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Abstract

Commercially processed food, drinking-water sources and effluent waters discharged into bodies of water from wastewater treatment plants are putative but yet poorly delineated sources of human exposure to chemical mutagens and oestrogen-like chemicals globally. To this end, this study was aimed at determining the current situation for a possible comparison between a European country (Finland) and an African country (Nigeria). A total of 116 commercially processed food items and ready-to-eat snacks (three lots each) were obtained from Finland (60) and Nigeria (36) for initial screening, as well as sachet-pure water (16 different brands) from Nigeria, bottled still and mineral waters (10 brands each), tap water (hot and cold collected over a 3-month period) and influent and effluent water samples from both a drinking-water treatment plant (collected over a 3-month period) and a wastewater treatment plant (collected over a 2-year period) in Finland.

All samples were collected in their respective countries and extracted by established methods. The mutagenic potential of the food extracts was first determined by the standard plate incorporation assay (Ames test), using two strains of *Salmonella enterica* sv. Typhimurium (TA 100 and TA 98) in the presence and absence of metabolic activation (S9 mix), and subsequently by a methylcellulose overlay, as well as treat-and-wash assays, while the oestrogenicity of the water and food samples, as well as food packaging materials, was determined by a yeast bioluminescent assay, using two recombinant yeast strains (*Saccharomyces cerevisiae* BMAEREIuc/ERα and *S. cerevisiae* BMA64/luc). The cytotoxicity of the food extracts was measured by the trypan blue and lactate dehydrogenase tests, using the HepG2 cell line, as well as by the boar sperm motility assay, while possible DNA damage was assessed by the comet assay.

The mutagenicity of commercially processed food items in Finland was generally low: 60% or 73% were non-mutagenic in *S. Typhimurium* strains TA 100 and TA 98, respectively. While the majority of the initially positive samples proved negative in the complementary assays, cold cuts of cold-smoked beef, grilled turkey and smoked chicken (a single batch of each) were also mutagenic in all
three assays with the TA 100 strain, with and without metabolic activation, indicating that the mutagenic effect was not secondary to histidine release from the food products. The low mutagenicity outcome of the Finnish food items was further confirmed by independent chemical analyses of similar food products for four polycyclic aromatic hydrocarbons.

In contrast to the outcome in Finland, the majority of food items from Nigeria (75%) were mutagenic in the Ames test, either in the presence or absence of the S9 mix and in either of the strains. Chin-chin, hamburger, suya and bean cake were mutagenic in all three assays with the *Salmonella* TA 100 strain, either in the presence or absence of the S9 mix. However, none of the food samples caused DNA damage in the comet assay. They were also not cytotoxic in any of the three assays measuring this aspect.

In all, 31% of the sachet-packed water samples in Nigeria were oestrogenic, with concentrations ranging from 0.79 to 44.0 ng/l oestradiol equivalent concentrations (EEQs), while the tap and bottled water samples from Finland showed no signs of oestrogenicity in the *in vitro* test. Similarly, the oestrogenic activity of the influent samples from the wastewater treatment plant in Helsinki were generally low (from below the limit of detection to 0.7 ng/l EEQ), except in March and August 2011, when relatively high levels (14.0 and 7.8 ng/l EEQ, respectively) were obtained. No oestrogenic activity was recorded in any of the treated effluent samples from the wastewater treatment plant, nor was any in the influent and effluent samples from the drinking-water plant. The outcome of this study implies that Nigerian food items and drinking-water sources are more likely to contain mutagenic and oestrogenic chemicals than their Finnish counterparts, and efforts should be made to reduce the level of human exposure to these chemicals in the diet.
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In loving memories of my father in-law, Deacon Wilfred Ajayi Osayamwen

&

Professor Anthony Uyiekpen Osagie

May your gentle soul continue to rest in perfect peace, Amen.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Acrylamide</td>
<td>MeIQ</td>
<td>2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline</td>
</tr>
<tr>
<td>aPAD</td>
<td>Acute population-adjusted reference dose</td>
<td>MeIQx</td>
<td>2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline</td>
</tr>
<tr>
<td>BaA</td>
<td>Benzo[a]anthracene</td>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>BbF</td>
<td>Benzo[b]fluoranthene</td>
<td>NA</td>
<td>N-nitrosoamine</td>
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<tr>
<td>BaP</td>
<td>Benzo[a]pyrene</td>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol A</td>
<td>NDMA</td>
<td>N-nitrosodimethylamine</td>
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<td>Ch</td>
<td>Chrysene</td>
<td>NPRO</td>
<td>N-nitrosopropine</td>
</tr>
<tr>
<td>DEHP</td>
<td>di(2-ethylhexyl)phthalate</td>
<td>NPYR</td>
<td>N-nitrosopyrrolidine</td>
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<td>DES</td>
<td>Diethylstilboestrol</td>
<td>NSAR</td>
<td>N-nitrososarcosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
<td>NVNA</td>
<td>Nonvolatile N-nitrosoamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine-disrupting chemical</td>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
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<td>EEQ</td>
<td>Oestradiol equivalent concentration</td>
<td>PCDDs</td>
<td>Polychlorinated dibenzodioxins</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
<td>PCDE</td>
<td>Polychlorinated diphenyl ether</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
<td>PCDFs</td>
<td>Polychlorinated dibenzofurans</td>
</tr>
<tr>
<td>ETU</td>
<td>Ethylenethiourea</td>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
<td>PVC</td>
<td>Polyvinylchloride</td>
</tr>
<tr>
<td>FCM</td>
<td>Food contact material</td>
<td>PhiP</td>
<td>2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>FIC</td>
<td>Fold induction-corrected</td>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>HAA</td>
<td>Heterocyclic aromatic amine</td>
<td>VNA</td>
<td>Volatile N-nitrosoamine</td>
</tr>
<tr>
<td>ITX</td>
<td>Isopropylthioxanthone</td>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
<td></td>
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<tr>
<td>MC</td>
<td>Methyl cellulose</td>
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1.0 Introduction

Recently, there has been increased debate on the effects of chemicals on humans, especially following long-term exposure and exposures at critical windows of life. Two major classes of chemicals involved in this debate are mutagens and oestrogens, and possible sources of human exposure to these chemicals include processed food, drinking-water sources and effluent waters discharged from wastewater treatment plants (WWTPs).

Processed food items may contain mutagens such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (HAAs) (Tikkanen et al., 1993; Sinha and Rothman, 1999; Wretling et al., 2010; Chung et al., 2011; Essumang et al., 2012), along with several other compounds (pesticides, food contact materials (FCMs) etc.) inadvertently contaminating the food. In Sweden, Finland and The Netherlands for example, product groups including potato crisps, French fries, coffee, bread, biscuits and breakfast cereals reportedly contribute more than 90% to the total daily intake of mutagenic acrylamide (AA) (Konings et al., 2003; Svensson et al., 2003; Eerola et al., 2007; Hirvonen et al., 2011). Epidemiological studies have substantially furthered our understanding of the links between diet, genetic toxicity and cancer (Doll and Peto, 1981; Akintowa et al., 2007; Sharif et al., 2008; Sinha et al., 2009; Francois et al., 2010; Hirvonen et al., 2010). These studies have revealed that diet is a key contributor to human cancer, with approximately 32% of cancers estimated as being attributable to dietary factors (Willett, 1998). In Finland, dietary AA intake by male smokers is positively associated with increased risk of lung cancer (Hirvonen et al., 2010). In Argentina, Hawaii and The Netherlands, process-generated contaminants in food are associated with increased risk of different types of cancer (Marchand et al., 2002; Voskuil et al., 2002; Navarro et al., 2004).

Regarding endocrine-disrupting chemicals (EDCs), most information is available on compounds possessing oestrogen-like activity. Examples of such EDCs include bisphenol A (BPA), polyvinylchloride (PVC), di(2-ethylhexyl)phthalate (DEHP), polychlorinated biphenyls (PCBs),
dioxin and dioxin-like chemicals. These chemicals have been reported in most of the daily products used by humans, including food, FCMs and plastic bottles (Stroheker et al., 2003; ter Veld et al., 2006; Behr et al., 2011; Plotan et al., 2013). The presence of EDCs in the human diet is worrisome, also due to an increase in certain cancer types (breast, stomach and colon cancer), especially in the industrialized countries, and the established linkage between hormonal disorder and EDC exposure (Fisher, 2004; Meeker, 2010; Walvoord, 2010). In Finland, for example, cancers of the oesophagus, stomach, lungs and colon appear to be on the increase (Weiderpass and Pukkala, 2006; Hirvonen et al., 2010). In Nigeria, there is a paucity of data on the incidence of various cancer types. However, the major forms of cancer in Nigeria that have been linked with EDC exposure (breast and prostate cancers) are on the increase (Jedy-Agba et al., 2012).

From the point of view of risk assessment, EDCs present a particular challenge, because they may have non-monotonous dose-response curves not adequately covered by conventional toxicological experimentation and may be capable of causing untoward impacts at environmentally prevailing, low concentrations (Fagin, 2012). The overall presence of these chemical compounds in processed food, drinking-water sources and the environment is alarming, in view of the fact that these items are a prerequisite for human life. In addition, most toxicological studies on processed food and drinking-water sources are targeted at only one or a specific class of compounds, neglecting the possibilities of unintentionally added substances and formation of new substances in the final product as a result of processing.
2.0 Literature review

2.1 Food and food processing

Food is any substance, usually of plant or animal origin that is consumed to provide nutritional support for the body. While most food can, at least in principle, be eaten in its raw form, many also undergo some form of processing for reasons ranging from palatability, safety, shelf life extension and texture to flavour (Ramaswamy and Marcotte, 2006). The methods used in food processing are largely dependent on the type of food being processed, the quantity, desired product and available resources/technology. The processing methods employed in most developed countries have evolved over the years, from the traditional use of coal to more sophisticated techniques such as electroheating, high-pressure processing, cold/liquid smoking, ultrasound, use of modified atmospheres and microfiltration. Despite current advances in food-processing techniques worldwide, these crude methods are still the methods of choice in most developing countries (Essumang et al., 2012). In addition, the major aims considered in most modified methods of food processing are on improving and preserving the flavour, texture and nutrients of food, as well as killing vegetative cells contaminating it (Farber, 1991).

2.2 Toxicological safety of processed food

Food safety issues are increasing in importance globally, due to significant increases in the number and outbreaks of foodborne diseases, as well as increased incidences of cancer. The outbreaks of foodborne diseases are well documented (Bean et al., 1990; O’Mahony et al., 1990; Mead et al., 1999; Maruzumi et al., 2005; Cleary et al., 2010; Rowlands et al., 2010; Centre for Disease Control (CDC), 2013; Coldea et al., 2013; Gould et al., 2014), and this has further strengthened strategies and awareness of microbiological food safety. On the other hand, toxicological food safety has suffered some setbacks with respect to awareness, especially among
consumers. This may be related to the fact that most of the biological endpoints (e.g. cancer) of chemicals in processed food are observed after lengthy periods of time and often from long-term exposure. Epidemiological studies have furthered our understanding of the links between diet and cancer (Akintowa et al., 2007; Sharif et al., 2008; Sinha et al., 2009; Francois et al., 2010). Recent comprehensive analysis of the available literature data shows convincing evidence for consumption of processed meat as a cause of increased risk of colorectal, prostate, breast, pancreatic and colon cancer (Anderson et al., 2002; Navarro et al., 2004; Corpet, 2011; Berjia et al., 2014). In the case of cancers of the oesophagus, stomach and lung, the causal relationship is suggestive (World Cancer Research Fund (WCRF)/American Institute for Cancer Research (AICR), 2007). It is also noteworthy that the causes of cancer are complex, and in addition to diet, general lifestyle habits and individual genetic make-up are important factors (King et al., 2000; WCRF and AICR, 2007; Alaejos et al., 2008).

2.3 Sources of toxic chemicals in processed food

Toxic chemicals/compounds reported in processed food are present and/or formed in different ways, as outlined in Figure 2.1 below. These sources can be divided into deliberate addition and unintentional contamination.
2.3.1 Substances deliberately added to food (food additives)

Food additives are substances added intentionally to food to improve or modify its taste, colour and shelf life (di Sotto et al., 2014). There are 363 different additives, with 23 recognized uses, permitted in food in the European Union (EU) (EU, 2008; EU, 2013). Over the years, the safety of many of these food additives has come into question, either as a result of their toxic properties or their ability to interact with electron-rich biological macromolecules, resulting in adverse health effects, including reactive genotoxicity and carcinogenicity (di Sotto et al., 2014). To this end, controversy exists both on the toxicological safety of food additives and the use of these additives in different countries. For example, while food additives such as amaranth and vegetable carbon (carbon black) have been banned in the United States of America (USA), these substances
are still in use within the EU, Australia and New Zealand (European Food Safety Authority EFSA, 2010; EFSA, 2012a). Similarly, azo dyes such as blue 1, blue 2, yellow 5 and yellow 6, which are banned within the EU for their hyperactive effects, are still heavily used in the USA.

Similar controversy also exists on the toxicity of food additives. While the genotoxicity of some food additives (amaranth, allura red, new coccine, tartrazine, erythrosine, phloxine and rose Bengal) has been reported at low doses (Tsuda et al., 2001; Sasaki et al., 2002; Shimada et al., 2010), more recent reports show that these food additives are not a source of worry (Poul et al., 2009; EFSA, 2010; EFSA, 2012a; di Sotto et al., 2014).

### 2.3.2 Substances inadvertently contaminating food

In addition to substances deliberately added to food during processing, a number of unwanted substances have also been reported in food products, with some of them having genotoxic and oestrogenic effects. Examples of such substances are included below.

#### 2.3.2i Pesticides

Global reliance on pest-control chemicals (pesticides) in agriculture is an essential component of food production, and this process often leaves toxic residual compounds in food products. The presence of pesticide residues in processed food items is dependent on factors such as the persistent nature of the pesticide, degradation product, type and portion of food material, processing technique and other environmental factors. Some pesticides (e.g. malathion, ethion, profenofos, fenpropathrin, chlorpyrifos, cypermethrin, 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane (DDT), lindane, aldrin and diazinon), the majority of which are known to have long-term toxicological effects, have been reported in a large variety of raw and processed food products.
(Saeed et al., 2001; Fenske et al., 2002; Bai et al., 2006; Lorenzin, 2007; Maver et al., 2007; Gonzalez-Curbelo et al., 2012; Ahmed et al., 2014; Lozowicka et al., 2014; Mohebbi-Nozar et al., 2014; Wong et al., 2014), years after their prohibition. For example, residues of chlorpyrifos and lindane pesticides have been reported in ready-to-eat foods in the USA (Fenske et al., 2002), Italy (Lorenzin, 2007), Ghana (Johnson and Yawson, 2000) and in trace amounts in Spain (Gonzalez-Curbelo et al., 2012). These pesticides impact the central nervous, cardiovascular and respiratory systems in humans (International Agency for Research on Cancer IARC, 1979; Agency for Toxic Substances and Disease Registry ATSDR 1989). They are also carcinogenic and teratogenic in mouse (IARC, 1979; Tian et al., 2005). A number of publications have reported increased and/or unchanged levels of pesticides during processing (Pietrino, 1991; El-Hoshy, 1997). However, recent evidence shows that the levels of pesticide residues in food decrease during processing (Lewis et al., 1996; Nagayami, 1996; Nagesh and Verma, 1997; Abou-Arab, 1999; Dikshit, 2002; El-Nabarawy et al., 2002), but the levels/quantities detected in the final products are still of concern.

In 2002, Fenske et al. reported a chlorpyrifos pesticide exposure through diet in preschool children from Washington at 2.5 μg kg⁻¹ day⁻¹, exceeding the recommended acute population-adjusted reference dose (aPAD) exposure of 1.7 μg kg⁻¹ day⁻¹. Similarly, varying levels of pesticide residues have been reported in human serum globally (Glynn et al., 2003; Heudorf et al., 2003; Axmon et al., 2008; Bachlet et al., 2011; Saoudi et al., 2014). Some pesticides also undergo various forms of transformation during processing, possibly due to heat reaction or reactions with other chemical agents in food, thereby leading to the formation of new compounds, usually with even more toxic potential. The fungicides ethylenebisdithiocarbamates, for example, are suspected endocrine disruptors, and when taken up by plants, can be transformed into carcinogenic ethylenethiourea (ETU) during processing, thereby rendering them of concern to human health (IARC, 2001; Kontou et al., 2004; Geetanjali and Santosh, 2009).
2.3.2ii Heavy metals and other environmental pollutants

Heavy metals such as arsenic, mercury and lead, as well as environmental pollutants (e.g. dioxins, polychlorinated dibenzodioxins PCDDs/polychlorinated dibenzofurans PCDFs, PCBs and dioxin-like PCBs) have become a public health issue worldwide, especially during recent decades (Rose et al., 2001; Thorpe et al., 2001; Foran et al., 2004; Hites et al., 2004; Ashizuka et al., 2005; Chen et al., 2008; Loran et al., 2010; Ibrahim et al., 2011). These toxic environmental contaminants have been reported in fresh animal products and in a number of commercially processed foods and drinking water (Kim et al., 2001; Mayer, 2001; Amakura et al., 2003; Schecter et al., 2003; Watanabe et al., 2003; Yang et al., 2004; Yoshida et al., 2004; Chen et al., 2008; Perello et al., 2010; Nostbakken et al., 2015). Despite their presence in food, the actual potential risk to humans is influenced by dietary practices, such as consumption patterns, food packaging and preparation methods. The long-term effect of repeated exposure, especially since childhood, could also be of concern (Vogt et al., 2012). Most heavy metals act as co-mutagens, increasing the risk and formation of cancer (Fischer et al., 2005). For example, co-exposure of mouse hepatoma Hepa-1 cells to low concentrations of arsenic and BaP increases BaP-deoxyribonucleic acid (DNA) adduct levels by as much as 18-fold (Maier et al., 2002). There are two divergent views as to the effect of processing on the levels of environmental pollutants in processed food. While Rose et al. (2001) reported that processing techniques such as frying, grilling and barbecuing significantly reduce the levels of selected PCDD/PCDFs in processed beef, Perello et al. (2010) argued that cooking processes are only of limited value as a means of reducing the concentrations of PCDD/PCDFs, PCBs and polychlorinated diphenyl ethers (PCDEs) in food. Another independent study showed that techniques such as the use of firewood considerably increase the levels of these contaminants in processed food (Yang et al., 2004).
2.3.2iii FCMs

FCMs are commonly used in the packaging and food industries. They are complex formulas of various substances, such as polymers, antioxidants, solvents, plasticizers, adhesion promoters etc. that provide specialized functions to FCMs (Canellas et al., 2015). In addition, FCMs can also contain impurities from raw materials or by-products from side reactions among various ingredients, which are often unknown to FCM producers. Volatile and nonvolatile compounds in FCMs are underestimated sources of chemical food contaminants and potentially relevant sources of human exposure to both mutagens and EDCs (Lionti et al., 2014; Mertl et al., 2014). These compounds can also migrate from FCMs to food through the various layers (Canellas et al., 2010; Aznar et al., 2011; Vera et al., 2011; Sendon et al., 2012; Mertl et al., 2014), and under normal conditions of use (ter Veld et al., 2006; Vandenberg et al., 2007; Le et al., 2008). The migration of these substances from FCMs into food is dependent on factors such as temperature, type of food and the nature of the packaging materials (Dabrowska et al., 2003; Pocas and Hogg, 2007; Westerhoff et al., 2008). Moreover, the constant introduction of novel packaging materials has increased the number of specific hazards to which humans are exposed via migration from packaging into food (Arvanitoyannis and Bosnea, 2004).

2.3.3 Process-generated contaminants

2.3.3i Polyaromatic hydrocarbons (PAHs)

PAHs are condensed compounds of linked aromatic rings and are formed by incomplete combustion of organic materials (Samanta et al., 2002; Farhadian et al., 2011). These chemicals have oestrogenic properties (Santodonato, 1997), are known carcinogens in humans (Samanta et al., 2002) and cause mammary tumours in laboratory animals (el-Bayoumy et al., 1995; Hecht, 2002). Exposure to PAHs in the general population occurs primarily through charred,
smoked and broiled foods (Djinovic et al., 2008; Farhadian et al., 2010; Wretling et al., 2010; Alomirah et al., 2011; Chung et al., 2011; Essumang et al., 2012; Aaslyng et al., 2013), leafy vegetables (Phillips, 1999), wood- and coal-burning stoves (Lewis et al., 1999), air pollution (Lioy and Greenberg, 1990) and tobacco smoke (Besaratinia et al., 2002). The deposition of PAHs in processed food items mainly occurs through processing techniques, such as grilling, barbecuing, smoking and frying (Djinovic et al., 2008; Farhadian et al., 2010; Wretling et al., 2010; Alomirah et al., 2011; Essumang et al., 2012), and their formation and concentration are dependent on the type/method of processing, processing time and the type of food being processed. For example, heating highly fatty food directly by smoking is known to produce high levels of PAHs (Djinovic et al., 2008; Chung et al., 2011; John et al., 2011). Of the known PAHs, 15 (Figure 2.2 and Table 2.1) are genotoxic and carcinogenic (Scientific Committee on Food (SCF), 2002; EFSA, 2008). Of the known genotoxic and carcinogenic PAHs, benzo[a]pyrene (BaP) is the most commonly studied and has shown various toxicological effects in experimental animals (SCF, 2002; Schneider et al., 2002). For this reason, BaP has been used as a marker of carcinogenic PAHs in food since 2002. However, this compound is not always detectable in foods containing PAHs, and in 2008, EFSA suggested the use of the sum of four carcinogenic PAHs (PAH4), which included BaP, benzo[b]fluoranthene (BbF), benzo[a]anthracene (BaA) and chrysene (Ch), as markers for PAH concentration (EFSA, 2008). This has recently been implemented in EU regulations (European Commission, 2011). The presence of these PAHs in processed food is worrisome, because epidemiological studies show an increased risk of intestinal, breast, bladder, prostate, stomach, oesophageal and pancreatic cancers after high levels of consumption of processed meat, particularly well-done fried and barbecued red meat (Navarro et al., 2004; Norat et al., 2005; Sinha et al., 2009; Ferguson, 2010; John et al., 2011; Berjia et al., 2014).
Figure 2.2. Structures of the 15 principal carcinogenic PAHs.
<table>
<thead>
<tr>
<th>PAHs</th>
<th>MF</th>
<th>MW (g/mol)</th>
<th>MP (°C)</th>
<th>BP (°C)</th>
<th>Log $K_{ow}$</th>
<th>Sol. at 25 °C (g/L)</th>
<th>VP at 25 °C (mm/Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]A</td>
<td>C_{18}H_{12}</td>
<td>228.3</td>
<td>160</td>
<td>437.6</td>
<td>5.79</td>
<td>9.4 x 10^{-6}</td>
<td>2.1 x 10^{-7}</td>
</tr>
<tr>
<td>B[b]F</td>
<td>C_{20}H_{12}</td>
<td>252.3</td>
<td>168</td>
<td>NR</td>
<td>5.78</td>
<td>1.2 x 10^{-6}</td>
<td>5.0 x 10^{-7}</td>
</tr>
<tr>
<td>B[j]F</td>
<td>C_{20}H_{12}</td>
<td>252.3</td>
<td>166</td>
<td>NR</td>
<td>6.11</td>
<td>2.5 x 10^{-6}</td>
<td>2.62 x 10^{-8}</td>
</tr>
<tr>
<td>B[k]F</td>
<td>C_{20}H_{12}</td>
<td>252.3</td>
<td>217</td>
<td>480</td>
<td>6.84</td>
<td>8.0 x 10^{-7}</td>
<td>9.7 x 10^{-10}</td>
</tr>
<tr>
<td>B[g,h,i]P</td>
<td>C_{22}H_{12}</td>
<td>276.3</td>
<td>273</td>
<td>545</td>
<td>6.78</td>
<td>2.6 x 10^{-7}</td>
<td>1.0 x 10^{-10}</td>
</tr>
<tr>
<td>B[a]P</td>
<td>C_{20}H_{12}</td>
<td>252.3</td>
<td>179-179.3</td>
<td>310-312</td>
<td>5.97</td>
<td>1.62 x 10^{-6}</td>
<td>5.49 x 10^{-9}</td>
</tr>
<tr>
<td>Chrysene</td>
<td>C_{18}H_{12}</td>
<td>228.3</td>
<td>255</td>
<td>448</td>
<td>5.5</td>
<td>2.0 x 10^{-6}</td>
<td>7.8 x 10^{-9}</td>
</tr>
<tr>
<td>CP[c,d]P</td>
<td>C_{18}H_{10}</td>
<td>226.3</td>
<td>NR</td>
<td>438 (at 760 mmHg)</td>
<td>6.70</td>
<td>3.7 x 10^{-6}</td>
<td>1.4 x 10^{-10}</td>
</tr>
<tr>
<td>DB[a,h]A</td>
<td>C_{22}H_{14}</td>
<td>278.3</td>
<td>266</td>
<td>524</td>
<td>6.5</td>
<td>2.49 x 10^{-6}</td>
<td>1.0 x 10^{-10}</td>
</tr>
<tr>
<td>DB[a,e]P</td>
<td>C_{22}H_{14}</td>
<td>302.4</td>
<td>233.5</td>
<td>NR</td>
<td>7.28</td>
<td>8.02 x 10^{-8}</td>
<td>7.03 x 10^{-11}</td>
</tr>
<tr>
<td>DB[a,h]P</td>
<td>C_{22}H_{14}</td>
<td>302.4</td>
<td>317</td>
<td>NR</td>
<td>7.28</td>
<td>3.5 x 10^{-8}</td>
<td>6.41 x 10^{-12}</td>
</tr>
<tr>
<td>DB[a,i]P</td>
<td>C_{22}H_{14}</td>
<td>302.4</td>
<td>280</td>
<td>275 (at 0.05 mmHg)</td>
<td>7.28</td>
<td>5.54 x 10^{-7}</td>
<td>1.78 x 10^{-11}</td>
</tr>
<tr>
<td>DB[a,j]P</td>
<td>C_{22}H_{14}</td>
<td>302.4</td>
<td>162.4</td>
<td>NR</td>
<td>7.71</td>
<td>3.6 x 10^{-7}</td>
<td>4.8 x 10^{-10}</td>
</tr>
<tr>
<td>I[1,2,3-c,d]P</td>
<td>C_{22}H_{12}</td>
<td>276.3</td>
<td>163.6</td>
<td>536</td>
<td>6.7</td>
<td>1.9 x 10^{-7}</td>
<td>1.25 x 10^{-10}</td>
</tr>
<tr>
<td>5-MC</td>
<td>C_{19}H_{14}</td>
<td>242.3</td>
<td>117.5</td>
<td>NR</td>
<td>6.07</td>
<td>6.2 x 10^{-5}</td>
<td>2.53 x 10^{-7}</td>
</tr>
</tbody>
</table>

MF: Molecular formula; MW: Molecular weight; MP: Melting point; BP: Boiling point; Sol.: Water solubility; VP: Vapour pressure; NR: Not reported.
2.3.3ii Heterocyclic aromatic amines (HAAs)

HAAs are formed in food through the condensation of creatine/creatinine and Strecker degradation radicals (pyridines and pyrazines) generated from the reaction of sugars and amino acids during the Maillard reaction (Jägerstad et al., 1998). The formation of these compounds (heterocyclic amines HCAs) in food is influenced by factors such as temperature, type of processing, cooking time, pH, precursor concentrations, fat, moisture content and types of amino acid present (Knize et al., 1994; Nagao and Sugimura, 1995; Pais et al., 1999). In general, cooking at high temperature (usually above 150 °C) and for longer periods of time increases HAA formation (Knize et al., 1994; Skog et al., 1995; Jägerstad et al., 1998; Persson et al., 2002; Solyakov and Skog, 2002). 2-Amino-3,4-dimethyl-imidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are the most potent HAAs and are classified as possible human carcinogens (IARC, 1993). They have been reported in processed meat and fish products (Nagao et al., 1977; Gross and Fay, 1995; Knize et al., 1997, 1998; Sanz et al., 2008) and are said to be over 100- and 2000-fold more mutagenic than the toxic aflatoxin B1 and BaP, respectively (Stavric, 1994). Among the known HAAs, some are not directly mutagenic, but co-mutagenic (e.g. 9H-pyrido[3,4-b]indole and 2-methyl-β-carboline), and thus enhance the mutagenicity of other HAAs in food (Jägerstad et al., 1998; Skog and Solyakov, 2002; Sugimura et al., 2004).

