawater (Danzl *et al.*, 2009) showing that the degradation varied with BPS having the least efficient degradation compared with the remaining analogues tested. Furthermore, ToxCast data for BPA, BPB, BPS, and HPP have been released by the U.S. Environmental Protection Agency (EPA) for effects on various CYP enzymes (U.S. EPA, 2014). Comparing these data with the QSAR predictions in the present study there were some deviations in CYP enzyme inhibition and activation data. The deviations may be due to reasons related to the basis and/or the performance of the models/assays. The CYP QSAR predictions are based on human clinical data and various human *in vitro* endpoints (Jonsdottir *et al.*, 2012), whereas the ToxCast data origin from *in vitro* experiments in human cell lines. Furthermore, the criteria for classification as active/inactive for ToxCast as well as QSAR data may explain differential outcomes.

The differences in the QSAR profiles for the test compounds indicate that the metabolism of the tested compounds differs. However, further studies have to be conducted with respect to metabolism of the BPA analogues to obtain firm knowledge regarding this issue.

CONCLUSIONS

In the present study, the most marked effects were on endocrine interference. Potent effects were observed on ER activity, AR activity, and steroid hormone levels, suggesting that the compounds may act by several modes of action within the endocrine system. When comparing the compounds, BPS had the lowest estrogenic and antiandrogenic activity, but had high efficacy on progestagen levels. The remaining BPA analogues had toxicological profiles that were generally similar to BPA. Finally, several of the compounds were flagged for genotoxicity, carcinogenicity, effects on metabolism and oxidative stress. Considering these findings, substituting BPA with any of the tested analogues should be carried out with caution.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

The Ministry of Food, Agriculture, and Fisheries of Denmark and The Danish Veterinary and Food Administration.

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

ACKNOWLEDGMENT

The authors wish to thank Dorothea Gilbert, Birgitte Møller Plesning, Kitt Lademann, Dorte Lykkegaard Korsbech, Lis Abildgaard Andersen, and Liljana Petrevska for technical assistance.

REFERENCES

Arlotto, M., Trant, J., and Estabrook, R. (1991). Measurement of steroid hydroxylation reactions by high-performance liquid-chromatography as indicator of P450 identity and function. *Methods Enzymol.* **206**, 454–462.

Atkinson, A., and Roy, D. (1995). In-vivo DNA adduct formation by bisphenol-A. *Environ. Mol. Mutagen.* **26**, 60–66.

Audebert, M., Dolo, L., Perdu, E., Cravedi, J. P., and Zalko, D. (2011). Use of the gamma H2AX assay for assessing the genotoxicity of bisphenol A and bisphenol F in human cell lines. *Arch. Toxicol.* **85**, 1463–1473.

Becerra, V., and Odermatt, J. (2012). Detection and quantification of traces of bisphenol A and bisphenol S in paper samples using analytical pyrolysis-GC/MS. *Analyst* **137**, 2250–2259.

Cabaton, N., Dumont, C., Severin, I., Perdu, E., Zalko, D., Cherkaoui-Malki, M., and Chagnon, M. (2009). Genotoxic and endocrine activities of bis(hydroxyphenyl)methane (bisphenol F) and its derivatives in the HepG2 cell line. *Toxicology* **255**, 15–24.

Calafat, A. M., Ye, X., Wong, L., Reidy, J. A., and Needham, L. L. (2008). Exposure of the US population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. *Environ. Health Perspect.* **116**, 39–44.

Cao, X., Zhang, J., Goodyer, C. G., Hayward, S., Cooke, G. M., and Curran, I. H. A. (2012). Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998–2008. *Chemosphere* **89**, 505–511.

Chen, M., Ike, M., and Fujita, M. (2002). Acute toxicity, mutagenicity, and estrogenicity of bisphenol-A and other bisphenols. *Environ. Toxicol.* **17**, 80–86.

Chou, W., Chen, J., Lin, C., Chen, Y., Shih, F., and Chuang, C. (2011). Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: A birth cohort study in Taiwan. *Environ. Health* **10**, 94.

Cobellis, L., Colacurci, N., Trabucco, E., Carpentiero, C., and Grumetto, L. (2009). Measurement of bisphenol A and bisphenol B levels in human blood sera from endometriotic women. *Biomed. Chromatogr.* **23**, 1186–1190.

Cunha, S. C., Almeida, C., Mendes, E., and Fernandes, J. O. (2011). Simultaneous determination of bisphenol A and bisphenol B in beverages and powdered infant formula by dispersive liquid-liquid micro-extraction and heart-cutting multidimensional gas chromatography-mass spectrometry. *Food Addit. Contam. Part A: Chem. Anal. Control. Expo. Risk Assess.* **28**, 513–526.

Danzl, E., Sei, K., Soda, S., Ike, M., and Fujita, M. (2009). Biodegradation of bisphenol A, bisphenol F and bisphenol S in seawater. *Int. J. Environ. Res. Public Health* **6**, 1472–1484.

Drake, A. J., Tang, J. I., and Nyirenda, M. J. (2007). Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease. *Clin. Sci.* **113**, 219–232.

Dybdahl, M., Nikolov, N. G., Wedebye, E. B., Jonsdottir, S. O., and Niemelä, J. R. (2012). QSAR model for human pregnane X receptor (PXR) binding: Screening of environmental chemicals and correlations with genotoxicity, endocrine disruption and teratogenicity. *Toxicol. Appl. Pharmacol.* **262**, 301–309.

Edlow, A. G., Chen, M., Smith, N. A., Lu, C., and McElrath, T. F. (2012). Fetal bisphenol A exposure: Concentration of conjugated and unconjugated bisphenol A in amniotic fluid in the second and third trimesters. *Reprod. Toxicol.* **34**, 1–7.

EFSA. (2013). Draft scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. Part: Exposure assessment. *EFSA J.* EFSA-Q-2012-00423, 1–314.

EFSA. (2006). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the Commission related to 2,2-BIS(4-HYDROXYPHENYL)PROPANE (Bisphenol A). *EFSA J.* **428**, 1–6.

Frederiksen, H., Aksglaede, L., Sorensen, K., Nielsen, O., Main, K. M., Skakkebaek, N. E., Juul, A., and Andersson, A. M. (2013). Bisphenol A and other phenols in urine from Danish children and adolescents analyzed by isotope diluted TurboFlow-LC-MS/MS. *Int. J. Hyg. Environ. Health* **216**, 710–720.

Gallart-Ayala, H., Moyano, E., and Galceran, M. T. (2011). Analysis of bisphenols in soft drinks by on-line solid phase extraction fast liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta* **683**, 227–233.

Geens, T., Goeyens, L., and Covaci, A. (2011). Are potential sources for human exposure to bisphenol-A overlooked? *Int. J. Hyg. Environ. Health* **214**, 339–347.

Gould, J., Leonard, L., Maness, S., Wagner, B., Conner, K., Zacharewski, T., Safe, S., McDonnell, D., and Gaido, K. (1998). Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol. Cell. Endocrinol.* **142**, 203–214.

Government of Canada. (2010). *Order Amending Schedule I to the Hazardous Products Act (Bisphenol A), Part II*, **144**,7. Government of Canada. Available at: http://www.chemicalsubstanceschimiques.gc.ca/challenge-defi/batch-lot-2/bisphenol-a/bpa-risk_hazard-eng.php. Accessed February 21, 2014

Grignard, E., Lapenna, S., and Bremer, S. (2012). Weak estrogenic transcriptional activities of Bisphenol A and Bisphenol S. *Toxicol. In Vitro* **26**, 727–731.

Grumetto, L., Montesano, D., Seccia, S., Albrizio, S., and Barbato, F. (2008). Determination of bisphenol A and bisphenol B residues in canned peeled tomatoes by reversed-phase liquid chromatography. *J. Agric. Food Chem.* **56**, 10633–10637.

Hadrup, N., Taxvig, C., Pedersen, M., Nellemann, C., Hass, U., and Vinggaard, A. M. (2013). Concentration addition, independent action and generalized concentration addition models for mixture effect prediction of sex hormone synthesis in vitro. *PLoS One* **8**, 1–13.

Horn, H. F., and Vousden, K. H. (2007). Coping with stress: Multiple ways to activate p53. *Oncogene* **26**, 1306–1316.

Ibuki, Y., Tani, Y., and Toyooka, T. (2008). UVB-exposed chlorinated bisphenol A generates phosphorylated histone H2AX in human skin cells. *Chem. Res. Toxicol.* **21**, 1770–1776.

Ike, M., Chen, M., Danzl, E., Sei, K., and Fujita, M. (2006). Biodegradation of a variety of bisphenols under aerobic and anaerobic conditions. *Water Sci. Technol.* **53**, 153–159.

Iso, T., Watanabe, T., Iwamoto, T., Shimamoto, A., and Furuichi, Y. (2006). DNA damage caused by bisphenol A and estradiol through estrogenic activity. *Biol. Pharm. Bull.* **29**, 206–210.

Jensen, G. E, Niemelä, J. R., Wedebye, E. B., and Nikolov, N. G. (2008). QSAR models for reproductive toxicity and endocrine disruption in regulatory use—A preliminary investigation. *SAR QSAR Environ. Res.* **19**, 631–41.

Jensen, G. E., Nikolov, N. G., Wedebye, E. B., Ringsted, T., and Niemela, J. R. (2011). QSAR models for anti-androgenic effect—A preliminary study. *SAR QSAR Environ. Res.* **22**, 35–49.

Jimenez-Diaz, I., Zafra-Gomez, A., Ballesteros, O., Navea, N., Navalon, A., Fernandez, M. F., Olea, N., and Vilchez, J. L. (2010). Determination of bisphenol A and its chlorinated derivatives in placental tissue samples by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **878**, 3363–3369.

Jonsdottir, S. O., Ringsted, T., Nikolov, N. G., Dybdahl, M., Wedebye, E. B., and Niemela, J. R. (2012). Identification of cytochrome P450 2D6 and 2C9

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

substrates and inhibitors by QSAR analysis. *Bioorg. Med. Chem.* **20**, 2042–2053.

Kitamura, S., Suzuki, T., Sanoh, S., Kohta, R., Jinno, N., Sugihara, K., Yoshihara, S., Fujimoto, N., Watanabe, H., and Ohta, S. (2005). Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. *Toxicol. Sci.* **84**, 249–259.

Krishnan, A., Stathis, P., Permuth, S., Tokes, L., and Feldman, D. (1993). Bisphenol-A—An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279–2286.

Kuruto-Niwa, R., Nozawa, R., Miyakoshi, T., Shiozawa, T., and Terao, Y. (2005). Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. *Environ. Toxicol. Pharmacol.* **19**, 121–130.

Laier, P., Cederberg, T., Larsen, J., and Vinggaard, A. (2003). Applicability of the CALUX bioassay for screening of dioxin levels in human milk samples. *Food Addit. Contam.* **20**, 583–595.

Lee, H. J., Chattopadhyay, S., Gong, E. Y., Ahn, R. S., and Lee, K. (2003a). Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. *Toxicol. Sci.* **75**, 40–46.

Lee, M., Kwon, J., and Chung, M. (2003b). Enhanced prediction of potential rodent carcinogenicity by utilizing comet assay and apoptotic assay in combination. *Mutat. Res.* **541**, 9–19.

Lee, Y. J., Ryu, H., Kim, H., Min, C. S., Lee, J. H., Kim, E., Nam, B. H., Park, J. H., Jung, J. Y., Jang, D. D., *et al.* (2008). Maternal and fetal exposure to bisphenol A in Korea. *Reprod. Toxicol.* **25**, 413–419.

Liao, C., and Kannan, K. (2011). Widespread occurrence of bisphenol A in paper and paper products: Implications for human exposure. *Environ. Sci. Technol.* **45**, 9372–9379.

Liao, C., Liu, F., Alomirah, H., Loi, Vu Duc, Mohd, M. A., Moon, H., Nakata, H., and Kannan, K. (2012a). Bisphenol S in urine from the United States and seven Asian countries: Occurrence and human exposures. *Environ. Sci. Technol.* **46**, 6860–6866.

Liao, C., Liu, F., and Kannan, K. (2012b). Bisphenol S, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol A residues. *Environ. Sci. Technol.* **46**, 6515–6522.

Ma, Q. (2008). Xenobiotic-activated receptors: From transcription to drug metabolism to disease. *Chem. Res. Toxicol.* **21**, 1651–1671.

Mark, M., Ghyselinck, N. B., and Chambon, P. (2009). Function of retinoic acid receptors during embryonic development. *Nucl. Recept. Signal.* **7**, 1–15.

Mendum, T., Stoler, E., VanBenschoten, H., and Warner, J. C. (2011). Concentration of bisphenol A in thermal paper. *Green Chem. Lett. Rev.* **4**, 81–86.

Miyawaki, J., Sakayama, K., Kato, H., Yamamoto, H., and Masuno, H. (2007). Perinatal and postnatal exposure to bisphenol A increases adipose tissue mass and serum cholesterol level in mice. *J. Atheroscler. Thromb.* **14**, 245–252.

Moral, R., Wang, R., Russo, I. H., Lamartiniere, C. A., Pereira, J., and Russo, J. (2008). Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. *J. Endocrinol.* **196**, 101–112.

Mortensen, S. K., and Pedersen, M. (2007). Confirmatory analysis of acetylgestagens in plasma using liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **586**, 217–222.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival—Application to proliferation and cyto-toxicity assays. *J. Immunol. Methods* **65**, 55–63.

Motohashi, H., and Yamamoto, M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* **10**, 549–557.

OECD. (2012). *Test no. 457: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists*. OECD Publishing. Available at: http://www.oecd-ilibrary.org/content/book/9789264185395-en. Accessed February 21, 2014

Paris, F., Balaguer, P., Terouanne, B., Servant, N., Lacoste, C., Cravedi, J., Nicolas, J., and Sultan, C. (2002). Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines. *Mol. Cell. Endocrinol.* **193**, 43–49.

Rivas, A., Lacroix, M., Olea-Serrano, F., Laios, I., Leclercq, G., and Olea, N. (2002). Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **82**, 45–53.

Rosenmai, A. K., Nielsen, F. K., Pedersen, M., Hadrup, N., Trier, X., Christensen, J. H., and Vinggaard, A. M. (2013). Fluorochemicals used in food packaging inhibit male sex hormone synthesis. *Toxicol. Appl. Pharmacol.* **266**, 132–142.

Sharpe, R. M., and Skakkebaek, N. E. (2008). Testicular dysgenesis syndrome: Mechanistic insights and potential new downstream effects. *Fertil. Steril.* **89**, 33–38.

Skakkebaek, N. E., Rajpert-De Meyts, E., and Main, K. M. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. *Hum. Reprod.* **16**, 972–978.

Sui, Y., Ai, N., Park, S., Rios-Pilier, J., Perkins, J. T., Welsh, W. J., and Zhou, C. (2012). Bisphenol A and its analogues activate human pregnane X receptor. *Environ. Health Perspect.* **120**, 399–405.

The European Commission. (2011). Commission directive 2011/8/EU of 28 January 2011 amending Directive 2002/72/EC as regards the restriction of use of Bisphenol A in plastic infant feeding bottles. *Off. J. Eur. Union* 10-05-2013.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Takahashi, M., Maizumi, N., Yamaguchi, F., and Barrett, J. (1998). Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. *Int. J. Cancer* **75**, 290–294.

U.S. EPA. (2014). Interactive Chemical Safety for Sustainable (iCSS) Dashboard. Available at: http://actor.epa.gov/dashboard/. Accessed February 21, 2014

Van der Linden, S. C., Von Bergh, A. R. M., Van Vugt-Lussenburg, B. M. A., Jonker, L. R., Brouwer, A., Teunis, M., Krul, C. A. M., and Van der Burg, B. (2014). Development of high throughput reporter gene assays to detect genotoxicity and oxidative stress. *Mutat. Res.* **760**, 23-32.

Vinas, P., Campillo, N., Martinez-Castillo, N., and Hernandez-Cordoba, M. (2010). Comparison of two derivatization-based methods for solid-phase microextraction-gas chromatography-mass spectrometric determination of bisphenol A, bisphenol S and biphenol migrated from food cans. *Anal. Bioanal. Chem.* **397**, 115–125.

Vinggaard, A., Korner, W., Lund, K., Bolz, U., and Petersen, J. (2000). Identification and quantification of estrogenic compounds in recycled and virgin paper for household use as determined by an in vitro yeast estrogen screen and chemical analysis. *Chem. Res. Toxicol.* **13**, 1214–1222.

Vinggaard, A., Nellemann, C., Dalgaard, M., Jorgensen, E., and Andersen, H. (2002). Antiandrogenic effects in vitro and in vivo of the fungicide prochloraz. *Toxicol. Sci.* **69**, 344–353.

Vinggaard, A. M., Niemela, J., Wedebye, E. B., and Jensen, G. E. (2008). Screening of 397 chemicals and development of a quantitative structure-activity relationship model for androgen receptor antagonism. *Chem. Res. Toxicol.* **21**, 813–823.

Willingham, E., Agras, K., de Souza, A. E., Jr, Konijeti, R., Yucel, S., Rickie, W., Cunha, G. R., and Baskin, L. S. (2006). Steroid receptors and mammalian penile development: An unexpected role for progesterone receptor? *J. Urol.* **176**, 728–733.

Xu, L. C., Sun, H., Chen, J. F., Bian, Q., Qian, J., Song, L., and Wang, X. R. (2005). Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. *Toxicology* **216**, 197–203.

Xu, X., Zhang, J., Wang, Y., Ye, Y., and Luo, Q. (2010). Perinatal exposure to bisphenol-A impairs learning-memory by concomitant down-regulation of N-

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

methyl-D-aspartate receptors of hippocampus in male offspring mice. *Horm. Behav.* **58**, 326–333.

Yamada, H., Furuta, I., Kato, E. H., Kataoka, S., Usuki, Y., Kobashi, G., Sata, F., Kishi, R., and Fujimoto, S. (2002). Maternal serum and amniotic fluid bisphenol A concentrations in the early second trimester. *Reprod. Toxicol.* **16**, 735–739.

You, L. (2004). Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chem. Biol. Interact.* **147**, 233–246.

Yue, W., Yager, J. D., Wang, J., Jupe, E. R., and Santen, R. J. (2013). Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. *Steroids* **78**, 161–170.

Zhai, Y., Pai, H. V., Zhou, J., Amico, J. A., Vollmer, R. R., and Xie, W. (2007). Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. *Mol. Endocrinol.* **21**, 138–147.

Zhang, B., Cheng, Q., Ou, Z., Lee, J. H., Xu, M., Kochhar, U., Ren, S., Huang, M., Pflug, B. R., and Xie, W. (2010). Pregnane X receptor as a therapeutic target to inhibit androgen activity. *Endocrinology* **151**, 5721–5729.

Zhang, X., Chang, H., Wiseman, S., He, Y., Higley, E., Jones, P., Wong, C. K. C., Al-Khedhairy, A., Giesy, J. P., and Hecker, M. (2011a). Bisphenol A disrupts steroidogenesis in human H295R cells. *Toxicol. Sci.* **121**, 320–327.

Zhang, Z., Alomirah, H., Cho, H., Li, Y., Liao, C., Minh, Tu Binh, Mohd, M. A., Nakata, H., Ren, N., and Kannan, K. (2011b). Urinary bisphenol A concentrations and their implications for human exposure in several Asian countries. *Environ. Sci. Technol.* **45**, 7044–7050.

Downloaded from http://toxsci.oxfordjournals.org/ at DTU Library on October 30, 2014

9.3 Manuscript 3

Rosenmai, A.K., Trier, X., Taxvig, C., van Vugt-Lussenburg, B.M.A., and Vinggaard, A.M. (2014). Fluorinated compounds and technical mixtures for use in food contact materials have estrogenic activity in an *in vitro* screening. Manuscript in preparation.

Comments to manuscript

We are still in the process of conducting additional experiments to verify some of the findings described in the paper. These additional experiments include,

- Cell viability tests:
  In a few cases we observed increased response in the cell viability test with exposure to test compounds. We are conducting additional cell viability measurements to shed light on this issue and these data will be included in the final Manuscript.

- H295R experiments:
  We obtained inconclusive results on 17β-estradiol levels with 6:2 FTOH exposure and testosterone levels with TM3 exposure in the H295R steroidogenesis assay. Additional experiments are being conducted to obtain conclusive results, which will be included in the final Manuscript.

1 **Fluorinated compounds and technical mixtures for use in food contact materials**
2 **have estrogenic activity *in vitro***

3 Anna Kjerstine Rosenmai[a], Xenia Trier[b], Camilla Taxvig[a], Barbara Medea Alice van Vugt-
4 Lussenburg[c] & Anne Marie Vinggaard[a]

5 *[a]Division of Toxicology & Risk Assessment, National Food Institute, Technical University of*
6 *Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*
7 *[b]Division of Food Chemistry, National Food Institute, Technical University of Denmark, DK-2860*
8 *Søborg, Denmark*
9 *[c]BioDetection Systems b.v., Science Park 406, 1098 XH Amsterdam, The Netherlands*

10 **Corresponding Author**
11 Anne Marie Vinggaard

12 annv@food.dtu.dk

13 Division of Toxicology & Risk Assessment
14 National Food Institute
15 Technical University of Denmark
16 Mørkhøj Bygade 19
17 DK-2860 Søborg
18 Denmark

19 Phone + 45 35 88 75 49

20 **Running Title**
21 *In vitro* toxicological screening of nineteen fluorine-containing compounds and technical mixtures

22 **Abstract**

23 Food packaging of paper and board often contain numerous chemicals with potential to contaminate
24 food and thus can represent a route for human chemical exposure. Fluorinated compounds are used
25 in technical mixtures (TMs) intended for food packaging due to their repellency properties, but the
26 fluorinated constituent of TMs are often not known. Polyfluorinated alkyl phosphate ester
27 surfactants (PAPs) are known constituents that can metabolize into perfluoroalkyl carboxylic acids
28 (PFCAs) and fluorotelomer alcohols (FTOHs). Humans are exposed to fluorinated compounds,
29 which are readily detectable in serum, but also in umbilical cord blood, suggesting foetal exposure.
30 Foetal exposure to endocrine disrupting chemicals may adversely affect normal development,
31 placing focus on fluorinated compounds as some have potential to interfere with hormone systems.

32 In this study, we aimed at gaining further knowledge on the endocrine activity of fluorinated
33 compounds including short chained PFCAs and three TMs, for which little toxicological knowledge
34 is publically available. We also analysed several PAPs, FTOHs, and longer chained PFCAs by
35 applying a broad panel of *in vitro* assays such as ER, AR, and AhR reporter gene assays and H295R
36 steroidogenesis assay.

37 Overall, the main effects were estrogenic. We found that some PAPs, FTOHs, and TMs were
38 estrogenic either through activation of the estrogen receptor and/or increasing 17β-estradiol levels
39 *in vitro*. Conversely, short chained PFCAs did not cause any discernible effects. As PAPs of longer
40 chains showed endocrine activity, future studies should include short chained PAPs and a further
41 investigation of the tested TMs is advised.

42 **Keywords**

43 Fluorinated compounds, polyfluorinated alkyl phosphate ester surfactants, perfluoroalkyl carboxylic
44 acids, fluorotelomer alcohols, estrogenicity, endocrine activity, steroidogenesis

45 **Introduction**

46 In modern society the road from food production to consumption can be long and complex. It
47 involves multiple steps, many of which have the potential to contaminate the food source with
48 unwanted substances. Food packaging materials are potential sources of such contamination
49 (Borchers et al. 2010). For example, food packaging of paper and board can contain a complex
50 mixture of chemicals, some of which stem from the material itself and others originating from
51 added printing inks, adhesives, and coatings. If these chemicals migrate to the food source, there is
52 a potential risk for human exposure and downstream health effects.

53 Among the chemicals used in food packaging materials of paper and board are fluorinated
54 compounds, which are typically added to technical mixtures (TMs) because of their ability to repel
55 water and oils (Kissa 2001). Polyfluorinated alkyl phosphate ester surfactants (PAPs) have been
56 used in such TMs (Trier et al. 2011), but TMs containing other fluorinated compounds are also
57 available on the market. The specific chemicals structures of the fluorinated constituents of some of
58 these TMs are unknown, which pose particular challenges with respect to assessing toxicity and
59 exposure.

60 PAPs can metabolize into the final metabolites perfluoroalkyl carboxylic acids (PFCAs), including
61 perfluorooctanoic acid (PFOA), likely through the intermediate metabolites, fluorotelomer alcohols
62 (FTOHs) (Figure 1) (D'eon and Mabury 2007; D'eon and Mabury 2011). Together with PAPs,
63 FTOHs and PFCAs may also be present in TMs as residuals (Prevedouros et al. 2006), and thus
64 potentially constituting a direct and indirect source of exposure. Indeed, both PAPs and the final
65 metabolites have been detected in human sera/plasma (Calafat et al. 2007; D'eon et al. 2009; Houde
66 et al. 2006; Olsen et al. 2012) and breast milk (Kubwabo et al. 2013; So et al. 2006), with PFCAs
67 also detected in umbilical cord blood (Kim et al. 2011; Monroy et al. 2008). Thus, humans may be
68 exposed to fluorinated chemicals in foetal, neonatal, as well as adult life. This is of general concern
69 considering the long serum half-life of PFOA in humans (Olsen et al. 2007), and of particular
70 concern in relation to foetal exposure, as endocrine disrupting chemicals may contribute to
71 disrupted development culminating in compromised health at birth or later in life (Skakkebaek et al.
72 2001).

73 The reported effects of PFOA exposure are many, including increased incidence of liver, pancreatic,
74 and testicular tumours, as well as liver toxicity in animal studies (Lau 2012). Fluorinated

75 compounds can also interfere with the endocrine system, including increased serum/plasma 17β-
76 estradiol levels following exposure to some PFCAs and FTOHs (Biegel et al. 1995; Biegel et al.
77 2001; Cook et al. 1992; Feng et al. 2009; Liu et al. 2009; Liu et al. 2010a) and decreased
78 testosterone levels following exposure to some PFCAs *in vivo* (Bookstaff et al. 1990; Cook et al.
79 1992; Shi et al. 2010). Finally, exposure to PAPs can result in increased estrogen and decreased
80 androgen levels *in vitro* (Rosenmai et al. 2013). Notably, these studies typically analysed longer
81 chained fluorinated compounds, and the potential for endocrine disruption of the shorter chained
82 PFCAs and fluorinated constituents of some TMs remain largely unknown.

83 In this study, we sought to gain further insight into the potential of fluorinated compounds to
84 interfere with the endocrine system. We chose a broad screening approach, as available data on
85 toxicity is very limited with regard to shorter chained PFCAs and fluorinated constituents in some
86 TMs. We analysed the effects of three TMs, PFCA of chain lengths 4-12, three FTOHs and four
87 PAPs, which also enabled comparison between toxicological profiles of substances. Through
88 various *in vitro* assays, we find effects on several endpoints, including estrogen receptor (ER) and
89 aryl hydrocarbon receptor (AhR) activity, as well as estrogen levels. Overall, the FTOHs, PAPs and
90 TMs showed some endocrine activity, whereas the shorter chained PFCAs did not cause any
91 discernible effects. Finally, we also analysed the potential of the fluorinated compounds and TMs to
92 affect receptors and biomarkers involved in oxidative stress (Motohashi and Yamamoto 2004),
93 genotoxicity (Horn and Vousden 2007), and embryonic development (Mark et al. 2009), but
94 observed no effects.

95 Materials and Methods

96 *Test compounds and technical mixtures*

97 All compounds used in the study are listed in Table 1 together with their respective CAS numbers
98 and reported purity. In addition, three commercially available fluorine-based technical mixtures
99 (TM) for use in food contact materials were tested and are denoted TM1, TM2, and TM3,
100 corresponding to Solvera PT5045 (fluoropolyether ammonium phosphate salt, Solvey Solexis,
101 Italy), Capstone (fluorinated acrylic cationic copolymer, Du Pont de Nemeurs, The Netherlands),
102 and Cartafluor CFI (fluorinated acrylic cationic copolymer, Clariant presently owned by Archroma,
103 Austria), respectively.

104 Stock solutions were prepared by dissolving PFdoDA and TM3 in ethanol, TM1 and TM2 in H_2O,
105 and all other compounds in dimethyl sulfoxide (DMSO). Concentrations of stock solutions were 20
106 mM for PAPs and 40 mM for all remaining compounds. TMs were of unknown concentration.
107 Stated intervals of compounds for example PFBA-PFHpA, denotes an interval of chain lengths, in
108 this case, the compounds PFBA, PFPA, PFHxA, and PFHpA.

109 *Steroidogenesis assay*

110 The steroidogenesis assay was performed using the NCI-H295R human adrenal cortico-carcinoma
111 cell line (ATCC, LGC standards, Boras, Sweden) as described previously (Rosenmai et al. 2013).
112 Positive controls were included in all experiments to assure assay performance (see (Rosenmai et al.
113 2014) for control specifications). Extraction and quantification of progesterone, testosterone, and
114 17β-estradiol (Time-resolved fluoroimmunoassays, PerkinElmer, Skovlunde, Denmark), as well as
115 cell viability assessment was performed as previously described (Rosenmai et al. 2013; Vinggaard
116 et al. 2002). Compounds causing increased 17β-estradiol levels were tested for immunoassay
117 interference and showed no interference (data not shown).

118 TMs, 6:2 FTOH, 4:2 FTOH, PFBA-PFHpA, and PFNA-PFdoDA were tested in triplicate reactions
119 in 2-5 experiments. Test compounds were tested at a maximum concentration of 50 μM and TMs
120 were tested in a maximum concentration of 0.25% of original material across seven two-fold
121 dilutions. Vehicle concentrations were constant within each cell plate. Testosterone and 17β-
122 estradiol levels were quantified for PFBA-PFHpA, 6:2 FTOH, and 4:2 FTOH, whereas all
123 hormones were quantified for PFNA-PFdoDA and the TMs.

124 *Reporter gene assays*

125 The estrogen receptor (ER), androgen receptor (AR), and aryl hydrocarbon receptor (AhR) reporter
126 gene assays were conducted as previously described (Laier et al. 2003; Rosenmai et al. 2014;
127 Vinggaard et al. 2002) with a few exceptions. The retinoic acid receptor (RAR), nuclear factor
128 (erythroid-derived 2)-like 2 (nrf2), p53, and glucocorticoid receptor (GR) CALUX reporter gene
129 assays were constructed by and performed at BioDetection Systems essentially as described
130 previously (Piersma et al. 2013; Rosenmai et al. 2014).

131 The stably transfected human ovarian adenocarcinoma cell line, BG1Luc4E2 (ER) (gift from Dr.
132 Michael Denison, University of California, USA), Chinese hamster ovary cells (AR) (ATCC, LGC

133 standards, Boras, Sweden), stably transfected rat hepatoma cells, H4IIE-CALUX (AhR) (gift from
134 Dr. Michael Denison, University of California, USA), and stably transfected human U2OS
135 osteosarcoma cell line (RAR CALUX, nrf2 CALUX, p53 CALUX, GR CALUX (BioDetection
136 Systems, The Netherlands)) were used. All assays and experiments were conducted with positive
137 controls to assure assay performance. The positive control was dexamethasone in the GR CALUX
138 reporter gene assay and specification for controls in the remaining reporter gene assays are given in
139 Rosenmai et al. (2014). The AR reporter gene assay was conducted in antagonist mode and thus
140 cells were co-exposed with 0.066 nM or 0.1 nM R1881. Plasmids for receptors and reporter gene
141 for transfection in the AR reporter gene assay were kind gifts from Albert Brinkmann (Erasmus
142 University, Rotterdam, The Netherlands). In the ER reporter gene assay, cell viability was assessed
143 by the previously described visual evaluation (Rosenmai et al. 2014) or by addition of resazurin at
144 3.3 µg/mL in wells, incubation for three hours, and fluorescence measurement.

145 All test compounds and TMs were tested in the ER and AhR reporter gene assays. TMs, 6:2 FTOH,
146 4:2 FTOH, and all PFCAs excluding PFOA were tested in the AR reporter gene assay and PFOA-
147 PFdoDA were tested in the nrf2 CALUX, p53 CALUX, RAR CALUX, and GR CALUX reporter
148 gene assays.

149 All assays were performed in 2-5 experiments in 2-6 replicate reactions across 7-10 concentrations.
150 The dilution factor was two in all reporter gene assays for all test compounds and TMs, with the
151 exception of a dilution factor of ten for test compounds in the ER reporter gene assay. The
152 maximum tested concentration for TM1 and TM2 was 0.25% of original material and 100 µM for
153 all PFCAs and FTOHs. A 1 mg/µL EtOH solution of TM3 was prepared, which was diluted by a
154 factor 400 for the tested maximum concentration in the applied assays. The maximum tested
155 concentration for PAPs was 100 µM in the ER reporter gene assay and 50 µM in the AR reporter
156 gene assays. The maximum tested concentration for the diPAPs was 25 µM and for tri- and
157 monoPAPs it was 50 µM in the AhR reporter gene assay. Vehicle concentrations were constant in
158 all wells within each experiment in the AR, AhR, RAR CALUX, GR CALUX, nrf2 CALUX, and
159 p53 CALUX reporter gene assays. In the ER reporter gene assays the vehicle concentration was
160 0.2-0.25% in all wells, except for PAPs for which vehicle was 0.5% but only at the maximum tested
161 concentration.

162 *Data processing and statistical analysis*

163 All statistical analysis was conducted on individual experiments. Within each experiment replicates
164 were normalized to the mean of the controls. Residuals to mean within each exposure group were
165 tested for whether means of these were significantly different by conducting a one-way ANOVA.
166 Further, residuals to mean within each exposure group were pooled and tested for normal
167 distribution by use of the D'agostino Pearson's Omnibus test. If pooled residuals were normally
168 distributed, a one-way ANOVA (post-test Dunnett) was performed. A Kruskal Wallis (post-test
169 Dunn) was performed on normalized data if pooled residuals were not normally distributed.

170 If the post-test led to a significant dose-dependent effect in the majority of experiments in either the
171 p53 CALUX, nrf2 CALUX, GR CALUX, RAR CALUX, AhR, AR, and ER reporter gene assays,
172 this was perceived as the dominant effect. If cytotoxicity was observed, the data was removed from
173 the dataset and if a significant increase was observed in cell viability this was reported in Table 2 if
174 this effect could not be ignored as a chance finding. In the steroidogenesis assay, effects were
175 reported if the majority of experiments showed significant changes in the post-test in response to
176 increasing exposure concentration, if these effects did not occur at concentrations leading to
177 statistically significant cytotoxicity.

178 The qualitative effects are reported in Table 2 and one representative experiment is shown for some
179 endpoints in which statistically significant effects were observed in the majority of experiments in
180 Figure 2, 3, and 4. All data processing and statistical analyses were performed in GraphPad Prism 5.

181 **Results**

182 *Effects on steroidogenesis*

183 TM2 exposure led to a significant increase in 17β-estradiol levels (Table 2, Figure 2), but no effects
184 were observed on testosterone and progesterone. Perfluoroalkyl carboxylic acids of chain lengths
185 10-12 (PFDA-PFdoDA), all led to increased 17β-estradiol levels. This increase was accompanied
186 by an increased response in one or all experiments of the cell viability assays. PFDA-PFdoDA did
187 not affect progesterone and testosterone levels with increasing exposure and PFBA-PFHpA, PFNA,
188 TM1, and 4:2 FTOH did not affect any of the hormones measured. 6:2 FTOH did not lead to
189 changes in testosterone levels and the effect on 17β-estradiol was inconclusive as noted in Table 2,
190 as two independent experiments showed significantly increased 17β-estradiol levels, but only one
191 experiments followed a classical dose-response relationship. TM3 caused no significant change in
192 progesterone and 17β-estradiol levels, but led to a decreasing trend in testosterone levels in both

193 experiments. This decrease was only significant in one experiment and thus results on testosterone
194 were deemed inconclusive in Table 2.

195 *ER activity*

196 TM2, TM3, 4:2 FTOH, 6:2 FTOH, 8:2 FTOH, and 8:2 monoPAPs led to increased activity in the
197 ER reporter gene assay (Table 2, Figure 3). For PFOA and PFNA an increased response was seen in
198 the ER reporter gene assay, which was accompanied by an increased response in the cell viability
199 assay in some cases (Table 2). TM1, 8:2 triPAPs, 8:2 diPAPs, 10:2 diPAPs, PFBA-PFHpA and
200 PFDA-PFdoDA exposure did not affect ER activity.

201 *AR antagonism*

202 A decreased response was observed with exposure to some test compounds and TMs in the AR
203 reporter gene assay, but in all cases lowered response was accompanied by cytotoxicity, and the
204 effect was attributed to compromised cell viability; stated as 'no effect' in Table 2.

205 *AhR activity*

206 8:2 triPAPs and 8:2 monoPAPs caused an increased AhR activity (Table 2, Figure 4). None of the
207 remaining compounds led to changes in activity of the receptor.

208 *GR CALUX, nrf2 CALUX, p53 CALUX and RAR CALUX reporter gene assays*

209 PFOA-PFdoDA did not lead to increased activity of the GR and RAR and did not lead to increased
210 levels of nrf2 and p53 in the reporter gene assays (data not shown).

211 **Discussion**

212 We have analysed the endocrine activity of a selection of fluorinated compounds including; i)
213 compounds used in food packaging materials (PAPs), ii) compounds occurring as metabolic product
214 of the functional coating ingredient or occurring as impurities in some TMs (FTOH and PFCAs),
215 and iii) three different commercially available TMs containing fluorinated compounds of unknown
216 chemical composition. An overview of major results for PAPs, FTOHs, and PFCAs are shown in
217 Figure 1. We tested for a variety of endocrine activities, including estrogenicity and
218 antiandrogenicity. Overall, the most prominent effect was estrogenicity, either by causing increased
219 17β-estradiol levels or increased ER activity.

220 *Estrogenicity of perfluorinated alkyl carboxylic acids and fluorotelomer alcohols*

221 Previous studies have shown that PFOA and PFNA exposure can cause increased serum 17β-
222 estradiol levels *in vivo* (Biegel et al. 1995; Biegel et al. 2001; Cook et al. 1992; Feng et al. 2009)
223 and *in vitro* (Kraugerud et al. 2011; Rosenmai et al. 2013). We observed increased 17β-estradiol
224 levels following PFDA-PFdoDA exposure, but PFNA did not cause an effect. PFOA and PFNA
225 stimulated ER transactivation in our ER reporter gene assay, whereas PFDA-PFdoDA did not cause
226 any effects. PFOA-PFdoDA were tested previously for ER mediated transactivation of a reporter
227 protein *in vitro*, in which only PFOA caused an effect (Kjeldsen and Bonefeld-Jorgensen 2013).
228 Thus, both studies suggest that PFOA can affect ER activity *in vitro*, whereas PFDA-PFdoDA
229 cannot. Notably, we observed divergent results for PFNA compared to that reported previously *in*
230 *vitro* on both ER activity and effects on 17β-estradiol levels. Although we have no clear explanation
231 for this, the use of different cell-lines or exposure regimes can be a parameter that has influenced
232 the output. Finally, an increase in response in the cell viability assay was observed occasionally in
233 both the ER reporter gene assay and the steroidogenesis assay, which may indicate stimulation of
234 cell proliferation or increased mitochondrial activity. Therefore, it is difficult to judge whether the
235 increased 17β-estradiol levels and ER activity are caused by direct interference with the endpoint of
236 interest, increased cells numbers or interference with mitochondria. Regardless, either modality
237 could be relevant *in vivo*, as the net result may be increased 17β-estradiol in the serum or increased
238 ER activity.

239 6:2 and 8:2 FTOH has shown estrogenic potential in the breast cancer cell line MCF-7 (Maras et al.
240 2006), which is in accordance with increased ER activity with 4:2, 6:2, and 8:2 FTOH exposure in
241 our study. These compounds further increased plasma 17β-estradiol levels *in vivo* (Liu et al. 2009;
242 Liu et al. 2010a) and *in vitro* for 8:2 FTOH (Rosenmai et al. 2013). However, 6:2 FTOH exposure
243 led to inconclusive results regarding 17β-estradiol levels, thus previous findings could not be
244 confirmed. Collectively however, the presented data together with previously published results
245 strongly suggests that most of the tested fluorinated compounds exert estrogenic effects.

246 *Antiandrogenicity of perfluorinated alkyl carboxylic acids and fluorotelomer alcohols*

247 Fluorinated compounds can exert antiandrogenic effects *in vivo* by decreasing serum/plasma
248 testosterone levels following exposure to PFOA (Cook et al. 1992), PFDA (Bookstaff et al. 1990),
249 and PFdoDA (Shi et al. 2009). We observed no decrease in testosterone *in vitro*, suggesting that the

250 *in vivo* effects are not caused by a direct interference with the intracellular steroidogenic pathway.
251 The *in vivo* studies report on decreased body weight, where Bookstaff et al. (1990) and Shi et al.
252 (2009) can establish effects on testosterone levels either at concentrations below that leading to
253 decreased body weight or by comparing to pair-fed controls, whereas Cook et al. (1992) observe a
254 significant decreasing trend, which cannot be established when compared to controls. The latter
255 study implies general toxicity with lowered testosterone as a function thereof, rather than directly
256 by antiandrogenic action of the compounds (Cook et al. 1992). As opposed to a previous study
257 (Kjeldsen and Bonefeld-Jorgensen 2013), we observed no AR antagonism upon PFOA-PFDA
258 exposure despite the experimental conditions being very similar. We speculate that this can be due
259 to differences in the agonists applied on the antagonist plates, the cell concentrations, and/or the
260 amount transfected of reporter vector and receptor plasmids.

261 It has been shown that 6:2 FTOH and 8:2 FTOH exposure *in vivo* increases testosterone levels in
262 females, whereas in males an increase is observed with 6:2 FTOH, whereas 8:2 FTOH cause a
263 decrease in testosterone (Liu et al. 2009; Liu et al. 2010a). The male findings are in line with other
264 results published with 8:2 FTOH exposure (Liu et al. 2010b; Rosenmai et al. 2013). However, we
265 detected no effect on testosterone levels by 6:2 FTOH in the present study. Collectively, the
266 presented data as well as data from previous studies on antiandrogenicity of the tested fluorinated
267 compounds are not completely in line and thus the mechanism behind an antiandrogenic effect
268 remains unclear.

269 *Comparison of endocrine activity of PAPs, FTOH, and PFCAs*

270 As mentioned, PAPs can metabolize *in vivo* into the final metabolites PFCAs, possibly through the
271 intermediate metabolite FTOH (D'eon and Mabury 2007; D'eon and Mabury 2011). Thus, our study
272 allowed comparison of effects across groups of compounds and assays to gain further insight into
273 mechanism-of-action and overall endocrine activity.

274 The mechanism of action causing altered hormone levels in the steroidogenesis assay is clearly
275 different between PAPs (shown previously in (Rosenmai et al. 2013)), FTOHs, and PFCAs (Table
276 2). Overall, PAPs lead to decreased androgen and progestagen levels, whereas 4:2 FTOH, 6:2
277 FTOH, and all the tested PFCAs do not. It is likely that the effect of PAPs is caused by a direct
278 effect on steroidogenesis, as indicated by altered mRNA expression of key steroidogenic genes by
279 some PAPs (Rosenmai et al. 2013). Although mRNA levels were not measured in this study, the

280 hormone data for PFDA-PFdoDA indicate a different mechanism, possibly involving interference
281 with CYP19 or 17β-estradiol clearance, both of which could lead to the observed increased estrogen
282 levels.