2.3.3iii N-nitrosamines (NAs)

N-nitroso compounds (NAs and N-nitrosamides) are formed in food by the reaction of nitrite with amines or amides. Dietary exposure of humans to these compounds mainly occurs from consumption of cured meat and fish products (Dietrich et al., 2005). Curing of meat inhibits
the growth of *Clostridium botulinum*, thereby decreasing the risk of this organism of producing toxins and heat-resistant spores (Herrmann et al., 2015). It also modifies the taste and preserves the colour of meat (Lijinsky, 1999). However, the curing agents (usually sodium nitrite) can nitrosate the amines in meat, resulting in the formation of NAs. This is favoured by factors such as heat, meat quality, fat content, storage and maturation conditions etc. NAs are divided into two forms: volatile (VNA) and nonvolatile (NVNA), with VNAs (e.g. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR)) being the most widely studied, and are often referred to as the most potent carcinogens of the two. However, there is a recent report that the NVNAs (e.g. *N*-nitrosoproline (NPRO) and *N*-nitrososarcosine (NSAR)) could undergo decarboxylation into their carcinogenic counterparts, either during heat treatment or by microbial activity in the large intestine (Herrmann et al., 2015). The carcinogenic properties of NAs have been extensively investigated. They induce tumours of the liver, lung, oesophagus, bladder, pancreas and other sites in various animal species (Bogovski and Bogovski, 1981; Preussmann and Stewart, 1984; Lijinsky, 1992; Hecht, 1997). Endogenous NAs account for 45—75% of the total exposure and can primarily be formed by bacteria in the oral cavity after ingestion of nitrate (Dubrow et al., 2010) or in the acidic environment of the stomach, where nitrite reacts with the degradation products of amino acids (Habermeyer and Eisenbrand, 2009).

2.3.3iv Acrylamide (AA)

AA is a colourless and odourless crystalline solid, used in its polymerized form as a flocculant in water purification, paper and fabric manufacturing and as a sealing adjuvant in tunnel construction (Curtis et al., 2014; Pedreschi et al., 2014). It shows both neurological and reproductive effects at high doses (Friedman, 2003; Tyl and Friedman, 2003), and it decreases litter size at low doses in rodents (Tyl and Friedman, 2003).
The presence of AA in processed food was propelled into the spotlight in 2002, following investigation of AA exposure resulting from an industrial accident in Sweden (Tareke et al., 2002). Since then, high concentrations of this genotoxic compound have been reported in processed carbohydrate-rich food globally (Rufian-Henares et al., 2007; Wenzl et al., 2007; Chen et al., 2012; Cheng et al., 2012; Shamla and Nisha, 2014; Omar et al., 2015; Pacetti et al., 2015; Wyka et al., 2015). AA is formed in high-carbohydrate food from the reaction of a free amino acid (asparagine) with reducing sugars during high-temperature cooking and processing, usually via the Maillard reaction (Figure 2.3). Considering the toxic nature of AA, the European Chemical Agency, in March 2010, added AA to its list of “very high concern” (Linback et al., 2012). Modification of processing conditions, such as lowering time, temperature, pH, presoaking in water, addition of antioxidants and use of asparaginase to reduce the asparagine concentration prior to cooking are some recommendations for possible reduction of AA in food products (Hendriksen et al., 2009; Food Drink Europe, 2011). However, their application in the food industry remains a pending issue.

Figure 2.3. Acrylamide formation from asparagine and glucose.
2.4 Health effects of mutagens in food

Process-generated contaminants in food, such as PAHs and HAAs, are all well-known human carcinogens. The PAH-induced contaminant BaP is known to induce oral cancer in humans, disrupt embryo development and induce significant DNA damage in the mouse (Walle et al., 2006; Einaudi et al., 2014; Zhan et al., 2014), while the HAA-induced contaminant PhIP causes lung, bladder and stomach cancer in humans, as well as colon and mammary cancer in other animals (IARC, 1993).

Recent comprehensive analysis of available literature data show convincing evidence for consumption of processed meat as a cause of increased risk of colorectal cancer; in the case of cancers of the oesophagus, stomach and lung, the causal relationship is suggestive (WCRF/AICR, 2007). Cooking meat at a relatively high temperature and close to the cooking source has been linked with increased incidence of colorectal cancer in Argentina, Hawaii and The Netherlands (Marchand et al., 2002; Voskuil et al., 2002; Navarro et al., 2004). Findings from a prospective European cohort study link HAA intake with increased risk of colorectal adenoma (Rohrmann et al., 2009). A number of process-generated contaminants (PAHs and HAAs) have been reported in varying concentrations in different types of smoked meat in several parts of Europe (Eerola et al., 2007; Stumpe-Viksna et al., 2008; Wretling et al., 2010) and Africa (Akpambang et al., 2009; Essumang et al., 2012; Amos-Tautua et al., 2013). However, no nexus has been established in relation to the increased incidence of cancer in these countries. In Finland, cancers of the oesophagus, lungs, stomach and colon are on the increase (Weiderpass and Pukkala, 2006), some of which have been linked with dietary AA intake (Hirvonen et al., 2010). In Nigeria, there is a paucity of data on the incidence of various cancer types. However, the major forms of cancer in Nigeria, breast and prostate cancers, may be
increasing (Jedy-Agba et al., 2012). Both have been associated with meat-cooking habits (Grover and Martin, 2002).

2.5 Mechanism of activity of mutagens

A mutagen is a biological, chemical or physical agent that alters or causes a permanent change (mutation) in the genetic material (usually DNA) of an organism. Such mutations are usually the first step in a sequence of events that ultimately leads to the development of cancer. The mechanisms of activity of mutagens in relation to cancer are complex and largely unclear. However, most mutagens generate reactive oxygen species (ROS), suggesting that oxidation of DNA may play a role in carcinogenesis (Fischer et al., 2005). Metabolic activation of PAHs and HAAs to reactive metabolites and the DNA adducts formed are postulated to be central to the carcinogenesis of PAH- and HAA-induced cancers (Hecht, 2002). PAHs and HAAs are metabolized through activation and detoxification pathways (Mordukhovich et al., 2010). When exposure is high or detoxification is insufficient, PAH-DNA or HAA-DNA adducts are formed in body tissues (Santella 1999; Gammon and Santella, 2008). These adducts persist when repair mechanisms are inadequate (Braithwaite et al., 1999).

Mutation in tumour suppressor genes, such as p53, caused by PAHs or HAAs induces cells to grow uncontrollably, thereby leading to cancer. The p53 gene is one of the most commonly mutated genes identified in various types of human tumours (Nakanishi et al., 2000). Codons with p53 genes are major binding sites for PAHs (Chakravarti et al., 1998). In addition to p53, metabolically activated PAH (PAH-diol-epoxides) often target oncogenic sequences within the ras family of proto-oncogenes, including Ha-ras, Ki-ras and N-ras (codons 12, 13 and 61). Mutation of ras at these particular codons converts it into an oncogene, capable of transforming cells into a malignant phenotype.
2.6 Methods for determining the presence of mutagens in food

The presence of mutagens in commercially processed food is mainly determined by chromatographic techniques (high-performance liquid chromatography HPLC, gas chromatography-mass spectrometry GC-MS etc.). Although these methods are effective, they are time-consuming, expensive and highly specific, identifying only targeted mutagens, which are further used for risk assessment, and often requiring additional toxicological studies. Most of the toxicological studies and risk assessments are carried out, based on studies with exposure to one chemical at a time or between one and four chemicals (in the case of PAHs). This is particularly challenging, considering that exposure to a mixture of different chemicals, some of which are co-mutagens, increase the mutagenic effects of already known mutagens. One way of elucidating the possible potential biologic effect of a mixture of compounds is by using the bacterial reverse-mutation assay (Ames test). The Ames test is over 90% accurate in predicting genotoxicity (Weisburger, 2001; Gatehouse, 2012). The principle of this bacterial reverse mutation test is that it detects mutations that revert to mutations present in the test strains (usually Salmonella) and restores the functional capability of the bacteria to synthesize an essential amino acid (usually histidine). The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain (Maron and Ames, 1983; Gatehouse, 2012). This test is rapid, inexpensive, specific and relatively easy to perform. The specificity of the test strains provides useful information on the types of mutations that are induced by genotoxic agents and gives insight into further studies.

2.7 Xenoestrogens and EDC exposure: An overview

Xenoestrogens are natural (e.g. phytoestrogens) and synthetic (e.g. PCBs, BPA and phthalates) chemical compounds that have oestrogenic effects on living organisms via the
endocrine system. The endocrine systems regulate metabolic processes, including nutritional, behavioural, reproductive, growth, intestinal, cardiovascular and kidney functions and also maintain homeostasis (World Health Organization WHO, 2002). For many endocrine systems, homeostasis programming is established during fetal/neonatal development, so that an imbalance at this stage of life, e.g. from exposure to a xenoestrogen, may result in permanent misprogramming (Seckl and Meaney, 2004; Owen et al., 2005). Hormones are the chemical messengers of the endocrine system and are released into the bloodstream from numerous glands and tissues (Figure 2.4). Hormonal imbalances are usually due to the effects of xenoestrogens.

Figure 2.4. Hormone-producing glands and tissues of the endocrine system, with sample hormones [in colour] (Modified from Norris, 1996).
EDCs are defined as exogenous substances that alter the functions of the endocrine system and thereby cause adverse health effects in an intact organism or its progeny (WHO/International Programme on Chemical Safety IPCS, 2002; Guo et al., 2013). EDCs are differentiated from other groups of substances with various modes of action by three criteria: the presence of i) an adverse effect in an intact organism or a (sub)population, ii) endocrine activity and iii) a plausible causal relationship between the two (EFSA, 2013). Controversies exist on the effects and ways of assessing EDC exposure, both within the scientific community and among regulators worldwide. Currently, no formal criteria have been established, internationally or at the EU level, for identifying EDCs (European Commission, 2014).

2.8 Xenoestrogen exposure via the food chain

Xenoestrogens are ubiquitous and heterogeneous and have gained notoriety, due to their association with certain reproductive disorders, developmental abnormalities and other adverse physiological effects in both humans and wildlife (Fisher, 2004; Bourguignon and Parent, 2010; Zama and Uzumcu, 2010). In addition, oestrogen mimics have also been reported among chemicals used for processing and preserving food (Sinha et al., 2009; Connolly et al., 2011; Zhang et al., 2012a), as well as in soy-based food products (Takamura-Enya et al., 2003; Behr et al., 2011). The sources of human exposure to xenoestrogens include phytoestrogens and synthetic xenoestrogens.

2.8.1 Phytoestrogens

Phytoestrogens are naturally occurring plant compounds found in numerous fruits and vegetables (Cederroth et al., 2012). They are nonsteroidal compounds that bind to and
activate oestrogen receptors (ERs) α and β by mimicking the conformational structure of 17β-
hydroxyoestradiol (Kuiper et al., 1997, 1998). Phytoestrogens are categorized into three classes:
the isoflavones, lignans and coumestans. Genistein and daidzin are the predominant isoflavones
found in soybeans and make up the most important dietary source of phytoestrogens for humans
(Cederroth et al., 2012).

2.8.1a Health effects of phytoestrogens

The safety of phytoestrogens is mainly controversial. A critical look into the
available literature also shows inconsistent results.

2.8.1ai Negative health effects of phytoestrogens

Exposures to phytoestrogens have a number of negative health effects in both
humans and experimental animals. Examples of such effects include enhancement of
proliferation or metastasis of some cancer types (Helferich et al., 2008; Martínez-Montemayer et
al., 2010; de la Parra et al., 2012), altered fertility and disruption of several aspects of
reproduction [e.g. sexual development, timing of puberty, sex-dependent behaviours, testicular
and ovarian endocrine functions, gamete production, pregnancy and lactation] (Cederroth et al.,
2012). The first reported case of the potential effect of phytoestrogens on reproduction was in the
1940s, when ewes grazing in clover pastures developed an infertility syndrome, due to exposure
to high levels of formononetin, an isoflavone present in red clover (Trifolium pratense; Bennetts
et al., 1946). This syndrome (later referred to as `clover disease`), resulted in reduced ovulation,
low lambing and structural defects in the reproductive tract (Adams, 1995). Furthermore,
phytoestrogens taken at high doses, or at critical stages of development in rodents, result in
severe reproductive tract disorders (Lamartiniere et al., 1995; Strauss et al., 1998; Tou et al., 1999), impair fertility in female mice (Nagao et al., 2001; Jefferson et al., 2005) and enhance vaginal cell maturation in female human infants (Bernbaum et al., 2008). In male marmoset monkeys, infants fed on isoflavone (soy)-containing formula milk for 1–1.5 months showed serum testosterone decreased by 50–70%, compared with co-twin counterparts fed on cow formula milk (Sharpe et al., 2002). In humans, epidemiological data showed an increase in the incidence of hypospadias in sons of women consuming vegetarian diets during pregnancy (North and Golding, 2000), possibly due to an increased exposure to phytoestrogens. However, this was not substantiated with blood levels of phytoestrogens for vegetarian versus non-vegetarian mothers (North and Golding, 2000).

2.8.1.ii Positive health effects of phytoestrogens

One of the notable beneficial impacts of phytoestrogens reported most often in the literature is their anticancer properties. The anticancer properties of phytoestrogens are a result of the presence of peptides such as lunasin and the Bowman-Birk inhibitor, a 43- and 71- amino-acid protease inhibitor (Galvez et al., 2001; Armstrong et al., 2003). These biological peptides are released during gastrointestinal digestion or during the fermentation of soy proteins by enzymatic proteolysis. For example, bioactive peptides encrypted in the amino-acid sequence of soy proteins exert anticancer, antihypertensive, hypocholesterolaemic, antiobesity and antioxidant activities (Martinez-Villaluenga et al., 2008, 2009, 2010; Wang et al., 2008). Epidemiological studies have shown that phytoestrogens may protect against breast cancer and cardiovascular diseases (Pilsakova et al., 2010; Zhang et al., 2012b). In addition, they possess antioxidant properties and influence malignant cell proliferation, differentiation and angiogenesis, making them potential anticancer agents (Adlercreutz, 1995). Studies amongst
Western populations have consistently shown null or inverse relationships between increasing intakes of phytoestrogens and the risk of different cancer types and reproductive health (Shultz et al., 1991; Mitchell et al., 2001; Linseisen et al., 2004; Zhang et al., 2004; Chang et al., 2007; Rossi et al., 2008; Bandera et al., 2011; Hedelin et al., 2011; Ollberding et al., 2012).

2.8.2 FCMs and unintentionally added substances

FCMs are unintentionally added substances in food, and their presence usually occurs from leaching of food packaging materials (Brotons et al., 1995; Stroheker et al., 2003; ter-Veld et al., 2006). Examples of chemicals in FCMs include BPA, 4-nonylphenol and several phthalates. The leaching of chemical substances into food and drinking water occurs under normal use conditions (ter-Veld et al., 2006; Vandenberg et al., 2007; Le et al., 2008) and could be influenced by factors such as storage conditions, sunlight exposure and ambient temperature (Dabrowska et al., 2003; Westerhoff et al., 2008). There are over 50 chemical compounds authorized for use in FCMs that have oestrogenic potential (Muncke, 2009). Interestingly, when FCMs are assessed for their health risk, they are not routinely tested for their endocrine-disrupting potential (Muncke, 2011). Currently, a number of substances used in FCMs are still being evaluated for their safety. However, the Endocrine Society has expressed its concern about the widespread exposure of humans to these chemicals, because they are capable of affecting multiple endpoints within a living system (Diamanti-Kandarakis et al., 2009). Drinking-water sources, as well as bottled mineral and flavoured waters, contain oestrogenic substances, largely due to leaching of several phthalates and other plasticizers, including BPA used in packaging materials (Wagner and Oehlmann, 2009, 2011; Li et al., 2010; Plotan et al., 2013). The use of BPA in FCMs generated intense debate over the past decade, leading to its ban for use in FCMs. Recently, the Food and Drug Administration (FDA) and EFSA reported that the use of BPA in
FCM is of no toxicological concern (FDA, 2014; EFSA 2014a). However, although not directly on BPA, EFSA has expressed concern over chronic dietary exposure to FCMs and other contaminants in food, particularly among the most vulnerable groups (EFSA, 2012; 2014a). This is also the opinion presented in the scientific literature (Schonfelder et al., 2002; Markey et al., 2005; Benachour and Aris, 2009; Angle et al., 2013; Cabaton et al., 2013; Weber et al., 2015). Similarly, polybrominated biphenyl ether (PBDE) flame retardants, which are applied in plastics used in FCMs, certain phthalate plasticizers, as well as isopropylthioxanthone (ITX), benzophenone and 4-methylbenzophenone, used in printing inks for (non-plastic) FCMs, are of concern, due to their possible oestrogenic effects and widespread occurrence in food (EFSA, 2014b).

2.9 Xenoestrogens in the environment

It has been widely recognized that treated and untreated effluents discharged from WWTPs are major sources of EDCs in the environment (Ahn et al., 2012; Bazin et al., 2012; Ferguson et al., 2013; Brockmeier et al., 2014; Long et al., 2014; Xu et al., 2014). 4-Nonylphenol, the main degradation product of alkylphenol polyethoxylates, is widely used as a surfactant in household, agriculture and industrial processes, and BPA, an industrial raw material mainly used in the plastic, rubber, adhesive and cable industries, are the two major contributors to the endocrine-disrupting activities in aquatic environments (Auriol et al., 2006). Pesticides from agricultural runoff have also been indicted for their endocrine-disrupting potentials (Liu et al., 2005; Zhao et al., 2008, 2009; Jin et al., 2009; Zhang et al., 2010a, b; Yaglova and Yaglov, 2014; Lu et al., 2015; Pandey and Mohanty, 2015). Moreover, studies have shown that metabolites of these pesticides sometimes possess more potent oestrogenic activity than their parent compounds (Lao et al., 2010; Zhang et al., 2010a; Lu et al., 2015), making them more of a
potential threat. Considering the rates and levels of these possible oestrogenic compounds in the environment and their reported effects on nonhuman species, it is, of course, more worrisome, because it is totally impossible to remove these contaminants from the environment within a short time.

2.10 Health effects of synthetic xenoestrogens

The health effects of xenoestrogens in rodents, reptiles and laboratory animals are well documented (Guillette and Iguchi, 2003; Kidd et al., 2007; Coe et al., 2010; Pollock et al., 2010; Marmugi et al., 2014; Sobolewski et al., 2014; van Esterik et al., 2014). In humans, the evidence is less compelling.

2.10.1 Effects of xenoestrogens in males

Xenoestrogens have profound effects on developmental functions and reproductive organs in both males and females. They have been implicated in declining human sperm count and quality, fertility impairment and in abnormalities of the male reproductive tract (Toppari et al., 1996). EDCs stimulate the proliferation of epithelial cells in the reproductive tract and mammary gland of females and in the prostate of males (Kushner et al., 2003). They interfere with various sperm functions and, therefore, could impair human fertilization (Schiffer et al., 2014), leading to low sperm count and declining male reproductive performance (Delbes et al., 2006). Moreover, xenoestrogenic performance PBDEs cause DNA damage and affect neurodevelopmental behaviour at the critical endpoint (EFSA 2014b).

Epidemiological studies have continuously linked exposure to chemicals (e.g. to phthalates, PCBs, dioxins and nonpersistent pesticides) with reduced semen quality. In a USA-
based study, Duty et al. (2003) found links between monobutyl phthalate exposure and poor sperm motility and concentrations. Exposure to persistent organochlorine pollutants (e.g. PCBs and \( p,p'-\)dichlorodiphenyldichloroethylene \([p,p'-\text{DDE}])\), which are notable EDCs, also has a negative impact on human sperm chromatin integrity (Spano et al., 2005).

Mocarelli et al. (2008) also furthered our understanding of the role timing plays in end-point effects, since exposure to dioxin at specific stages in life significantly impacted semen quality. This study was based on men exposed to high levels of 2,3,7,8-tetrachlorodibenzo-\( p \)-dioxin (TCDD), the most toxic dioxin, as a result of a chemical plant explosion in 1976 in Seveso, Italy. Men exposed prepubertally (1–9 years of age) demonstrated poor semen quality as adults. Interestingly, men exposed between 10–17 and 18–27 years of age showed slightly positive or no differences in semen quality, respectively. Several occupational studies have also found associations between pesticide exposure and reduced semen quality (Whorton et al., 1979; Larsen et al., 1998; Juhler et al., 1999; Abell et al., 2000; Padungtod et al., 2000; Oliva et al., 2001; Lifeng et al., 2006).

The onset of puberty is regulated by gonadotropins that stimulate increases in oestrogen levels in both boys and girls, resulting in the pubertal growth spurt. The average age of puberty has decreased over the last century, presumably due to improved nutrition, but perhaps also due to exposure to EDCs (Teilmann et al., 2002). Environmental chemicals, including phthalate plasticizers, \( p,p' \)-DDE and polybrominated biphenyls, have been suggested as potential causative agents for precocious puberty (Blanck et al., 2000; Colon et al., 2000; Krstevska-Konstantinova et al., 2001).
2.10.2 Effects of xenoestrogens in females

Human studies in 1999 showed a possible link between maternal dietary exposure to fish from the contaminated Great Lakes and reduced fertility (Buck et al., 1999). Increased risk of infertility and spontaneous abortion for women working in the agricultural industry has also been reported (Rupta et al., 1991; Fuortes et al., 1997). This implies that exposure to PCBs and pesticides via agricultural activities could account for this reduced fertility and spontaneous abortion. In another independent study, blood samples from 2000 American and 50 Southeast Asian women found a link between PCB and \( p,p'\)-DDE levels and altered menstrual cycles in women (Cooper et al., 2005; Windham et al., 2005). The suggestive implication is that such alterations may influence other endpoints (e.g. fertility, pregnancy and reproductive cancers). In a case-control study, women with ovarian diseases showed higher levels of BPA in their sera than did normal healthy women (Takeuchi and Tsutsumi, 2002; Takeuchi et al., 2004). A study of 1188 women found a significant relationship between diethylstilboestrol (DES) exposure and uterine fibroids (Baird and Newbold, 2005). In analysis of these studies, Baird and Newbold (2005) concluded that there was a definitive increase in uterine fibroids in DES-exposed daughters, and the discrepancies between the studies were due to the differences and sensitivities of the methods used to detect the tumours. Increase in hormone-related disorders, especially in the industrialized countries, is also linked to EDC exposure (Fisher, 2004; Meeker, 2010).

2.11 Mechanism of activity of EDCs

EDCs can impart adverse effects by a number of different mechanisms, including, but not limited to, the following:

1. Activating or suppressing expression of ERs via a genomic or non-genomic pathway
2. Activating or suppressing expression of the androgen receptor

3. Activating the aryl hydrocarbon (AH) receptor

4. Inhibition of steroid hormone synthesis, transport or metabolism

5. Modulation of neurotransmitter receptors

6. Other less-defined mechanisms (WHO, 2002; SCOPE/IUPAC, 2003).

A single EDC may also affect multiple target cells by multiple mechanisms.

Activation or suppression of the ER (ERα or ERβ) is the most widely studied mechanism of EDC effect. ERs are nuclear receptors present in many cell types. They are large protein molecules with a specific binding site that has conformational specificity for the 17 β-oestradiol molecule (Thomson, 2005). Despite the specificity of the binding site, it is sufficiently generic to allow binding by a wide range of natural and synthetic compounds. Oestrogenic compounds act by diffusing into the cell nucleus and binding there to the ligand-binding domain of the receptor, whereupon the ER-ligand undergoes a conformational change and dimerizes. The dimers, with cofactors, form a complex that binds to specific sequences of DNA, known as oestrogen-response elements (EREs), of oestrogen-responsive genes. Binding to the ERE stimulates transcription and messenger-ribonucleic acid (mRNA) synthesis, leading to increased or decreased expression of oestrogen-responsive genes that encode specific proteins (Gruber et al., 2002; WHO, 2002). This is the classic pathway of oestrogenicity at the cellular and whole-body levels and is shown schematically in Figure 2.5.
Figure 2.5 Schematic of the (xeno)estrogen nuclear receptor-mediated mechanism (modified from Schug et al., 2011). 1. Xenoestrogens circulate in the plasma and enter the cells. 2. The oestrogenic compound diffuses into the nucleus and binds to the ER located in the nucleus (3). 4. The receptor occupied undergoes conformational change, dimerizes and binds with cofactors to specific sequences on oestrogen-responsive genes. 5. mRNA is transcribed. 6. The mRNA is transported from the nucleus to the cytoplasm to act as a template for protein synthesis.
3.0 Aims of the study

Several independent lines of evidence indicate that heat-processed food and drinking-water sources contribute to human cancer risk through the ingestion of genotoxic and oestrogenic compounds, thereby causing a number of adverse effects (Jägerstad and Skog, 2005; Sinha et al., 2009). Pollution of bodies of water through discharge of inadequately treated sewage waters has also been linked to oestrogenic effects in fish, with man being the final recipient of such pollution. Most of the toxicological studies carried out on food and water sources are based on exposure to one chemical at a time, neglecting the possibility of co-exposure to a mixture of chemicals, as well as the formation of new complex and even more toxic compounds during processing. This is usually the case in reality, since exposure from food is to a mixture of different chemicals.

This research work therefore was primarily aimed at the following:

i. To investigate to what degree commercially employed methods in the processing of foods and ready-to-eat snacks in Finland and Nigeria can inadvertently result in the occurrence of genotoxic substances in the final products.

ii. To determine whether commercially processed food items possess oestrogenic potential, using a yeast bioreporter assay.

iii. To determine whether drinking-water sources from both Finland and Nigeria contain oestrogenic chemicals, using an assay similar to that in ii above.

iv. To determine the oestrogenic activity of treated and untreated water from both a drinking-water plant and WWTP in Helsinki.
4.0 Materials and methods

4.1 Materials

All chemicals used in this study were of analytical grade. The β-nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate used were obtained from Roche Biochem (Stockholm, Sweden). The Aroclor-induced S9 from rat liver was purchased from Trinova Biochem (Giessen, Germany). The histidine, potassium chloride, magnesium sulphate, Celite 545, potassium phosphate dibasic anhydrous and sodium ammonium phosphate were purchased from Merck AG (Darmstadt, Germany). The magnesium chloride hexahydrate, ethyl acetate and citric acid monohydrate were obtained from VWR International (Leuven, Belgium). The dichloromethane, hexane, acetone, cyclohexane, methanol (MeOH) and toluene were purchased from J.T. Baker (Deventer, The Netherlands). The biotin, tryptophan, methylcellulose (MC), dimethyl sulphoxide (DMSO), BaP, 2-aminoanthracene, sodium azide, oestradiol, BPA, Florisil, progesterone and testosterone were purchased from Sigma-Aldrich (Steinheim, Germany). The D-luciferin was obtained from Biotherma (Handen, Sweden). The yeast nitrogen base medium without amino acids was obtained from Becton Dickinson and Company (East Rutherford, NJ, USA). The Supelclean ENVI-Chrom P (6-ml, 500-mg) solid-phase extraction (SPE) cartridges were purchased from Supelco (Bellefonte, PA, USA), while the strata-X 33u polymeric reversed-phase (500 mg/5 ml) was obtained from Phenomenex (Aschaffenburg, Germany).

4.2 Microorganisms

The bacteria, Salmonella enterica sv. Typhimurium strains TA 100 and TA 98, were obtained from the Pasteur Institute (Paris Cedex, France). Two recombinant yeast strains
Saccharomyces cerevisiae BMAERE\textsubscript{Luc/ER$\alpha$} and S. cerevisiae BMA64/luc (Leskinen et al., 2005) were used in this study. In the yeast bioluminescent assay, the BMAERE\textsubscript{Luc/ER$\alpha$} served as a reporter strain in which the ER$\alpha$ is expressed. In ligand binding, the dimerized receptor binds the EREs in the promoter region of the luc reporter gene. In S. cerevisiae BMA64/luc, the luciferase is expressed constitutively, and this strain was used for determination of cytotoxicity in the test samples. Both yeast strains were kind gift donations by Dr. Johanna Rajasärkkä of the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki. The yeasts were grown on Difco Yeast Nitrogen Base Medium (Difco Laboratories Inc. (Becton Dickinson), Franklin Lakes, NJ, USA) without amino acids, supplemented with 40% glucose and their respective amino acids.

4.3 Cell line

The human hepatocellular carcinoma-derived cell line (HepG2) was obtained from the American Type Culture Collection through LGC Standards (Boras, Sweden) and cultured in Eagle’s Minimum Essential Medium (LGC Standards) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$ in an air atmosphere incubator (NuAire Inc., Plymouth, MN, USA).

4.4 Food samples

A total of 116 food samples, representing 39 varieties of three lots each, obtained from both Finland and Nigeria, were used in this study. The food samples from Finland were classified as commercially processed food products and ready-to-eat snacks (II). Here, 45 samples of industrially processed and packaged food products were purchased from a popular
supermarket in Helsinki (Prisma, Viikki), while the ready-to-eat food samples were acquired from a local representative (at SOKOS City Centre) of a global chain of hamburger restaurants also located in Helsinki. All samples were collected at separate times, and the cooking conditions such as time and temperature were not available.

A total of 36 samples (three lots of 12 varieties), representing commonly consumed, commercially processed food items in Nigeria, were also evaluated for their mutagenic potential. All products were extracted before the expiry date shown on the packages. Twenty food samples were also obtained from Prisma for confirmatory testing by chemical analysis.

4.5 Drinking-water samples

A total of 60 water samples, representing tap water (hot and cold) collected bimonthly in Finland, both from the premises of the University of Helsinki, Viikki campus and a residential building in Vantaa, Finland, over a 3-month (March–May) period, 10 different brands each of bottled still and mineral waters, purchased from a local grocery store (Prisma, Viikki) and 16 sachet-pure water samples sold in Benin City metropolis, Edo State, Nigeria were used in this study.

4.6 Wastewater samples

Treated and untreated water samples, taken bimonthly, were obtained from the WWTP in Viikinmäki, Helsinki over the preceding 20-month period on two occasions in 2011 and 2014. The Viikinmäki WWTP is the largest of such plant in Finland, serving over 1 million inhabitants and processing both household (85%) and industrial (15%) wastewater from five different districts (Helsinki, Kerava, Tuusula, Järvenpää, Sipoo and of the central and
eastern districts of Vantaa) in Finland. It receives approximately 270 000 m$^3$ of wastewater per
day and an average of 100 million m$^3$ each year. Equivalent samples from a household water
purification plant (located in the same region) were also obtained in March, April and June of
2014.

4.7 Sample preparation: Food samples

Possible mutagenic compounds in Finnish food products were extracted, using the
method described by Peters et al. (2004), with slight modifications. Briefly, 20 g of food sample
were homogenized with 80 ml of 1-M NaOH at 24 000 revolutions per minute (rpm). The
homogenate was mixed with 20 g of Extrelut refill material (VWR International, Helsinki,
Finland) and then poured into an empty Extrelut 20 column. The organics were eluted from the
Extrelut column with 40 ml of dichloromethane/toluene solution (95:5 v/v) into a cartridge. The
organics were finally eluted with 5 ml of MeOH-NH$_2$OH (9:1) solution and evaporated to
dryness under a gentle stream of nitrogen in a fume hood.

The Nigerian food samples were extracted in Nigeria (Tikkanen, 1991), and approximately 2 ml
of the final extracts were shipped on ice to the Department of Food Hygiene and Environmental
Health, Faculty of Veterinary Medicine, University of Helsinki for analysis. For determination of
recovery, fresh meat samples were spiked with 250 μg of BaP and 2-aminoanthracene in two
different cases and extracted in the same way as above. The food samples for confirmatory
chemical analysis were extracted by accelerated solvent extraction and SPE (Jira et al., 2008),
using super-clean ENVI-Chrom P (6-ml, 500-mg) cartridges.
**4.8 Sample preparation: Water samples**

All water samples (500 ml) obtained from Finland were extracted by the SPE method, as described by Kopperi et al. (2013), using Phenomenex strata-X 33u polymeric reversed phase 500 mg/5 ml (Phenomenex), while pure water samples from Nigeria (1000 ml each) were extracted by the liquid-liquid extraction (LLE) method, as described by Barber et al. (2000). The percentage recovery of both extraction methods was determined by spiking distilled water samples with 2.5 mg of BPA and extracting them in the same way as the test samples.

**4.9 Sample preparation: Food packaging material**

Samples that were oestrogenic were unwrapped and their packaging materials reused for negative samples to disclose possible leaching of xenoestrogens. The repackaging was done in such a way as to maintain close contact between the food sample and packaging material. The repackaged food samples were kept in an oven at 80 °C for 30 min and then at 4 °C for 7 days before extraction to maximize the leaching effect without spoiling the foodstuffs. For extraction, these packaging materials were embedded in MeOH in a mechanical shaker at 60 °C overnight. This is a slight modification of a previous method used with success to extract oestrogenic compounds from food samples (Behr et al., 2011).

**4.10 Cytotoxicity assays**

The cytotoxic effect of the food extract concentrations used in this study was investigated, using three independent assays measuring trypan blue exclusion, lactate dehydrogenase (LDH) activity and boar sperm motility.
4.10.1 Trypan blue test

HepG2 cells were grown in 24-well plates (VWR, Finland) until semi-confluent cells were obtained (48 h). This was followed by exposure of the cells to different concentrations of food extracts for 4, 24 or 48 h. After exposure, the cells were trypsinized, using a 0.25% (w/v) trypsin–0.53 mM ethylenediaminetetraacetic acid (EDTA) solution (LGC Standards). The trypsinized cells were transferred to 1.5-ml Eppendorf tubes and centrifuged for 5 min at 2500 rpm. The pellets were then resuspended in phosphate-buffered saline (PBS), after which 10 μl of the cells were mixed with 5 μl (0.8 mM) of trypan blue dye before microscopic observation. The lysis solution served as positive controls for both the trypan blue test and LDH assay.

4.10.2 LDH assay

The activity of LDH was determined in HepG2 cells exposed to the same concentrations of the food extracts used in the genotoxicity assays. The LDH test measures plasma membrane integrity and was performed according to the instructions provided in the Cytotoxicity Detection KitPLUS (LDH), version 6 (Roche Biochem).

4.10.3 Boar sperm motility inhibition bioassay

Extracts of selected food samples were assessed for their mitochondrial toxicity, using the boar sperm motility assay (Andersson et al., 2010). Briefly, 2 ml of boar semen in screw-capped exposure vials were exposed to 10 μl of food extracts for 30 min, 24 or 48 h at 20 °C. Vehicle (DMSO) exposure was prepared simultaneously with the test samples for each time point. After exposure, the vials were shaken gently to disperse the sperm cells, and 200 μl of the
suspension were drawn into a warmed test tube and placed in a heating block (30 °C) for approximately 5 min to activate sperm motility. Sperm motility was assessed by dispensing the warmed sperm suspension onto a microscopic slide, using a pre-warmed capillary tube, and immediately observed with a 40-x inverted phase-contrast objective.

4.11 Mutagenicity assay

The mutagenic potential of the food extracts was determined initially by the standard plate incorporation assay. Samples showing mutagenic potential in this assay were subjected to treat-and-wash as well as MC overlay assays to ascertain to what degree a localized release of proteins, peptides or histidine from the samples contributed to the outcome.

4.11.1 Standard plate incorporation assay

The standard plate incorporation assay was performed as described by Maron and Ames (1983), using *Salmonella* strains TA 100 and TA 98 with and without metabolic activation (S9 mix). The amount of S9 used in the S9 mix was 10%. Water and DMSO were used as negative controls for both strains, while sodium azide (0.04 mg/ml) and 2-aminoanthracene (0.02 mg/ml) served as positive controls for TA 100 and TA 98, respectively. BaP (0.1 mg/ml) was also used as a positive control for both strains. The volume of the controls used was 50 μl per plate in triplicate plates. Sodium azide is a known direct mutagen in *Salmonella* TA 100 (Mortelmans and Zeiger, 2000), whereas 2-aminoanthracene is metabolically activated by monooxygenases of the cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A family) in rat liver (Carrière et al., 1992). Likewise, BaP requires metabolic activation for mutagenicity (Gabbani et al., 1998).
For all samples, four different concentrations of the food extracts (25, 50, 100 and 200 mg/ml) were tested in triplicate plates (50 μl per plate). The highest concentration (200 mg/ml) was equivalent to 1 g of the food sample. The plates were incubated at 37 °C for 48 h.

4.11.2 Treat-and-wash assay

The treat-and-wash assay was conducted according to the method described by Thompson et al. (2005). The protocol applied was as per the standard plate incorporation assay, with the exception that the S9 mix, bacteria and sample extract were incubated for 90 min prior to the addition of molten top agar. Briefly, a 500 μl aliquot of S9 mix/phosphate buffer (0.2 M, pH 7.4) was combined with 100 μl each of late-log bacterial culture and sample extract solution in a sterile 15 ml tube. The mixture was incubated for 90 min in a mechanical shaker (180 rpm) at 37 °C. The extended duration of the bacterial exposure compensated for the absence of bacterial exposure on the plates, since the test sample was washed away prior to plating. After a 90 min pre-incubation, 10 ml of washing solution (Oxoid No. 2 nutrient broth; Oxoid (ThermoFisher Scientific Inc.), Waltham, MA, USA in PBS [1:7 v/v]) was added, and the washed bacteria were collected by centrifugation at 2000 g for 30 min. All but approximately 700 μl of the supernatant was removed and discarded, and the bacteria were resuspended in the residual supernatant prior to plating via the top agar.

4.11.3 Methylcellulose overlay assay

The MC overlay assay was performed, as previously described (Thompson et al., 2005). Briefly, a 500 μl aliquot of S9 mix/phosphate buffer (0.2 M, pH 7.4) was combined with 100 μl of late-log bacterial culture in a sterile 15 ml tube. A 2 ml aliquot of the MC overlay
suspension was added to the tube, and a 100 μl aliquot of the sample extract solution was added immediately afterward. The mixture was overlaid on a pre-warmed (37 °C) minimal glucose plate. The plates were held at 4 °C for 1 h after plating to ensure gelling of the MC overlay, and subsequently incubated (not inverted) at 37 °C for 48—72 h. The MC overlay was prepared on the day of the test (Thompson et al., 2005), and the mixture was stirred at 50–60 °C throughout use.

4.12 Comet assay

The comet assay (single-cell gel electrophoresis) was used to evaluate possible breakage in the single-stranded DNA of HepG2 cells, following treatment with extracts of food samples showing mutagenic potential. The comet assay was performed under alkaline conditions (pH > 13), as described previously (Singh et al., 1998). Hydrogen peroxide and 0.7% DMSO served as positive and negative controls, respectively.

4.13 Yeast bioluminescent assay

The yeast bioluminescent assay was performed, as previously described (Leskinen et al., 2005; Rajasäärkkä and Virta, 2011). The optimized parameters for the 384 well plates that were used in this study have also been reported (Rajasäärkkä and Virta, 2011), except that 5% ethanol was used as the vehicle, because the 10% DMSO reported by Rajasäärkkä and Virta (2011) was cytotoxic in the test system.
4.14 Chemical analysis: GC-MS/MS

Gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis was performed, using a gas chromatograph (Agilent, 6890N; Agilent Technologies, Santa Clara, CA, USA) coupled with a Micromass Quattro Micro GC triple-quadrupole analyser (Waters, Micromass; Waters Corp., Milford, MA, USA), using an Agilent J and W Select PAH (30 m x 0.25 mm x 0.15 μm) column, helium as the carrier gas, at a flow rate of 1.0 ml/min, with the following conditions:

Injection: Splitless injection, injection volume, 1 μl. Electron ionization (EI). Injector temperature 300 °C, transfer-line temperature 300 °C, ion source temperature 275 °C. The column temperature programme was as follows: Initial temperature 110 °C (0.7 min), 85 °C/min to 180 °C, 3 °C/min to 230 °C (7 min), 28 °C/min to 280 °C (15 min), 14 °C/min to 350 °C (5 min).

4.15 Statistical analysis/Interpretation of data

The mutagenic potency of each food sample was determined from the slope of the linear portion of the dose-response curve by linear-regression analysis, using the software program Prisma 4.0 (GraphPad Software Inc., San Diego, CA, USA). In addition to the requirement for a statistically significant ($P < 0.05$) dose-response effect, only those samples were considered mutagenic whose highest test concentration generated at least twice as many revertants as the negative control (DMSO). For proper interpretation and clarity, the number of revertants obtained was compared with both their experiment-specific controls and aggregate controls across all experiments. The $P$ values of these comparisons in the tables, figures and supplementary tables are derived from the regression analyses. In the oestrogenic activity assays,
the fold induction, fold induction-corrected (FIC) and limit of detection (LOD) were calculated, as described previously (Leskinen et al., 2005). The sigmoidal dose-response curves for increasing concentrations of oestradiol and BPA were obtained, using Prisma 4.0. The oestradiol and BPA equivalents of the food samples showing oestrogenic activity were calculated from the probit transformation of the curves.
5.0 RESULTS

5.1 Recovery experiment

The percentage recoveries of the chemicals added in the spiking experiments were relatively high, ranging from 68 % to 89 % (Table 5.1). They were also comparably high between the alternative methods employed.

Table 5.1 Percentage recoveries from the spiking experiments.

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Test substance</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE (food samples)</td>
<td>BaP</td>
<td>73</td>
</tr>
<tr>
<td>SPE (food samples)</td>
<td>2-aminoanthracene</td>
<td>71</td>
</tr>
<tr>
<td>SE (food samples)</td>
<td>BaP</td>
<td>89</td>
</tr>
<tr>
<td>SE (food samples)</td>
<td>2-aminoanthracene</td>
<td>77</td>
</tr>
<tr>
<td>SPE (water samples)</td>
<td>BPA</td>
<td>72</td>
</tr>
<tr>
<td>LLE (water samples)</td>
<td>BPA</td>
<td>68</td>
</tr>
</tbody>
</table>

**Key:** SPE: solid-phase extraction; SE: soxlet extraction; LLE: liquid-liquid extraction

5.2 Mutagenicity test results

5.2i Standard plate incorporation assays

The mutagenic activity of the commercially processed food items expressed as the number of revertants per gram original product, obtained by the standard plate incorporation
assay, are presented in Tables 2a, b, 3a, b (II) and Tables 2 and 3 (III) for the Finnish and Nigerian food samples, respectively. Both commercially processed food items and ready-to-eat snacks in Finland generally exhibited fairly low levels of mutagenic activity, in both *Salmonella* TA 100 and TA 98 (II). Extracts of industrially processed grilled chicken, smoked fish, cold-smoked fish, grilled beef, French fries, mashed potato, hamburger (beef) and hamburger (chicken) showed no discernible mutagenic responses in any of the three batches examined in either strain of *Salmonella*, regardless of the S9 status. Meanwhile, extracts from seven food products (smoked chicken, honey-roasted chicken, grilled turkey, cold-smoked beef, cold-smoked fish, sausage and pepper salami) generated revertants at least twice as high as those of the negative control (DMSO) in at least one of the three batches investigated, and in one or both strains of *Salmonella*. Of interest was the extract of cold cuts of grilled turkey, which showed clear mutagenic activity in all three batches examined with *Salmonella* TA 98 (Table 2b; II). In two cases, this occurred without metabolic activation (S9 mix), and once in its presence. One of these lots (batch 1) also proved mutagenic in TA 100, both in the presence and absence of the S9 mix. This particular batch also yielded a drastic mutagenic response in a modified Ames test (see below). With the ready-to-eat snacks, three of the snack products examined generated a more than twofold higher number of revertants than the control compounds, and produced a significant dose response. Chicken nuggets were particularly mutagenic in all the batches examined with *Salmonella* TA 100, mainly in the presence of the S9 mix. In *Salmonella* TA 98, the numbers of revertants produced by chicken nuggets were either marginally elevated or, if they were over twice as high as the control, lacked a significant dose response (Table 3b; II).

The results from commercially processed food items in Nigeria were more alarming and revealing, since the majority of food samples investigated (75 %) exhibited fairly high mutagenic activity (the maximal response being comparable to those elicited by the positive control, BaP), mainly in the *Salmonella* TA 100 strain (III). Chin-chin, hamburger, suya and
bean cake were the most mutagenic. For all of these food products, the number of revertants generated was over two-fold as that of DMSO in all the batches analysed and mostly independent of metabolic activation. A somewhat surprising result was found with extracts of the potato products (French fries and potato chips) from Nigeria, since at least one of the lots of both products proved directly mutagenic with the TA 100 strain (III). This was certainly not the case with similar food products in Finland (II). Meanwhile, roasted maize, plantain chips and coconut candy were the only food products without mutagenic activity in the TA 100 strain (Table 2; III).

In *Salmonella* TA 98, only three food or snack varieties (potato chips, peanut and suya) exhibited mutagenic potency in at least one of the batches investigated (Table 3; III). Suya displayed the most consistent outcome, with all its batches being mutagenic in the presence of the S9 mix. In support of the result with TA 100, the same batch of potato chips (number 3) also exhibited direct mutagenicity in TA 98.

**5.2ii Modified Ames test**

To ascertain to what degree a localized release of proteins, peptides or histidine contributed to the mutagenicity test results obtained with the standard plate incorporation assay, ‘treat-and-wash’ as well as MC overlay assays were performed. The outcome was dependent on the bacterial strain, type of food, S9 status and assay. For some food extracts initially mutagenic in the standard plate incorporation assay, the number of revertants decreased below the two-fold limit level in comparison with the negative control. Hence, the original Ames test result was, in these cases, interpreted to be of secondary nature and not due to genuine mutations. For some commercially processed Finnish food items and ready-to-eat snacks, the complementary assays tended to reduce the number of revertants per gram obtained, compared with the results obtained with the standard plate incorporation assay (II). Several surprises also emerged in these
complementary assays with the Finnish food samples, since extracts of industrially processed food (cold cuts of smoked chicken, honey-roasted chicken and cold-smoked beef) that initially required metabolic activation for their mutagenicity in the standard plate incorporation assay were, unexpectedly, directly mutagenic with the *Salmonella* TA 100 strain in the MC overlay assay (Supplementary Table 2; II). One of these samples (cold-smoked beef) also showed similar results in the treat-and-wash assay (Supplementary Table 6; II). Interestingly, none of the ready-to-eat food samples exhibited any form of mutagenicity in either of these auxiliary assays with *Salmonella* TA 100 (Supplementary Tables 4 and 8; II) in contrast to their outcome in the standard plate incorporation assay.

The most striking result was obtained with an extract of industrially processed cold cuts of grilled turkey (batch 1). Although this item showed clear mutagenic activity in all three assays both in the presence and absence of the S9 mix with the *Salmonella* TA 100 strain, both the MC overlay and treat-and-wash procedures tended to diminish the revertant number, compared with the standard assay (Figure 1B; II). With the *Salmonella* TA 98 strain, however, by far the most conspicuous mutagenic response was recorded in the treat-and-wash assay, in which it produced revertants over 13- and 5-fold higher than its control with and without the S9 mix, respectively (Supplementary Table 7 and Figure 2; II). The two other batches of grilled turkey were also mutagenic with the TA 98 strain in this assay, both in the presence and absence of the S9 mix, but no such drastic enhancement by the treat-and-wash procedure was seen (Supplementary Table 7; II).

In contrast to the situation in Finland, a large number of food items from Nigeria were mutagenic in all three assays, both in the presence and absence of the S9 mix. For some food items (hamburger, suya and bean cake), a single lot was mutagenic in all three assays, but only in the presence of the S9 mix. In the absence of S9, the outcome with these three products varied. In contrast to this pattern, a single batch of hamburger (batch 2) was mutagenic in all
three assays, both in the presence and absence of the S9 mix. Meanwhile, extracts of fried chicken (batch 2) and bean cake (batch 2) that required metabolic activation for their mutagenicity in the standard plate incorporation assay were, unexpectedly, directly mutagenic in the treat-and-wash assay in the *Salmonella* TA 100 strain (Table 4; III). One of these samples (bean cake, batch 2) also showed similar results in the MC overlay assay (Table 4; III).