283 8:2 monoPAPs, FTOHs, PFOA, and PFNA harbour the potential to increase ER activity in the ER
284 reporter gene assay (Table 2). This highlights three points: 1) as none of the tri- or di-alkylated
285 PAPs caused any effect, it indicates that the degree of alkylation may play a role in ER activity. 2)
286 8:2 monoPAPs may contribute significantly to estrogenicity as parent compound (8:2 monoPAPs),
287 intermediate metabolite (8:2 FTOH), and final metabolite (PFOA and PFNA), all led to ER activity.
288 3) short chained FTOH may be an important contributor to effects exerted by short chained
289 fluorinated compounds, as 6:2 FTOH and 4:2 FTOH led to activity, whereas none of the short
290 chained PFCAs did. These points are only valid if test compounds did not metabolize within the
291 cells, as the effects then could be caused by metabolites and not the test compound *per se*. However,
292 as the tri- and di-alkylated PAPs did not cause any effects, it is unlikely that PAPs dealkylated into
293 FTOH in the cells. Likewise, since the shorter chained PFCAs did not activate the ER, but their
294 upstream metabolites 4:2 and 6:2 FTOH did, it is also unlikely that the FTOHs metabolized into
295 PFCAs.

296 The AhR plays a role in regulation of several enzymes involved in metabolism of xenobiotics (Ma
297 2008). As 8:2 triPAPs and 8:2 monoPAPs activated the AhR, this may add to the overall endocrine
298 activity of these compounds, as it could affect the clearance rate of compounds exerting effects.

299 From this and a previous study (Rosenmai et al. 2013), it becomes clear that the mechanism and
300 pathway of endocrine effects of the PAPs, FTOH, and PFCAs is different. Overall, PAPs induce
301 effects across several endpoints, and are thus more potent than the FTOHs and PFCAs. However,
302 these metabolites also cause effects, which may lead to greater endocrine disruptive potential of
303 PAPs, as metabolism will not deactivate the compound, but rather form other active compounds.

304 *Estrogenicity of technical mixtures – what is next?*

305 The three TMs that we tested are all commercially available and intended for coating of food
306 packaging of paper and board. However, information on the specific fluorinated chemical structures
307 of the TMs, fluorinated impurities, or other chemicals for that matter, is not publically available or
308 is very difficult to find. The characterization of impurities of the TMs is in progress (Trier &

309 Eschauzier, Manuscript in preparation). These data gaps limit our ability to conclude on the active
310 compounds causing the effects, but it does not exclude the fluorinated constituents as being
311 causative agents.

312 Both TM2 and TM3 showed estrogenic potential by affecting 17β-estradiol levels and/or ER
313 activity. As no effect was observed on testosterone or progesterone levels, it is unlikely that the
314 dominating fluorinated constituents in the mixtures exhibit similar activities as PAPs. On the other
315 hand, it is possible that impurities such as FTOHs and PFCAs are present in the TMs and thus
316 contribute to the effects, as these also affected ER activity and 17β-estradiol levels. As we have
317 limited knowledge about the concentrations of such impurities, it is only possible to speculate on
318 such contributions. Regardless, the fact that the TMs show estrogenic potential warrants further
319 investigations into composition of the mixture. It would also be informative to study the ability of
320 the specific fluorinated compounds to migrate into foods to obtain further information regarding
321 exposure risk.

322 *Is short-chained chemistry a better alternative?*

323 To our knowledge little is known about the endocrine activity of shorter-chained fluorinated
324 compounds. As the industry is shifting towards such compounds (Scheringer et al. 2014), which
325 potentially metabolize into short-chained PFCAs, we also included PFBA-PFHpA in our study.

326 In view of hazard potentials, we would deem PFCAs with chain lengths of 4-7 (PFBA-PFHpA)
327 better alternatives than the remaining test compounds, as they caused no effects in any of the
328 applied assays. However, we need to acknowledge that the compounds were tested *in vitro*, which
329 has certain limitations. Furthermore, as the tested PAPs had a greater endocrine activity overall than
330 the PFCAs, we need to include shorter-chained PAPs in future investigations to assess, if these also
331 have greater potential than their final metabolites.

332 *Genotoxicity and oxidative stress*

333 PFOA has previously been reported to increase the incidence of several types of tumours in animal
334 models (Lau 2012). Thus, we included two tests, nrf2 CALUX and p53 CALUX reporter gene
335 assays, to assess the ability to cause oxidative stress and genotoxicity, respectively. As we observed
336 no effects with test compound and TM exposure, this study provides no further evidence for the
337 potential of genotoxic effects or oxidative stress.

Summary

We have shown that fluorinated compounds can be estrogenic and that parent compounds seem to be more potent than their metabolites. The compounds likely act through different mechanisms of action and target several pathways, which may add to the overall potential for endocrine activity. In some cases the TMs showed estrogenicity, whereas the short chained PFCAs did not, which to our knowledge is the first report of endocrine activities or lack thereof for these compounds and TMs. We suggest initiating further studies within this area, starting foremost with characterizing the composition of the TMs. It would likewise be prudent to investigate the endocrine activity of shorter-chained PAPs to obtain further knowledge on whether short-chained chemistry is a better alternative to long-chained fluorinated compounds.

Funding

The Ministry of Food, Agriculture, and Fisheries of Denmark and the Danish Veterinary and Food Administration.

Acknowledgements

The authors would like to thank Birgitte Møller Plesning, Kitt Lademann, Lene Sofie Dahl Svensson, and Dorthe Lykkegaard Korsbech for technical assistance and Terje Svingen for giving indispensable feedback on the written work.

355 ## References

356 Biegel LB, Liu RCM, Hurt ME, Cook JC. 1995. Effects of ammonium perfluorooctanoate on leydig-cell
357 function - in-vitro, in-vivo, and ex-vivo studies. Toxicol Appl Pharmacol 134(1):18-25; doi:
358 10.1006/taap.1995.1164.

359 Biegel L, Hurtt M, Frame S, O'Connor J, Cook J. 2001. Mechanisms of extrahepatic tumor induction by
360 peroxisome proliferators in male CD rats. Toxicological Sciences 60(1):44-55; doi: 10.1093/toxsci/60.1.44.

361 Bookstaff RC, Moore RW, Ingall GB, Peterson RE. 1990. Androgenic deficiency in male-rats treated with
362 perfluorodecanoic acid. Toxicol Appl Pharmacol 104(2):322-333.

363 Borchers A, Teuber SS, Keen CL, Gershwin ME. 2010. Food safety. Clin Rev Allergy Immunol 39(2):95-141;
364 doi: 10.1007/s12016-009-8176-4.

365 Calafat AM, Wong L, Kuklenyik Z, Reidy JA, Needham LL. 2007. Polyfluoroalkyl chemicals in the US
366 population: Data from the national health and nutrition examination survey (NHANES) 2003-2004 and
367 comparisons with NHANES 1999-2000. Environ Health Perspect 115(11):1596-1602; doi:
368 10.1289/ehp.10598.

369 Cook JC, Murray SM, Frame SR, Hurtt ME. 1992. Induction of leydig-cell adenomas by ammonium
370 perfluorooctanoate - a possible endocrine-related mechanism. Toxicol Appl Pharmacol 113(2):209-217; doi:
371 10.1016/0041-008X(92)90116-A.

372 D'eon JC, Crozier PW, Furdui VI, Reiner EJ, Libelo EL, Mabury SA. 2009. Observation of a commercial
373 fluorinated material, the polyfluoroalkyl phosphoric acid diesters, in human sera, wastewater treatment plant
374 sludge, and paper fibers. Environ Sci Technol 43(12):4589-4594; doi: 10.1021/es900100d.

375 D'eon JC, Mabury SA. 2007. Production of perfluorinated carboxylic acids (PFCAs) from the
376 biotransformation of polyfluoroalkyl phosphate surfactants (PAPS): Exploring routes of human
377 contamination. Environ Sci Technol 41(13):4799-4805; doi: 10.1021/es070126x.

378 D'eon JC, Mabury SA. 2011. Exploring indirect sources of human exposure to perfluoroalkyl carboxylates
379 (PFCAs): Evaluating uptake, elimination, and biotransformation of polyfluoroalkyl phosphate esters (PAPs) in
380 the rat. Environ Health Perspect 119(3):344-350; doi: 10.1289/ehp.1002409.

381 Feng Y, Shi Z, Fang X, Xu M, Dai J. 2009. Perfluorononanoic acid induces apoptosis involving the fas death
382 receptor signaling pathway in rat testis. Toxicol Lett 190(2):224-230; doi: 10.1016/j.toxlet.2009.07.020.

383 Horn HF, Vousden KH. 2007. Coping with stress: Multiple ways to activate p53. Oncogene 26(9):1306-1316;
384 doi: 10.1038/sj.onc.1210263.

385 Houde M, Martin JW, Letcher RJ, Solomon KR, Muir DCG. 2006. Biological monitoring of polyfluoroalkyl
386 substances: A review. Environ Sci Technol 40(11):3463-3473; doi: 10.1021/es052580b ER.

387 Kim S, Lee KT, Kang CS, Tao L, Kannan K, Kim K et al. 2011. Distribution of perfluorochemicals between
388 sera and milk from the same mothers and implications for prenatal and postnatal exposures. Environmental
389 Pollution 159(1):169-174; doi: 10.1016/j.envpol.2010.09.008.

390 Kissa E. 2001. Fluorinated Surfactants and Repellents. 2nd ed. New York:Marcel Dekker.

391 Kjeldsen LS, Bonefeld-Jorgensen EC. 2013. Perfluorinated compounds affect the function of sex hormone
392 receptors. Environmental Science and Pollution Research 20(11):8031-8044; doi: 10.1007/s11356-013-
393 1753-3.

394 Kraugerud M, Zimmer KE, Ropstad E, Verhaegen S. 2011. Perfluorinated compounds differentially affect
395 steroidogenesis and viability in the human adrenocortical carcinoma (H295R) in vitro cell assay. Toxicol Lett
396 205(1):62-68; doi: 10.1016/j.toxlet.2011.05.230.

397 Kubwabo C, Kosarac I, Lalonde K. 2013. Determination of selected perfluorinated compounds and
398 polyfluoroalkyl phosphate surfactants in human milk. Chemosphere 91(6):771-777; doi:
399 10.1016/j.chemosphere.2013.02.011.

400 Laier P, Cederberg T, Larsen J, Vinggaard A. 2003. Applicability of the CALUX bioassay for screening of
401 dioxin levels in human milk samples. Food Addit Contam 20(6):583-595; doi:
402 10.1080/0265203031000107820.

403 Lau C. 2012. Perfluorinated compounds. In: Molecular, Clinical and Environmental Toxicology, Volume 3:
404 Environmental Toxicology (Luch A, ed), 47.

405 Liu C, Deng J, Yu L, Ramesh M, Zhou B. 2010a. Endocrine disruption and reproductive impairment in
406 zebrafish by exposure to 8:2 fluorotelomer alcohol. Aquatic Toxicology 96(1):70-76; doi:
407 10.1016/j.aquatox.2009.09.012.

408 Liu C, Yu L, Deng J, Lam PKS, Wu RSS, Zhou B. 2009. Waterborne exposure to fluorotelomer alcohol 6:2
409 FTOH alters plasma sex hormone and gene transcription in the hypothalamic-pituitary-gonadal (HPG) axis of
410 zebrafish. Aquatic Toxicology 93(2-3):131-137; doi: 10.1016/j.aquatox.2009.04.005.

411 Liu C, Zhang X, Chang H, Jones P, Wiseman S, Naile J et al. 2010b. Effects of fluorotelomer alcohol 8/2
412 FTOH on steroidogenesis in H295R cells: Targeting the cAMP signalling cascade. Toxicol Appl Pharmacol
413 247(3):222-228; doi: 10.1016/j.taap.2010.06.016.

414 Ma Q. 2008. Xenobiotic-activated receptors: From transcription to drug metabolism to disease. Chem Res
415 Toxicol 21(9):1651-1671; doi: 10.1021/tx800156s.

416 Maras M, Vanparys C, Muylle F, Robbens J, Berger U, Barber J et al. 2006. Estrogen-like properties of
417 fluorotelomer alcohols as revealed by MCF-7 breast cancer cell proliferation. Environ Health Perspect
418 114(1):100-105; doi: 10.1289/ehp.8149.

419 Mark M, Ghyselinck NB, Chambon P. 2009. Function of retinoic acid receptors during embryonic
420 development. Nucl Recept Signal 7:1-15; doi: 10.1621/nrs.07002.

421 Monroy R, Morrison K, Teo K, Atkinson S, Kubwabo C, Stewart B et al. 2008. Serum levels of perfluoroalkyl
422 compounds in human maternal and umbilical cord blood samples. Environ Res 108(1):56-62; doi:
423 10.1016/j.envres.2008.06.001.

424 Motohashi H, Yamamoto M. 2004. Nrf2-Keap1 defines a physiologically important stress response
425 mechanism. Trends Mol Med 10(11):549-557; doi: 10.1016/j.molmed.2004.09.003.

426 Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL et al. 2007. Half-life of serum
427 elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired
428 fluorochemical production workers. Environ Health Perspect 115(9):1298-1305; doi: 10.1289/ehp.10009.

429 Olsen GW, Lange CC, Ellefson ME, Mair DC, Church TR, Goldberg CL et al. 2012. Temporal trends of
430 perfluoroalkyl concentrations in american red cross adult blood donors, 2000-2010. Environ Sci Technol
431 46(11):6330-6338; doi: 10.1021/es300604p.

432 Piersma AH, Bosgra S, van Duursen MBM, Hermsen SAB, Jonker LRA, Kroese ED et al. 2013. Evaluation of
433 an alternative in vitro test battery for detecting reproductive toxicants. Reproductive Toxicology 38:53-64; doi:
434 10.1016/j.reprotox.2013.03.002.

435 Prevedouros K, Cousins I, Buck R, Korzeniowski S. 2006. Sources, fate and transport of
436 perfluorocarboxylates. Environ Sci Technol 40(1):32-44; doi: 10.1021/es0512475.

437 Rosenmai AK, Nielsen FK, Pedersen M, Hadrup N, Trier X, Christensen JH et al. 2013. Fluorochemicals
438 used in food packaging inhibit male sex hormone synthesis. Toxicol Appl Pharmacol 266(1):132-142; doi:
439 10.1016/j.taap.2012.10.022.

440 Rosenmai AK, Dybdahl M, Pedersen M, van Vugt-Lussenburg BMA, Wedebye EB, Taxvig C et al. 2014. Are
441 structural analogues to bisphenol A safe alternatives? Toxicological Sciences 139(1):35-47; doi:
442 10.1093/toxsci/kfu030.

443 Scheringer M, Trier X, Cousins IT, de Voogt P, Fletcher T, Wang Z et al. 2014. Helsingor statement on poly-
444 and perfluorinated alkyl substances (PFASs). Chemosphere 114:337-339; doi:
445 10.1016/j.chemosphere.2014.05.044.

446 Shi Z, Ding L, Zhang H, Feng Y, Xu M, Dai J. 2009. Chronic exposure to perfluorododecanoic acid disrupts
447 testicular steroidogenesis and the expression of related genes in male rats. Toxicol Lett 188(3):192-200; doi:
448 10.1016/j.toxlet.2009.04.014.

449 Shi Z, Zhang H, Ding L, Feng Y, Wang J, Dai J. 2010. Proteomic analysis for testis of rats chronically
450 exposed to perfluorododecanoic acid. Toxicol Lett 192(2):179-188; doi: 10.1016/j.toxlet.2009.10.016.

451 Skakkebaek NE, Rajpert-De Meyts E, Main KM. 2001. Testicular dysgenesis syndrome: An increasingly
452 common developmental disorder with environmental aspects. Hum Reprod 16(5):972-978.

453 So MK, Yamashita N, Taniyasu S, Jiang QT, Giesy JP, Chen K et al. 2006. Health risks in infants associated
454 with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. Environ Sci
455 Technol 40(9):2924-2929; doi: 10.1021/es060031f.

456 Trier X, Granby K, Christensen JH. 2011. Polyfluorinated surfactants (PFS) in paper and board coatings for
457 food packaging. Environmental Science and Pollution Research; doi: 10.1007/s11356-010-0439-3.

458 Vinggaard A, Nellemann C, Dalgaard M, Jorgensen E, Andersen H. 2002. Antiandrogenic effects in vitro and
459 in vivo of the fungicide prochloraz. Toxicol Sci 69(2):344-353; doi: 10.1093/toxsci/69.2.344.

460

461 **Tables**

462 *Table 1: Names, abbreviations, CAS numbers and purity of test compounds.*

Abbreviation	Name	CAS	Purity
4:2 FTOH	4:2 fluorotelomer alcohol	2043-47-2	97 %
6:2 FTOH	6:2 fluorotelomer alcohol	647-42-7	97 %
8:2 FTOH	8:2 fluorotelomer alcohol	678-39-7	97 %
PFBA	Perfluorobutanoic acid	375-22-4	98 %
PFPA	Perfluoropentanoic acid	375-85-9	97 %
PFHxA	Perfluorohexanoic acid	307-24-4	97 %
PFHpA	Perfluoroheptanoic acid	375-85-9	99 %
PFOA	Perfluorooctanoic acid	335-67-1 3825-26-1	96 % 98 %
PFNA	Perfluorononanoic acid	375-95-1	97 %
PFDA	Perfluorodecanoic acid	335-76-2	98 %
PFunDA	Perfluoroundecanoic acid	2058-94-8	95 %
PFdoDA	Perfluorododecanoic acid	307-55-1	97 %
8:2 monoPAPs	8:2 polyfluoroalkyl phosphate ester surfactants	57678-03-2	99.5 %
8:2 diPAPs	8:2/8:2 polyfluoroalkyl phosphate ester surfactants	678-41-1	98.3 %
10:2 diPAPs	10:2/10:2 polyfluoroalkyl phosphate ester surfactants	1895-26-7	94.6 %
8:2 triPAPs	8:2/8:2/8:2 polyfluoroalkyl phosphate ester surfactants	NA	99.5 %

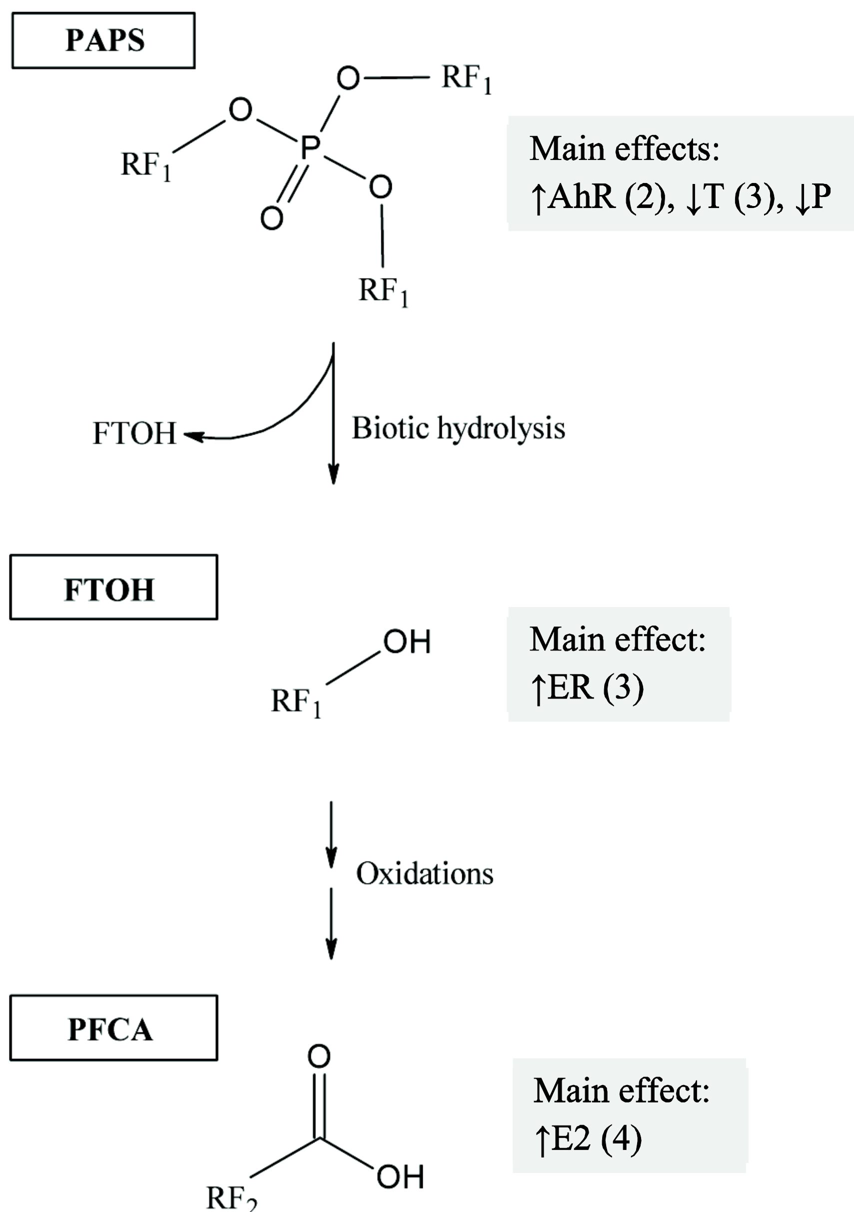
463 NA: Not available

464 *Table 2: Qualitative effects of fluorinated compounds and technical mixtures (TMs) on*
465 *progesterone (P), testosterone (T), and 17β-estradiol levels in the steroidogenesis assay (H295R) as*
466 *well as effects on human estrogen receptor (hER), human androgen receptor (hAR), and aryl*
467 *hydrocarbon receptor (AhR) activity. Results are based on statistically significant exposure-related*
468 *effects observed in the majority of independent experiments within each assay. Increased responses*
469 *are indicated with an upward arrow (↑, green), decreased responses are indicated with a*
470 *downward arrow (↓, red), and no effect is indicated with the marking (-).*