5.3 Cytotoxicity assay: Trypan blue exclusion assay

The percentage viability of the HepG2 cells in the trypan blue exclusion test exhibited an inverse correlation with the concentration of some food extracts (smoked chicken, grilled turkey and cold-smoked beef) from Finland (II), following a 48-h exposure. Meanwhile, the non-survival percentage of the HepG2 cells following treatment with extracts of commercially processed food items from Finland and Nigeria in all cases did not exceed 50% (II and III) following 4-, 24- or 48-h exposures. Therefore, they were considered non-cytotoxic in this assay at all time points.

5.4 Cytotoxicity assay: LDH assay

In both studies (II and III), there was a direct correlation between the test samples and LDH release. However, the maximum released never exceeded 25% of that induced by the positive control substance applied (lysis solution). Hence, the extracts were classified as non-cytotoxic in this assay, following exposure for 4, 24 or 48 h.
5.5 Cytotoxicity assay: Boar sperm motility assay

The effect of the food extracts on boar sperm motility was also investigated, following a 4-h exposure. However, none of the food extracts tested affected boar sperm motility in a statistically significant manner.

5.6 Comet assay

As a follow-up of the results obtained in the conventional Ames test, samples showing mutagenic potential in at least one strain of *Salmonella* were screened for single-strand breaks, DNA-DNA/DNA-protein cross-linking and alkali-labile sites by the comet assay. None of the samples investigated tested positive when compared with both positive and negative controls. In the positive controls (treatment with hydrogen peroxide), the expected DNA migration towards the anode was observed by microscopic examination.

5.7 GC-MS/MS

The majority of Finnish food samples produced low levels of PAHs (BaP, BaA, Ch and BbF), except for a single batch of smoked fish (Table 5.2). In this lot, all four PAHs, exceeded the acceptable limit of 5 μg/kg. The concentration of BaP was 8.2 μg/kg, while those of BaA and Ch were three-fold greater than the acceptable limit. For another lot of this same product, the levels of all PAHs were less striking, slightly lower than the acceptable limit.
Table 5.2 Levels of PAHs in commercially processed Finnish foods and their mutagenic potential only in the presence of the S9 mix.

<table>
<thead>
<tr>
<th>Food item</th>
<th>PAHs (μg/kg)</th>
<th>Ames test (revt/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BaP</td>
<td>BaA</td>
</tr>
<tr>
<td>Smoked ham</td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Honey-roasted chicken</td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Grilled turkey</td>
<td>ND</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pepper salami</td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Cold-smoked beef</td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td></td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sauna-smoked ham</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Smoked fish</td>
<td>4.7</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Key: ND: Not detected; LOQ: Limit of quantification (0.78 μg/kg); LOD: Limit of detection (0.26 μg/kg); NA: Not applicable; asterisk (*): Significantly different from control ($P < 0.05$). Sum: The total sum of benzo[a]pyrene, benzo[a]anthracene, chrysene and benzo[b]fluoranthene.
5.8 Oestrogenic activity assay: Commercially processed food

The majority of commercially processed food samples and ready-to-eat snacks in Finland failed to exhibit detectable oestrogenic activity. However, extracts of industrially processed and packaged hamburgers (beef and chicken) and of pepper salami were consistently oestrogenic in all batches examined (Table 2; I). Pepper salami showed the most potent oestrogenic activity, with oestradiol equivalents varying between 1.6 and 443 pg/g. Extracts of chicken hamburger were the least oestrogenic of the positive samples (0.2--0.6 pg/g), while the oestradiol equivalents of beef hamburger fell between these two. There was wide variation in the oestrogenic activities among different batches of the same products, amounting to almost 1000-fold for pepper salami. Meanwhile, equivalent burger products obtained from a hamburger restaurant showed no oestrogenic activity in the test system. When it was revealed that all the positive food samples contained a soy-based ingredient (Table 3; I), while none of the negative samples purchased from the supermarket harboured it, two different brands of soy sauces were examined to verify soy oestrogenicity in this assay system; both generated an intense signal (Table 4; I).

5.9 Oestrogenic activity assay: Bottled water, mineral water, tap and sachet-pure water

In all, 31% of the sachet-pure water samples from Nigeria were oestrogenic in this assay. The oestrogenicity of the positive samples ranged from 0.79 to 44.0 ng/l (median: 23.0 ng/l) oestradiol equivalent concentrations (EEQs). In contrast, no brands of bottled still and mineral waters, as well as tap water, from Finland yielded any oestrogenic activity (IV).
5.10 Oestrogenic activity assay: Food packaging material

Commercially processed food samples that were non-oestrogenic in the assay were repackaged in the packaging materials of the positive oestrogenic samples of equivalent products to ascertain whether the oestrogenic activities in these foodstuffs actually originated from the wrappers. To complement this approach, the oestrogenic activities of the wrappers of all positive food samples were also directly determined. Both the repackaged Finnish food items and the wrappers exhibited no oestrogenic activity in our test system (I). Meanwhile, 40% of the sachet-pure water samples generated positive signals in the yeast-based assay (Table 8; III).

5.11 Oestrogenic activity assay: Influent and effluent samples

The oestrogenic activities of the influent samples from the WWTP were low (approximately 0.5 ng/l EEQ) throughout this period, except for 2 months in 2011, March and August, when they peaked at 14 and 7.8 ng/l, respectively (Table 2; IV). The influent and effluent samples from an equivalent household water purification plant, as well as all treated effluent waters from the WWTP, likewise showed no oestrogenic activity in this test system.
6.0 Discussion

Commercially processed food and drinking-water sources are prerequisites for human life and are consumed in increasing amounts globally. Therefore, it is of the utmost importance to ensure that, in addition to their microbiological safety, they do not contain chemicals that may pose a toxicological risk to consumer health. A conceivable potential risk in this regard is the presence of genotoxic compounds deliberately added to, inadvertently contaminating or arising in the processing of food. Environmental exposure to xenoestrogens also occurs via indiscriminate discharge of inadequately treated sewage water into lakes and streams, and the terrestrial environment. Regular screening studies are necessary to verify that the methods used by food vendors, bottled/sachet water companies and in the treatment of influent and drinking water are also appropriate and sound from this point of view. The present investigation was aimed at exploring the current situation in Finland and Nigeria.

6.1 Recoveries and sample representativeness

As observed in 4.7 and 4.8 above, two different methods each were used for the extraction of food and water samples in both countries, due to differences in the types of laboratory material available. However, the recoveries of potentially mutagenic and oestrogenic compounds in the two assays were comparable. Both methods have also previously been used for the extraction of mutagenic and oestrogenic compounds in food and water samples.

The carefully selected Finnish food products investigated represent those commonly consumed in Finland and probably in Europe. The sampling covered food products processed by various methods (grilled, smoked, cold-smoked and fried) and of differing origins (fish, meat and plant). Ready-to-eat snacks from a fast-food centre were also examined to cover a broader range of
commonly consumed food products (II). In Nigeria, the situation is different from that in Finland regarding the types of food consumed, and Nigerian foodstuffs do not faithfully represent the varieties commonly consumed elsewhere in Africa. The food products analysed encompassed snacks, quick meals and delicacies, which are widely consumed across all social classes. Bean cake, for example, is a popular quick meal among most Nigerians, and its preparation is time-consuming. Ready-to-eat bean cake vendors are preferably patronized, based on the customer’s perceived hygienic standards of the food handlers, and not on the processing technique, although this food item can only be prepared by deep frying. The same also holds for the special delicacy suya (red meat processed by direct smoking with wood or coal).

6.2 Mutagenicity studies of commercially processed food

The Ames test is a rapid *in vitro* assay for detection of the genotoxic potential of chemicals (Maron and Ames, 1983) and has a high predictability for rodent carcinogens (McCann et al., 1975; Zeiger, 1998; Mortelmans and Zeiger, 2000; Hakura et al., 2005). Although there is evidence that this test system does not detect some genotoxic chemicals, such as AA, either in the presence or absence of metabolic activation (Knaap et al., 1988; Dearfield et al., 1995), it is still considered reliable for the detection of most mutagens and potentially genotoxic chemicals (Hakura et al., 2005). Metabolic activation is also regarded as a critical step in mutation (Guengerich, 2000), since many potential carcinogens remain inactive until they are enzymatically transformed into electrophilic species that are capable of covalently binding to DNA and thereby leading to mutation. To this end, the rat Arochlor-induced liver S9 fraction offers a more complete representation of the metabolic profile than other S9 fractions (Hakura et al., 2005), because it contains both phase I and phase II activities (Brandon et al., 2003).
The mutagenic activity observed in this study with some extracts of commercially processed food items in Finland and Nigeria showed that such products cannot be completely ignored as health hazards. The levels/number of revertants per gram obtained for some food items may be of toxicological significance, due to the magnitude of mutagenic activities (2.5—13-fold over those obtained with negative controls). For some food products (both in Finland and Nigeria), there was a notable (to over 10-fold) variation in mutagenic potencies between batches and among equivalent products and various products processed in the same way. There was also variation in mutagenic activity among equivalent food products with the three assays (standard plate incorporation, MC overlay and treat-and-wash assays), casting doubt on the significance of some findings. Only a fraction of the commercially processed food items in Finland (smoked chicken, grilled turkey and cold-smoked beef), initially mutagenic in the standard plate incorporation assay, were also mutagenic in the complementary assays. In these cases, the mutagenic effect was considered genuine (i.e. not secondary to histidine release from the food products) and thus of concern. Meanwhile, the mutagenicity test results of processed food items from Nigeria gave insight into the probable differences in processing techniques used in the developed and developing countries. A total of 75% of commercially processed food items in Nigeria were mutagenic in the standard plate incorporation assay with the TA 100 strain; likewise, this was almost the case in the complementary assays as well. This is in contrast to the study of commercial food items in Finland, where generally only a single batch of each product was mutagenic.

Considered together with a study published in Finland over 20 years ago (Tikkanen, 1991), the current findings imply that processing techniques in Finland have improved remarkably, because the majority of food items investigated in that study were mutagenic, which is in contrast to that reported here (II). It is also noteworthy that Tikkanen (1991) did not consider the possible release of histidine from the food products. In that study and in others, only Salmonella TA 98 was
used, and sometimes only in the presence of the S9 mix. Based on findings from the present study, TA 98 is far less sensitive to food mutagens than is TA 100.

The low levels of mutagenic activities observed in Finnish food samples were surprising and somewhat unexpected, compared with the results of Nigerian food items, foodstuffs from elsewhere (Stavric et al., 1995; Sharif et al., 2008) and previously in Finland (Tikkanen, 1991). To this end, an independent chemical analysis of the four principal PAHs was carried out on similar food products (three lots of each) previously examined in the mutagenicity assays, albeit using a slightly different method of extraction. Interestingly, the results of the chemical analysis lend further credence to the mutagenicity test results, because the majority of food items contained non-detectable levels of PAHs. This finding reinforces the view that the food industry in Finland has recognized its responsibility and taken appropriate steps towards producing products that do not pose genotoxic hazards to consumers. However, much still needs to be done with the various smoking techniques. In accordance with the results of the mutagenicity assays, the majority of food items did not contain significant levels of PAHs, while those harbouring any of the mutagenic PAHs showed variation among batches of the same and equivalent products. All meat products (smoked ham, honey-roasted chicken, grilled turkey, pepper salami, cold-smoked beef and sauna-smoked pork) mostly contained PAHs below the limit of quantification (LOQ) and LOD of 0.78 and 0.26 μg/kg, respectively. Djinovic et al. (2008) reported similar findings from Serbia, while in Sweden a significant number of processed meat and meat products (9 out of 38) contained BaP, ranging from 6.6 to 36.9 μg/kg, exceeding the 5.0 μg/kg maximum level established by the EU (Wretling et al., 2010). Elsewhere, varying concentrations of PAHs have been reported in commercially processed meat, meat products, fish and fish products (Farhadian et al., 2010; Alomirah et al., 2011; Chung et al., 2011; Essumang et al., 2012; Aaslyng et al., 2013). The results of both the Ames test and GC-MS/MS analysis in Finland are in agreement with two recent independent studies (Jägerstad and Skog, 2005; EFSA, 2008). Jägerstad and Skog (2005) reported
that the daily intake of nitrosodimethylamine was significantly lower in Finland (0.08 μg/day) than in other European countries (0.12–0.38 μg/day). A report submitted to EFSA by 16 participating EU countries and compiled/released in 2008 showed that the total dietary exposure to BaP was also lower (185 ng/day) in Finland than in other participating countries (188—255 ng/day). For PAH2, PAH4 and PAH8, the levels obtained in Finland were slightly higher than those recorded in the United Kingdom (EU country with the lowest levels of PAH2, PAH4 and PAH8).

As for the Nigerian food samples, the cause of the high number of revertants obtained with most food products is suggestive. The processing techniques (charcoal-grilled, deep-frying) commonly used in Nigeria have been implicated in generating high levels of both PAHs and HAAs in the final products (Iwasaki et al., 2010; Liao et al., 2010, 2012; Chung et al., 2011; Essumang et al., 2012). Double-heat treatment/reuse of cooking oil, which is always the case in Nigeria and probably in most African countries, results in an increase in the genotoxic activity of food products (Isidor and Parrella, 2009; Srivastava et al., 2010). During frying, cooking oil undergoes deterioration through various chemical and physical processes such as oxidation, polymerization, hydrolysis and cyclization, leading to the formation of both volatile and nonvolatile undesirable by-products (Isidor and Parrella, 2009). These derivatives are partially absorbed by the fried food, which thus becomes carcinogenic (Alomirah et al., 2010). For example, the PAH compounds BaP, BaA and Ch are all well-known human carcinogens that have been detected in different types of cooking oil (Alomirah et al., 2010). Meat and fish products grilled and/or smoked traditionally are also usually heavily contaminated with the PAH compound BaP (Akpambang et al., 2009).

Most of the food items (except suya) investigated in this study have not been previously screened for either PAHs or HAAs in Nigeria, and the PAH compound BaP was first reported in suya (8.5 μg/kg) from Nigeria in 1982 (Bababunmi et al., 1982). The probable reason for the high level in that case, in addition to the processing method, was the use of car tyres for smoking. This method also increased the levels of PAHs in food products in Ghana, compared with
other methods (Abdul et al., 2014). After the initial study by Bababunmi et al. (1982), even higher levels of PAHs have been reported. Duke and Albert (2007) found BaP contents ranging from 6.5 to 21.5 μg/kg in suya meat from four different selling points in the Niger Delta axis of Nigeria, while Akpambang et al. (2009) reported the levels of BaP, Ch, BbF and BaA in commercially processed suya in Nigeria to be 10.1, 25.6, 12.3 and 15.4 μg/kg dry weight, respectively. More recently, Amos-Tauta et al. (2013) reported a mean concentration of BaA in suya of 7.23 μg/kg. In all of these studies, the concentrations of all PAHs were greater than the acceptable limit of 5.0 μg/kg.

6.3 Genotoxicity of drinking water

Considerable mutagenicity in drinking water in Finland has previously been reported (Vartiainen and Liimatainen, 1986; Vartiainen et al., 1988). This was largely attributed to high levels of the by-products of disinfection (mainly chlorination) stemming from chemical reactions with humic substances. For example, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and several other chlorinated hydroxyl furanones were found in 71% of Finnish drinking-water samples with mutagenic outcomes (Kronberg and Vartianien, 1988; Smeds et. al., 1997). Consequently, a linear relationship was reported between exposure to Finnish drinking-water mutagenicity and the risk of bladder, kidney, stomach and pancreatic cancers, as well as lymphomas in people consuming the water (Koivusalo et al., 1994, 1995). Realization of these ensuing health risks led to substantial changes in water treatment practices by increased use of artificial groundwater, chloramine disinfection and ozonation, use of chlorine dioxide as post-disinfectant, as well as improved coagulation/flocculation techniques, thereby rapidly decreasing mutagenicity (Nissinen et al., 2002). Two recent studies in Nigeria also reported drinking-water sources to be genotoxic (Adelanwa et al., 2011; Olorunfemi et al., 2014). Thus, household water can also be a genotoxic risk factor, but it was not specifically addressed in this thesis.
6.4 Oestrogenic activities of commercially processed food items and drinking water

We investigated the presence of xenoestrogens in commercially processed food items (mainly meat and fish products) arising from substances deliberately added to or inadvertently contaminating the food, formed as a result of food processing or leaching from food packaging materials. Overall, food packaging materials do not seem likely to be a notable source of human exposure to xenoestrogens through intake/consumption of commercially processed Finnish foods. In addition, no significant oestrogenic activity emerged as a result of processing. However, three varieties of commercially processed Finnish food items tested positive in this assay. Interestingly, they all shared one property: a soy-based ingredient. The oestrogenic activity of soy as well as of soy-based products has been reported previously (Takamura-Enya et al., 2003; Behr et al., 2011). Since the two soy sauces analysed in this study were also potentially oestrogenic (I), it was concluded that the soy-based components are likely to be at least a contributing factor for the oestrogenic activity in these food products (pepper salami, chicken and beef hamburger). The calculated oestradiol and genistein equivalents of the soy-containing products investigated present pepper salami as the most oestrogenic of the three, with its oestradiol equivalents ranging from 1.6 to 443 pg/g. Although no information is available on the oestrogenic activity of pepper salami, its oestradiol equivalent recorded here is almost of the same magnitude (150—1544 ng/kg original product) obtained for related products (Behr et al., 2011). Soy-free products were also reported to be non-oestrogenic in their test system. As for soy-based hamburgers, the oestradiol equivalent levels obtained here are similar to (although slightly lower than) those found by Behr et al. (2011).

In Nigeria, drinking water is mainly sold in small polyethylene bags and is usually referred to by the populace as sachet-pure water. To the best of my knowledge, this is the first study to investigate the possible oestrogenic activity of sachet-packed drinking water in Nigeria. The
microbiological and physiochemical quality of sachet-packed water in Nigeria has been studied quite extensively (Orisakwe et al., 2006; Olaoye and Onilude, 2009; Oyedeji et al., 2010; Edema et al., 2011; Adebayo et al., 2012; Muazu et al., 2012; Omalu et al., 2012; Onilude et al., 2013). All of these studies reported that the majority of sachet-packed pure waters sold in Nigeria were heavily tainted by pathogens and, therefore, unsafe for consumption. A similar situation has also been reported in sachet waters from Ghana (Addo et al., 2009; Ackah et al., 2012). Two studies from both countries particularly reported high levels of lead, far above the recommended limit (Orisakwe et al., 2006; Ackah et al., 2012).

The outcome of this study shows that sachet-packed pure water may also contain oestrogen-like chemicals. A total of 31% of such water samples investigated were oestrogenic, with EEQs ranging from 0.79 to 44.0 ng/l. However, both the frequency of positive samples and their concentrations were actually lower than was feared. In recent studies carried out in Europe, using a comparable in vitro yeast assay, Pinto and Reali (2009) analysed mineral waters packed in polyethylene terephthalate (PET) bottles in Italy. The levels they detected varied from 0.03 to 23.1 ng/l (mode 9.5 ng/l) EEQs. Somewhat surprisingly, tap water comprising either surface water or groundwater contained approximately 15 ng/l EEQ. In another study, Wagner and Oehlmann (2009) determined the oestrogenic activities in 20 major brands of bottled water in Germany. Twelve of these samples proved positive, with the levels ranging from 2.64 to 75.2 ng EEQ/l (mean 18.0 ng/l). More recently, Real et al. (2015) reported that 79.3% of the bottled water (including water in plastic and glass) in southern Spain was oestrogenic (mean: 0.113 ± 0.07 pmol/l oestradiol-17β equivalent E2EQ). Thus, substances exhibiting oestrogen-like activity are common in water samples in both the industrialized and developing countries. Despite the above information, water samples taken from drinking-water sources (tap water, bottled still and mineral water) and drinking-water plants (treated and untreated) in Finland again showed no notable oestrogenic activity. This outcome is similar to three recent findings in South America, Europe and Asia. Using a similar in
vitro assay and by complimentary chemical analysis, no oestrogenic activity was reported in tap water in Brazil (Bergamasco et al., 2011). Similarly, Maggioni et al. (2013) observed no oestrogenic activity in five different brands of PET-bottled mineral water in Italy. In contrast, Zheng et al. (2013) reported oestrogenic activity in tap water collected over a 1-year period in China, ranging from 35.2 to 1511 pg/l EEQ. The negative outcome of the Finnish study stems from legislation, favourable administration, extensive research and follow-up approaches aimed at sustainable use of clean water resources (Ministry of Agriculture and Forestry, Finland, 2009). In 2002, Finnish drinking-water quality was ranked by the United Nations as the best in the world (World Water Council, 2003), which is in line with the current findings.

The contamination of drinking water by oestrogenic chemicals is multifaceted. These chemicals may arise from plastic or glass bottles, bottle caps, transport pipelines, disinfection agents, the bottling process itself or from environmental pollution of water sources (Real et al., 2015). A recent study in the southeastern USA showed that drinking-water sources may be contaminated by EDCs, with average total concentrations of pharmaceutical and personal care products and EDCs averaging 360 and 98 ng/l in source water and drinking water, respectively (Padhye et al., 2014). A similar situation was also reported in China (Hu et al., 2013). Exposure of PET-bottled water to sunlight and high temperature (60 °C) in the presence of carbon dioxide increases the migration of substances (formaldehyde, acetaldehyde and antimony) from PET bottles into drinking water (Bach et al., 2014, 2013). Antimony was previously reported in water from PET bottles (Shotyk et al., 2006) and is said to be oestrogenic in vitro (Sax, 2010).

6.5 Oestrogenic activity of food packaging material

Food packaging materials are a putative and controversial source of human exposure to xenoestrogens globally (Brotons et al., 1995; Stroheker et al., 2003; ter Veld et al., 2006). The
oestrogenic activity of packaging materials results from the leaching of plasticizers used in FCMs. Several of these plasticizers, such as a tris(2-ethylhexyl)trimellitate and benzoate mixture, are oestrogenic (ter Veld et al., 2006). However, in the material consisting of commercially processed and packaged food items in Finland, no evidence of this was obtained (I), except for sachet materials used in packaging pure water in Nigeria (III). The latter finding suggests the presence of oestrogenic compounds in the packaging material. These compounds usually leach under normal use conditions (ter Veld et al., 2006; Vandenberg et al., 2007; Le et al., 2008).

6.6 Oestrogenic activity of wastewater samples

The concentration rates found in influent samples from the Viikinmäki WWTP were lower than those reported elsewhere. Using a similar in vitro bioreporter assay, influent wastewater samples showed oestrogenic activity ranging from below the detection limit to 25 ng/l EEQ in France (Bellet et al. 2012), while a recent report revealed strikingly high levels [1136 ± 269 ng/l EEQ] in China (Zhao et al., 2015). In the latter study, the value increased (1417 ± 320 ng/l EEQ) after primary treatment. In Spain, Germany and China, the concentrations measured by MS were also far higher than those determined in the present study (Petrovic et al., 2002; Andersen et al., 2003; Zhou et al., 2012). Varying levels of EDCs and oestrogenicity have also been reported in effluent samples globally (Petrovic et al., 2002; Andersen et al., 2003; Smit et al., 2011; Ahn et al., 2012; Bazin et al., 2012; Jarosova et al., 2012; Schiliro et al., 2012; Ferguson et al., 2013). However, this is in contrast to the case in Finland. The oestrogenic outcome of the effluent samples reported here is in accordance with a recent study from 16 European countries, including Finland, in which effluent samples were screened for possible oestrogenic activity (Jarosova et al., 2014). This implies that the treatment method (activated sludge with mechanical, chemical and biological purification) currently employed in Helsinki is effective in removing oestrogenic compounds from
wastewater during treatment. Activated sludge and/or an upflow anaerobic sludge blanket reactor, followed by chlorination steps, were also effective in removing EDCs from wastewater (Rahman et al., 2009; Pessoa et al., 2014).

In Nigeria, there are no working sewage treatment plants (Daily Trust, 2014). Industrial and domestic wastewater collections are decentralized, with each individual building having its respective septic tank for the collection of its waste. When such septic tanks are emptied, the contents are often discharged untreated into bodies of water. These bodies of water serve as water supplies for drinking and irrigation purposes. In addition to untreated waste discharged into bodies of water, high levels of rainfall, agricultural runoff and increased use of pesticides in Nigeria further increase exposure to toxic chemicals from bodies of water. Studies have shown varying concentrations of insecticides and herbicides in surface and river waters (Behfar et al., 2013; Scott et al., 2014; Stone et al., 2014; Agbohessi et al., 2015; Rose et al., 2015), which in Nigeria are crude drinking-water sources and are also used for fishing. In particular, fish from such polluted bodies of water were recently reported to contain high levels of pesticides (Agbohessi et al., 2015; Rose et al., 2015), some of which are known to have oestrogenic potential (Agbohessi et al., 2015). This implies that rivers in Nigeria could be a potential reason for concern of exposure to both pathogenic organisms and oestrogenic chemicals.
7.0 Conclusions

We arrived at the following conclusions from the current studies:

1. Commercially processed foods are potential sources of human exposure to genotoxic chemicals. These chemicals are often difficult to regulate/control, because they are formed in food as a result of food processing. However, appreciable progress has been made in Finland towards reducing the levels of these contaminants in commercial foodstuffs.

2. In Nigeria, much still needs to be done, since the majority of food items (chin-chin, hamburger, suya and bean cake) investigated were proven to be mutagenic. The dissimilar mutagenic outcome in the two countries may largely be due to differences in processing techniques.

3. Drinking-water sources (tap water, bottled still and mineral waters) and water from drinking-water treatment plants in Finland are not sources of concern, with respect to their oestrogenic potentials.

4. Meanwhile, sachet-pure water samples from Nigeria, as well as packaging materials, could pose grave problems for consumers, because 31% of the samples were oestrogenic, of which 40% were attributed to FCMs.

5. A 2-year study of both influent and effluent wastewater samples from Viikinmäki WWTP in Finland showed that the treatment process (activated sludge coupled with mechanical, chemical and biological purification) used in the treatment of wastewater is effective in removing oestrogenic chemicals. In Nigeria, there are no centralized WWTPs. This may impair proper waste treatment and also increases exposure to EDCs.
8.0 References


Commercial processed food may have endocrine-disrupting potential: soy-based ingredients making the difference

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Commercial processed food may have endocrine-disrupting potential: soy-based ingredients making the difference

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Processed and packaged food items as well as ready-to-eat snacks are neglected and poorly characterised sources of human exposure to endocrine-disrupting chemicals (EDCs). In this study we investigated the presence of xenoestrogens in commercially processed and packaged Finnish foods, arising from substances deliberately added or inadvertently contaminating the food, substances formed as a result of food processing, or substances leaching from food packaging materials. Samples were obtained in three separate batches of equivalent products from both a supermarket and a local representative of a global chain of hamburger restaurants and extracted by a solid-phase extraction method. Their endocrine-disrupting potential was determined by yeast bioluminescent assay, using two recombinant yeast strains Saccharomyces cerevisiae BMAERElu/ERa and S. cerevisiae BMA64/luC. In this test system, the majority of samples (both foodstuffs and wrappers) analysed proved negative. However, all batches of industrially prepared hamburgers (but not those obtained from a hamburger restaurant) as well as pepper salami significantly induced luciferase activity in the BMAERElu/ERa yeast strain indicating the presence of xenoestrogens, with estradiol equivalents of these products ranging from 0.2 to 443 pg g⁻¹.

All three products contained soy-based ingredients, which apparently accounted for, or at least contributed to, their high estrogenic activity, since no signal in the assay was observed with extracts of the packaging material, while two different soy sauces tested yielded an intense signal (28 and 54 pg ml⁻¹ estradiol-equivalent). These findings imply that by and large chemicals arising in the processing or packaging of foodstuffs in Finland constitute an insignificant source of xenoestrogens to consumers. However, soy-derived ingredients in certain food items might render the entire products highly estrogenic. The estrogenic activity of soy is attributed to isoflavones whose health effects – though widely considered beneficial – are controversial. As hamburgers are a popular type of food among children, the findings are noteworthy and possibly of concern.

Keywords: endocrine-disrupting compounds; processed food; ready-to-eat snacks; soy; xenoestrogens

Introduction

Certain compounds known to interfere adversely with the human endocrine system have been reported in the immediate environment, especially in natural water bodies (Eggen et al. 2003; Sumpter 2005). Such substances are usually referred to as endocrine-disrupting chemicals (EDCs). EDCs that mimic the activity of estrogen within the human organism are ubiquitous, and have gained notoriety due to their association with certain reproductive disorders, developmental abnormalities and other adverse physiological effects in both humans and wildlife (Fisher 2004; Bourguignon & Parent 2010; Zama & Uzumcu 2010). Incidentally, estrogen mimics have also been reported among chemicals used for processing and preserving food (Sinha et al. 2009; Connolly et al. 2011; Zhang, Jia et al. 2012) as well as in soy-based food products (Takamura-Enya et al. 2003; Behr et al. 2011). Food packaging materials are also reported sources of human exposure to EDCs (Brotons et al. 1995; Stroheker et al. 2003; ter-Veld et al. 2006). Known EDCs from food packaging materials include bisphenol A, nonylphenol and several phthalates. Similarly, bottled mineral and flavoured waters are also sources of exposure to EDCs (Plotan et al. 2012).

The overall presence of EDCs in the human diet is worrisome due to an increase in certain cancer types (breast, stomach, colon), especially in industrialised countries, and the associated linkage between cancer and EDC exposure (Fisher 2004; Meeker 2010; Walvoord 2010). In Finland, for example, cancers of the oesophagus, stomach and colon appear to be on the increase (Weiderpass & Pukkala 2006). From the point of view of risk assessment, EDCs present a particular challenge because they may have non-monotonic dose–response curves not adequately covered by conventional toxicological experimentation, and because of their capability of causing untoward impacts at environmentally prevailing low concentrations (Fagin 2012).

Despite the global concern and awareness of human exposure to EDCs, processed food items and ready-to-eat snacks are yet a poorly delineated source of such...
exposure. This is evidenced by the limited number of studies done on the presence of EDCs in processed foods. Moreover, such studies are totally lacking in Finland. Hence, the present work sought to investigate the abundance of xenoestrogens in commercially processed Finnish foods (mainly meat and fish products), arising from substances deliberately added to or inadvertently contaminating the food, substances formed as a result of food processing, or substances leaching from food packaging materials.

Materials and methods

Chemicals and medium

Estradiol, progesterone, testosterone and bisphenol A were purchased from Sigma-Aldrich (Steinheim, Germany). D-luciferin was obtained from Biotherma (Handen, Sweden). Yeast nitrogen base medium without amino acids was obtained from Becton Dickinson (Franklin Lanes, NJ, USA) while genistein was obtained from LC Laboratories (Woburn, MA, USA).

Microorganisms

Two recombinant yeast strains Saccharomyces cerevisiae BMAEREluc/ERα and S. cerevisiae BMA64/luc (Leskinen et al. 2005) were used. BMAEREluc/ERα served as a reporter strain, in which the ERα is expressed. Upon ligand binding, the dimerised receptor binds the estrogen response elements in the promoter region of the luc reporter gene. In BMA64/luc, luciferase is expressed constitutively, and this strain was used for the determination of cytotoxicity of the samples. Both yeast strains were kindly provided by Johanna Rajasärkkä of the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki. Yeasts were grown on Difco Yeast Nitrogen Base medium without amino acids, supplemented with glucose and the amino acids alanine, histidine and leucine.

Samples

Forty-five samples (three lots of 15 different products) of industrially processed and packaged food products and 15 samples of ready-to-eat snacks (three lots of five different snack varieties) were evaluated for their possible estrogenic activities. The industrially processed and packaged food products were purchased from a popular supermarket in Helsinki (Prisma, Viikki), while the ready-to-eat snacks were acquired from a local representative (Sokos City Centre) of a global chain of hamburger restaurants (McDonald’s) also located in Helsinki. The sample types and manufacturers are listed in Table 1.

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Smoked chicken</td>
<td>Company A</td>
</tr>
<tr>
<td>Honey roasted chicken</td>
<td>Company B</td>
</tr>
<tr>
<td>Grilled chicken</td>
<td>Company C</td>
</tr>
<tr>
<td>Grilled turkey</td>
<td>Company B</td>
</tr>
<tr>
<td>Smoked fish</td>
<td>Company D</td>
</tr>
<tr>
<td>Cold-smoked fish</td>
<td>Company E</td>
</tr>
<tr>
<td>Fried fish</td>
<td>Company E</td>
</tr>
<tr>
<td>Cold-smoked beef</td>
<td>Company B</td>
</tr>
<tr>
<td>Grilled beef</td>
<td>Company C</td>
</tr>
<tr>
<td>Pepper salami</td>
<td>Company F</td>
</tr>
<tr>
<td>Sausage</td>
<td>Company G</td>
</tr>
<tr>
<td>French fries</td>
<td>Company G</td>
</tr>
<tr>
<td>Mashed potatoes</td>
<td>Company G</td>
</tr>
<tr>
<td>Hamburger (beef)</td>
<td>Company H</td>
</tr>
<tr>
<td>Hamburger (chicken)</td>
<td>Company A</td>
</tr>
</tbody>
</table>

We carefully ensured that all products were extracted before the expiry date shown on the packages.

Extraction of food samples

Possible estrogenic compounds were extracted from food samples using the method described by Peters et al. (2004) with slight modifications. Briefly, 20 g of food sample were homogenised with 80 ml of 1 M NaOH at 24,000 rpm for approximately 10 min. The homogenate was mixed with 20 g of Extrelut refill material (VWR international, Helsinki, Finland) and then poured into an empty Extrelut 20 column. The organics were eluted from the Extrelut column with 40 ml of dichloromethane/toluene solution (95:5 v/v) into a cartridge. The organics were finally eluted with 5 ml of MeOH-NH₄OH (9:1) solution and evaporated to dryness under a gentle stream of nitrogen. For determination of recovery, fresh meat was spiked with 250 ng of estradiol and extracted in the same way as above. The percentage recovery was approximately 78%.

Repackaging and extraction of packaging materials

The samples found to be estrogenic were unwrapped and their packaging materials reused for negative samples to disclose possible leaching of xenoestrogens. The repackaging was done in such a way that there was a close contact between the food sample and packaging material. The repackaged food samples were kept in an oven at 80°C for 30 min and then at 4°C for 7 days before extraction to maximise the leaching effect without spoiling the foodstuffs. For extraction, these packaging materials were embedded in methanol in a
mechanical shaker at 60°C overnight. This is a slight modification of a previous method used with success to extract estrogenic compounds from food samples (Behr et al. 2011).

**Bioassay**

The yeast bioluminescent assay was performed as previously described (Leskinen et al. 2005; Rajasärkkä & Virta 2011). The optimised parameters for 384-well plates used in this study have also been reported (Rajasärkkä & Virta 2011), except that 5% ethanol was used as the vehicle because the 10% DMSO reported by Rajasärkkä and Virta (2011) was cytotoxic in the test system. Estradiol, genistein and bisphenol A were used as positive controls, while progesterone and testosterone served as negative controls.

**Data analysis**

The fold induction, fold induction corrected (FIC) and LOD were calculated as described previously (Leskinen et al. 2005). The sigmoidal dose–response curves for increasing concentrations of estradiol, genistein and bisphenol A were obtained using the software program Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). The estradiol and genistein-equivalents of food samples showing estrogenic activity were calculated from probit transformation of the curves.

**Results**

With *S. cerevisiae* BMAEREucl/ERα, all positive controls (estradiol, genistein and bisphenol A) produced sigmoidal dose–response curves (Figure 1). In this test system, estradiol proved to be 10³–10⁵-fold as potent as bisphenol A and genistein. This is in agreement with data gathered from other assays (Leskinen et al. 2005; ter Veld et al. 2006; Rajasärkkä & Virta 2011). Meanwhile, there was no luciferase activity detectable in the BMAEREucl/ERα yeast strain when it was treated with increasing concentrations of either progesterone or testosterone (data not shown). The LOD in the estrogenic assay was 2.4 FIC and corresponded to 76 fM, 1.8 pM and 1.2 nM of estradiol, genistein and bisphenol A, respectively.

The majority of food samples analysed failed to exhibit detectable estrogenic activity. However, extracts of industrially processed and packaged hamburgers (beef and chicken) and of pepper salami were consistently estrogenic in all their batches examined (Table 2). Pepper salami showed the most potent activities, with its estradiol-equivalent varying between 1.6 and 443 pg g⁻¹. Extracts of chicken hamburger were the least estrogenic of the positive samples (0.2–0.6 pg g⁻¹), while the estradiol-equivalent of beef hamburger fell between the two. An important observation was the wide variation in the estrogenic activities among different batches of the same products amounting to almost 1000-fold for pepper salami. Meanwhile, equivalent burger products obtained from a hamburger restaurant did not show any estrogenic activity in the test system.

Samples previously found to be non-estrogenic in the assay where repackaged in the packaging materials of positive estrogenic samples of equivalent products to ascertain if the estrogenic activities in these foodstuffs actually originated from the wrappers. To complement this approach, the estrogenic activities of the wrappers of all positive samples were also directly determined. Both repackaged food samples and the wrappers exhibited no estrogenic activity in the test system. When it turned out that all the positive food samples contained a soy-based ingredient (Table 3) while none of the negative samples purchased from the supermarket harboured it, two different brands of soy sauces were examined to verify soy estrogenicity in this assay system. They both generated an intense signal (Table 4).

The recombinant yeast strain, *S. cerevisiae* BMA64/luc, produced an intense signal with all samples in the assay conducted, indicating the viability of the cells. This implies that neither the vehicle (5% ethanol) used in the study nor the test samples were cytotoxic to the cells.

**Discussion**

The present study investigated the presence of xenoestrogens in commercially processed Finnish foods (mainly meat and fish products), arising from substances deliberately added to or inadvertently contaminating the food, substances formed as a result of food processing, or substances leaching from food packaging materials. The
results show that, overall, food packaging materials are not likely to be a notable source of xenoestrogens in commercially processed Finnish foods. Also, we observed that no significant estrogenic activity may have emerged as a result of processing. However, three different varieties of foodstuffs tested positive in this assay. Interestingly, they all shared one property: a soy-based ingredient. As the two soy sauces analysed also proved potently estrogenic, the soy-based components are likely to be at least a contributing factor for the estrogenic activity in these food products (pepper salami, chicken and beef hamburger) obtained from a supermarket. The calculated estradiol and genistein-equivalents of the soy-containing products investigated present pepper salami as the most estrogenic of the three, with its estradiol-equivalents ranging from 1.6 to 443 pg g$^{-1}$. The estrogenic activity of soy as well as of soy-based products has been reported previously (Takamura-Enya et al. 2003; Behr et al. 2011), and stems from its isoflavones (mainly genistein) which are also considered antioxidants. At present, controversy remains as to the health impacts of isoflavones. While epidemiological studies have shown that soy and soy foods may protect against breast cancer and cardiovascular diseases (Pilsakova et al. 2010; Zhang, Kang et al. 2012), experimental studies have suggested that soy isoflavones could actually enhance the proliferation or metastasis of some types of cancer (Helferich et al. 2008; Martínez-Montemayer et al. 2010; de la Parra et al. 2012). Moreover, soy-based isoflavones have been reported to impair fertility in female mice by neonatal exposure (Nagao et al. 2001; Jefferson et al. 2005), and enhance vaginal cell maturation in female human infants (Bernbaum et al. 2008). In male marmoset monkeys, feeding of infants with soy formula milk for 1.0–1.5 months decreased serum testosterone by 50–70% compared with co-twin counterparts fed on cow’s formula milk (Sharpe et al. 2002).

Estradiol and several other sex hormones are widely used as growth enhancers in cattle, especially in the United States, Australia and Canada (Preston 1999). Because estradiol is thousands of times more potent than estrogenic pesticides and other industrial food contaminants, its use as an implant in animals has largely been condemned. Studies have shown that estradiol may pose a risk of serious negative effects at very low concentrations, especially in infants and young children (Saenz de Rodriguez et al. 1985; Andersson & Skakkebaek 1999). This reinforces the view that the estradiol equivalence levels found in this study may be of concern. The USFDA has established a maximum safe tissue level of estradiol in muscle, liver, kidney and animal fat at 0.12,
0.48, 0.36 and 0.24 μg kg⁻¹, respectively (Doyle 2000). However, two dissenting reviews (Andersson & Skakkebaek 1999; SCVMRPH 1999) have expressed concern about the safety of such concentrations. Although the estradiol-equivalent concentrations detected in the present study were mostly (except for a single batch of pepper salami) lower than the residual limits set by the USFDA, they suggest that for people favouring soy-based food products in their nutrition the exposure to compounds with estrogenic activity may be high.

In contrast to the hamburgers purchased from a supermarket, the equivalent products (cheese, beef and chicken hamburgers) obtained from a hamburger restaurant proved, however, not to be estrogenic with the BMAEREEluc/ERα yeast strain. Surprisingly, both cheese burgers and beef hamburgers obtained from the restaurant were informed to contain soy. A possible explanation for the divergent outcome with soy-based burger products obtained from a supermarket and a restaurant is a different concentration of the soy ingredients (this was not revealed in the product labels). The soy content might be so low in the restaurant burgers that the estrogenic activity fell below the detection limit. In addition to the soy-based ingredient, pepper salami (which exhibited the highest estrogenic activity of all) contained an unidentified antioxidant (Table 4). This may further have contributed to the outcome, since some antioxidants (propyl gallate, propandiolphosphite and butylated hydroxyanisole) have been reported to be estrogenic in both ERα and ERβ reporter gene cell lines (ter Veld et al. 2006). In agreement with this, butylated hydroxytoluene and hydroxyanisole as well as propyl gallate (compounds extensively used as antioxidants) were recently demonstrated to be estrogenic in Wistar prepubescent rats (Pop et al. 2013).

The findings of the present study are in keeping with previously published data on similar food products. Although no information exists on the estrogenic activity of pepper salami, its estradiol-equivalent recorded here is almost of the same magnitude (150–1544 ng kg⁻¹ original product) obtained for related products (Behr et al. 2011). Soy-free products were also reported to be non-estrogenic in their test system. As to soy-based hamburgers, the estradiol-equivalent levels obtained here are similar to (although slightly lower than) those found by Behr et al. (2011).

Food packaging materials are a putative and controversial source of human exposure to xenoestrogens (Brotons et al. 1995; Strohkeer et al. 2003; ter Veld et al. 2006). The estrogenic activity of packaging materials might result from leaching of plasticisers used in softening the wrappers. Several of these plasticisers such as tris(2-ethylhexyl)trimellitate and benzoate mixture have been reported to be estrogenic (ter Veld et al. 2006). However, in our material consisting of commercially processed and packaged Finnish foods, no evidence of this was obtained.

Food packaging materials of estrogenic samples as well as negative food items repackaged in wrappers of positive samples were not estrogenic in the test system.

In conclusion, the findings imply that, by and large, chemicals arising in the processing or packaging of food-stuffs in Finland constitute an insignificant source of xenoestrogens to consumers. However, soy-derived ingredients and antioxidants in certain food items may render the entire products highly estrogenic. The estrogenic activity of soy is attributed to isoflavones whose health effects – though widely considered beneficial – are controversial. As hamburgers are a popular type of food among children, the findings are noteworthy and possibly of concern.

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1. Introduction

Food processing is the set of methods and techniques used to transform raw ingredients into food or to modify food properties for consumption by humans or animals. There has been a growing demand for produced foods globally due to changes in the lifestyle of the world population (Akintowa et al., 2007). The majority of these foods is processed and preserved using methods (physical and chemical) that have the potential to trigger formation of mutagenic, genotoxic and carcinogenic substances. It has been estimated that 1–2 g of potentially mutagenic substances are consumed by humans every day from food and beverages alone (Ames & Gold, 1990). Previous epidemiological studies have substantially furthered our understanding of the links between diet and genetic toxicity, and cancer (Akintowa et al., 2007; Francois et al., 2010; Sharif, Ghazali, Rajab, Haron, & Osman, 2008; Sinha et al., 2009). These studies have revealed that diet is a key contributor to human cancer with approximately 32% of cancers estimated as being attributable to dietary factors as a whole (Willett, 1998), although the contribution of dietary genotoxic substances is apparently far less prominent (World Cancer Research Fund/American Institute for Cancer Research, 2007).

According to a recent comprehensive analysis of the available literature data, the evidence is convincing for consumption of processed meat as a cause of increased risk of colorectal cancer; in the case of cancers of the oesophagus, stomach and lung, the causal relationship is suggestive (World Cancer Research Fund/American Institute for Cancer Research, 2007). High temperature cooking methods such as pan-frying and grilling/barbecuing produce compounds such as heterocyclic amines and polycyclic aromatic hydrocarbons (PAHs), which are well-known animal carcinogens (Ferguson, 2010). Highly carcinogenic N-nitroso compounds are also known to be formed during frying of nitrite-treated bacon and meat (Cross & Sinha, 2004), although humans are mainly exposed to N-nitroso compounds via endogenous synthesis in the stomach (Lutz, 1990).

Acrylamide, a compound that has gained considerable attention in recent years due to its high toxicity (Erkekoglu & Baydar, 2010), foremost its carcinogenicity (Hogervorst et al., 2010), and common occurrence in, for example, a variety of snacks, has been shown to form in carbohydrate-rich foods as a result of heat processing (Hogervorst et al., 2010). In the daily diet of the Swedes and the Dutch, product groups including potato crisps, French fries, coffee, bread, biscuits and breakfast cereals contribute more than 90% to the total intake of acrylamide (Konings et al., 2003; Svensson et al., 2003). This has also been reported for Finnish foodstuffs (Eerola, Hollebekkers, Hallikainen, & Peltonen, 2007). On the other hand, heterocyclic aromatic amines are often present in hamburgers (Knize et al., 1998), and commercially sold hamburgers have been reported to possess variable levels of mutagenic activity (Gabbani et al., 1998; Stavric, Matula, Klassen, & Downie, 1995). There are also reports of mutagenic activity in urine of subjects who have consumed foodstuffs processed at a high temperature (Gabbani et al., 1998; Peters et al., 2004).
The mutagenic potential of commercially processed food products depends on a number of factors related to cooking conditions, such as the equipment used, ingredients, temperature and cooking time, as attested by considerable differences in the mutagenic activity among equivalent products from different manufacturers or restaurants (Knize, Dolbear, Carroll, Moore, & Felton, 1994; Peters et al., 2004; Tikkanen, 1991; Tikkanen, Sauri, & Latva-Kala, 1993). For example, the Ames/Salmonella test shows a correlation between meat-processing temperature and the number of revertant colonies per gram of meat (Peters et al., 2004; Tikkanen, 1991). Also, industrial processing of food has a marked effect on the mutagenic activity of the final product, based on the variations recorded in equivalent products from various manufacturers (Tikkanen et al., 1993).

The presence of mutagenic compounds in commercially heat-processed foods such as meat, fish and poultry products was observed in Finland over twenty years ago (Tikkanen, 1991). Thereafter, a number of toxic compounds have been extracted at varying concentrations from such products in Finland (Eerola et al., 2007; Tikkanen et al., 1993). However, since the methods of preparing these products have probably undergone marked changes over the years, screening of the overall mutagenic potential of commercially produced foodstuffs is warranted to ensure that they do not represent a genotoxic hazard to consumers. To this end, the present study set out to use the Ames test together with complementary assays to investigate the mutagenicity of processed and preserved foodstuffs as well as some ready-to-eat snacks in Finland.

2. Materials and methods

2.1. Materials

All materials used in this study were of analytical grade. The NADP and glucose-6-phosphate used were obtained from Roche Biochem (Stockholm, Sweden). Aroclor-induced S9 from rat liver was purchased from Merck AG (Darmstadt, Germany). Salmonella enterica sv. typhimurium strains TA 100 and TA 98 were obtained from Pasteur's Institute (Paris Cedex, France). Histidine, potassium chloride, magnesium sulphate, potassium phosphate dibasic anhydrous and sodium ammonium phosphate were obtained from Pasteur's Institute (Paris Cedex, France). Histidine, potassium chloride, magnesium sulphate, potassium phosphate dibasic anhydrous and sodium ammonium phosphate were purchased from Merck AG (Darmstadt, Germany). Magnesium chloride hexahydrate and citric acid monohydrate were acquired from VWR international (Leuven, Belgium). Biotin, tryptophan, methylcellulose (MC), dimethyl sulfoxide, benz(a)pyrene, 2-aminoanthracene and sodium azide were obtained from Sigma–Aldrich (Steinheim, Germany).

2.2. Cell line

Human hepatocellular carcinoma-derived cell line (HepG2) was obtained from American Type Culture Collection through LGC standards (Boras, Sweden) and cultured in Eagle's Minimum Essential Medium (LGC standards, Boras, Sweden) containing 10% heat-inactivated fetal bovine serum (Sigma–Aldrich, Steinheim, Germany). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air atmosphere incubator (NuAire Inc., Plymouth, USA).

2.3. Sampling

Forty-five samples of industrially processed and packaged food products and 15 samples of ready-to-eat snacks were evaluated for their mutagenic potential. The industrially processed and packaged food products were purchased from a popular supermarket in Helsinki (Prisma, Viikki), while the ready-to-eat food samples were acquired from a local representative (at SOKOS City Centre) of a global chain of hamburger restaurants also located in Helsinki, Finland. The sample types and manufacturers are listed in Supplementary Table 1. A total of three batches were collected at separate times. The cooking conditions such as time and temperature were not available. We carefully ensured that all products were extracted before the expiry date shown on the packages.

2.4. Extraction

Possible mutagenic compounds were extracted from the food samples using the method described by Peters et al. (2004) with slight modifications. Briefly, 20 g of food sample was homogenized with 80 ml of 1 mol/l NaOH at 24,000 rpm. The homogenate was mixed with 20 g of Extrelut refill material (VWR international, Helsinki, Finland) and then poured into an empty Extrelut 20 column. The organics were eluted from the Extrelut column with 40 ml of dichloromethane/toluene solution (95:5 v/v) into a cartridge. The organics were finally eluted with 5 ml of MeOH–NH₄OH (9:1) solution and evaporated to dryness under a gentle stream of nitrogen in a fume hood. For determination of recovery, fresh meat was spiked with 50 μl of benzo(a)pyrene and 250 ng of 2-aminoanthracene in two different cases and extracted in the same way as above.

2.5. Cytotoxicity assays

The cytotoxic effect of the concentrations of food extracts used in this study was investigated by three independent assays measuring trypan blue exclusion, lactate dehydrogenase activity, and boar sperm motility.

2.5.1. Trypan blue test

HepG2 cells were grown in 24-well plates (VWR, Finland) until semi-confluent cells were obtained (48 h). This was followed by exposure of the cells to different concentrations of food extracts for 4, 24 or 48 h. After exposure, the cells were trypsinised using 0.25% (w/v) trypsin–0.53 mmol/l EDTA solution (LGC standards, Boras, Sweden). Trypsinised cells were transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 2,500 rpm. Pellets were then re-suspended in PBS, after which 10 μl of the cells were mixed with 5 μl (0.8 mmol/l) trypan blue dye before microscopic observation.