	H295R			hER	hAR	AhR
	P	**T**	**E2**			
TM1	-	-	-	-	-	-
TM2	-	-	↑	↑	-	-
TM3	-	a	-	↑	-	-
8:2 triPAPs	↓	↓	-	-	-	↑
10:2 diPAPs	↓	-	-	-	-	-
8:2 diPAPs	↓	↓	-	-	-	-
8:2 monoPAPs	↓	↓	-	↑	-	↑
8:2 FTOH	↓	↓	↑	↑	-	-
6:2 FTOH		-	a	↑	-	-
4:2 FTOH		-	-	↑	-	-
PFdoDA	-	-	↑[b]	-	-	-
PFunDA	-	-	↑[b]	-	-	-
PFDA	-	-	↑[c]	-	-	-
PFNA	-	-	-	↑[c]	-	-
PFOA	-	-	↓↑[d]	↑[c]	-	-
PFHpA		-	-	-	-	-
PFHxA		-	-	-	-	-
PFPA		-	-	-	-	-
PFBA		-	-	-	-	-

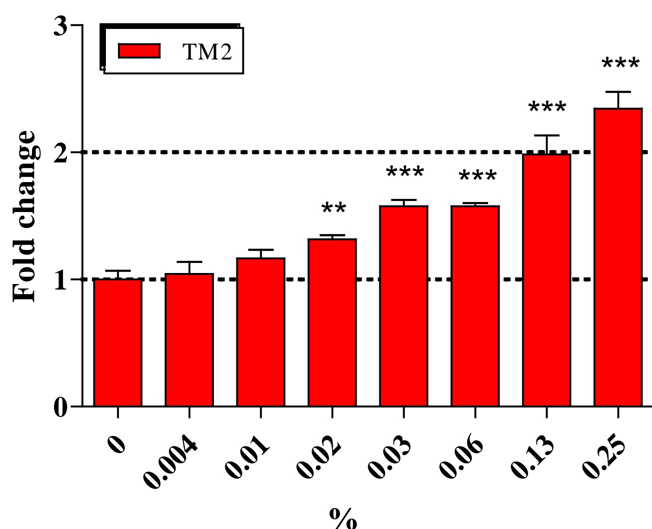
471 Grey areas: not tested/measured
472 Striped areas: results from Rosenmai et al. (2013)
473 (a) inconclusive
474 (b) increased response in cell viability plate observed in concentrations giving response in all
475 experiments leading to significant effects
476 (c) increased response in cell viability plate observed in concentrations giving response in one
477 experiments of those leading to significant effects
478 (d) a U-shaped concentration-response curve was reported with a decrease in E2 levels at lower
479 exposure concentrations and an increase at higher exposure concentrations

480 **Figures**



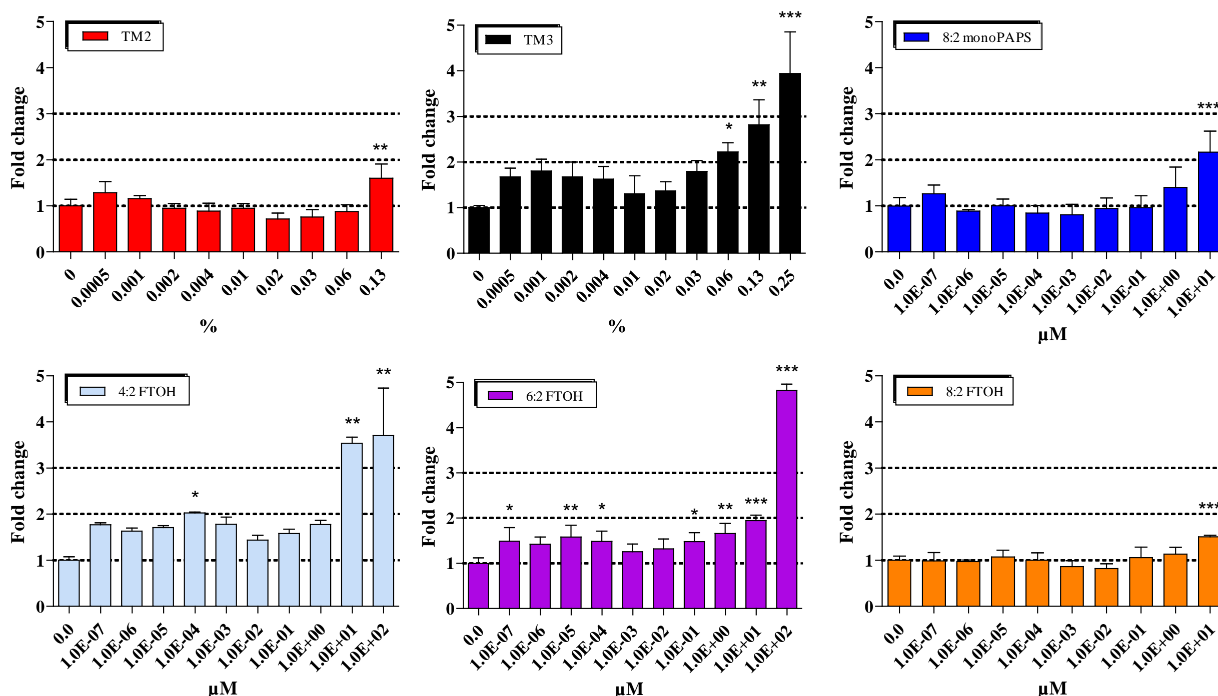
481

482 *Figure 1: Metabolic pathway of PAPs into the intermediate metabolites FTOH and the final*
483 *metabolite PFCAs (D'eon et al., 2007, 2011). The main effects for the groups of fluorinated*
484 *compounds are shown in the grey boxes based on results from this study and Rosenmai et al.*
485 *(2013). Number of compounds leading to effects is indicated in brackets. $RF_1 = F(CF_2)_nCH_2CH_2$.*
486 *$RF_2 = F(CF_2)_n$. In this study: diPAPs (n = 8 or 10), monoPAPs and triPAPs (n = 8), FTOHs (n = 4,*
487 *6, or 8), PFCAs (n = 3-11).*



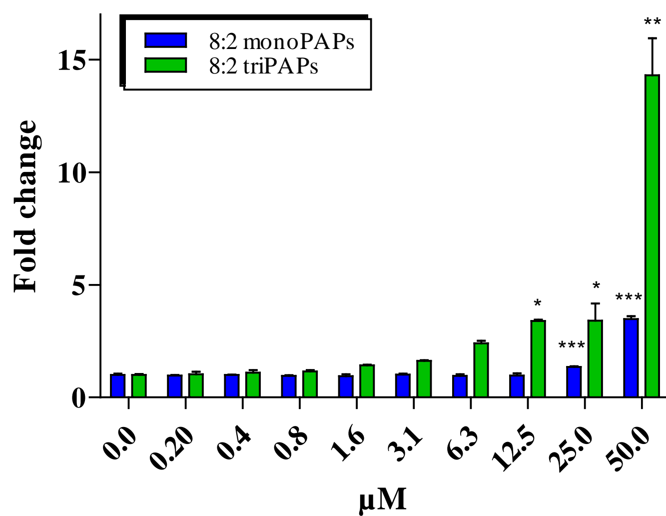
488

489 *Figure 2: Increased 17β-estradiol levels following exposure to TM2 based on data from the*
490 *steroidogenesis assay. Fold change (±SD) as a function of percent (%) original material provided.*
491 *Data is based on one representative experiment.*



492

493 *Figure 3: Increased estrogen receptor activity following exposure to TM2, TM3, 8:2 monoPAPs,*
494 *and FTOHs based on data from the ER reporter gene assay. Fold change (±SD) as a function of*
495 *percent (%) original material provided for TMs and exposure concentration (µM) for FTOHs and*
496 *monoPAPs. Data is based on one representative experiment.*



497

498 *Figure 4: Increased aryl hydrocarbon receptor activity following exposure to 8:2 monoPAPs and*
499 *8:2 triPAPs in the AhR reporter gene assay. Fold change (±SD) as a function of exposure*
500 *concentration (µM). Data is based on one representative experiment.*

9.4 Manuscript 4

Rosenmai, A.K., Bengtstrøm, L., Taxvig, C., Trier, X., Petersen, J.H., Granby, K. and Vinggaard, A.M. (2014). A strategy to identify problematic chemicals in food contact materials of paper and board. Manuscript in preparation.

Comments to manuscript

At present only data from AR, ER, and AhR reporter gene assays are included in the Manuscript. Six other assays, namely nrf2, p53, RAR, GR CALUX reporter gene assays as well as PPARa/y reporter gene assays and data on cytotoxicity will be part of the final Manuscript. Furthermore, data from the Comet assay and Ames test is part of the overall strategy, but will be included in another Manuscript.

We are still in the process of conducting additional experiments to verify some of the findings described in the paper. These include,

- Cell viability tests:
  In some cases we observed increased responses in the cell viability plates in the ER reporter gene assay. We are in the process of conducting additional experiments to shed light on this issue, which will be included in the final paper.

A strategy to identify problematic chemicals in food contact materials of paper and board

Anna Kjerstine Rosenmai[a], Linda Bengtström[b], Camilla Taxvig[a], Xenia Trier[b], Jens Højslev Petersen[b], Kit Granby[b] & Anne Marie Vinggaard[a]

[a] *Division of Toxicology & Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*

[b] *Division of Food Chemistry, Toxicology & Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*

Corresponding Author

Anna Kjerstine Rosenmai

akjro@food.dtu.dk

Division of Toxicology & Risk Assessment
National Food Institute
Technical University of Denmark
Mørkhøj Bygade 19
DK-2860 Søborg
Denmark

Phone + 45 35 88 76 00

Running Title

Bioassay guided analysis of extracts from food contact materials

Abstract

Food contact materials (FCMs) constitute a source of human exposure to chemicals. Lists of compounds intended for use in these materials exist, but limited knowledge is available on the potential toxicological effects of many of the compounds allowing for appropriate risk assessment.

In this study, we aimed at developing a strategy which allows for screening of FCMs and identification of potentially problematic compounds in these materials by means of applying analytical chemistry tools as well as bioassay guided analysis in combination.

A step-by-step approach was developed in which extracts from FCMs were tested *in vitro*, active extracts underwent fractionation, fractions were tested *in vitro*, and tentative identification of compounds was conducted in active fractions. Selected tentatively identified compounds were tested individually *in vitro* and quantified in the extracts. The battery of *in vitro* assays covered endpoints related to endocrine disruption, oxidative stress, cytotoxiticy and genotoxicity.

All 20 extracts led to effects on aryl hydrocarbon receptor activity, whereas only a subset of extracts led to effects on estrogen receptor (ER) and androgen receptor (AR) activity. Two extracts were selected for further investigation by use of the step-by-step strategy, one extract from a pizza box and one from a sandwich wrapper. By doing so, we successfully identified di-butyl phthalate, butyl-benzyl phthalate, and bisphenol A (BPA) as causing ER activity in the pizza box of which BPA mainly caused the effect. In the sandwich wrapper extract, which caused AR antagonism, we identified two causative agents, dehydroabietic acid (DHAA) and abietic acid, of which the latter was present at high concentrations and thus mainly caused the effect in the extract. This is to our knowledge the first reporting of the antiandrogenicity of DHAA.

Collectively these data suggest that applying the strategy is a useful tool to assess potential hazards posed by chemicals in FCMs of paper and board, as well as identifying causative agents.

Keywords

Biodirected analysis, bioassay guided analysis, food contact material, paper, board, food packaging

Introduction

Assuring food safety is a challenge in modern society as the potential sources of food contamination are many, one of which is compounds migrating from food contact materials (FCMs) (Borchers et al. 2010). We already know of several compounds, which are used in FCMs and which have the potential to cause adverse effects, such as bisphenol A (BPA) and phthalates. BPA is used in applications such as polycarbonate plastic bottles, as a monomer used for epoxy resin coatings in cans for foods and drink (EFSA 2006), and have been found in recycled paper (Geens et al. 2011; Vinggaard et al. 2000), whereas phthalates are used in applications such as PVC tubing, food-packaging films, and have been measured in paper and board (Cao 2010). BPA and phthalates are known endocrine disruptors, and have shown adverse effects *in vivo* (Christiansen et al. 2014; Foster 2006; Miyawaki et al. 2007).

Besides these known compounds there are multiple other compounds used in FCMs. A compiled list of indirect additives including up to 3000 compounds, some of which are intended for FCMs, have recently been compared to available toxicity data (Neltner et al. 2013). This comparison revealed that the majority of the indirect additives are not sufficiently examined for toxicological effects (Neltner et al. 2013). Though no specific EU regulation exists on the use of chemicals in FCMs of paper and board, there is a framework directive, which states that compounds used in FCMs should not migrate into foods in amounts, which can adversely affect human health (The European Commission 2004). Based on the aforementioned data, it becomes apparent that a strategy is needed to obtain information on potential effects of compounds present in FCMs.

The vast numbers of compounds, for which little is known on adverse effects pose challenges as testing all the compounds would be extremely time consuming. Thus, several previous studies have investigated effects of mixtures of compounds originating from FCMs of paper and board by applying a bioassay guided analysis approach. In these studies extracts from FCMs of paper and board were tested *in vitro* assessing endpoint such as genotoxicity, cell toxicity, and endocrine disruption (Binderup et al. 2002; Lopez-Espinosa et al. 2007; Ozaki et al. 2004; Ozaki et al. 2005; Vinggaard et al. 2000; Weber et al. 2006). On several occasions compounds were identified by use of these strategies, which could explain the observed effects either fully or partly. These strategies can be applied to enable a future prioritization of studies to be conducted on the identified compounds.

In this study, we wanted to develop a test strategy to obtain information on the potential effects of compounds present in FCMs of paper and board by applying both analytical chemistry tools and bioassay guided analysis. A broad battery of *in vitro* assays was used to examine effects of compounds present in the materials including endpoints associated with endocrine disruption, oxidative stress, cytotoxiticy and genotoxicity. Additional data obtained by the Comet assay and Ames test will be published elsewhere, but is part of the overall strategy. Extracts from 20 FCMs were tested in these assays and two of the extracts underwent further investigation by *in vitro* testing of fractions of the extracts. Finally the compounds that most likely were responsible for the *in vitro* effects in question were identified.

85 **Materials and Methods**

86 The step-by-step procedure in the FCM strategy is shown in Figure 1. The strategy involved several
87 steps including, 1) preparation of extracts from FCMs of paper and board, 2) testing of extracts *in*
88 *vitro*, 3) fractionation of selected active extract, 4) testing of fractions in the assay in which the
89 extract led to effect, 5) tentative identification of compounds in active fractions, 6) evaluation to
90 narrow down the list of tentatively identified compounds, 7) *in vitro* testing of final list of
91 tentatively identified compounds, 8) verification of presence of substances in the extract as well as
92 quantification of active identified compounds, and 9) evaluation of contribution to effect in extract
93 of identified compounds by calculating equivalence factors (EQs).

94 *Test compounds and chemicals*

95 Ethanol (99.9 %) used for the extraction and re-dissolving of evaporated extracts was purchased
96 from Merck (Darmstadt, Germany). Methanol (99.9 %) used for mobile phases for HPLC
97 fractionation was purchased from Rathburn (Walkerburn, Scotland). All aqueous solutions were
98 prepared using ultrapure water obtained from a Millipore Milli-Q Gradient A10 system (Millipore,
99 Bedford, MA, USA). HPLC MS grade formic acid and a water solution of 25 % ammonium
100 hydroxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was
101 obtained from Merck (Darmstadt, Germany). Standards for the LC-qTOF method; bisphenol A
102 (BPA), methylparaben, perfluorooctanoic acid (PFOA), bisphenol A diglycidyl ether (BADGE) as
103 well as deuterated BPA (d_{16}-BPA) used for the quantitative determination of bisphenol A were
104 obtained from Sigma-Aldrich. Di-butyl phthalate (DBP), deuterated di-butyl phthalate (d_4-DBP),
105 butyl-benzyl phthalate (BBP), and di-isobutyl phthalate (DiBP) were used for quantitative
106 determination in fractions and extracts. Abietic acid (AA), dehydroabietic acid (DHAA),
107 isorhamnetin and rhamnetin (Sigma-Aldrich) as well as 4-oxo-retinoic acid (Santa Cruz
108 Biotechnology, TX, USA) were used for quantification in extracts and fractions thereof.

109 Stock solutions for testing *in vitro* of DBP, BBP, DiBP, BPA, AA, DHAA, isorhamnetin,
110 rhamnetin, and 4-oxo-retinoic acid were prepared in DMSO at 40-50 mM.

111 *Paper and board samples*

112 In total 20 different paper and board samples were applied in the test strategy. See Table 1 for paper
113 and board characteristics.

114 *Production of extracts and fractions*

115 The paper and board extracts and fractions were produced as described previously (Bengtstrom et
116 al. 2014). Selected FCM extracts exhibiting *in vitro* activity in the initial screening phase were
117 fractionated (indicated in Table 1 with the marking *) by liquid chromatography (LC). New extracts
118 were produced before fractionation, which was performed in two separate rounds, one under
119 alkaline and one under acidic conditions.

120 *Tentative identification of compounds in fractions of extracts by HRMS*

121 The extracts and corresponding fractions exhibiting *in vitro* activity were analysed by high
122 resolution mass spectrometry (HRMS) techniques (time-of-flight mass spectrometry coupled to
123 either gas or liquid chromatography (GC-qTOF or LC-qTOF, Agilent Technologies, Santa Clara,
124 CA, USA)). The methods for the tentative identification process are presented in full detail in
125 Bengtström et al. (Manuscript in preparation). In brief, the highly concentrated extracts and
126 fractions were diluted 1:100 *v/v* prior to analysis. Cut-offs for peaks were based on the threshold of
127 toxicological concern for substances with suspected genotoxic effects (Cramer Class III) (EFSA
128 Scientific Committee 2012), corresponding to 25 ng/dm^2, for both tentative identification methods.
129 Peaks with areas below this threshold were not further investigated.
130
131 In GC-qTOF, the extracts and fractions were ionized by electron ionization (EI) at 70 eV. Only
132 peaks above the area corresponding to one tenth d_4-DBP were analysed by the GC-qTOF to
133 compensate for differences in detector response. Analysis was performed in the Agilent
134 MassHunter Qualitative software with the NIST library v.1.1.
135
136 Data obtained by the LC-qTOF analysis was analysed by using MassHunter Qualitative software as
137 well as ProGenesis QI software (Nonlinear Dynamics Limited, UK). Only peaks with area above
138 one per cent of that corresponding to BADGE for positive mode and PFOA for negative mode were
139 considered. Differences in detector response and ion suppression were compensated for by this cut-
140 off. Compounds were tentatively identified by using a customized library containing approximately
141 2300 matrix specific entries (Bengtström et al., Manuscript in preparation) as well as the
142 ChemSpider and PubMed database.

143 *Quantitative determination of phthalates by GC-qTOF*

144 Phthalates were quantified by the accredited method described previously (Petersen and Jensen
145 2010). All quantified phthalates showed good linearity ($R^2 > 0.98$, not weighted, not forced through
146 0). The phthalate standards used for an external calibration curve were run using the same
147 parameters and settings as described in Bengtström et al. (Manuscript in preparation). Extracts were
148 diluted 1:1000 *v/v* with ethanol prior to analysis. The data was analysed by the Agilent MassHunter
149 Quantitative software.

150 *Quantitative determination by LC-MS/MS*

151 All extracts were diluted 1:1000 *v/v* with ethanol before analysis. Quantification of BPA was based
152 on an accredited LC tandem mass spectrometry (LC-MS/MS) method, described in Table 2. The
153 eight-point calibration curve (0, 7.5, 15, 30, 75, 150, 225 and 300 ng mL^{-1}) consisted of BPA
154 standard in methanol and water (75:25, *v/v*). Internal standard, d_{16}-BPA at 150 ng mL^{-1}, was added
155 to both calibration standards and extracts. The mass transition reactions used for BPA quantification
156 were *m/z* 227.2 > 212.1 as quantifier, *m/z* 227.2 > 133.1 as qualifier and *m/*z 241.2 > 223.1 for d_{16}-
157 BPA.

158 A seven point calibration curve (0, 10, 20, 50, 100, 200 and 500 ng mL^{-1}) with a standard mixture in
159 ethanol of AA, DHAA, 4-oxo-retinoic acid, isorhamnetin, rhamnetin recognized as the most likely

to cause AR activity was analysed according to Table 2. Masses used for quantification of AA were *m/z* 301.5 > 301.5 and for DHAA *m/z* 299.5 > 299.5. The mass transition reactions used for 4-oxo-retinoic acid quantification were *m/z* 313.2 > 254.2 as quantifier, *m/z* 313.2 > 163.1 as qualifier. For isorhamnetin *m/*z 315 > 300 and *m/*z 315 > 151 was used as quantifier and qualifier respectively; and for rhamnetin *m/*z 315 > 300 and *m/*z 315 > 165 was used as quantifier and qualifier.

The calibration curves for all methods were obtained by plotting the peak area versus concentrations. All compounds quantified showed good linearity (R^2 > 0.98, not weighted, not forced through 0) in their respective method and investigated range. Data from both quantifications were analysed by the Waters QuanLynx (v 4.1) software.