2.5.2. Lactate dehydrogenase (LDH) assay

The activity of lactate dehydrogenase (LDH) was determined in HepG2 cells exposed to the same concentrations of the food extracts used in genotoxicity assays. The LDH test measures plasma membrane integrity and it was performed according to the instructions provided in the Cytotoxicity Detection KitPLUS (LDH); version 6 (Roche Biochem, Stockholm, Sweden).

2.5.3. Boar sperm motility inhibition bioassay

Extracts of selected food samples were assessed for their mitochondrial toxicity using the boar sperm motility assay (Andersson et al., 2010). Briefly, 2 ml of boar semen in screw-capped exposure vials was exposed to 10 μl of food extracts for 30 min, 24 or 48 h at 20 °C. Vehicle (DMSO) exposure was prepared simultaneously with the test samples for each time point. After exposure, the vials were shaken gently to disperse the sperm cells, and 200 μl of the suspension was drawn into a warmed test tube and placed in a heating block (30 °C) for approximately 5 min to activate sperm motility. Sperm motility was assessed by dispensing warmed sperm suspension onto a microscopic slide using a pre-warmed capillary tube, and immediately observed with a 40× inverted phase contrast objective.
2.6. Mutagenicity assay

The mutagenic potential of food extracts was determined initially by the standard plate incorporation assay. Samples showing mutagenic potential in this assay were subjected to treat-and-wash as well as methylcellulose overlay assays to ascertain to what degree a localized release of proteins, peptides or histidine from the samples contributed to the outcome.

2.6.1. Standard plate incorporation assay

The standard plate incorporation assay was performed as described by Maron and Ames (1983) using Salmonella strains TA 100 and TA 98 with and without metabolic activation (59 mix). The amount of 59 used in the 59 mix was 10%. Water and DMSO were used as negative controls for both strains while sodium azide (0.04 mg/ml) and 2-aminoanthracene (0.02 mg/ml) served as positive controls for TA 100 and TA 98, respectively. Benzo(a)pyrene (0.1 mg/ml) was also used as a positive control for both strains. The volume of controls used was 50 µl/plate in triplicate plates. Sodium azide is a known direct mutagen in Salmonella TA 100 (Mortelmans & Zeiger, 2000), whereas 2-aminoanthracene is metabolically activated by mono-oxygenases of the CYP1A family in rat liver (Carrière, de Waziers, Courtois, Leroux, & Beaune, 1992). Likewise, benzo(a)pyrene requires metabolic activation for mutagenicity (Gabbani et al., 1998).

For all samples, four different concentrations of the food extracts (25, 50, 100 and 200 mg/ml) were tested in triplicate plates (50 µl/plate). The highest concentration (200 mg/ml) was equivalent to 1 g of the food sample. The plates were incubated at 37 °C for 48 h.

The results of the mutagenic activities are presented as the number of revertant colonies per gram of food sample. Only the mean and standard deviation of the highest concentration for all food extracts, and the dose–response curve for extracts showing mutagenic activity are shown.

2.6.2. Treat-and-wash assay

The treat-and-wash assay was conducted according to the method described by Thompson, Morley, Kirkland, and Proudlock (2005). The protocol applied was as per the standard plate incorporation assay with the exception that the 59 mix, bacteria and sample extract were incubated for 90 min prior to the addition of the residual supernatant prior to plating via top agar. The mixture was overlaid on a pre-warmed (37 °C) bacterial culture and sample extract solution in a sterile 15 ml broth in phosphate-buffered saline [1:7 v/v].

The mixture was incubated at 37 °C for 1 h after plating to ensure gelling of the MC overlay, and subsequently incubated (not inverted) at 37 °C for 48–72 h. The MC overlay was prepared on the day of the test (Thompson et al., 2005), and the mixture was stirred at 50–60 °C throughout use.

2.7. Comet Assay

The Comet Assay (single-cell gel electrophoresis) was used to evaluate possible breakage of single- or double-stranded DNA in HepG2 cells following treatment with extracts of food samples showing mutagenic potential. The Comet Assay was performed in alkaline conditions (pH > 13) as described previously (Singh et al., 1988). Hydrogen peroxide and 0.7% DMSO served as positive and negative controls, respectively.

2.8. Statistical analysis/interpretation of data

The mutagenic potency of each food sample was determined from the linear slope of the dose-response curve by linear regression analysis using Prisma 4.0 (GraphPad software Inc. San Diego, CA). In addition to a statistically significant (p < 0.05) dose–response effect, samples were only considered mutagenic where the highest test concentration generated at least twice as many revertants as the negative control (DMSO). For proper interpretation and clarity, the number of revertants obtained was compared with both experiment-specific controls and aggregate controls across all experiments. The p values of these comparisons presented in Tables, Figs. and Supplementary Tables are derived from the regression analyses.

3. Results

3.1. Plate incorporation assay: Control substances

In the conventional Ames test, the negative control substances used elicited 5–6 times as many colonies in the TA 100 strain as in the TA 98 strain (Table 1). In the presence of 59 mix, benzo[a]pyrene elevated colony number from this level to 2.5- (TA 100) or 4-fold (TA 98) higher. In TA 100, benzo[a]pyrene caused an almost 2-fold increase in the absence of 59, which did not occur in TA 98. Also, sodium azide instigated a larger increase in the colony number with 59 (7-fold) than without (4-fold), despite being the recommended direct control mutagen for TA 100 (Mortelmans & Zeiger, 2000). On the other hand, 2-aminoanthracene performed as expected in TA 98 generating 15- and 2.5-fold increments in colony abundances with and without 59, respectively.

3.2. Plate incorporation assay: Industrially processed food items

The mutagenic activity of industrially processed food products expressed as the number of revertants per gram original product, obtained using the standard plate incorporation assay, is presented in Tables 2a and 2b. Overall, the products exhibited fairly low mutagenic activity in both Salmonella strains. However, the results showed some variation in the mutagenic potency both among equivalent products processed differently and among different products processed in the same way.

Extracts of industrially processed grilled chicken, smoked fish, cold-smoked fish, grilled beef, French fries, mashed potatoes, hamburger (beef) and hamburger (chicken) did not show discernible mutagenic potential in any of the three batches examined in either strain of Salmonella, disregard of the 59 status (Tables 2a and 2b). Seven of the other products examined produced revertants at least two-fold higher than negative control (DMSO) in at least one of the three batches screened, and in one or both strains. The number of revertants produced by
cold-smoked beef on Salmonella TA 100 (with S9) ranged from 420.3 to 583.3 revertants per gram, showing clear mutagenic activity in two of the batches (Table 2a). In the third batch investigated, the number of revertants (420.3 ± 4.6) was more than twice that yielded by negative controls, but there was no dose–response, so it was not considered mutagenic. The extract of cold cuts of grilled turkey showed clear mutagenic activity in all the three batches examined with Salmonella TA 98 (Table 2b). In two cases, this occurred without S9 mix and once in its presence. One of these lots (batch 1) proved mutagenic also in TA 100, both in the presence and absence of S9 mix. This particular batch also yielded a significant mutagenic response in the modified Ames test (see below). Some batches of pepper salami and sausage were mutagenic only in the TA 100 strain, and one lot of fried fish only in the TA 98 strain.

Supplementary Fig. 1 presents the dose–response curves of those industrially processed food products, which displayed significant mutagenic activity. The number of revertants increased, in most cases in a similar fashion, as a function of extract concentration in both strains and irrespective of S9 status.

### Table 1

Ranges for revertant colonies obtained with control substances in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Number of revertant colonies</th>
<th>Aggregate controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salmonella TA 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+S9</td>
<td>-S9</td>
</tr>
<tr>
<td>Water</td>
<td>166.7 ± 17.8</td>
<td>128.0 ± 15.7</td>
</tr>
<tr>
<td>DMSO</td>
<td>145.7 ± 18.5</td>
<td>120.7 ± 17.9</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>10000.0–1104.0</td>
<td>553.0–569.7</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>327.3–442.3</td>
<td>239.0–247.3</td>
</tr>
<tr>
<td>Salmonella TA 98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waters</td>
<td>25.3–34.3</td>
<td>19.7–27.7</td>
</tr>
<tr>
<td>DMSO</td>
<td>22.0–34.0</td>
<td>19.7–24.0</td>
</tr>
<tr>
<td>2-Aminonaphthalene</td>
<td>400.3–449.3</td>
<td>54.0–58.0</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>82.6–116.7</td>
<td>18.0–33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Range.

** Mean ± SD.

### Table 2a

Ranges for revertants generated by the highest concentrations (1.0 per g of food sample) of extracts of industrially processed food products on Salmonella TA 100 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Revertants per gram</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9</td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
</tr>
<tr>
<td>Chicken</td>
<td>524.7 ± 99.6</td>
<td>239.3 ± 20.8</td>
<td>266.7 ± 27.4</td>
<td>258.0 ± 8.5</td>
</tr>
<tr>
<td>Chicken*</td>
<td>544.7 ± 43.1</td>
<td>255.0 ± 18.5</td>
<td>398.7 ± 9.0</td>
<td>274.7 ± 23.5</td>
</tr>
<tr>
<td>Chicken†</td>
<td>241.7 ± 23.7</td>
<td>201.0 ± 13.3</td>
<td>272.7 ± 20.1</td>
<td>224.0 ± 23.1</td>
</tr>
<tr>
<td>Turkey</td>
<td>709.7 ± 68.0</td>
<td>394.0 ± 18.4</td>
<td>272.7 ± 20.1</td>
<td>224.0 ± 23.1</td>
</tr>
<tr>
<td>Fish†</td>
<td>243.7 ± 10.2</td>
<td>198.3 ± 6.7</td>
<td>223.0 ± 10.4</td>
<td>230.7 ± 18.0</td>
</tr>
<tr>
<td>Fish†</td>
<td>291.7 ± 5.2</td>
<td>256.0 ± 22.7</td>
<td>236.3 ± 12.0</td>
<td>234.3 ± 21.4</td>
</tr>
<tr>
<td>Fish†</td>
<td>648.0 ± 9.15</td>
<td>281.0 ± 58.3</td>
<td>306.7 ± 25.1</td>
<td>254.0 ± 23.6</td>
</tr>
<tr>
<td>Beef†</td>
<td>294.3 ± 29.0</td>
<td>211.7 ± 22.8</td>
<td>318.0 ± 25.2</td>
<td>257.7 ± 42.6</td>
</tr>
<tr>
<td>Beef†</td>
<td>583.3 ± 153.4</td>
<td>291.3 ± 54.7</td>
<td>420.3 ± 14.6</td>
<td>376.3 ± 13.4</td>
</tr>
<tr>
<td>Sausage†</td>
<td>337.3 ± 19.0</td>
<td>310.7 ± 22.8</td>
<td>2213.3 ± 22.5</td>
<td>172.7 ± 15.9</td>
</tr>
<tr>
<td>Pepper salami</td>
<td>398.3 ± 68.1</td>
<td>282.0 ± 20.1</td>
<td>371.0 ± 46.5</td>
<td>289.7 ± 8.5</td>
</tr>
<tr>
<td>French fries</td>
<td>260.0 ± 23.5</td>
<td>200.7 ± 15.0</td>
<td>197.3 ± 8.1</td>
<td>177.0 ± 26.0</td>
</tr>
<tr>
<td>Mashed Potatoes</td>
<td>219.7 ± 14.5</td>
<td>154.0 ± 9.4</td>
<td>274.3 ± 8.7</td>
<td>145.3 ± 16.7</td>
</tr>
<tr>
<td>Hamburger</td>
<td>244.3 ± 11.6</td>
<td>200.7 ± 11.2</td>
<td>232.7 ± 6.1</td>
<td>192.0 ± 7.2</td>
</tr>
<tr>
<td>Hamburger†</td>
<td>272.0 ± 22.9</td>
<td>198.0 ± 10.4</td>
<td>220.3 ± 5.1</td>
<td>188.0 ± 29.6</td>
</tr>
</tbody>
</table>


* Chicken

** Beef

† Significantly different from respective controls (P < 0.05).

‡ Significantly different from aggregate control (P < 0.05).

### 3.3. Plate incorporation assay: Ready-to-eat snacks

Tables 3a and 3b show the results of the mutagenic potential of ready-to-eat snacks from a popular hamburger retailer. At the highest concentration, three out of five snack products examined generated a more than twice the number of revertants found in control, and produced a significant dose–response. Extracts of chicken nuggets were mutagenic in all the batches examined with Salmonella TA 100, mainly in the presence of S9 mix (Table 3a). In Salmonella TA 98, the numbers of revertants produced by chicken nuggets were either marginally elevated or, if they were greater than two-fold compared with the control, they lacked a significant dose–response (Table 3b).

Similarly, extracts of beef hamburger obtained from hamburger retailer were repeatedly mutagenic (Tables 3a and 3b) whereas industrially-processed beef burgers were negative (Tables 2a and 2b). All potato products (French fries and mashed potatoes) as well as burgers containing chicken, from a supermarket or the hamburger retailer, proved not to be mutagenic.
Table 2b
Number of revertants generated by the highest concentrations (200 mg/ml) of extracts of industrially processed food products on Salmonella TA 98 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Revertants per gram</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9/C0</td>
<td>-S9/C0</td>
<td>+S9/C0</td>
<td>-S9/C0</td>
</tr>
<tr>
<td>Chicken¹</td>
<td>62.7 ± 5.0</td>
<td>41.3 ± 8.1</td>
<td>46.3 ± 3.2</td>
<td>34.0 ± 7.9</td>
</tr>
<tr>
<td>Chicken²</td>
<td>68.3 ± 3.2</td>
<td>47.3 ± 2.9</td>
<td>38.3 ± 3.2</td>
<td>37.3 ± 1.5</td>
</tr>
<tr>
<td>Chicken³</td>
<td>46.3 ± 8.3</td>
<td>29.7 ± 3.5</td>
<td>45.0 ± 7.9</td>
<td>39.3 ± 9.0</td>
</tr>
<tr>
<td>Turkey⁴</td>
<td>71.7 ± 24.8</td>
<td>57.3 ± 13.7</td>
<td>45.7 ± 3.1</td>
<td>47.3 ± 7.6</td>
</tr>
<tr>
<td>Fish³</td>
<td>36.3 ± 0.6</td>
<td>29.3 ± 1.2</td>
<td>33.3 ± 3.1</td>
<td>28.7 ± 2.5</td>
</tr>
<tr>
<td>Fish⁴</td>
<td>66.7 ± 16.8</td>
<td>45.7 ± 4.7</td>
<td>33.7 ± 3.8</td>
<td>28.3 ± 2.9</td>
</tr>
<tr>
<td>Beef³</td>
<td>72.0 ± 9.0</td>
<td>56.7 ± 7.8</td>
<td>42.7 ± 1.5</td>
<td>39.0 ± 2.0</td>
</tr>
<tr>
<td>Beef⁴</td>
<td>43.0 ± 6.4</td>
<td>29.7 ± 10.2</td>
<td>42.3 ± 4.9</td>
<td>32.7 ± 0.6</td>
</tr>
<tr>
<td>Sausage</td>
<td>76.3 ± 8.7</td>
<td>42.3 ± 3.3</td>
<td>48.7 ± 7.6</td>
<td>42.0 ± 3.5</td>
</tr>
<tr>
<td>Pepper salami</td>
<td>49.7 ± 3.5</td>
<td>36.0 ± 7.0</td>
<td>32.7 ± 1.5</td>
<td>24.7 ± 5.5</td>
</tr>
<tr>
<td>French fries</td>
<td>75.3 ± 1.6</td>
<td>36.3 ± 5.5</td>
<td>67.0 ± 7.6</td>
<td>47.0 ± 8.7</td>
</tr>
<tr>
<td>Mashed potatoes</td>
<td>34.0 ± 1.9</td>
<td>29.0 ± 2.5</td>
<td>31.0 ± 1.0</td>
<td>28.7 ± 4.0</td>
</tr>
<tr>
<td>Hamburger²</td>
<td>41.7 ± 5.7</td>
<td>31.0 ± 4.5</td>
<td>38.0 ± 3.6</td>
<td>24.3 ± 3.5</td>
</tr>
<tr>
<td>Hamburger³</td>
<td>46.3 ± 9.0</td>
<td>31.3 ± 7.6</td>
<td>42.0 ± 3.6</td>
<td>30.0 ± 3.6</td>
</tr>
</tbody>
</table>

¹ Chicken.
² Beef.
*C160 Significantly different from respective controls (P < 0.05).
*a Significantly different from aggregate control (P < 0.05).

Table 3a
Number of revertants generated by the highest concentrations (10 per g of food sample) of extracts of ready-to-eat food snacks on Salmonella TA 100 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Revertants per gram</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9/C0</td>
<td>-S9/C0</td>
<td>+S9/C0</td>
<td>-S9/C0</td>
</tr>
<tr>
<td>Cheese Burger</td>
<td>334.0 ± 19.5</td>
<td>279.7 ± 24.5</td>
<td>325.3 ± 25.5</td>
<td>213.7 ± 27.0</td>
</tr>
<tr>
<td>Chicken¹</td>
<td>319.0 ± 12.0</td>
<td>236.0 ± 34.0</td>
<td>292.3 ± 15.0</td>
<td>254.3 ± 3.1</td>
</tr>
<tr>
<td>French fries</td>
<td>200.7 ± 6.7</td>
<td>174.7 ± 23.6</td>
<td>150.0 ± 6.6</td>
<td>159.7 ± 12.1</td>
</tr>
<tr>
<td>Hamburger²</td>
<td>253.7 ± 10.2</td>
<td>222.7 ± 22.2</td>
<td>158.7 ± 21.9</td>
<td>109.7 ± 10.4</td>
</tr>
<tr>
<td>Hamburger³</td>
<td>330.7 ± 25.2</td>
<td>269.3 ± 23.0</td>
<td>244.3 ± 45.6</td>
<td>202.3 ± 15.6</td>
</tr>
</tbody>
</table>

Key.
¹ Nugget.
² Chicken.
³ Beef.
*C224 Significantly different from respective controls (P < 0.05).
*a Significantly different from aggregate control (P < 0.05).

Table 3b
Number of revertants generated by the highest concentrations (1.0 per g of food sample) of extracts of ready-to-eat food snacks on Salmonella TA 98 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Revertants per gram</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9/C0</td>
<td>-S9/C0</td>
<td>+S9/C0</td>
<td>-S9/C0</td>
</tr>
<tr>
<td>Cheese Burger</td>
<td>53.7 ± 4.0</td>
<td>17.3 ± 3.2</td>
<td>52.7 ± 3.2</td>
<td>35.7 ± 4.5</td>
</tr>
<tr>
<td>Chicken¹</td>
<td>55.7 ± 3.2</td>
<td>35.0 ± 4.6</td>
<td>61.0 ± 13.1</td>
<td>40.0 ± 4.6</td>
</tr>
<tr>
<td>French fries</td>
<td>43.0 ± 2.7</td>
<td>19.3 ± 2.3</td>
<td>34.7 ± 1.5</td>
<td>20.3 ± 2.1</td>
</tr>
<tr>
<td>Hamburger²</td>
<td>41.7 ± 6.4</td>
<td>23.3 ± 6.8</td>
<td>40.7 ± 7.6</td>
<td>26.7 ± 5.7</td>
</tr>
<tr>
<td>Hamburger³</td>
<td>66.7 ± 06.0</td>
<td>37.3 ± 5.1</td>
<td>53.3 ± 8.6</td>
<td>37.0 ± 4.0</td>
</tr>
</tbody>
</table>

Key.
¹ Nugget.
² Chicken.
³ Beef.
*C160 Significantly different from respective controls (P < 0.05).
*a Significantly different from aggregate control (P < 0.05).
3.4. Modified Ames tests

In the modified Ames tests (Supplementary Tables 2–9), the outcome proved to depend on bacterial strain, type of food, S9 status, and assay, albeit for most food samples the complementary assays tended to reduce the number of revertants per gram obtained compared with the standard plate incorporation assay (some examples are shown in Figs. 1 and 2).

Extracts of industrially processed food (cold cuts of smoked chicken, honey-roasted chicken and cold-smoked beef) which required metabolic activation for their mutagenicity in the standard plate incorporation assay, were, unexpectedly, directly mutagenic in *Salmonella* TA 100 strain in the MC overlay assay (Supplementary Table 2). One of these samples (cold-smoked beef) produced the same outcome with the treat-and-wash assay (Supplementary Table 6). None of the ready-to-eat food samples exhibited any form of mutagenicity in either of these auxiliary assays with *Salmonella* TA 100 (Supplementary Tables 4 and 8) in contrast to the outcome in the standard plate incorporation assay. Extract of ready-to-eat beef burger, however, exhibited indirect mutagenic activity with the *Salmonella* TA 98 strain in the treat-and-wash assay (Supplementary Table 9).

Although cold cuts of grilled turkey (batch 1) showed clear mutagenic activity in all three assays in the presence or absence of S9 mix with the *Salmonella* TA 100 strain, both the MC overlay and treat-and-wash procedure produced fewer revertants compared with the standard assay (Fig. 1B). With the *Salmonella* TA 98 strain, however, by far the most conspicuous mutagenic response was recorded in the treat-and-wash assay, in which it produced revertants more than 13- and 5-fold control levels, with and without S9 mix, respectively (Supplementary Table 7 and Fig. 2). The other batches of grilled turkey were also mutagenic with the TA 98 strain in the presence or absence of S9 mix, but not to the same extent (Supplementary Table 7).

3.5. Cytotoxicity assays

The cytotoxicity of the four concentrations of all food extracts was determined by both trypan blue exclusion and LDH secretion of HepG2 human hepatocellular carcinoma cells, as well as by
the boar sperm motility assay. The percentage viability of HepG2 cells in the trypan blue exclusion test exhibited an inverse correlation with the concentration of extracts from smoked chicken, grilled turkey and cold-smoked beef following 48 h exposure (Supplementary Fig. 2). However, the 50 ± 5% cytotoxicity limit (Nymark et al., 2012) was not reached at any concentration. Although there was a direct correlation between extract concentration and LDH release, the maximum release never exceeded 25% of that induced by the positive control substance applied (lysis solution; Supplementary Fig. 2A). Hence, the extracts were classified non-cytotoxic in this assay following exposure for 4, 24 or 48 h. Moreover, none of the food extracts tested affected boar sperm motility in a statistically significant manner. These samples were also not cytotoxic in another independent cytotoxicity assay carried out on the yeast strain Saccharomyces cerevisiae BMA64/luc (Omoruyi, Kabiersch, & Pohjanvirta, 2013).

3.6. Comet Assay

As a follow-up of the results obtained in the conventional Ames test, samples showing mutagenic potential in at least one strain of Salmonella were screened for single-strand breaks, DNA-DNA/ DNA-protein cross-linking and alkali-labile sites by the Comet Assay. None of the samples investigated tested positive when compared with both positive and negative controls. In the positive controls (treatment with hydrogen peroxide), the expected DNA migration towards the anode was observed by microscopic examination.

3.7. Recovery analysis

The percentage recovery for both benzo[a]pyrene and 2-aminoanthracene was approximately 73%.

4. Discussion

Industrially processed and packaged foodstuffs as well as ready-to-eat snacks are consumed in increasing quantities all over the world. Therefore, it is important to ensure in addition to microbial safety, that products do not contain chemicals that might pose a toxicological risk to consumer health. A conceivable potential risk in this regard is the formation of genotoxic compounds during the processing of industrial foodstuffs or cooking of snacks. Regular screening studies are necessary to verify that the methods used by food industry and for example, fast-food outlets are appropriate from this point of view. The present investigation aimed to explore the current situation in Finland.

While the majority of the samples analyzed in our study proved negative, the mutagenic activity observed with extracts from some batches of industrially processed foods (smoked chicken, honey-roasted chicken, grilled turkey, cold-smoked beef, fried fish, and pepper salami) and ready-to-eat snacks (cheese burger, hamburger containing beef, and chicken nuggets) indicates that exposure to mutagenic compounds arising from commercial food products in Finland cannot be completely excluded. Levels may be toxicologically significant, because the magnitude of mutagenic activities was 2.5- to 13-fold those of negative controls. However, for some products there was a notable (up to over 10-fold) variation in mutagenic potency between batches, and among equivalent products and different products processed in the same way. There was also variation in mutagenic potency among equivalent food products with the three assays (standard plate incorporation, MC overlay as well as treat-and-wash assays), in particular for ready-to-eat snacks, casting doubt on the significance of some findings. Conversely, the positive results obtained with the MC overlay and treat-and-wash assays for some industrially processed food products (smoked chicken, grilled turkey and cold-smoked beef) coupled with their mutagenic response in the standard plate incorporation assay imply the effect is genuine (i.e., not secondary to histidine release from the food products) and thus of concern.

It is also noteworthy that extracts of certain food items (e.g. cold-smoked beef, Batch 3) exhibited mutagenic activity without metabolic activation in all three assays, strongly suggesting direct mutagenicity. Based on the classification of Abbas, Mirocha, and Shier (1984), no cytotoxic effects were observed in the assays conducted with different concentrations of extracts from food samples used in the mutagenicity studies. This is in keeping with a previous report (Sharif et al., 2008), further strengthening the validity and relevance of the mutagenicity findings and, concurrently, justifying our requirement for dose-response across the entire range of concentrations examined. On the other hand, none of the samples was discernibly different from the negative control using the Comet Assay. This has mechanistic implications, since the Comet Assay detects mainly DNA strand breaks (He, Chen, Jin, & Jin, 2000), whereas the Salmonella strains TA 100 and TA 98 are sensitive to base-pair substitutions and frame-shift mutations, respectively (Mortelmans & Zeiger, 2000).