Reporter gene assays for testing of extracts, fractions, and tentatively identified compounds

Nine reporter gene assays were applied to test the extracts from FCMs. These assays included the androgen receptor (AR), estrogen receptor (ER), aryl hydrocarbon receptor (AhR), and peroxisome proliferator-activated receptors (PPARα/γ) reporter gene assays. Furthermore, the glucocorticoid receptor (GR), retinoic acid receptor (RAR), nuclear factor (erythroid-derived 2)-like 2 (nrf2), and p53 CALUX reporter gene assays were applied. The experimental procedures and materials used for these assays have been described previously (Piersma et al. 2013; Rosenmai et al. 2014; Taxvig et al. 2012; Vinggaard et al. 2002). The AR reporter gene assay was conducted in both agonist and antagonist mode (0.1 nM R1881 added) on extracts. Extracts were tested in a maximum concentration of 0.25-1 % of extract provided in 2-3.3 fold dilutions. The vehicle concentrations were constant except in the nrf2, p53, RAR and GR CALUX reporter gene assays, in which the vehicle was diluted accordingly. The experiments were repeated 1-3 times. Data from the nrf2, p53, RAR, and GR CALUX as well as PPARα/γ reporter gene assays are currently not included in this paper, but will be included before submission of the manuscript. Data from the Comet assay and Ames test obtained from testing the extracts will be published elsewhere.

All fractions of extract S4 and S8 were tested in the AR and ER reporter gene assay, respectively, in one concentration of 0.25 % of fraction provided. 1-2 experiments were conducted in 2-4 replicates.

Tentatively identified compounds were tested in the ER and AR reporter gene assays. Compounds included DHAA, AA, rhamnetin, isorhamnetin, and 4-oxo-retinoic acid in the AR reporter gene assay and BPA, DiBP, BBP, and DBP in the ER reporter gene assay.

All *in vitro* experiments were conducted with a positive control, which was 17β-estradiol in the ER reporter gene assay and R1881 or hydroxyflutamide (OHF) in the AR reporter gene assay in the agonism or antagonism mode, respectively.

In vitro data processing and calculation of equivalence factors

For extracts and tentatively identified compounds statistical analysis was conducted on individual experiments in which data was normalized to vehicle controls. Residuals to means within each exposure group was tested for whether means of these were statistically different (ANOVA) and successively pooled and tested for normal distribution (D'agostino Pearson's Omnibus test). Data

197 for which pooled residuals were normally distributed were analysed by a one-way ANOVA (post-
198 test Dunnett) and all other data were analysed by the Kruskal Wallis test (post-test Dunn). Exposure
199 groups, in which cell toxicity was observed, were removed before statistical analysis was
200 conducted. Data obtained from testing fractions underwent no further data processing.

201 The overall criteria for reporting *in vitro* effects for extracts and selected tentatively identified
202 compounds were that means between exposure groups should exhibit statistically significant
203 differences, the effects should be dose-dependent, and should be reflected in the majority of
204 conducted experiments.

205 In AR antagonism mode the tentative lowest observable effect concentrations (LOECs) were
206 reported as the concentration at which a ≥ 25% decrease was observed. In agonist mode tentative
207 LOECs were reported if ≥ 50% increase was observed. The greatest response at non-cytotoxic
208 concentrations in the ER, AR, and AhR reporter gene assays were reported as the tentative
209 maximum efficacy (E_{max}).

210 Dose-response data for selected extracts, selected tentatively identified compounds in fractions, and
211 positive controls in the respective assay were fitted to a four parameter sigmoidal curve fit in which
212 the limits were fixed at 1 and the tentative E_{max}. Based on these curve fits Hill slopes and EC_{50}
213 values were obtained, which were used for determination of estrogen equivalence factors (EEQs)
214 and androgen equivalence factors (AEQs) for both extracts (EQ_{meas}) and for identified compounds
215 in fractions (EQ_{calc}). The following equations were used to calculate EQs,

216 (1) $$response = bottom * \frac{top-bottom}{1+10^{(\log(EC_{50})-concentration)*hillslope}}$$

217

218 (2) $$concentration = log(EC_{50}) - \frac{log\left(\frac{top-response}{response-bottom}\right)}{hillslope}$$

219 Here top and bottom represent the highest and lowest y-values, respectively, and Hill slope and
220 EC_{50} values are estimated from the curve fit.

221 The tentatively identified compounds were quantified in the extract and based on the parameters
222 obtained from the individual dose-response curves, the predicted response was calculated by using
223 equation (1). The concentration of the identified compound inserted were those leading to the
224 tentative E_{max} in the extract. Successively this calculated response was inserted in equation (2), in
225 which all parameters inserted were based on the positive control; leading to the identified
226 compounds being converted into EQs of the positive control. The EQs for individual compounds
227 were summed to obtain the EQ_{calc}. For the extract the EQ_{meas} was calculated by inserting the
228 tentative E_{max} for the extract into equation (2) with all the parameters in the equation being based on
229 the positive control.

230 **Results**

231 *In vitro effects of extracts*

232 An overview of data obtained for the extracts in the AR, ER, and AhR reporter gene assay are given
233 in Table 3 with the determined tentative LOECs (dm^2/mL) and tentative efficacies (E_{max}) (%).
234 Concentration-response relationships for extracts S4 and S8 that were further investigated for
235 determination of causative agents are shown in Figure 2.

236 Several of the extracts led to AR agonism (S2, S3, S4, S11, S16, and S18) and antagonism (S2, S3,
237 S4, S5, S13, and S18). Notably, extract S4, which was further examined by fractionation and
238 identification of causative agents both led to agonism and antagonism in the AR reporter gene
239 assay. This dual modality on AR activity was also observed for extract S2, S3, and S18. S4 was
240 selected for further examination as it showed a tentative E_{max} of 52 % in the antagonist mode with a
241 tentative LOEC of $2.3*10^{-2}$ (dm^2/mL). Twelve extracts did not affect AR activity.

242 Nine of the extracts led to increased ER activity with tentative E_{max} values ranging from 63-245%
243 increase compared to the control and tentative LOECs ranging from $1.3*10^{-3}$-$5.9*10^{-2}$ (dm^2/mL).
244 Extract S8 was chosen for further investigation as it showed high potency and materials were
245 available for fractionation. 11 extracts did not increase ER activity. Indications of cytotoxicity were
246 observed in some cases at higher extract concentrations.

247 All extracts caused increased AhR activity in the reporter gene assay of which S8 and S14 exhibited
248 marked potency and efficacy in the range of 2.3-4.8 dm^2/mL and more than 1000 % increase in
249 response. Extract S2-S4 were among the less active samples leading to approximately 100 %
250 increase in response and tentative LOECs of around $2.1*10^{-1}$-$6.3*10^{-2}$ dm^2/mL.

251 *In vitro effects of fractions*

252 Data from the ER and AR reporter gene assays for the fractions, both alkaline and acidic, are shown
253 in Figure 2. The fractions selected to undergo further identifications were those giving the greatest
254 change in response in the respective assay. Fraction 8 of extract S4 showed a marked decrease in
255 response in both the acidic and alkaline mode and was chosen for further identification. Acidic
256 fraction 9 and 10 also led to a decreasing trend compared to the remaining fractions, but were not
257 further tested. Fractions of extract S8 led to a marked increase in acidic fraction 7 and alkaline
258 fraction 6, and these were chosen for further investigation. Several other acidic fractions led to
259 increased responses, but were not further tested.

260 *Tentative identification of compounds in selected fractions*

261 A comprehensive list of all peaks and compounds tentatively identified for fractions with activity in
262 the ER or AR reporter gene assays is submitted as Supplementary Materials. The selection of
263 compounds for further investigation *in vitro* was based on an expert judgment including information
264 on previously reported effects, read-across, and commercial availability of tentatively identified
265 compounds.

266 In extract S8, BPA, DBP, and DiBP tentatively identified in both the acidic and alkaline fraction
267 with ER activity, were selected for further investigation. Moreover, BBP found in the acidic
268 fraction with ER activity, was also selected for additional testing. For the fractions of extract S4

269 causing AR activity, tentatively identified compounds, namely DHAA, AA, isorhamnetin,
270 rhamnetin, and 4-oxo-retinoic acid were selected for further *in vitro* testing.

271 *In vitro testing of tentatively identified compounds*

272 Of the four selected compounds tested for ER activity, BPA, BBP, and DBP led to increased
273 activity, whereas DiBP did not lead to any effect. Among the five selected compounds tested for
274 AR antagonism, four caused marked effects, namely isorhamnetin, 4-oxo-retinoic acid, AA, and
275 DHAA. Marked cytotoxicity accompanied the decreased response for rhamnetin and thus we could
276 not establish whether this compound had antagonistic effects on AR activity. Results for tentatively
277 identified compounds affecting the ER and AR activity are shown in Figure 2.

278 *Quantification of identified compounds*

279 Confirmation of tentatively identified compounds was conducted in both fractions and extracts,
280 whereas the quantification of these was only done in the extracts. In order to confirm the identity
281 and to quantify the compounds, standards of the selected tentatively identified compounds were
282 analysed by either GC-qTOF or LC-MS/MS. Relative retention times and fragmentation patterns
283 for both BPA and the phthalate standards, analysed by GC-qTOF, confirmed the initial
284 identification as correct, according to criteria set previously (The European Commission 2002),
285 whereas only two of five tentatively identified compounds in the AR active extract and fractions of
286 this, DHAA and AA, had matching relative retention times between standards and fraction (> 0.2
287 min) when analysed by LC-MS/MS. Moreover, DHAA and AA were the only compounds out of
288 the five tested that had an entry in the customized database. Concentrations for quantified
289 compounds causing effects are given in Table 4.

290 *Calculated and measured equivalence factors*

291 The EEQs calculated based on identified compounds were higher than that measured, EEQ_{calc} =
292 $1.42*10^{-5}$ µM versus $EEQ_{meas} = 2.23*10^{-6}$ µM. The same was evident for AEQs based on identified
293 compounds in the AR reporter gene assay, $AEQ_{calc} = 1.49*10^{-1}$ µM versus $AEQ_{meas} = 8.84*10^{-2}$
294 µM. The calculated and measured EQs are shown in Table 4.

295 **Discussion**

296 In this study, we aimed at developing a strategy to identify potentially problematic compounds in
297 FCMs of paper and board by applying a step-by-step approach, as illustrated in Figure 1. 20 FCMs
298 were investigated of which several caused effects in the *in vitro* assays, Table 3. The full strategy
299 was applied on two extracts, the sandwich wrapper consisting of virgin pulp (extract S4) and the
300 pizza box consisting of recycled material (extract S8), which were active in the AR and ER reporter
301 gene assays, respectively. Applying the work-flow to these extracts illustrated that the strategy
302 could be used to identify causative agents present in the extracts, as we successfully identified
303 DHAA, AA, BPA, DBP, and BBP, which could more than explain the responses of the extracts.

304 ***The food contact material strategy***

305 *In vitro effects of extracts*

306 The extracts tested in the three *in vitro* assays showed activity to a varying degree depending on the
307 assay. All extracts led to effects in the AhR reporter gene assay, whereas in the ER and AR reporter
308 gene assays effects were only observed for some extracts.

309 Several of the paper and board extracts induced ER activity, which is in accordance with findings of
310 estrogenicity of kitchen rolls reported previously (Vinggaard et al. 2000). Kitchen rolls made from
311 recycled materials in particular led to effects, which was suggested primarily to be caused by the
312 occurrence of BPA in these materials (Vinggaard et al. 2000). In the present study eight of 14
313 extracts fully or partly based on recycled materials caused effects and one extract out of six made of
314 virgin paper caused effect. The results suggest that estrogenic potential is prevalent in recycled
315 compared to virgin paper, though recycled paper in itself is not a clear marker of estrogenicity, as
316 six recycled FCMs did not lead to effects.

317 The AhR activity was induced by all the extracts tested suggesting that common compounds are
318 present in the extracts leading to effects, or that many different compounds present in FCMs have
319 the ability to activate the receptor. Four extracts were previously examined for ability to activate the
320 AhR, which all led to effects to varying degrees (Binderup et al. 2002), supporting the findings of
321 the current study. Binderup et al. (2002) suggested mono-ortho PCBs as contributing to the effects
322 to a minor degree. However, other well-known AhR activators were not examined, such as dioxins,
323 non ortho-PCBs and PAHs (Binderup et al. 2002). We are in the process of investigating the
324 extracts further to shed light on potential reasons for the activities observed (Bengtström et al.,
325 Manuscript in preparation).

326 *Identification of causative agents*

327 The list of tentatively identified compounds in active fractions underwent an expert evaluation. This
328 evaluation was based on previously reported effects, known biophores for the effect in question,
329 read-across, and commercial availability of compounds, leading to a final candidate list. This
330 process was successful as most of the final candidate compounds led to effects in the assays and
331 were present in the extract. Of the nine tentatively identified compounds on the final list, seven
332 caused effects in the respective assay, namely DBP, BBP, BPA, DHAA, AA, isorhamnetin, and 4-
333 oxo-retinoic acid, of which five were also identified and quantified in the extracts.

334 The tentative identification was conducted by use of high resolution mass spectrometry (HRMS),
335 qTOF, which is necessary when examining a complex matrix as paper and board. HRMS has high
336 sensitivity when scanning broad mass ranges as well as high mass resolution. The advantages of
337 this is two-fold: 1) broad ranges of compounds can be identified, which is essential, as we wanted
338 as many compounds as possible to be identified and 2) high mass resolution allows for accurate
339 identification, causing a higher degree of certainty in the tentative identification, ultimately leading
340 to a reduced list of candidate compounds.

341 We further applied two chromatographic separation methods, GC and LC. This enabled analysis of
342 a broader spectrum of compounds, compared to using only one of the methods, as GC-MS separates
343 small, non-polar and thermostable volatiles, whereas LC-MS separates larger, more polar, thermo-
344 labile compounds. The four compounds selected for further investigation from fractions active in
345 ER were tentatively identified by GC-qTOF, whereas the five compounds selected for further
346 investigation from AR active fractions were tentatively identified by LC-qTOF. These data
347 illustrate that by using these two methods in combination, we obtain more knowledge on
348 compounds present in the extracts, as none of the AR active compounds were identified by GC-
349 qTOF.

350 Utilizing GC-qTOF in combination with EI ionization allowed for tentative identification by use of
351 a vast commercially available mass spectral library. In contrast, no such library is available for
352 identification using LC-qTOF, which is a major disadvantage, and thus we build a customized
353 database for this purpose (Bengtström et al., Manuscript in preparation). Only DHAA and AA had
354 matches in the customized database, whereas the other AR active compounds did not, and further
355 these were not confirmed in the extract when compared to standards. This illustrates that using a
356 customized database enhances the likelihood for correct identification, as DHAA and AA was
357 found in both the database and in the extracts, which is in-line with that previously reported (Kind
358 and Fiehn 2007).

359 *Equivalence factors*

360 In the AR reporter gene assay, four of the five tentatively identified compounds in the sandwich
361 wrapper made from virgin pulp (extract S4), namely DHAA, AA, isorhamnetin, and 4-oxo-retinoic
362 acid, all inhibited AR activity, whereas no AR antagonism at non-cytotoxic concentrations of
363 rhamnetin could be detected. Only the identities of DHAA and AA were confirmed in the extract.
364 The AEQ_{calc} values for these two compounds were higher than the AEQ_{meas} of the extract,
365 suggesting that AA and DHAA can explain the response observed.

366 In the ER reporter gene assay the sum of the calculated EEQs for identified compounds were higher
367 than the EEQ measured for the pizza box (extract S8), suggesting that we have identified the
368 causative agents. The somewhat higher EEQ_{calc} compared to the EEQ_{meas} could be caused by other
369 compounds being present in the extract, which inhibit the ability of BPA, DBP, and BBP to activate
370 the ER. Furthermore, an assumption for the calculated EEQ is that dose-addition occurs in the
371 extract, however if this is not the case, this could add to the observed differences in EEQs. Finally,
372 the lesser EEQ measured in the extract could also be caused by cytotoxicity. Overall, we were
373 successful in identifying well-known EDs in the extract of the pizza box (extract S8) made from
374 recycled fibres.

375 ***Identified causative agents***

376 BPA is used in thermal paper (Liao and Kannan 2011; Mendum et al. 2011) and has previously
377 been reported in recycled paper (Vinggaard et al. 2000), which may explain the observed presence
378 of this compound in the pizza box (extract S8) in our study. Furthermore, phthalates are used in

inks, lacquers, and adhesives, and are considered general environmental contaminants and therefore occur in recycled paper and board (Fasano et al. 2012; Pocas et al. 2010; Suciu et al. 2013), possibly explaining the presence of BBP and DBP in this study. Besides being used intentionally or non-intentionally in paper and board, human exposure through the diet to BPA and DBP is believed to be a major source (EFSA 2013; Wormuth et al. 2006) and the compounds or their metabolic products are routinely measured in human urine (Calafat et al. 2008; CDC 2014), suggesting widespread exposure.

The ability of BPA, DBP, and BBP to exhibit estrogenicity *in vitro* has been reported previously (Ghisari and Bonefeld-Jorgensen 2009; Gould et al. 1998; Grignard et al. 2012; Kitamura et al. 2005; Krishnan et al. 1993; Mankidy et al. 2013; Paris et al. 2002; Shen et al. 2009; Zhang et al. 2011) and thus these data are in-line with the activities observed here in the ER reporter gene assay. In our study the potency of BPA is greater than BBP and DBP and thus despite the higher concentrations of DBP in the extract, it is BPA that drives the effect, which is illustrated by the higher EEQ for BPA, than that of the phthalates.

Besides the *in vitro* estrogenicity of BPA, this compound has led to low-dose effects *in vivo* such as disturbed mammary gland development (Moral et al. 2008), behavioural changes (Xu et al. 2010), as well as decreased anogenital distance (Christiansen et al. 2014). Whereas phthalates have led to effects such as hypospadias, cryptorchidism, changes in nipple retention, and reduced anogenital distance (Foster 2006). Based on these findings it is of concern that these compounds are present in FCMs of paper and board.

DHAA and AA are resin acids present in different types of resins, and thus the compounds may very well be present in paper and board materials, as they are naturally occurring constituents of wood (Roberts 1996; Sjöström and Alen 1998). During the pulping process, DHAA and AA can be removed, but they can also be present in the final paper product (Roberts 1996). Besides the natural occurrence in wood, these compounds are also used in the paper making process (Leach and Pierce 1993; Roberts 1996). In the BIOSAFEPAPER project and a study by Ozaki et al. (2006), DHAA and AA were identified in several of the FCMs investigated (Ozaki et al. 2006; Weber et al. 2006). In the latter study the compounds were detected in 15 out of 20 FCMs of paper and board and were shown capable of migrating into food simulants (Ozaki et al. 2006). Furthermore, DHAA and AA were detected in foods packed in FCM of paper (Mitani et al. 2007), suggesting that human exposure may occur through food consumption.

In the AR reporter gene assay DHAA and AA have similar dose-response relationships, Figure 2, however as AA is present in higher amounts, this compound drives the effect in the extract. To our knowledge, these results are the first to show antiandrogenicity of DHAA. AA however has been reported to exhibit antiandrogenic potential on AR activity in two *in vitro* assays (Rostkowski et al. 2011) in accordance with our findings. Furthermore, AA has shown the ability to inhibit 5α-reductase activity (Roh et al. 2010), which is responsible for converting testosterone into its more potent form, dihydrotestosterone. This mechanism-of-action is different from that examined in the present study, AR activity, however these two mechanisms might lead to a greater overall

418 antiandrogenic potential of AA. Various endocrine disrupting effects have further been reported in
419 studies on fish (Christianson-Heiska et al. 2008; Orrego et al. 2010). Overall, these studies imply
420 that DHAA and AA have the potential to interfere with hormone systems, which could be a concern
421 if human exposure occurs.

422 In the present study, we have illustrated that bioassay guided analysis is a valuable tool for
423 examining FCMs of paper and board for presence of potentially problematic compounds. In the
424 strategy several *in vitro* assays were included in which several of the extracts caused effects. This
425 highlights that the 'contamination level' of FCMs may be high and that we need to focus more on
426 this potential source of human exposure to chemicals. However, it is noteworthy that rejecting
427 FCMs as potentially problematic based on *in vitro* assays solely is not straight-forward, and we
428 need more experience in interpreting the *in vitro* outcome before recommendations can be given.
429 By applying the strategy, we successfully identified three compounds with estrogenic potential as
430 well as two compounds with antiandrogenic potential, which were present in the selected FCMs of
431 paper and board. We recommend further studies to be conducted applying this strategy on FCMs of
432 paper and board in order to improve and refine the strategy further. Large numbers of FCMs still
433 remain to be tested in this set-up and many more could contain potentially problematic compounds.

434 **Acknowledgements**

435 The authors would like to thank Birgitte Møller Plesning, Kitt Lademann, Lene Sofie Dahl
436 Svensson, Dorthe Lykkegaard Korsbech, Lisbeth Krüger Jensen, Anni Helleskov and Annie
437 Foverskov for technical assistance in the laboratory. Thanks to Hopi Popi, CZ and Henrik
438 Kjellgren at Nordic paper for their generous donation of samples. Also, thanks to Malcolm Driffield
439 at the Food and Environmental Research Agency (FERA), York, UK for contributing to the
440 customized database, as well as Non-linear Dynamics for supplying the Prognenesis Qi.

441 **References**

442 Bengtstrom L, Trier X, Granby K, Rosenmai AK, Petersen JH. 2014. Fractionation of extracts from paper and
443 board food contact materials for in vitro screening of toxicity. Food Additives and Contaminants Part A-
444 Chemistry Analysis Control Exposure & Risk Assessment 31(7):1291-1300; doi:
445 10.1080/19440049.2014.912357.

446 Binderup M, Pedersen G, Vinggaard A, Rasmussen E, Rosenquist H, Cederberg T. 2002. Toxicity testing
447 and chemical analyses of recycled fibre-based paper for food contact. Food Addit Contam 19:13-28; doi:
448 10.1080/02652030110089878.

449 Borchers A, Teuber SS, Keen CL, Gershwin ME. 2010. Food safety. Clin Rev Allergy Immunol 39(2):95-141;
450 doi: 10.1007/s12016-009-8176-4.

451 Calafat AM, Ye X, Wong L, Reidy JA, Needham LL. 2008. Exposure of the US population to bisphenol A and
452 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect 116(1):39-44; doi: 10.1289/ehp.10753.

453 Cao X. 2010. Phthalate esters in foods: Sources, occurrence, and analytical methods. Comprehensive
454 Reviews in Food Science and Food Safety 9(1):21-43; doi: 10.1111/j.1541-4337.2009.00093.x.

455 CDC. 2014. Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables,
456 March 2013. http://www.cdc.gov/exposurereport/ ed. .

457 Christiansen S, Axelstad M, Boberg J, Vinggaard AM, Pedersen GA, Hass U. 2014. Low-dose effects of
458 bisphenol A on early sexual development in male and female rats. Reproduction 147(4):477-487; doi:
459 10.1530/REP-13-0377.

460 Christianson-Heiska I, Haavisto T, Paranko J, Bergelin E, Isomaa B. 2008. Effects of the wood extractives
461 dehydroabietic acid and betulinol on reproductive physiology of zebrafish (danio rerio) - A two-generation
462 study. Aquatic Toxicology 86(3):388-396; doi: 10.1016/j.aquatox.2007.12.001.

463 EFSA. 2006. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in
464 contact with food on a request from the commission related to 2,2-BIS(4-HYDROXYPHENYL)PROPANE
465 (bisphenol A). 428:The EFSA Journal.

466 EFSA. 2013. Draft scientific opinion on the risks to public health related to the presence of bisphenol A
467 (BPA) in foodstuffs - part: Exposure assessment. EFSA-Q-2012-00423:EFSA Journal 2013.

468 EFSA Scientific Committee. 2012. Scientific opinion on exploring options for providing advice about possible
469 human health risks based on the concept of threshold of toxicological concern (TTC).

470 Fasano E, Bono-Blay F, Cirillo T, Montuori P, Lacorte S. 2012. Migration of phthalates, alkylphenols,
471 bisphenol A and di(2-ethylhexyl)adipate from food packaging. Food Control 27(1):132-138; doi:
472 10.1016/j.foodcont.2012.03.005.

473 Foster P. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to
474 phthalate esters. Int J Androl 29(1):140-147; doi: 10.1111/j.1365-2605.2005.00563.x.

475 Geens T, Goeyens L, Covaci A. 2011. Are potential sources for human exposure to bisphenol-A overlooked?
476 Int J Hyg Environ Health 214(5):339-347; doi: 10.1016/j.ijheh.2011.04.005.

477 Ghisari M, Bonefeld-Jorgensen EC. 2009. Effects of plasticizers and their mixtures on estrogen receptor and
478 thyroid hormone functions. Toxicol Lett 189(1):67-77; doi: 10.1016/j.toxlet.2009.05.004.

479 Gould J, Leonard L, Maness S, Wagner B, Conner K, Zacharewski T et al. 1998. Bisphenol A interacts with
480 the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142(1-2):203-214; doi:
481 10.1016/S0303-7207(98)00084-7.

482 Grignard E, Lapenna S, Bremer S. 2012. Weak estrogenic transcriptional activities of bisphenol A and
483 bisphenol S. Toxicol In Vitro 26(5):727-731; doi: 10.1016/j.tiv.2012.03.013.

484 Kind T, Fiehn O. 2007. Seven golden rules for heuristic filtering of molecular formulas obtained by accurate
485 mass spectrometry. BMC Bioinformatics 8:105; doi: 10.1186/1471-2105-8-105.

486 Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K et al. 2005. Comparative study of the
487 endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84(2):249-259; doi:
488 10.1093/toxsci/kfi074.

489 Krishnan A, Stathis P, Permuth S, Tokes L, Feldman D. 1993. Bisphenol-a - an estrogenic substance is
490 released from polycarbonate flasks during autoclaving. Endocrinology 132(6):2279-2286; doi:
491 10.1210/en.132.6.2279.

492 Leach RH, Pierce RJ. 1993. The Printing Ink Manual. 5th ed. Dordrecht, The Netherlands:Kluwer Academic
493 Publishers.

494 Liao C, Kannan K. 2011. Widespread occurrence of bisphenol A in paper and paper products: Implications
495 for human exposure. Environ Sci Technol 45(21):9372-9379; doi: 10.1021/es202507f.

496 Lopez-Espinosa M-, Granada A, Araque P, Molina-Molina J-, Puertollano M-, Rivas A et al. 2007.
497 Oestrogenicity of paper and cardboard extracts used as food containers. Food Additives and Contaminants
498 Part A-Chemistry Analysis Control Exposure & Risk Assessment 24(1):95-102; doi:
499 10.1080/02652030600936375.

500 Mankidy R, Wiseman S, Ma H, Giesy JP. 2013. Biological impact of phthalates. Toxicol Lett 217(1):50-58;
501 doi: 10.1016/j.toxlet.2012.11.025.

502 Mendum T, Stoler E, VanBenschoten H, Warner JC. 2011. Concentration of bisphenol A in thermal paper.
503 Green Chemistry Letters and Reviews 4(1):81-86; doi: 10.1080/17518253.2010.502908.

504 Mitani K, Fujioka M, Uchida A, Kataoka H. 2007. Analysis of abietic acid and dehydroabietic acid in food
505 samples by in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry.
506 Journal of Chromatography a 1146(1):61-66; doi: 10.1016/j.chroma.2007.01.118.

507 Miyawaki J, Sakayama K, Kato H, Yamamoto H, Masuno H. 2007. Perinatal and postnatal exposure to
508 bisphenol a increases adipose tissue mass and serum cholesterol level in mice. J Atheroscler Thromb
509 14(5):245-252.

510 Moral R, Wang R, Russo IH, Lamartiniere CA, Pereira J, Russo J. 2008. Effect of prenatal exposure to the
511 endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. J
512 Endocrinol 196(1):101-112; doi: 10.1677/JOE-07-0056.

513 Neltner TG, Alger HM, Leonard JE, Maffini MV. 2013. Data gaps in toxicity testing of chemicals allowed in
514 food in the United States. Reproductive Toxicology 42:85-94; doi: 10.1016/j.reprotox.2013.07.023.

515 Orrego R, Guchardi J, Krause R, Holdway D. 2010. Estrogenic and anti-estrogenic effects of wood
516 extractives present in pulp and paper mill effluents on rainbow trout. Aquatic Toxicology 99(2):160-167; doi:
517 10.1016/j.aquatox.2010.04.016.

518 Ozaki A, Ooshima T, Mori Y. 2006. Migration of dehydroabietic and abietic acids from paper and paperboard
519 food packaging into food-simulating solvents and tenax TA. Food Addit Contam 23(8):854-860; doi:
520 10.1080/02652030600743813.

521 Ozaki A, Yamaguchi Y, Fujita T, Kuroda K, Endo G. 2004. Chemical analysis and genotoxicological safety
522 assessment of paper and paperboard used for food packaging. Food and Chemical Toxicology 42(8):1323-
523 1337; doi: 10.1016/j.fct.2004.03.010.

524 Ozaki A, Yamaguchi Y, Fujita T, Kuroda K, Endo G. 2005. Safety assessment of paper and board food
525 packaging: Chemical analysis and genotoxicity of possible contaminants in packaging. Food Addit Contam
526 22(10):1053-1060; doi: 10.1080/02652030500090885.

527 Paris F, Balaguer P, Terouanne B, Servant N, Lacoste C, Cravedi J et al. 2002. Phenylphenols, biphenols,
528 bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in
529 reporter cell lines. Mol Cell Endocrinol 193(1-2):43-49; doi: 10.1016/S0303-7207(02)00094-1.

530 Petersen JH, Jensen LK. 2010. Phthalates and food-contact materials: Enforcing the 2008 European Union
531 plastics legislation. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk
532 Assessment 27(11):1608-1616; doi: 10.1080/19440049.2010.501825.

533 Piersma AH, Bosgra S, van Duursen MBM, Hermsen SAB, Jonker LRA, Kroese ED et al. 2013. Evaluation of
534 an alternative in vitro test battery for detecting reproductive toxicants. Reproductive Toxicology 38:53-64; doi:
535 10.1016/j.reprotox.2013.03.002.

536 Pocas MF, Oliveira JC, Pereira JR, Hogg T. 2010. Consumer exposure to phthalates from paper packaging:
537 An integrated approach. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure &
538 Risk Assessment 27(10):1451-1459; doi: 10.1080/19440049.2010.490790.

539 Roberts JC. 1996. The Chemistry of Paper . 1st ed. Cambridge, England:Royal Society of Chemistry
540 Paperbacks.

541 Roh S, Park M, Kim Y. 2010. Abietic acid from resina pini of pinus species as a testosterone 5 alpha-
542 reductase inhibitor. J Health Sci 56(4):451-455.

543 Rosenmai AK, Dybdahl M, Pedersen M, van Vugt-Lussenburg BMA, Wedebye EB, Taxvig C et al. 2014. Are
544 structural analogues to bisphenol A safe alternatives? Toxicological Sciences 139(1):35-47; doi:
545 10.1093/toxsci/kfu030.

546 Rostkowski P, Horwood J, Shears JA, Lange A, Oladapo FO, Besselink HT et al. 2011. Bioassay-directed
547 identification of novel antiandrogenic compounds in bile of fish exposed to wastewater effluents. Environ Sci
548 Technol 45(24):10660-10667; doi: 10.1021/es202966c.

549 Shen O, Du G, Sun H, Wu W, Jiang Y, Song L et al. 2009. Comparison of in vitro hormone activities of
550 selected phthalates using reporter gene assays. Toxicol Lett 191(1):9-14; doi: 10.1016/j.toxlet.2009.07.019.

551 Sjöström E, Alen R. 1998. Analytical Methods in Wood Chemistry, Pulping, and Papermaking. Berlin,
552 Heidelberg:Springer-Verlag.

553 Suciu NA, Tiberto F, Vasileiadis S, Lamastra L, Trevisan M. 2013. Recycled paper-paperboard for food
554 contact materials: Contaminants suspected and migration into foods and food simulant. Food Chem
555 141(4):4146-4151; doi: 10.1016/j.foodchem.2013.07.014.

556 Taxvig C, Dreisig K, Boberg J, Nellemann C, Schelde AB, Pedersen D et al. 2012. Differential effects of
557 environmental chemicals and food contaminants on adipogenesis, biomarker release and PPAR gamma
558 activation. Mol Cell Endocrinol 361(1-2):106-115; doi: 10.1016/j.mce.2012.03.021.

559 The European Commission. 2002. COMMISSION DECISION of 12 August 2002 Implementing Council
560 Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results.
561 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF ed. .

562 The European Commission. 2004. REGULATION (EC) no 1935/2004 OF THE EUROPEAN PARLIAMENT
563 AND OF THE COUNCIL of 27 October 2004 on Materials and Articles Intended to Come into Contact with
564 Food and Repealing Directives 80/590/EEC and 89/109/EEC. http://eur-
565 lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:338:0004:0017:en:PDF ed. .

566 Vinggaard A, Korner W, Lund K, Bolz U, Petersen J. 2000. Identification and quantification of estrogenic
567 compounds in recycled and virgin paper for household use as determined by an in vitro yeast estrogen
568 screen and chemical analysis. Chem Res Toxicol 13(12):1214-1222; doi: 10.1021/tx000146b.

569 Vinggaard A, Nellemann C, Dalgaard M, Jorgensen E, Andersen H. 2002. Antiandrogenic effects in vitro and
570 in vivo of the fungicide prochloraz. Toxicol Sci 69(2):344-353; doi: 10.1093/toxsci/69.2.344.

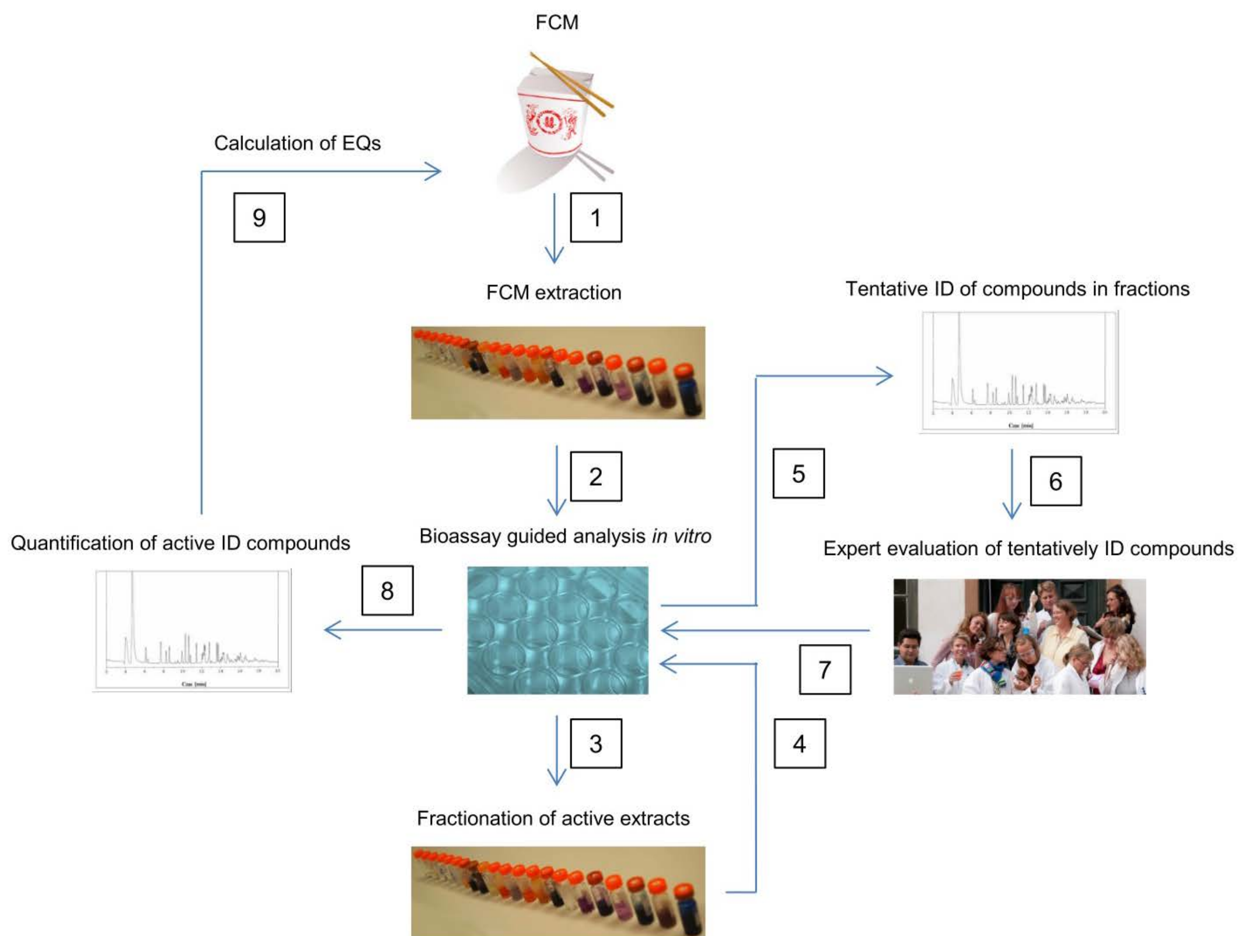
571 Weber A, von Wright A, Honkalampi-Hämäläinen U, Järvinen M, Lhuguenet JC, Severin I et al. 2006.
572 Biosafepaper - application of bioassays for safety assessment of paper and board for food contact. QLK1-
573 CT-2001-00930.

574 Wormuth M, Scheringer M, Vollenweider M, Hungerbuhler K. 2006. What are the sources of exposure to
575 eight frequently used phthalic acid esters in europeans? Risk Analysis 26(3):803-824; doi: 10.1111/j.1539-
576 6924.2006.00770.x.

577 Xu X, Zhang J, Wang Y, Ye Y, Luo Q. 2010. Perinatal exposure to bisphenol-A impairs learning-memory by
578 concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice.
579 Horm Behav 58(2):326-333; doi: 10.1016/j.yhbeh.2010.02.012.

580 Zhang Z, Hu Y, Zhao L, Li J, Bai H, Zhu D et al. 2011. Estrogen agonist/antagonist properties of dibenzyl
581 phthalate (DBzP) based on in vitro and in vivo assays. Toxicol Lett 207(1):7-11; doi:
582 10.1016/j.toxlet.2011.08.017.

583



584

585 *Figure 1: Step-by-step workflow for the FCM strategy. Numbers indicate, 1) production of extracts from FCMs of paper and board,*
586 *2) in vitro testing of extracts, 3) fractionation of extracts exhibiting in vitro activity, 4) in vitro testing of fraction from active extracts,*
587 *5) tentative identification of compounds based on in vitro activity of fractions, 6) selection of tentatively identified compounds by*
588 *expert judgment for further in vitro examination, 7) testing of selected tentatively identified compounds in vitro, 8) quantification of*
589 *selected tentatively identified compounds causing in vitro activity, 9) calculation of equivalence factors (EQs). Abbreviations: ID =*
590 *identification, FCM = food contact material.*

591 *Table 1: Sample overview of the tested FCMs of paper and board with indication of usage or intended use, material type, suppliers, pulp type, whether the materials were printed and the*
592 *mass volumes.*

Extract no.	Usage	Material	Supplier	Pulp type	Printing	Grammage (g/m^2)
S2	Plain paper	Paper	Paper industry	Virgin pulp	No	45
S3	Baking paper	Paper	Retail	Virgin pulp	No	45
S4	Sandwich wrapper[*]	Paper	Retail	Virgin pulp	No	40
S5	Baking paper	Paper	Retail	Virgin pulp	No	40
S6	Baking mold	Paper	Retail	Virgin pulp	Yes	40
S7	Flour bag[a]	Paper	Retail	Virgin pulp	Yes	80
S8	Pizza box[*]	Corrugated fibreboard	Retail	Recycled	Yes	550
S9	Susceptor for microwave popcorn	Paperboard	Printing industry	Virgin and recycled	Yes	190
S10	Sausage tray	Paperboard	Printing industry	Virgin and recycled	Yes	270
S11	Microwave pizza tray	Paperboard	Printing industry	Virgin and recycled	Yes	475
S12	Frozen fish box	Paperboard	Printing industry	Virgin and recycled	Yes	400
S13	Cake tray	Paperboard	Printing industry	Virgin and recycled	Yes	420
S14	Tomato punnet	Paperboard	Printing industry	Virgin and recycled	Yes	400
S16	TEPP Chinese Zineth	Paperboard	Printing industry	Recycled	Yes	310
S17	Microwave popcorn bag	Paper	Printing industry	Recycled	Yes	90
S18	TEPP Chinese Spark	Paperboard	Printing industry	Recycled	Yes	330
S19	Paperboard with UV print	Paperboard	Printing industry	Recycled	Yes	280
S20	Paperboard with water soluble print	Paperboard	Printing industry	Recycled	Yes	230
S21	Paperboard with offset print	Paperboard	Printing industry	Recycled	Yes	280
S22	Cereal box[a]	Paperboard	Retail	Recycled	Yes	380

593 [a]Contained food at purchase. [*]Samples for further fractionation.

Table 2: Methods used for quantification of a) bisphenol A and b) for compounds found in AR active fractions.

a)

LC conditions			
Instrument	Waters Acquity UPLC System (Waters, Milford, USA)		
Column	Kinetex C18 (2.6 µm, 2.1x100 mm) (Waters)		
Column temperature	40°C		
Injection volume	3 µL		
Mobile phase	A: water		
	B: methanol		
Flow rate	0.3 mL min^{-1}		
Gradient	Time (min)	%A	%B
	0	80	20
	1	55	45
	4.5	20	80
	5.5	2	98
	13	2	98
Post time	2 min		
Total run time	15		

MS conditions	
Instrument	Micromass Quattro Ultima (Waters, Milford, USA) in MRM mode
Ionization mode	ESI -
Voltage	- 3 kV
Source temperature	120°C
Cone voltage	55 V
Cone gas flow	100 L h^{-1}
Desolvation temperature	500°C
Desolvation gas flow	775 L h^{-1}

b)

LC conditions			
Instrument	Agilent 1200 Series HPLC (Agilent Technologies, CA, US)		
Column	Gemini C18 (3µm 2 x 200 mm) (Phenomenex, CA, USA)		
Column temperature	40°C		
Injection volume	3 µL		
Mobile phase	A: water + 0.01 formic acid		
	B: methanol		
Flow rate	0.2 mL min^{-1}		
Gradient	Time (min)	%A	%B
	0	10	90
Total run time	10 min		

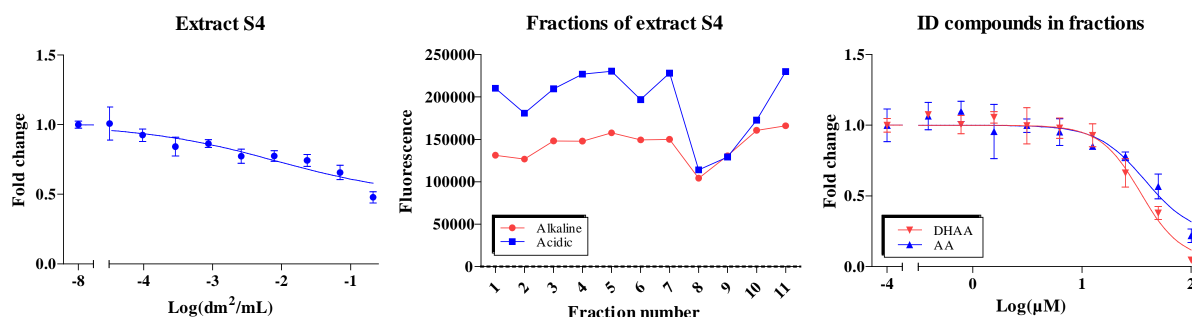
MS conditions	
Instrument	Micromass Quattro Ultima (Waters, Milford, USA) in MRM mode
Ionization mode	ESI-
Voltage	-2.5 kV
Source temperature	120°C
Cone voltage	20 V
Cone gas flow	135 L h^{-1}
Desolvation temperature	380°C
Desolvation gas flow	575 L h^{-1}

Table 3: Tentative lowest observable effect concentrations (LOECs) (dm^2/mL) and tentative efficacies (E_{max}) (%) for the 20 paper and board extracts examined in the AR reporter gene assay in agonist and antagonist mode, the ER reporter gene assay and the AhR reporter gene assay.