Pepper salami has not previously been regarded as a potential source of mutagens. In our study, this product exhibited both direct and indirect mutagenicity in one batch, and indirect in another when examined using TA 100 strain in the standard plate
incorporation assay. The number of revertants obtained with Salmonella TA 100 ranged from 371.00 ± 0.45 to 433.00 ± 30.05 rev/g. All the values were at least twice their respective negative controls but the lack of dose–response in one of the batches resulted in its classification as non-mutagenic. Both positive samples of pepper salami (batch 2 and 3) were also found to be mutagenic with the MC overlay assay, in the presence of S9 mix, but not with the treat-and-wash assay (209.3 ± 13.4 and 204.0 ± 0.00 rev/g). Thus, the true mutagenicity of these samples remains unclear. Further studies on this food item are warranted.

Three out of five products of ready-to-eat snacks examined were found to be mutagenic, in at least two batches, using the conventional Ames test. However, except for beef burger in the treat-and-wash assay (TA 98, without S9), all these samples were negative in the modified Ames tests suggesting their initial positive results stemmed from local or general histidine release. In this context, it is noteworthy that in contrast to hamburgers purchased from the fast-food outlet, hamburgers acquired from the supermarket were all non-mutagenic as measured by the standard plate incorporation test. Overall, these results imply the industrial cooking methods currently used in Finland are carefully considered also from the toxicological point of view. Previous studies have reported mutagenic compounds in a hamburger extract (Stavric et al., 1995) and in the urine of subjects after a hamburger meal (Gabbani et al., 1998). However, in both of these studies, the subjects prepared the hamburgers without monitoring the cooking temperature and time, which could account for the high level of mutagenicity observed. Homemade food products cooked at a high temperature are reported sources of human exposure to genotoxic compounds (Tikkkanen, 1991). Also, the authors did not take into consideration the possible release of histidine from the burger products, which apparently may have accounted for the high number of revertants observed (Khandoudi et al., 2009; Thompson et al., 2005).

The negative results obtained for French fries (both those processed industrially and those obtained from a hamburger restaurant) as well as mashed potatoes with the standard Ames test and Comet Assays were not unexpected. Acrylamide is the principal mutagen formed in both potato products, and studies have shown that this compound is not mutagenic in the Ames test, either in the presence or absence of metabolic activation (Dearfield et al., 1995; Knaap et al., 1988). Furthermore, two independent studies have shown that acrylamide is not mutagenic in Chinese hamster V79 cells (Baum et al., 2005; Tsuda et al., 1993). Acrylamide is formed when food high in carbohydrate is processed at a high temperature, usually between 120 and 180 °C. While we may attribute the negative results obtained to such findings, it is also worth mentioning that modified processing methods for potatoes, at relatively low temperatures for shorter periods of time, can significantly reduce, or eliminate, the formation of genotoxic compounds (Peters et al., 2004). Moreover, the use of a potato variety low in carbohydrate can have the same effect (Sowokinos, 1990).

The localized release of proteins, peptides or histidine from food samples has been reported as a source of false positive results in the Ames test (Khandoudi et al., 2009; Thompson et al., 2005). To prevent this potential misinterpretation and exaggeration of ostensible mutagenicity, treat-and-wash as well as MC overlay assays were performed for all samples eliciting a positive outcome in the conventional Ames test. The results from these complementary assays for some samples (cold cuts of smoked chicken, grilled turkey and cold-smoked beef) were consistent, further reinforcing our initial findings with the Ames test. Among these, extracts of grilled turkey stood out for their ability to induce revertants in the presence and absence of metabolic activation system in both bacterial strains. Grilled turkey also yielded the highest number of colonies in the MC overlay assay in both strains (with S9 mix). Interestingly, one of the lots of grilled turkey (batch 1), substantially enhanced the generation of revertants, with colonies more than 13- and 5-fold their controls, produced in the presence and absence of S9 mix, respectively, as determined using the treat-and-wash assay. Possible reasons for this finding are the long pre-incubation period in the presence of a nutrient broth, increased viability of the bacteria, and removal of substances increasing or inhibiting reverse mutation. It is notable that two other batches of grilled turkey also produced significant effects in the treat-and-wash assay in TA 98 strain (in TA 100, only batch 1 was examined), both in the presence and absence of S9 mix.

Although it is challenging to compare results for processed food products worldwide, the data obtained in our studies are similar to those reported elsewhere (Peters et al., 2004; Stavric et al., 1995) but somewhat at odds with those of a previous study in Finland, where the majority of ready-made industrial food samples were mutagenic and, for example, grilled chicken generated up to 1400 revertants in the TA 98 strain (Tikkkanen, 1991). Moreover, Tikkkanen (1991) did not control for possible histidine release. It should also be noted that most of the earlier studies in Finland and elsewhere have employed Salmonella TA 98 alone (and sometimes only in the presence of S9). Based on our findings, TA 98 strain may be more or less sensitive to food mutagens than TA 100, depending on sample type and assay conditions. On the other hand, the observed generally low levels of mutagenic activity in industrially processed food products in Finland today are in agreement with the more recent data of Jägerstad and Skog (2005). They reported the daily intake of nitrosodimethylamine (a known mutagen formed as a result of processing especially at high temperature) to be significantly lower in Finland (0.08 μg/day) when compared with other European countries (0.12–0.38 μg/day). This together with our data, suggest the food industry in Finland has recognized its responsibilities and taken appropriate steps towards producing products that do not pose genotoxic hazards to consumers. However, the process has clearly not been brought to completion yet.

In conclusion, our study demonstrated that, while in most cases, the risk of genotoxicity associated with consumption of industrially processed and ready-to-eat foodstuffs is low in Finland, there are still products the production of which should be refined further to reduce the potential risk for consumer. Our data may have toxicological implications and further studies are, therefore, needed.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.04.055.

References

Dietary Exposure of Nigerians to Mutagens and Estrogen-Like Chemicals

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Abstract: Food and drinking water are poorly delineated sources of human exposure to chemical food mutagens and endocrine-disrupting chemicals. In this study, we investigated the presence of mutagens and chemicals exhibiting estrogenic activity in the daily diet of Nigerians, using in vitro assays. Commercially processed foods or snacks and various brands of pure water sachets were extracted by solid-phase extraction and liquid-liquid extraction, respectively. Mutagenicity was determined by the conventional Ames test and two complementary assays on two strains of Salmonella (TA 100 and TA 98), while the estrogenic activity was assessed by a yeast bioluminescent assay, using two recombinant yeast strains (Saccharomyces cerevisiae BMAERE1uc/ERα and S. cerevisiae BMA64/luc). A third of the food varieties investigated (chin-chin, hamburger, suya and bean cake) were mutagenic in all three assays, either in the presence or absence of S9 mix. Of the packed water samples, five out of the sixteen investigated (31%), were found to be estrogenic, with estradiol and bisphenol A equivalents ranging from 0.79 to 44.0 ng/L and 124.2 to 1,000.8 ng/L, respectively. Hence, although the current situation in Nigeria does not appear to be substantially worse than, e.g., in Europe, regular monitoring is warranted in the future.
1. Introduction

Food and drinking water are major sources of human exposure to both mutagens and endocrine-disrupting chemicals (EDCs) globally [1–7]. This is alarming in view of the fact that food and water are prerequisites of human life.

The sources of chemical mutagens in food vary remarkably, depending on the foodstuff and processing methodology. However, emphasis has traditionally been placed on reducing the levels of possible mutagenic residues in meat, grain, vegetables etc. prior to processing, neglecting the possibility of a less clear-cut risk: the formation of these mutagenic compounds in food as a result of processing. Yet, processed food items are reported to contain chemical substances known to have mutagenic, genotoxic and carcinogenic effects, and thus acting as a key global contributor to human cancer risk [8–11]. Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines have been reported in processed food (mainly meat and fish products) at various concentrations all over the world [12–15]. The formation of these chemical mutagens during food processing has been demonstrated to depend on a number of factors such as cooking time, method of cooking and type of heat source [3,8,12]. For example, the Ames test shows a correlation between meat-processing temperature and the number of revertants generated per gram of meat [3,4]. Chung et al. [12] also reported that charcoal-grilled pork contained higher levels of PAHs (10.2 μg/kg) compared with other methods of processing. Likewise, high concentrations of PAHs have been found in smoked-cured fish in Ghana [13].

The contamination by mutagenic PAHs of thermally treated high-protein foods such as charcoal-grilled meat products is mainly due to the direct pyrolysis of food fats and the deposition of PAHs from smoke produced through incomplete combustion of the thermal agents [16]. Unfortunately, this method of food processing is the method of choice in most developing nations, including Nigeria. Although knowledge of proper processing techniques would help reduce the risk of generating mutagenic compounds in food, a recent study showed that only 4.76% of 63 subjects involved in food processing in Nigeria had a formal training in a food safety/hygiene-related discipline [17]. Similar percentages have also been reported in Kenya and Ghana [18,19].

Regarding EDCs, the bulk of information available is on compounds possessing estrogen-like activity. Phytoestrogens and food contact materials are the main sources of human exposure to xenoestrogens in food [20–22]. While the health effects of phytoestrogens remain controversial, synthetic xenoestrogens have been associated with certain cancer types, reproductive disorders, developmental abnormalities and other adverse physiological effects in both humans and wildlife [23–25]. In this light, it is quite worrisome that drinking water sources as well as bottled mineral and flavored waters have been reported to contain estrogenic substances [5–7,26]. The estrogenic activity in bottled mineral and still water is mainly attributed to the prevailing use of several phthalates and other plasticizers including bisphenol A in packaging materials [5,6]. These chemicals are increasingly
raising concern, because they may leach into consumer products in normal use [27–29]. There are over 50 chemical compounds authorized for use in food contact materials which are known to have endocrine-disrupting potential [30]. Interestingly, when food contact materials are assessed for their health risk, they are not routinely tested for their endocrine-disrupting potential [31]. However, the Endocrine Society has expressed its concern about the widespread exposure of humans to these chemicals, as they are capable of affecting multiple endpoints within a living system [32].

Chemical mutagens and EDCs in food and water samples have usually been determined by various methods of analytical chemistry. However, these methods suffer from a number of limitations in their ability to elucidate the entire range of chemical mutagens and EDCs in a single experiment, including an unknown number of yet-to-be identified compounds. In vitro assays offer the advantage of detecting all substances that contribute to the functional property (mutagenicity or estrogenic activity) being assessed in food, water and environmental samples. Therefore, in the present study, we sought to determine the genotoxic and estrogenic properties of food and water samples by in vitro assays. We focused on Nigerian products, because the customary food processing methods there are potentially risky in this regard (see above) and because, to the best of our knowledge, such information does not yet exist in the body of scientific literature.

2. Materials and Methods

2.1. Materials

All chemicals used in this study were of analytical grade. The NADP and glucose-6-phosphate used were obtained from Roche Biochem (Stockholm, Sweden). Aroclor-induced S9 from rat liver was purchased from Trinova Biochem (Giessen, Germany). Histidine, potassium chloride, magnesium sulfate, potassium phosphate dibasic anhydrous and sodium ammonium phosphate were purchased from Merck AG (Darmstadt, Germany). Magnesium chloride hexahydrate and citric acid monohydrate were acquired from VWR international (Leuven, Belgium). Biotin, tryptophan, methylcellulose (MC), dimethyl sulfoxide (DMSO), benzo[a]pyrene, 2-aminoanthracene, sodium azide, estradiol, bisphenol A, progesterone and testosterone were purchased from Sigma-Aldrich (Steinheim, Germany). D-Luciferin was obtained from Biotherma (Handen, Sweden). Yeast nitrogen base medium without amino acids was obtained from Becton Dickinson (Franklin Lakes, NJ, USA).

2.2. Microorganisms

The bacteria, *Salmonella enterica* sv. *typhimurium* strains TA 100 and TA 98, were obtained from Pasteur’s Institute (Paris Cedex, France). Two recombinant yeast strains *Saccharomyces cerevisiae* BMAEREEluc/ERα and *S. cerevisiae* BMA64/luc [33] were used in this study. In the yeast bioluminescent assay, BMAEREEluc/ERα served as a reporter strain, in which the ERα is expressed. Upon ligand binding, the dimerized receptor binds the estrogen response elements in the promoter region of the luc reporter gene. In *S. cerevisiae* BMA64/luc, luciferase is expressed constitutively, and this strain was used for determination of cytotoxicity of the test samples. Both yeast strains are kind gift donations by Dr. Johanna Rajasärkkä of the Department of Food and Environmental Sciences, Faculty of Agriculture.
and Forestry, University of Helsinki, Finland. Yeasts were grown on Difco Yeast Nitrogen Base medium without amino acids, supplemented with 40% glucose and their respective amino acids.

2.3. Cell Line

Human hepatocellular carcinoma-derived cell line (HepG2) was obtained from American Type Culture Collection through LGC standards (Boras, Sweden) and cultured in Eagle’s Minimum Essential Medium (LGC standards) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, Steinheim, Germany). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air atmosphere incubator (NuAire Inc., Plymouth, MA, USA).

2.4. Sampling and Sample Preparation

A total of 36 samples (3 lots of 12 varieties) representing commonly consumed, commercially processed food items in Nigeria were evaluated for their mutagenic potential. All varieties were obtained from different vendors since no quality control is carried out in the production of these food items. Moreover, equivalent food products obtained from the same manufacturer have been previously reported to vary in their mutagenic potential [2,4]. Since the major source of xenoestrogens in processed food items are phytoestrogens and food contact materials, and all our food samples were informed to be free of soy (a highly significant source of phytoestrogens) and mostly unpackaged, we targeted water samples as possible sources of exposure to EDCs. Sixteen sachet pure water samples sold in Benin City metropolis, Edo State, Nigeria were acquired for the purpose of this study. Food samples were extracted by solid phase extraction method [4], while possible estrogenic compounds were extracted from the water samples (1000 mL each) by liquid–liquid extraction as described by [34]. The final extracts were concentrated to approximately 2 mL using a rotary evaporator, and the concentrates were shipped on ice to the Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland. Upon arrival, samples were further concentrated to dryness under nitrogen. Food samples were reconstituted in DMSO, while water samples were reconstituted in 5% ethanol for in vitro analyses. Food packaging materials were extracted for possible estrogenic activity as described previously [1].

2.5. Cytotoxicity Assays

The cytotoxic effect of the concentrations of food extracts used in this study was investigated by two independent assays measuring trypan blue exclusion and lactate dehydrogenase (LDH) activity as previously described [2]. Briefly, HepG2 cells were grown in 24-well plates (VWR, Finland) for 48 h, and further exposed to different concentrations of food extracts for 4, 24 or 48 h. After exposure, the cells were trypsinized and centrifuged for 5 min at 2500 rpm. Pellets were then resuspended in PBS, after which 10 μL of the cells were mixed with 5 μL (0.8 mM) trypan blue dye for microscopic observation. LDH activity was performed according to the instructions provided in the Cytotoxicity Detection Kit PLUS (LDH), version 6 (Roche Biochem, Stockholm, Sweden).
2.6. Mutagenicity Assay

The mutagenic potential of food extracts was initially determined by the standard plate incorporation assay. Samples showing mutagenic potential in this assay were subsequently subjected to “treat-and-wash” as well as methylcellulose overlay assays to ascertain to what degree a localized release of proteins, peptides or histidine from the samples contributed to the outcome.

2.6.1. Standard Plate Incorporation Assay

The standard plate incorporation assay was performed as described by Maron and Ames [35] using *Salmonella* strains TA 100 and TA 98 with and without metabolic activation (S9 mix). The amount of S9 used in the S9 mix was 10%. Water and DMSO were used as negative controls for both strains while sodium azide (0.04 mg/mL) and 2-aminoanthracene (0.02 mg/mL) served as positive controls for TA 100 and TA 98, respectively. Benzo[a]pyrene (0.1 mg/mL) was also used as a positive control for both strains. The volume of controls used was 50 μL/plate in triplicate plates. Sodium azide is a known direct mutagen in *Salmonella* TA 100 [36], whereas 2-aminoantracene is metabolically activated by mono-oxygenases of the CYP1A family in rat liver [37]. Likewise, benzo[a]pyrene requires metabolic activation for mutagenicity [38].

For all samples, four different concentrations of the food extracts (25, 50, 100 and 200 mg/mL) were tested in triplicate plates (50 μL/plate). The highest concentration (200 mg/mL) was equivalent to 1 g of the food sample. The plates were incubated at 37 °C for 48 h.

The results of the mutagenic activities are presented as the number of revertant colonies per gram of food sample. Only the mean and standard deviation of the highest concentration for all food extracts are shown.

2.6.2. Treat-and-Wash Assay

The treat-and-wash assay was conducted according to the method described by Thompson et al. [39]. The protocol applied was as per the standard plate incorporation assay with the exception that the S9 mix, bacteria and sample extract were incubated for 90 min prior to the addition of molten top agar. Briefly, a 500 μL aliquot of S9 mix/phosphate buffer (0.2 M, pH 7.4) was combined with 100 μL each of late-log bacterial culture and sample extract solution in a sterile 15 mL tube. The mixture was incubated for 90 min in a mechanical shaker (180 rpm) at 37 °C. The extended duration of bacterial exposure compensated for the absence of bacterial exposure on plates, as the test sample was washed away prior to plating. After a 90-min preincubation, 10 mL of wash solution (Oxoid No. 2 nutrient broth in phosphate-buffered saline (1:7 v/v)) was added, and the washed bacteria were collected by centrifugation at 2,000 g for 30 min. All but approximately 700 μL of the supernatant was removed and discarded, and the bacteria were resuspended in the residual supernatant prior to plating via top agar.

2.6.3. Methylcellulose Overlay Assay

Methylcellulose overlay assay was performed as previously described [39]. Briefly, a 500 μL aliquot of S9 mix/phosphate buffer (0.2 M, pH 7.4) was combined with 100 μL of late-log bacterial culture in a sterile 15 mL tube. A 2 mL aliquot of the MC overlay suspension was added to the tube,
and a 100 μL aliquot of the sample extract solution was added immediately afterward. The mixture was overlaid on a pre-warmed (37 °C) minimal glucose plate. Plates were held at 4 °C for 1 h after plating to ensure gelling of the MC overlay, and subsequently incubated (not inverted) at 37 °C for 48–72 h. The MC overlay was prepared on the day of the test, and the mixture was stirred at 50–60 °C throughout use.

2.7. Yeast Bioluminescent Assay

The yeast bioluminescent assay was performed as previously described [1]. Estradiol and bisphenol A were used as positive controls, while progesterone and testosterone served as negative controls.

2.8. Statistical Analysis/Interpretation of Data

The mutagenic potency of each food sample was determined from the slope of the linear portion of the dose-response curve by linear regression analysis using the software program Prisma 4.0 (GraphPad software Inc., San Diego, CA, USA). In addition to the requirement of a statistically significant ($p < 0.05$) dose-response effect, only those samples were considered mutagenic whose highest test concentration generated at least twice as many revertants as the negative control (DMSO). For proper interpretation and clarity, the number of revertants obtained was compared with both their experiment-specific controls and aggregate controls across all experiments. The $p$ values of these comparisons in the tables are derived from the regression analyses. In the estrogenic activity assays, the fold induction, fold induction corrected (FIC) and limit of detection (LOD) were calculated as described previously [33]. The sigmoidal dose-response curves for increasing concentrations of estradiol and bisphenol A were obtained using Prisma 4.0. The estradiol and bisphenol A equivalents of food samples showing estrogenic activity were calculated from probit transformation of the curves.

3. Results

3.1. Plate Incorporation Assay: Control Substances

The results obtained with the control substances on both strains of *Salmonella* are presented in Table 1. In the test system, the number of revertants generated by sodium azide was 4–5 fold the negative control (DMSO) in TA 100 strain, both in the presence and absence of metabolic activation (S9 mix). This was an expected outcome, because sodium azide is the recommended direct chemical mutagen for TA 100 strain [36]. Meanwhile, the number of revertants generated by benzo[a]pyrene was 3–4 and 2–3 fold that of DMSO with and without metabolic activation, respectively. In *Salmonella* TA 98 strain, 2-Aminoanthracene behaved as expected, with the number of revertant colonies being 17–23-fold higher than that of the control substance, in the presence of S9 mix. On the other hand, only a 2–3-fold increment was observed in the absence of S9 mix. No mutagenic effect/potency was observed with benzo[a]pyrene in the absence of metabolic activation; co-incubation with S9 resulted in an approximately three-fold increment in colony formation.
Table 1. Ranges for revertant colonies obtained with control substances in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Number of Revertant Colonies</th>
<th>Respective Controls *</th>
<th>Aggregate Controls **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9</td>
<td>−S9</td>
<td>+S9</td>
</tr>
<tr>
<td><strong>Salmonella TA 100</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>134.3–177.7</td>
<td>112.7–134.3</td>
<td>161.8 ± 22.4</td>
</tr>
<tr>
<td>DMSO</td>
<td>138.7–166.3</td>
<td>111.0–128.3</td>
<td>155.3 ± 17.9</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>568.7–651.0</td>
<td>432.7–516.3</td>
<td>621.6 ± 78.8</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>438.0–517.3</td>
<td>248.0–270.0</td>
<td>471.6 ± 48.5</td>
</tr>
<tr>
<td><strong>Salmonella TA 98</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>32.3–42.7</td>
<td>19.0–34.0</td>
<td>37.4 ± 3.1</td>
</tr>
<tr>
<td>DMSO</td>
<td>35.7–36.7</td>
<td>19.0–24.0</td>
<td>36.2 ± 2.8</td>
</tr>
<tr>
<td>2-Aminoanthracene</td>
<td>624.3–837.7</td>
<td>55.0–69.3</td>
<td>754.2 ± 68.2</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>102.0–122.0</td>
<td>20.3–29.7</td>
<td>116.8 ± 10.4</td>
</tr>
</tbody>
</table>

Notes: * Range; ** Mean ± SD.

3.2. Plate Incorporation Assay: Test Substances/Food Samples

The mutagenic activity of commercially processed food items, obtained by the standard plate incorporation assay, is presented in Tables 2 and 3. The majority of samples investigated (75%) exhibited fairly high mutagenic activity (the maximal responses being comparable to those elicited by benzo[a]pyrene), mainly in Salmonella TA100 strain. However, there was notable lot-to-lot variation.

In TA 100 strain, chin-chin, hamburger, suya and bean cake were the most mutagenic food samples investigated. The number of revertants generated by these samples was over twice that of DMSO in all the batches analyzed and mostly independent of the S9 mix. A somewhat surprising result was found with the potato products (french fries and potato chips), as at least one of the lots of both products proved directly mutagenic. Roasted maize, plantain chips and coconut-candy did not show any evidence of mutagenic potency in this strain (Table 2).