Extract no.	Usage	AR Agonism		AR Antagonism		AhR agonism		ER agonism	
		LOEC	E_{max}	LOEC	E_{max}	LOEC	E_{max}	LOEC	E_{max}
S2	Plain paper	$8.0*10^{-3}$	290	$2.2*10^{-1}$	45	$2.2*10^{-1}$	115	-	-
S3	Baking paper	$6.3*10^{-2}$	73	$2.3*10^{-3}$	65	$6.3*10^{-2}$	73	-	-
S4	Sandwich wrapper*	$7.8*10^{-3}$	532	$2.3*10^{-2}$	52	$2.1*10^{-1}$	81	-	-
S5	Baking paper	-	-	$2.5*10^{-2}$	28	$7.4*10^{-2}$	224	-	-
S6	Baking mold	-	-	-	-	$2.0*10^{-2}$	174	-	-
S7	Flour bag[a]	-	-	-	-	$6.5*10^{-3}$	568	$5.9*10^{-2}$	82
S8	Pizza box*	-	-	-	-	$4.8*10^{-5}$	1040	$2.6*10^{-3}$	103
S9	Susceptor for microwave popcorn	-	-	-	-	$1.1*10^{-2}$	505	-	-
S10	Sausage tray	-	-	-	-	$3.9*10^{-3}$	992	$2.3*10^{-2}$	69
S11	Microwave pizza tray	$3.7*10^{-3}$	327	-	-	$3.7*10^{-3}$	260	-	-
S12	Frozen fish box	-	-	-	-	$3.6*10^{-3}$	223	$2.1*10^{-2}$	63
S13	Cake tray	-	-	$5.1*10^{-3}$	42	$1.7*10^{-3}$	274	-	-
S14	Tomato punnet	-	-	-	-	$2.3*10^{-5}$	1069	$5.7*10^{-3}$	105
S16	Imported Chinese 1	$1.3*10^{-2}$	104	-	-	$1.4*10^{-3}$	670	$1.3*10^{-2}$	160
S17	Microwave popcorn bag	-	-	-	-	$4.4*10^{-4}$	229	-	-
S18	Imported Chinese 2	$1.1*10^{-1}$	141	$1.1*10^{-1}$	36	$3.9*10^{-3}$	701	$3.5*10^{-2}$	132
S19	Paperboard with UV print	-	-	-	-	$6.5*10^{-4}$	634	$1.3*10^{-3}$	245
S20	Paperboard with water soluble print	-	-	-	-	$8.9*10^{-4}$	430	$1.8*10^{-3}$	226
S21	Paper board with offset print	-	-	-	-	$1.1*10^{-3}$	869	-	-
S22	Cereal box[a]	-	-	-	-	$4.3*10^{-4}$	329	-	-

Values are based on one representative experiment in cases where more than one was conducted. *Samples for further fractionation.

AR antagonism



ER agonism

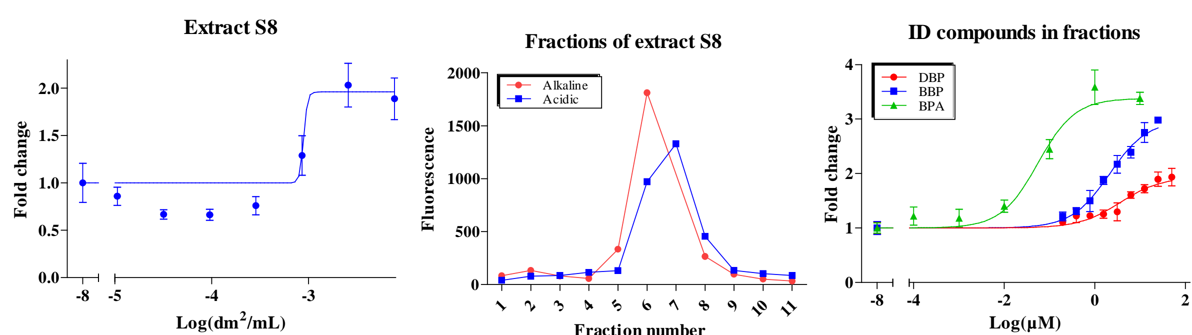


Figure 2: Androgen receptor (AR) antagonism of sandwich wrapper (extract S4) (top, left), fractions of extract S4 (top, middle), and identified (ID) compounds in active fractions of extract S4 (top, right). Estrogen receptor (ER) agonism of the pizza box (extract S8) (bottom, left), fraction of extract S8 (bottom, middle), and identified compounds in fraction 6 alkaline and 7 acidic of extract S8 (bottom, right). Graphs are based on one representative experiment in extract, fractions, and ID compounds. Data from extract and identified compounds were normalized to controls and fitted to a sigmoidal dose-response model. Error bars represent standard deviations (SD).

Table 4: Estimated and measured equivalence factors (EQs) in µM of hydroxyflutamide and 17β-estradiol in the AR and ER reporter gene assay, respectively, for the pizza box (extract S8) and the sandwich wrapper (extract S4), as well as identified compounds causing changes in activity in extracts including bisphenol A (BPA), di-butylphthalate (DBP), butyl-benzylphthalate (BBP), dehydroabietic acid (DHAA), and abietic acid (AA). [a]Concentrations (µM) for identified compounds in extract at maximum response.

ESTROGEN RECEPTOR ACTIVITY								
EXTRACT	BPA		DBP		BBP		TOTAL EEQ	
	µM[a]	EEQ	µM[a]	EEQ	µM[a]	EEQ	EEQ_{calc}	EEQ_{meas}
S8	0.08	$1.11*10^{-5}$	0.19	$1.89*10^{-7}$	0.07	$1.99*10^{-7}$	$1.42*10^{-5}$	$2.23*10^{-6}$

ANDROGEN RECEPTOR ACTIVITY						
EXTRACT	DHAA		AA		TOTAL EEQ	
	µM[a]	AEQ	µM[a]	AEQ	AEQ_{calc}	AEQ_{meas}
S4	3.91	$2.14*10^{-4}$	485.2	$1.49*10^{-1}$	$1.49*10^{-1}$	$8.84*10^{-2}$

Supplementary materials: Tentatively identified compounds

Data presented in this Supplementary material are the results obtained from the tentative identification process of fractions with positive toxicological response.

Indicated in the columns are:

Compound name

CAS number: if available

Molecular formula

Retention time: In respective method

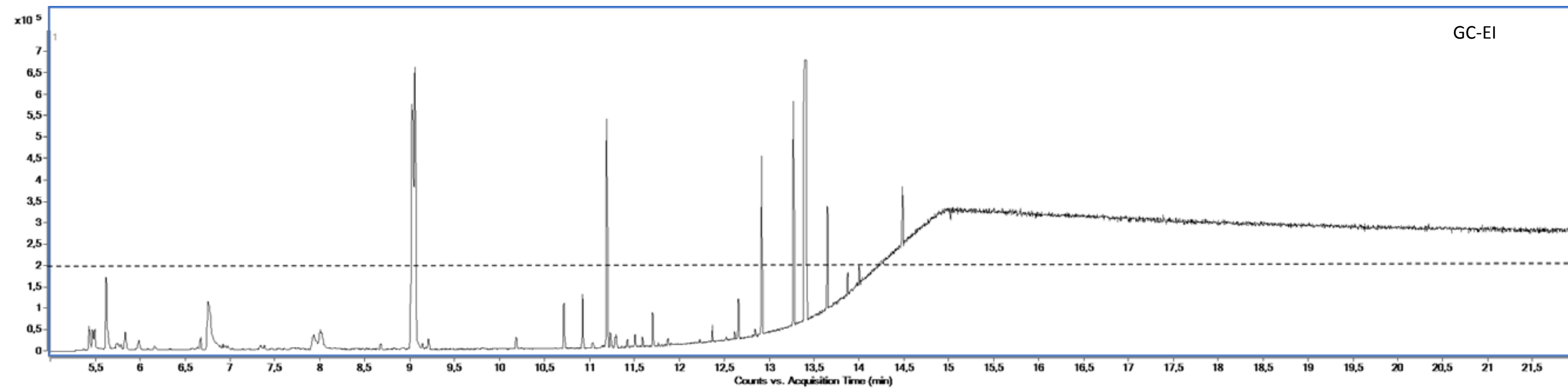
Ionization mode: GC-EI, LC-ESI+ and LC-ESI-

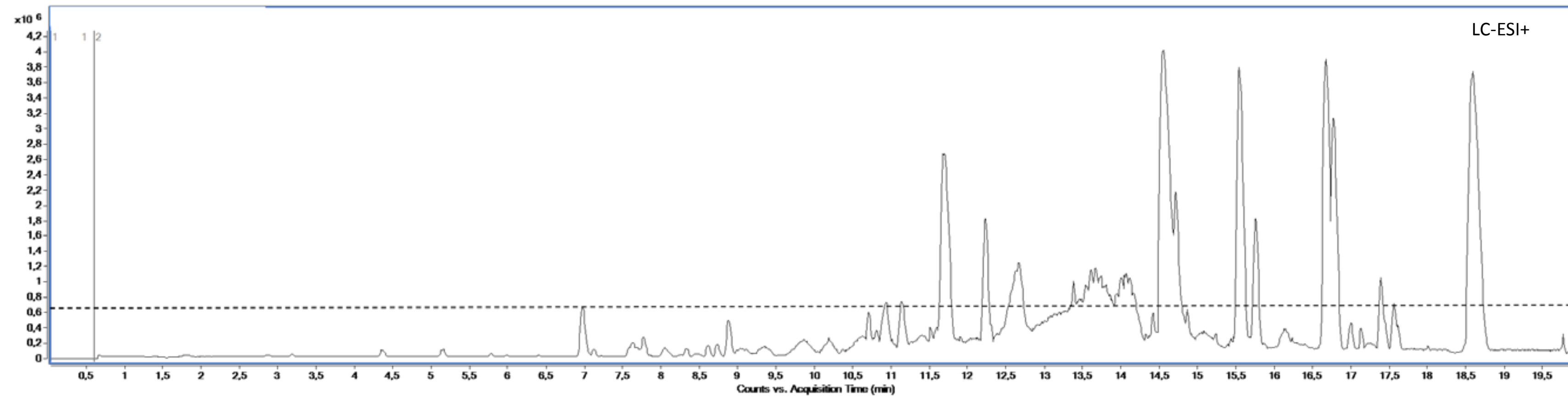
Customized database hit: only used for compounds identified in LC-ESI+ or LC-ESI-

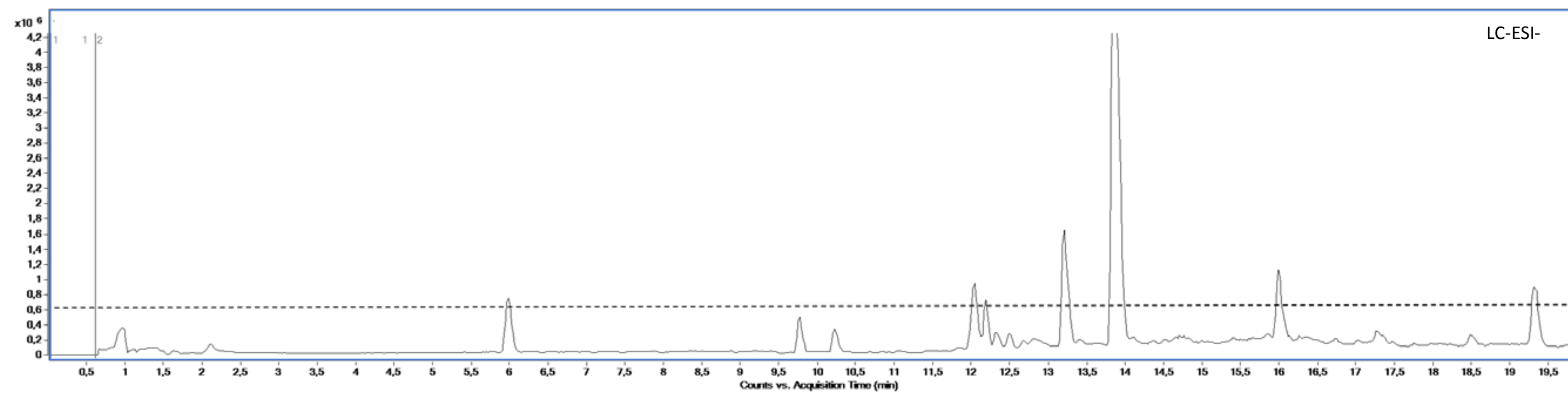
Number of ions: only used for compounds identified in LC-ESI+ or LC-ESI-. Adducts (if present) are also registered as these could facilitate localization of molecular ion.

Additional information: Fragmentation, relevant usage in paper and board

S4 acidic fraction 8



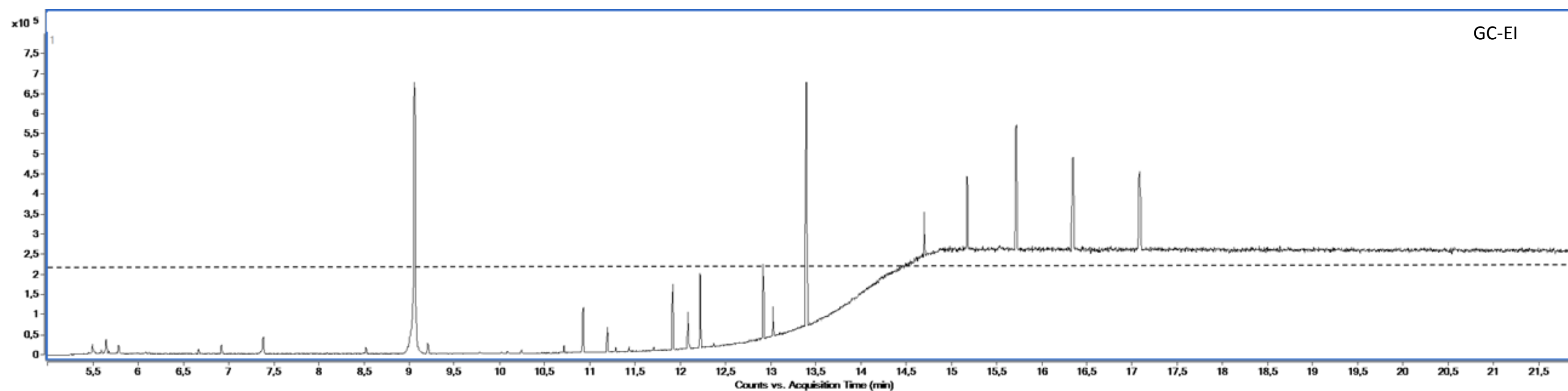


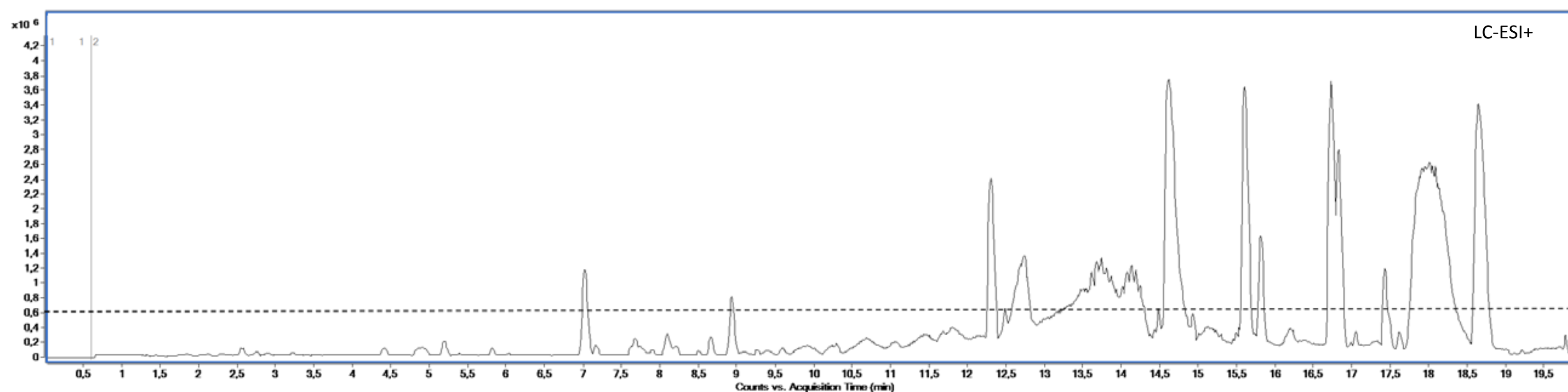


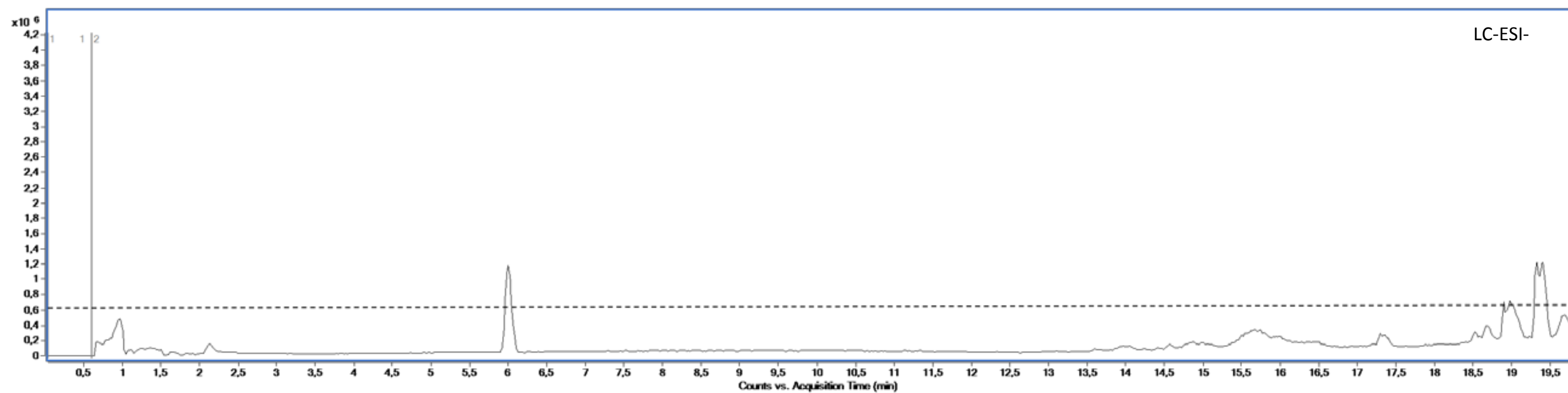
Compound	CAS number	Molecular formula	Retention time	GC-EI	LC-ESI+	LC-ESI-	Customized database hit	Number of ions (for LC only)	Additional information
1-Isocyanatooctadecane	112-96-9	C19H37NO	9.32	x					Used in coatings, adhesives and in printing
Phthalic acid, butyl cyclohexyl ester	84-64-0	C18H24O4	11.12	x					Used as plasticizer
			12.98	x					Aliphatic fragmentation pattern
			13.27	x					Aliphatic fragmentation pattern
2,2-diethoxyacetophenone	C12H16O3	6175-45-7	13.38	x					Used in resin compositions for ink jet printing
Methyl 6-methoxy-2,3-dihydro-1-benzofuran-2-carboxylate	C11H12O4	20166-65-8	13.64	x					
			14.48	x					Aliphatic fragmentation pattern
Propanamide, 3-(2-benzothiazolylthio)-	132605-19-7	C10H10N2OS2	6.95		x			2	Fragment into C3H5NO
			11.67		x			1	*m/z* 300.9967
		C18H30N4S	12.15		x			1	
Dehydroabietic acid	1740-19-8	C20H28O2	12.3		x		x	1	Resin acid
Abietic acid	514-10-3	C20H30O2	12.3		x		x	1 (-H2O adduct)	Resin acid
Isorhamnetin	480-19-3	C16H12O7	12.66		x			1	Naturally occurring flavonol
Rhamnetin	90-19-7	C16H12O7	12.66		x			1	Naturally occurring flavonol
		C23H40O5	12.66		x			2 (NH4+ adduct)	Fragments into C12H18O. No matching structures available

3,6,9,12-Tetraazaoctadeca-14,16-dien-18-oic acid, 1-amino-, ethylester	61347-03-3	C16H33N5O2	14.50	x			4	
4-oxo-retinoic acid	38030-57-8	C20H26O3	14.77	x			1 (NH4+ adduct)	
2-(8-Heptadecenyl)-2-oxazoline		C20H37NO	15.51 +15.81	x			2	Fragments into C3H5NO
			16.60	x			3	Molecular ion at *m/z* 1120.8380, fragments at *m/z* 569.4367 and *m/z* 284.2982 (C18H37NO)
		C26H55NO4S	17.40	x			7	Fragments into C24H48O2S, C13H28S, C11H16, C8H12, C7H10, C6H8
2-Mercaptobenzothiazole	149-30-4	C7H5NS2	5.98		x	x	3	Fragments into C7H5NS , used in rubber and latex production as well as in paper manufacturing, production of litographic plates and two-part cyanoacrylate adhesives
		C21H26N4	9.77		x		1	
		C16H32N4	12.05		x		1	
		C18H34N4S	12.05		x		1	
		C17H30N14O	13.21		x		2	
		C1736O10	13.82		x		2	Fragments into C13H28O8
(2E)-3-(Tetradecylsulfanyl)acrylic acid		C17H32O2S	15.98		x		1	

S4 alkaline fraction 8

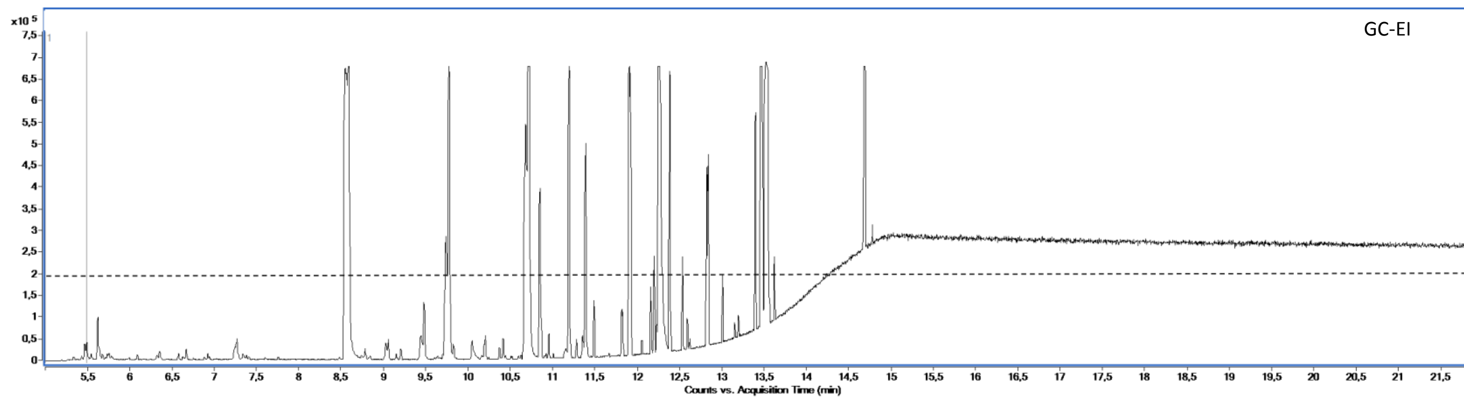


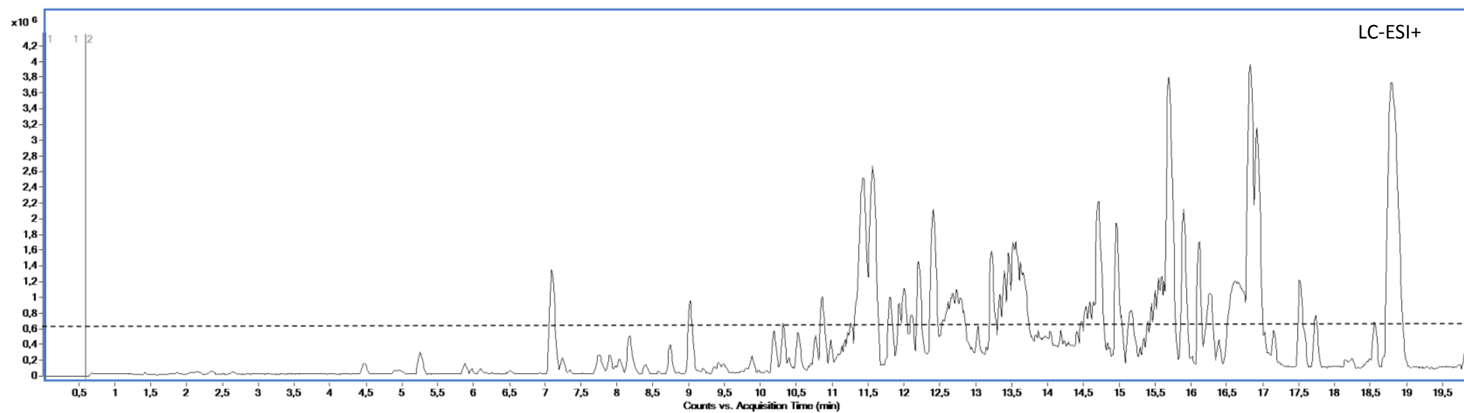


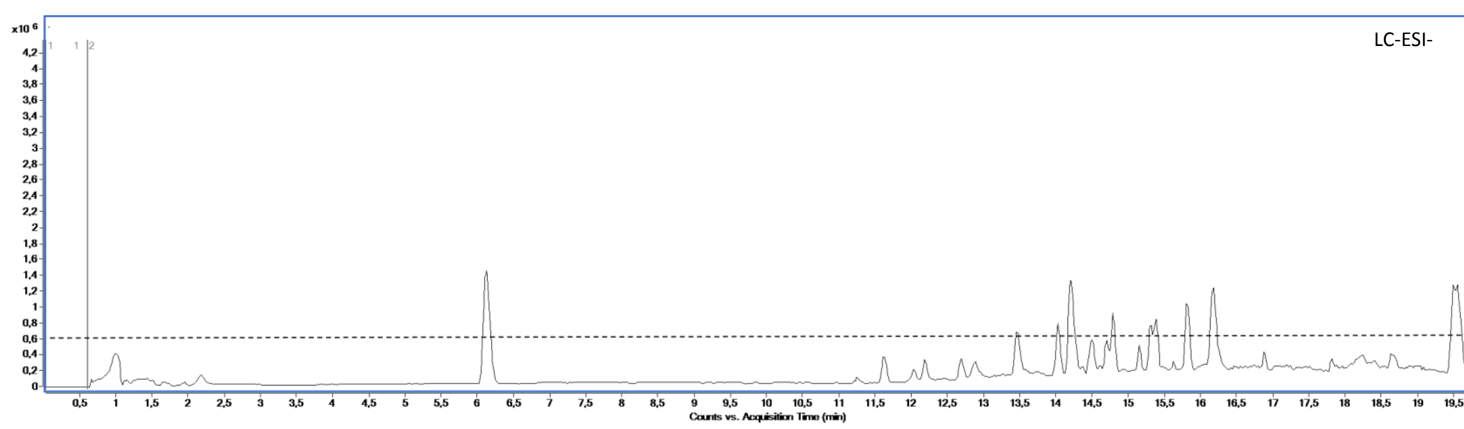


Compound	CAS number	Molecular formula	Retention time	GC-EI	LC-ESI+	LC-ESI-	Customized database hit	Number of ions (for LC only)	Additional information
1-Isocyanatooctadecane	112-96-9	C19H37NO	9.32	x					Used in coatings, adhesives and in printing
4-hydroxy-3a,7a-dimethyl-4,5-dihydro-3H-2-benzofuran-1-one	54346-06-4	C10H14O3	12.91	x					
2,2-diethoxyacetophenone	C12H16O3	6175-45-7	13.39	x					Used in resin compositions for ink jet printing
			14.7	x					Aliphatic fragmentation pattern
			15.17	x					Aliphatic fragmentation pattern
			15.71	x					Aliphatic fragmentation pattern
			16.34	x					Aliphatic fragmentation pattern
			17.07	x					Aliphatic fragmentation pattern
		C10H10N2OS2	7.02		x			2	Fragments into C3H5NO
Benzyl dimethylcarbamodithioate	7250-18-2	C10H13NS2	8.93		x			2	Fragments into C3H5NS,used in ink compositions
Dehydroabietic acid	1740-19-8	C20H28O2	12.3		x		x	1 (M+H)	Resin acid
Abietic acid	514-10-3	C20H30O2	12.3		x		x	1 (M+H-H2O)	Resin acid
			14.55		x			4	328.2714, 286.2235, 117.0741, 75.0262
2-(8-Heptadecenyl)-2-oxazoline		C20H37NO	15.51, 15.8		x			2	Fragments into C3H5NO
			16.6		x			3	*m/z* 1120.8380, fragments into *m/z* 569.4367 and *m/z* 284.2982 (C18H37NO)
		C26H55NO4S	17.40		x			7	Fragments into C24H48O2S, C13H28S, C11H16, C8H12, C7H10, C6H8
2-Mercaptobenzothiazole	149-30-4	C7H5NS2	5.98			x	x	2	Fragments into C7H5NS, used in rubber and latex production as well as in paper manufacturing, production of litographic plates and two-part cyanoacrylate adhesives

S8 acidic fraction 6



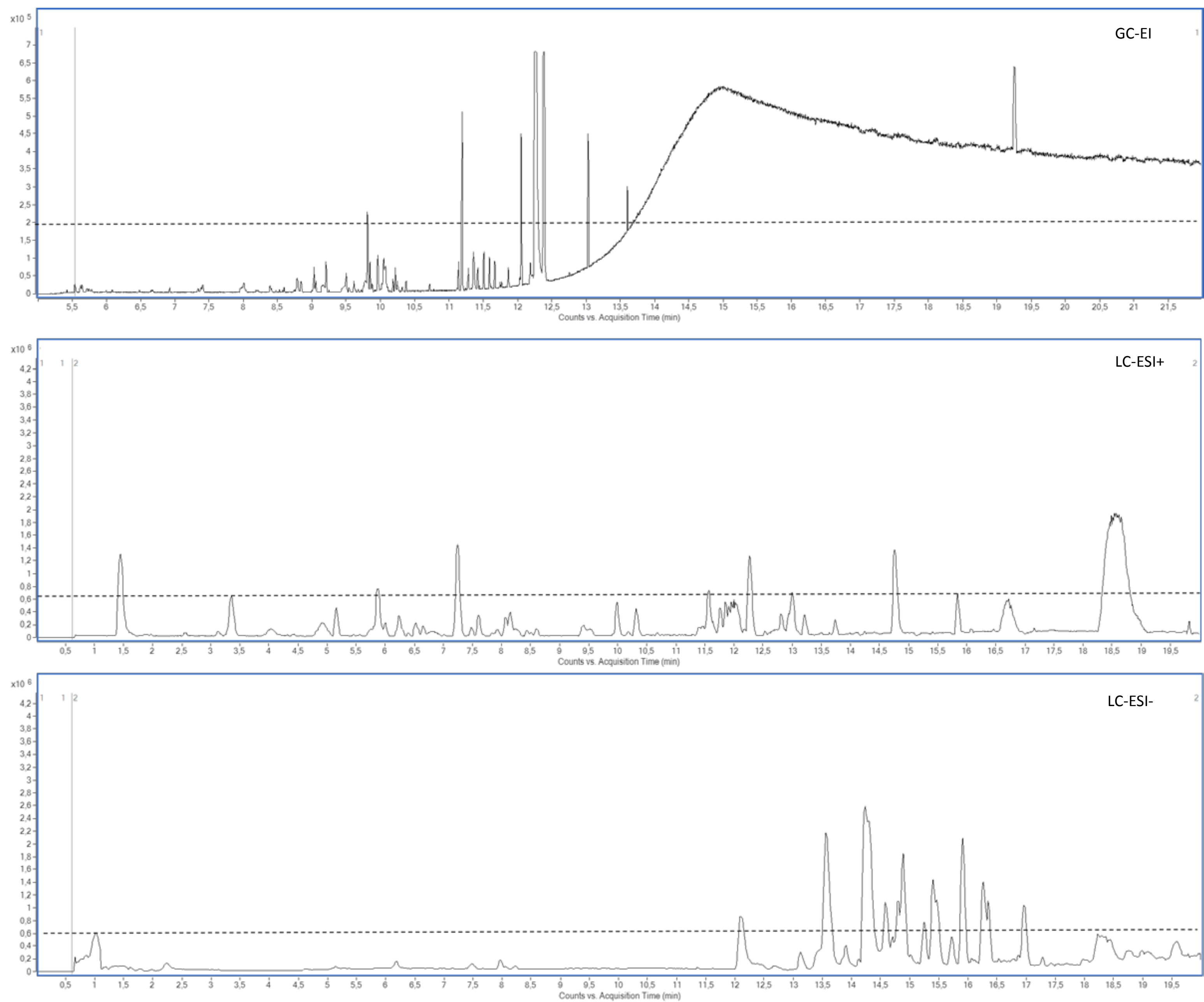




Compound	CAS number	Molecular formula	Retention time	GC-EI	LC-ESI+	LC-ESI-	Customized database hit	Number of ions (for LC only)	Additional information
N,N-Dimethyldodecylamine	112-18-5	C14H31N	8.58	x					
Vinyl benzoate	769-78-8	C9H8O2	9.48	x					Monomer for polyvinyl
Di-isobutyl phthalate	84-69-5	C16H22O4	10.68	x					Used in in printing inks, resin solvent, paper coatings and in adhesives
Dibutyl phthalate	84-74-2	C16H22O4	11.17 (GC); 12.25 (LC)	x	x		x	3	Used in in printing inks, resin solvent, paper coatings and in adhesives
2,2-Dimethoxy-1,2-diphenylethanone	24650-42-8	C16H16O3	10.85	x					Photo initiator. Associated with printing inks.
4a,9a-Dihydro-9,10-anthracenedione	84-65-1	C14H8O2	11.387	x					Digester additive in paper making process. Protecting cellulose (and hemicellulose) from alkaline degradation.
Methyl 4-(6-methyl-4-oxo-2-heptanyl)-1-cyclohexene-1-carboxylate	17904-27-7	C16H26O3	11.49	x					
Benzidine	92-87-5	C12H12N2	11.9	x					Used in production of dyes.
Bisphenol A	80-05-7	C15H16O2	12.25	x					Used for manufacturing epoxy, polycarbonate and other resins
Tributyl acetylcitrate	77-90-7	C20H34O8	12.53	x					Biodegradable plasticizer used in cellulose resin.
4'-Methoxy-2-hydroxystilbene	-	C15H14O2	12.84	x					Naturally occurring substance formed in hardwood.
Bis(2-ethylhexyl) (2E)-but-2-enedioate	141-02-6	C20H36O4	12.38	x					Fumarate. Possibly used to facilitate dye setting.
Benzyl Butyl Phthalate	85-68-7	C19H20O4	13.00	x					Used in in printing inks, resin solvent, paper coatings and in adhesives
1,3-Diphenyl isothianaphthene	16587-39-6	C20H14S	13.40	x					Used in production of vinyl polymer
2-(2-(Benzoyloxy)propoxy)propyl benzoate	20109-39-1	C20H22O5	13.46	x					Naturally occurring substance formed in hardwood.
Oxydi-2,1-ethanediyl dibenzoate	120-55-8	C18H18O5	13.52 (GC); 9.1 (LC)	x	x			2	Fragments into C10H11O2 in LC. Naturally occurring substance formed in hardwood.
Dipropyleneglycol dibenzoate	20109-39-1	C20H22O5	13.59	x					Used in photo resistant layers as well as adhesives
Diglycol dibenzoate	120-55-8	C18H18O5	14.69	x					Used as a plasticizer, in adhesives and in ink jet inks
Michler's ketone	90-94-8	C17H20N2O	7.1		x		x	2	Fragments into C8H11N, intermediate in the synthesis of dyes and pigments for paper
4,4-Dimethylandrost-5-ene		C21H34	10.85		x			1 (NH4+ adduct)	
2,4-Xylenol, 6,6'-isobutylidenedi-	33145-10-7	C20H26O2	11.4		x			1	Used in printing inks (for black)
			11.6		x			3	317.2086, 295.2264, 277.2186
Dipropylene glycol benzoate	27138-31-4	C20H22O5	11.8-12.0		x			2	Fragments into C10H11O2, used as plasticizer
		C26H26N2O2	12.5		x			1	

		C31H38N2	13.2	x			1	
		C19H37NOS	14.75	x			3 (-H20 adduct)	Fragments into C6H12S and C3H6S
			14.9	x			1	*m/z* 298.3151
			15.7	x			1	*m/z* 308.2898
			15.9	x			1	*m/z* 308.3001
		C40H36O	16.1	x			1	
			16.8	x			1	*m/z* 284.2992
Stearamide	124-26-5	C18H37NO	16.9	x		x	1	Used in toner pigments, as defoamer and in flexographic printing forms
		C27H44O2	17.55	x			4	Fragments into C25H40, C16H24, C11H16
N-Isopropylhexadecanamide	189939-61-5	C19H39NO	17.75	x			1	Used in amide containing copolymers
2-Mercaptobenzothiazole		C7H5NS2	6.15		x		2	Fragments into C7H5NS, used in rubber and latex production as well as in paper manufacturing, production of lithographic plates and two-part cyanoacrylate adhesives
Polyglycerol ricinoleate	29894-35-7	C18H33O3	13.45		x		2	Fragments into C12H20O2, printing ink
Nonidet P-40	11130-43-1	C18H30O3	14.03		x		2	Approved use as a component of articles intended for use in packaging, transporting, or holding food (US FDA)
Ricinoleic acid	141-22-0	C18H34O3	14.21		x	x	1 (-H2O)	Used in printing ink
Methyl 9,10-Dihydroxystearate	1115-01-1	C19H38O4	14.78		x		2	Fragments into C18H34O3, used in ink jet printing
Polyglycerol ricinoleate	29894-35-7	C18H33O3	15.31		x	x	1	Used in printing ink
12-Hydroxystearic acid	106-14-9	C18H36O3	15.82		x		1	Used as printing ink, resin composition and laminates
Dehydroabietic acid	1740-19-8	C20H28O2	16.21		x	x	1	Resin acid

S8 alkaline fraction 6



Compound	CAS number	Molecular formula	Retention time	GC-EI	LC-ESI+	LC-ESI-	Customized database hit	Number of ions (for LC only)	Additional information
4-Diethylaminobenzaldehyde	120-21-8	$C_{11}H_{15}NO$	9.81	x					Used in colorants
Isobutyl Benzoate	120-50-3	$C_{12}H_{16}O_2$	9.95	x					Used in olefin production, and in defoaming processes
N-Propylbenzamide	10546-70-0	$C_{10}H_{13}NO$	10.0	x					Found in curable compositions in printing
Dibutyl phthalate	84-74-2	$C_{16}H_{22}O_4$	11.12 (GC); 12.25 (LC)	x	x		x	3	Phthalate fragment at 149.023480. Also fragment at 205.086153 ($C_{12}H_{13}O_3$). Used in in printing inks, resin solvent, paper coatings and in adhesives
Bis(2-ethylhexyl) (2E)-but-2-enedioate	142-02-6	$C_{20}H_{36}O_4$	12.05	x					Used to facilitate dye setting
Bisphenol A	80-05-7	$C_{15}H_{16}O_2$	12.27	x					Used in resins, paper coatings and in epoxy adhesives
Fumaric acid, 3-methylbut-2-yl, undecyl ester	-		12.37	x					
Methyl 8,11,13-abietatrien-18-oate	1235-74-1	$C_{21}H_{30}O_2$	13.03	x					
2,4-Bis(1-phenylethyl)phenol	2769-94-0	$C_{22}H_{22}O$	13.9	x					Used in thermoplastics
Bisomer Amine D 700		$C_5H_{13}NO$	1.05		x			2	Fragments into C_3H_9N. Used in dye-containing curable compositions.
		$C_{20}H_{13}N_5$	3.35		x			1	
		$C_{22}H_{40}N_4O_4$	4.93		x			4 (+NH_4^+ adduct)	Fragments at $C_9H_{18}O_3$, $C_6H_{12}O_2$,C_3H_6O and $C_{22}H_{43}N_5O_4$
		$C_{20}H_{23}NO$	5.16		x			3	Fragment: $C_{11}H_{13}N$ and C_8H_9N
1,3-dibenzylurea	1466-67-7	$C_{15}H_{16}N_2O$	5.88		x			2	Fragments into C_8H_7NO
4-Nonylphenyl dihydrogen phosphate		$C_{15}H_{25}O_4P$	6.23		x		x	2 (-H_2O adduct)	Used as surfactant and thermal transfer dye sheets
N-Benzylbenzamide	1485-70-7	$C_{14}H_{13}NO$	7.2		x			2	Fragments into C_7H_4O
		$C_{11}H_{15}NO$	7.2		x			2	Fragments into $C_{10}H_{15}N$
Solvent Violet 8	52080-58-7	$C_{24}H_{27}N_3$	7.61		x		x	1	Used as a dye
		$C_{33}H_{29}N_3O_8$	8.0		x			1	
1-benzyl-3-tert-butyl-1H-pyrazole-5-carboxylic acid	100957-85-5	$C_{15}H_{18}N_2O_2$	9.98		x			3	Fragments into $C_{12}H_{10}N_2O_2$, and $C_{12}H_{10}N_2O$
N-Benzyl-1-tetradecanamine		$C_{21}H_{37}N$	10.34		x			3	Fragments into $C_{14}H_{29}N$ and C_7H_6, used in thermal dye transfer
			11.56		x			3	317.2086, 295.2264, 277.2186

Linolenic acid		C18H30O2	12.99	x			1 (-H2O adduct)	
		C19H37NOS	14.75	x			3 (-H2O adduct)	Fragments into C6H12S and C3H6S
			14.9	x				m/z 298.3151
			15.83	x				m/z 298.2812
		C13H22N4S	12.14		x		1	
Polyglycerol ricinoleate	29894-35-7	C18H33O3	13.51		x		2	Fragments into C12H20O2, used in printing ink
		C21H34N4O3S	14.23		x		1	
2-Dodecylbenzenesulfonic acid	27176-87-0	C18H30O3S	14.9		x		1 (-H2O adduct)	Used as binder resin in toners
3-tetradecyldihydrofuran-2,5-dione	47165-57-1	C18H32O3	15.2-15.5		x	x	1	Used in paper size compositions
12-Hydroxystearic acid	106-14-9	C18H36O3	15.88		x	x	1	Used as printing ink, resin composition and in laminates
Ricinoleic acid	141-22-0	C18H34O3	14.2; 16.25		x	x	1 (-H2O adduct)	Used in printing ink
Dehydroabietic acid	1740-19-8	C20H28O2	16.35		x	x	1	Resin acid
		C23H22O3	16.95		x		2	Fragments into C22H22O

National Food Institute
Technical University of Denmark
Mørkhøj Bygade 19
DK - 2860 Søborg

Tel. 35 88 70 00
Fax 35 88 70 01

www.food.dtu.dk

ISBN: 978-87-93109-29-2