In Salmonella TA 98, only three food or snack varieties (potato chips, peanut and suya) exhibited mutagenic potency in at least one of the batches investigated (Table 3). Suya displayed the most coherent outcome with all its three batches being mutagenic in the presence of S9 mix. In support of the result with TA 100, the same batch of potato chips (number 3) exhibited direct mutagenicity also in TA 98.
Table 2. Number of revertants generated by the highest concentrations (1.0 per g of food sample) of food extracts on *Salmonella* TA 100 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food Products</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9</td>
<td>−S9</td>
<td>+S9</td>
</tr>
<tr>
<td>Doughnut</td>
<td>319.0 ± 12.1 †φ</td>
<td>254.0 ± 14.0 †φ</td>
<td>201.3 ± 07.1</td>
</tr>
<tr>
<td>Chin-chin</td>
<td>285.7 ± 15.5 †</td>
<td>191.3 ± 10.3</td>
<td>460.3 ± 28.3 †φ</td>
</tr>
<tr>
<td>Hamburger</td>
<td>353.3 ± 43.5 †φ</td>
<td>248.3 ± 79.2 †φ</td>
<td>469.3 ± 44.4 †φ</td>
</tr>
<tr>
<td>Coconut-candy</td>
<td>265.7 ± 12.4</td>
<td>198.0 ± 06.9</td>
<td>245.7 ± 18.9</td>
</tr>
<tr>
<td>French fries</td>
<td>255.0 ± 47.8</td>
<td>304.3 ± 33.8 †φ</td>
<td>208.0 ± 26.9</td>
</tr>
<tr>
<td>Potato chips</td>
<td>159.0 ± 10.8</td>
<td>159.3 ± 04.6</td>
<td>210.7 ± 15.1</td>
</tr>
<tr>
<td>Plantain chips</td>
<td>124.7 ± 11.8</td>
<td>109.3 ± 01.5</td>
<td>159.3 ± 27.3</td>
</tr>
<tr>
<td>Peanut</td>
<td>293.3 ± 30.6 †</td>
<td>242.7 ± 04.6 †φ</td>
<td>339.0 ± 62.6 †φ</td>
</tr>
<tr>
<td>Roasted maize</td>
<td>252.3 ± 23.0</td>
<td>187.3 ± 04.7</td>
<td>176.7 ± 29.9</td>
</tr>
<tr>
<td>Suya</td>
<td>383.0 ± 20.7 †φ</td>
<td>240.7 ± 10.0 †φ</td>
<td>401.7 ± 12.1 †φ</td>
</tr>
<tr>
<td>Fried chicken</td>
<td>138.7 ± 06.0</td>
<td>133.0 ± 08.5</td>
<td>394.7 ± 14.7 †φ</td>
</tr>
<tr>
<td>Bean cake</td>
<td>312.0 ± 25.4 †φ</td>
<td>241.7 ± 36.8 †φ</td>
<td>365.0 ± 22.6 †φ</td>
</tr>
</tbody>
</table>

Notes: †: Significantly different from respective controls (*p < 0.05*); φ: Significantly different from aggregate control (*p < 0.05*).
Table 3. Number of revertants generated by the highest concentrations (1.0 per g of food sample) of food extracts on *Salmonella* TA 98 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food Products</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S9 −S9</td>
<td>+S9 −S9</td>
<td>+S9 −S9</td>
</tr>
<tr>
<td>Doughnut</td>
<td>40.3 ± 8.4</td>
<td>22.0 ± 8.2</td>
<td>30.7 ± 11.6</td>
</tr>
<tr>
<td>Chin-chin</td>
<td>34.0 ± 1.7</td>
<td>23.3 ± 2.1</td>
<td>31.0 ± 7.9</td>
</tr>
<tr>
<td>Hamburger</td>
<td>46.0 ± 14.0</td>
<td>26.3 ± 3.1</td>
<td>31.7 ± 4.5</td>
</tr>
<tr>
<td>Coconut-candy</td>
<td>38.7 ± 4.9</td>
<td>24.3 ± 1.6</td>
<td>40.7 ± 6.1</td>
</tr>
<tr>
<td>French fries</td>
<td>32.3 ± 1.5</td>
<td>36.0 ± 6.6</td>
<td>30.3 ± 3.5</td>
</tr>
<tr>
<td>Potato chips</td>
<td>36.3 ± 5.9</td>
<td>26.7 ± 3.8</td>
<td>33.0 ± 4.6</td>
</tr>
<tr>
<td>Plantain chips</td>
<td>29.0 ± 5.6</td>
<td>29.0 ± 2.6</td>
<td>32.3 ± 2.9</td>
</tr>
<tr>
<td>Peanut</td>
<td>69.7 ± 5.6</td>
<td>34.7 ± 4.0</td>
<td>78.0 ± 12.5</td>
</tr>
<tr>
<td>Roasted maize</td>
<td>24.3 ± 2.1</td>
<td>20.0 ± 2.0</td>
<td>31.7 ± 4.9</td>
</tr>
<tr>
<td>Suya</td>
<td>86.7 ± 5.8</td>
<td>27.7 ± 2.5</td>
<td>83.0 ± 3.5</td>
</tr>
<tr>
<td>Fried chicken</td>
<td>32.0 ± 5.0</td>
<td>28.3 ± 5.1</td>
<td>24.0 ± 4.0</td>
</tr>
<tr>
<td>Bean cake</td>
<td>32.0 ± 5.3</td>
<td>11.0 ± 1.7</td>
<td>28.7 ± 8.5</td>
</tr>
</tbody>
</table>

Notes: †: Significantly different from respective controls (*p* < 0.05); ‡: Significantly different from aggregate control (*p* < 0.05).
3.3. Modified Ames Tests

To ascertain to which degree a localized release of proteins, peptides or histidine contributed to the mutagenicity test results obtained with the standard plate incorporation assay, “treat-and-wash” as well as MC overlay assays were performed (Tables 4 and 5). The outcome proved to depend on bacterial strain, type of food, S9 status, and assay. For some food extracts initially found to be mutagenic in the standard plate incorporation assay, the number of revertants decreased below the two-fold limit level in comparison with the negative control. Hence, the original Ames test result was in these cases interpreted to be of secondary nature and not due to genuine mutations. However, in a large number of cases, the food extracts were mutagenic in all three assays both in the presence and absence of S9 mix. For some food items (hamburger, suya and bean cake), a single lot was mutagenic in all three assays but only in the presence of S9 mix. In the absence of S9, the outcome with these three products varied. In contrast to this pattern, a single batch of hamburger (batch 2) was mutagenic in all three assays, both in the presence and absence of S9 mix. A couple of surprises also emerged in these complementary assays. Extracts of fried chicken (batch 2) and bean cake (batch 2) that required metabolic activation for their mutagenicity in the standard plate incorporation assay, were, unexpectedly, directly mutagenic in the treat-and-wash assay in *Salmonella* TA 100 strain (Table 4). One of these samples (bean cake, batch 2) behaved the same way also in the MC overlay assay (Table 4). An identical shift from indirect to direct mutagen was recorded in *Salmonella* TA 98 strain for extracts of peanut (batch 2) and suya (batch 2) (Table 5).

3.4. Cytotoxicity Assays

The cytotoxicity of the four concentrations of all food extracts was determined by both trypan blue exclusion and LDH secretion assays in HepG2 human hepatocellular carcinoma cells. The non-survival percentage of HepG2 cells in the trypan blue exclusion test did not exceed 50%. Also, there was a significant difference between the positive control (lysis solution) and the test samples in the amount of LDH released. Hence, the extracts were classified non-cytotoxic in these assays following exposure for 4, 24 or 48 h.

3.5. Estrogenic Activity Assay: Control Substances

The positive and negative control compounds used in this study behaved as expected with the *S. cerevisiae* BMAEREluc/ERα yeast strain. Both positive controls (estradiol and bisphenol A) produced a sigmoidal dose-response curve (Figure 1), while the negative controls (progesterone and testosterone) did not elicit any luciferase activity in the test system. This is in keeping with previously published data [29,40]. The limit of detection (LOD) in the yeast bioluminescent assay was 2.4-fold induction corrected (FIC), corresponding to 76 fM and 1.2 nM of estradiol and bisphenol A, respectively.
Table 4. Number of revertants in the treat-and-wash as well as methylcellulose overlay assays generated by the highest concentrations (1.0 per g of food sample) of food extracts showing mutagenic potential on *Salmonella* TA 100 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food Product</th>
<th>Batch</th>
<th>Revertants per Gram</th>
<th>Treat-and-Wash Assay</th>
<th>Methylcellulose Overlay Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>−S9</td>
</tr>
<tr>
<td>Doughnut</td>
<td>1</td>
<td>115.0 ± 4.2</td>
<td>84.3 ± 8.7</td>
<td>215.0 ± 34.6</td>
</tr>
<tr>
<td>Chin-chin</td>
<td>1</td>
<td>192.7 ± 17.5</td>
<td>58.7 ± 7.8</td>
<td>128.3 ± 9.8</td>
</tr>
<tr>
<td>Hamburger</td>
<td>1</td>
<td>633.0 ± 23.3 *</td>
<td>124.7 ± 10.6</td>
<td>330.0 ± 10.4 *</td>
</tr>
<tr>
<td>French fries</td>
<td>1</td>
<td>121.3 ± 11.7</td>
<td>103.0 ± 1.4</td>
<td>84.3 ± 22.6</td>
</tr>
<tr>
<td>Peanut</td>
<td>1</td>
<td>108.0 ± 7.1</td>
<td>123.5 ± 13.4</td>
<td>182.3 ± 26.2</td>
</tr>
<tr>
<td>Suya</td>
<td>1</td>
<td>366.0 ± 22.6 *</td>
<td>113.0 ± 1.4</td>
<td>382.0 ± 17.2</td>
</tr>
<tr>
<td>Bean cake</td>
<td>1</td>
<td>736.3 ± 85.1 *</td>
<td>156.0 ± 13.9</td>
<td>401.0 ± 28.4</td>
</tr>
<tr>
<td>Chin-chin</td>
<td>2</td>
<td>618.7 ± 58.7 *</td>
<td>32.3 ± 12.5</td>
<td>126.0 ± 8.5</td>
</tr>
<tr>
<td>Hamburger</td>
<td>2</td>
<td>397.7 ± 21.2 *</td>
<td>408.0 ± 32.5 *</td>
<td>304.7 ± 19.4</td>
</tr>
<tr>
<td>Peanut</td>
<td>2</td>
<td>141.3 ± 23.3</td>
<td>42.0 ± 11.3</td>
<td>344.3 ± 31.8</td>
</tr>
<tr>
<td>Suya</td>
<td>2</td>
<td>181.0 ± 16.9</td>
<td>240.0 ± 42.4</td>
<td>165.0 ± 10.6</td>
</tr>
<tr>
<td>Fried chicken</td>
<td>2</td>
<td>174.3 ± 12.4</td>
<td>150.3 ± 12.8</td>
<td>192.3 ± 13.9</td>
</tr>
<tr>
<td>Bean cake</td>
<td>2</td>
<td>470.0 ± 16.3 *</td>
<td>260.7 ± 33.2 *</td>
<td>324.7 ± 28.6</td>
</tr>
<tr>
<td>Chin-chin</td>
<td>3</td>
<td>126.3 ± 12.0</td>
<td>131.7 ± 10.6</td>
<td>183.3 ± 9.9</td>
</tr>
<tr>
<td>Hamburger</td>
<td>3</td>
<td>135.0 ± 18.4</td>
<td>126.3 ± 9.2</td>
<td>166.7 ± 0.7</td>
</tr>
<tr>
<td>French fries</td>
<td>3</td>
<td>139.7 ± 6.8</td>
<td>97.0 ± 8.5</td>
<td>110.0 ± 3.5</td>
</tr>
<tr>
<td>Potato chips</td>
<td>3</td>
<td>194.7 ± 11.6</td>
<td>108.3 ± 9.4</td>
<td>90.7 ± 10.0</td>
</tr>
<tr>
<td>Suya</td>
<td>3</td>
<td>179.0 ± 9.9</td>
<td>209.0 ± 19.7 *</td>
<td>194.3 ± 7.8</td>
</tr>
<tr>
<td>Bean cake</td>
<td>3</td>
<td>371.3 ± 12.1 *</td>
<td>228.0 ± 23.3 *</td>
<td>267.7 ± 14.3</td>
</tr>
<tr>
<td>Fried chicken</td>
<td>3</td>
<td>117.7 ± 12.3</td>
<td>108.3 ± 11.4</td>
<td>158.0 ± 12.8</td>
</tr>
</tbody>
</table>

Note: *: Significantly different from control (p < 0.05).

Table 5. Number of revertants in the treat-and-wash as well as methylcellulose overlay assays generated by the highest concentrations (1.0 per g of food sample) of food extracts showing mutagenic potential on *Salmonella* TA 98 (mean ± SD) in the standard plate incorporation assay.

<table>
<thead>
<tr>
<th>Food Product</th>
<th>Batch</th>
<th>Revertants per Gram</th>
<th>Treat-and-Wash Assay</th>
<th>Methylcellulose Overlay Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>−S9</td>
</tr>
<tr>
<td>Suya</td>
<td>1</td>
<td>78.0 ± 8.5 *</td>
<td>17.0 ± 1.4</td>
<td>48.0 ± 4.8</td>
</tr>
<tr>
<td>Peanut</td>
<td>2</td>
<td>30.0 ± 8.5</td>
<td>158.0 ± 51.0 *</td>
<td>31.3 ± 5.0</td>
</tr>
<tr>
<td>Suya</td>
<td>2</td>
<td>33.3 ± 5.0</td>
<td>154.0 ± 0.0 *</td>
<td>29.3 ± 1.9</td>
</tr>
<tr>
<td>Potato chips</td>
<td>3</td>
<td>48.3 ± 7.2</td>
<td>21.7 ± 4.1</td>
<td>33.0 ± 2.4</td>
</tr>
<tr>
<td>Suya</td>
<td>3</td>
<td>42.0 ± 9.9</td>
<td>18.0 ± 3.1</td>
<td>39.7 ± 5.2</td>
</tr>
</tbody>
</table>

Note: *: Significantly different from control (p < 0.05).
3.6. Estrogenic Activity of Pure Water Sachets

The estrogenic activities of the 16 pure water samples investigated ranged from below LOD to 44.0 ng/L (median: 23.0 ng/L) estradiol equivalent (the amount of estradiol needed to bring about the same effect as the sample analyzed in an assay specific for estrogens) concentrations (EEQs). Five out of the 16 sachets produced luciferase activities greater than the LOD. The positive water samples were coded W1 to W5 (Table 6). W1 had the lowest value of 0.79 ng/L or 124.2 ng/L estradiol vs. bisphenol A equivalent concentrations, respectively. Concurrently, the highest values found (for sample W2) extended to 44.0 ng/L (estradiol equivalent) or 1000.8 ng/L (bisphenol A equivalent).

Table 6. Estradiol (EEQ) and bisphenol A (BPAEQ) equivalent concentrations of sachet water samples.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Water Samples</th>
<th>Sachet/Packaging Material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EEQs (ng/L)</td>
<td>BPAEQs (ng/L)</td>
</tr>
<tr>
<td>W1</td>
<td>0.79</td>
<td>124.2</td>
</tr>
<tr>
<td>W2</td>
<td>44.0</td>
<td>1000.8</td>
</tr>
<tr>
<td>W3</td>
<td>28.0</td>
<td>597.8</td>
</tr>
<tr>
<td>W4</td>
<td>23.0</td>
<td>442.8</td>
</tr>
<tr>
<td>W5</td>
<td>15.0</td>
<td>269.7</td>
</tr>
<tr>
<td>Median</td>
<td>23.0</td>
<td>443.0</td>
</tr>
<tr>
<td>Average</td>
<td>7.0</td>
<td>152.0</td>
</tr>
</tbody>
</table>

As an attempt to further trace the origins of the estrogenic activities observed, the sachets themselves were analyzed for possible leaching of estrogenic substances into the water. The packaging material of three out of the five positive samples did not generate any positive signal in the yeast-based assay. However, a feeble response was obtained from the packaging material of the two other samples (Table 6).
4. Discussion and Conclusions

Processed food items and bottled water are consumed in increasing quantities all over the world. Therefore, it is of utmost importance to ensure that in addition to their microbial safety, the products do not contain chemicals which might pose a toxicological risk to consumer health. A conceivable potential risk in this regard is the formation of genotoxic compounds during the processing of foodstuffs and leaching of food contact materials into food and water. Regular screening studies are necessary to verify that the methods used by food vendors are appropriate and sound also from this point of view. The present investigation aimed at exploring the current situation in Nigeria.

To the best of our knowledge, this is the first study on mutagenicity of food products from Africa. To compare our data with those of previous studies is challenging because of the paucity of published data on mixture effects combined with the wide variation in food types in different parts of the world. However, it is possible to compare foodstuffs based on the number of revertants their extracts generate in the Ames test and its derivatives, and we will utilize this approach.

Food processing methods as well as the sales of processed food items in Nigeria are poorly—if ever—regulated. Furthermore, it has been reported that the majority of Nigerians involved in food processing do not have formal training on food safety issues or related techniques [17,41]. This may bear on the present finding that the majority of food items (75%) investigated were mutagenic in the standard plate incorporation assay for at least one of the three batches when studied in Salmonella TA 100 strain. On the other hand, in Salmonella TA 98 strain only 25% of food extracts were found to yield a mutagenic response, possibly due to a weaker sensitivity of this strain compared with TA 100 or to the type of mutagens present.

The conventional Ames test outcome cannot, however, be taken at its face value in the case of food extracts as these may be sources of localized release of proteins, peptides or histidine itself onto the bacterial plates [2]. To prevent this potential misinterpretation of ostensible mutagenicity, “treat-and-wash” as well as methylcellulose overlay assays were performed for all samples eliciting a positive outcome in the conventional Ames test. The results of these complementary assays were consistent for some samples (bean cake, suya, hamburger, fried chicken and chin-chin), further reinforcing our initial findings with the Ames test. Extracts of bean cake and suya stood out from among the positive samples. All batches of bean cake exhibited mutagenic activity in the treat-and-wash assay with the Salmonella TA 100 strain, both in the presence and absence of S9 mix. One of these lots (batch 1) generated a conspicuously high number of revertants, almost five-fold its control (DMSO), with metabolic activation. A similar situation was observed with the MC overlay assay, as all bean cake samples were mutagenic in Salmonella TA 100 strain in the presence of S9 mix. Similarly, all batches of suya were consistently mutagenic in the treat-and-wash assay, with the Salmonella TA 100 strain, either in the presence or absence of S9 mix.

Bean cake is commonly consumed in different parts of Nigeria, irrespective of ethnicity, religion or social status. A probable explanation for the mutagenicity test results observed with extracts of bean cake is in the method of its processing. Bean cake is processed by deep-frying for several minutes. Deep-frying has previously been reported to result in the formation of mutagenic and genotoxic compounds in the final product [42]. Food vendors in Nigeria are also known to repeatedly reuse their frying oil, which is often already of questionable quality, for several days or weeks. This may have
contributed to the high number of revertants obtained with extracts of bean cake and fried chicken. Double heat-treatment of cooking oil has been shown to cause an increase in the genotoxic activity of food products [43,44]. During frying, cooking oil undergoes deterioration through various chemical and physical processes such as oxidation, polymerization, hydrolysis and cyclization, leading to the formation of both volatile and non-volatile undesirable by-products [43]. These derivatives are partially absorbed by the fried food, which thus becomes carcinogenic [45]. For example, the PAH compounds benzo[a]pyrene and benzo[a]anthracene are all well-known human carcinogens which have been detected in different types of cooking oil [45].

The positive mutagenicity test results obtained with suya were not unexpected. Suya is 100% beef, and it is a special type of delicacy, mainly consumed in Nigeria, irrespective of social status. All suya products are processed the same way: by charcoal-grilling. After processing, the products are left to be heated on the charcoal for several hours, until they are purchased. This processing method typically explains the reason for the mutagenicity test results obtained with extracts of suya in our study. The contamination of thermally treated high-protein foods, such as charcoal-grilled meat products, by PAHs and heterocyclic aromatic amines is well established [12,46–48]. The building up of PAHs in this case is due to their generation by direct pyrolysis of food fats and the direct deposition of PAHs from smoke produced through incomplete combustion of the thermal agent [16]. Heterocyclic aromatic amines, in turn, are formed through the condensation of creatine/creatine and the strecker degradation radicals (pyridines and pyrazines) generated from the reaction of sugars and amino acids during the Maillard reaction [49].

The present findings are worrisome, because meat-cooking habits have been linked with several forms of cancer [50–53]. In Argentina, for example, cooking meat at a high temperature and close to the cooking source has been linked with increased incidence of colorectal cancer [54]. This is also the case in Hawaii and the Netherlands [55,56]. More recently, a number of PAHs have been reported in different types of smoked meat in Serbia, Latvia and Sweden [14,57,58]. However, no nexus has been established in relation to increased incidence of cancer in these countries. In Nigeria, there is a paucity of data on the incidence of different cancer types, but the two major forms, breast and prostate cancers, may be increasing [59]. Both of them have been associated with meat-cooking habits [60].

Hamburger products have previously been reported as a major source of chemical food mutagens to consumers [38,61]. The results obtained in our study further reinforce this view, as two different lots of hamburgers examined were found to be mutagenic in all three assays in Salmonella TA 100 strain, with one of the lots (batch 2) being both directly and indirectly mutagenic in all three assays. Stavric et al. [61] previously reported hamburger products purchased in Ontario, Canada, to be mutagenic in a similar assay, but only with Salmonella TA 98 strain. The number of revertants generated in that study ranged from 63 to 1042 rev/g (average: 199 rev/g). This is in contrast to our study, in which hamburger products were only mutagenic with the Salmonella TA 100 strain, and not TA 98. In a recent study in Finland [2], the number of revertants generated with extracts of hamburger products were slightly lower than those obtained in this study, both with Salmonella TA 100 and 98 strains. Although these findings might seem to implicate the current cooking methods of hamburgers in Nigeria, the present outcome may not be entirely attributable to the processing methods. This is due to the fact that high levels of potassium bromate, a well-known mutagen and human carcinogen, have been detected in bread in different parts of Nigeria [62–64]. In one of these cases, Alli et al. [62]
found that even the lowest level of potassium bromate in their bread samples was over 150 times higher than the maximal permissible limit.

Overall, the mutagenicity test outcome of our study is in keeping with previously published data on food mutagenicity elsewhere [3,4,61], but somewhat at odds with a recent study published in Finland, where only 40% of the processed food items investigated showed mutagenic properties in the conventional Ames test [2]. In further contrast with the current findings, for most food varieties in the study by Omoruyi and Pohjanvirta [2], only a single batch proved positive. This may reflect more refined food processing techniques in Finland as compared with Nigeria.

In Nigeria, it is estimated that about 25% and 53% of people living in urban and rural areas, respectively, lack access to pure, portable water [65]. This is related to recent outbreaks of several water-borne diseases in major states of the country, specifically cholera [66,67]. It has prompted entrepreneurs to continuously establish water plants, in which pure water samples are mainly packaged in plastic sachets.

Our study demonstrates that pure water sachets may contain estrogen-like chemicals. Five of the 16 samples investigated were discovered to be estrogenic in our in vitro test system, with EEQs ranging from 0.79–44.0 ng/L. Both the frequency of positive samples and their concentrations were actually lower than we feared, considering that the proprietors of pure water sachet factories in Nigeria are principally entrepreneurs with little or no knowledge of water quality (microbiological, physicochemical or toxicological). There are two recent studies carried out in Europe in which estrogenic activity of water samples was assessed by a comparable in vitro yeast assay to that of ours. Pinto and Reali [68] analyzed mineral waters packed in polyethylene terephthalate (PET) bottles in Italy. The levels they detected varied from 0.03 through 23.1 ng/L (mode 9.5 ng/L) EEQs. Somewhat surprisingly, tap water made of either surface water or ground water contained approximately 15 ng/L EEQs. In another study, Wagner and Oehlmann [6] determined estrogenic activities in 20 major brands of bottled water in Germany. Twelve of these samples proved positive with the levels ranging from 2.64 to 75.2 ng EEQ/L (average 18.0 ng/L). Interestingly, in their material, the highest estrogenic activities were recorded for waters packaged in either non-reusable PET or reusable glass bottles, and even water packed in Tetra Pak™ bricks contained levels that were similar to those found in our study (14–44 ng/L). Thus, substances exhibiting estrogen-like activity are common in water samples in both industrialized and developing countries.

It is widely believed that the decline in male reproductive functions, increased incidence of different cancer types amongst young men and women and neurobehavioural diseases observed in the population of different countries may, at least partly, be attributable to exposure to estrogenic compounds, particularly during the intrauterine phase or during critical periods of postnatal development [23–25,69]. Studies in recent years have shown, for example, that the commonly used plasticizer, di(2-ethylhexyl)phthalate (DEHP), alters gene expression in rats and that, at appropriate concentrations, it alters the development of the central nervous system in the fetus [70]. Similarly, certain compounds, such as benzophenone used as food contact material, are reported to almost completely block the 17β-hydroxysteroid dehydrogenase type 3 enzymes that are required for testosterone synthesis [71].

The presence in or leaching into water samples of endocrine-disrupting chemicals is influenced by a number of factors such as storage conditions, exposure to sunlight and ambient temperature [72,73]. Unfortunately, the environmental conditions in Nigeria (abundant sunlight and high temperature) tend
to favor the migration of endocrine-disrupting chemicals from the packaging materials into water, as, for example, during transport of water containers. Therefore, sachets of water stored or transported in less appropriate conditions than our samples would be at risk of containing higher concentrations of estrogenic substances.

The bulk of dietary xenoestrogen exposure for adults has been proposed to emanate from dairy products, and total daily intake of estrogens has been estimated to be 80–100 ng [74]. Assuming an average daily water consumption of 3 L at Nigerian latitude [75], in the worst-case scenario based on our sample material, the intake from pure water sachets would double the estimated exposure. Hence, every effort should be taken to reduce the estrogen levels in these waters in the future.

In conclusion, the results obtained in our study show that both commercially processed food items and sachet-packed pure water sold in Nigeria, are sources of mutagen and estrogen-like chemicals, respectively. Although their concentrations are not alarming in the light of food and water analyses from other countries, measures should be taken to reduce them further and monitor their levels regularly. Since the number of samples examined here was relatively low, further survey studies are also warranted.

Acknowledgments

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Author Contributions

Iyekhoetin Matthew Omoruyi was involved in planning the study, conducting the research and writing the manuscript; Derek Ahamioje did the extraction of food and water samples; Raimo Pohjanvirta was involved in planning the study, supervising the research and writing the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References


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Estrogenic activity of wastewater, bottled waters and tap water in Finland as assessed by a yeast bio-reporter assay

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Abstract

Aims: Environmental pollutants appearing in wastewater, bottled mineral water, tap water, and bottled drinking water are potential, but yet poorly characterized, sources of human exposure to endocrine disrupting chemicals globally. Here, we investigated the current situation in the most densely populated region in Finland. Methods: Influent and effluent bi-monthly samples from a major wastewater treatment plant in Helsinki were obtained over a preceding 2-year period at two time-points (in 2011 and 2014). Equivalent samples from a household water purification plant (located in the same region) were also analyzed, together with various brands of bottled still and mineral water as well as tap water from residential buildings. Samples were obtained in one liter sterile containers, extracted by solid-phase extraction method, and their estrogenic potential determined by a yeast bioluminescent assay. Results: The estrogenic activities of influent samples from the wastewater treatment plant in Helsinki were generally low (from less than limit of detection to 0.7 ng/L estrogen equivalent quantities (EEQ)), except in March and August 2011, when relatively high levels (14.0 and 7.8 ng/L EEQ, respectively) were obtained. Meanwhile, no estrogenic activity was recorded in any of the treated effluent samples from the wastewater treatment plant, influent and effluent samples from the drinking water plant, as well as tap water, bottled still, and mineral waters. Conclusions: These findings indicate that the purification method applied in Helsinki wastewater treatment plant, activated sludge with mechanical, chemical and biological purification steps, is effective in reducing estrogenic activity, and that tap or bottled waters are not a significant source of these compounds to the population in this region.

Key Words: Endocrine-disrupting chemicals, wastewater, estrogenic activity, drinking water, tap water, mineral water, still water, bioassays

Introduction

In recent times, there has been an increased awareness of human exposure to exogenous estrogenic chemicals and an intensified debate on the effects of such exposure on humans over time. Examples of known xenoestrogens include bisphenol A, polyvinylchloride (PVC), di(2-ethylhexyl)phthalate (DEHP), polychlorinated biphenyls (PCBs), dioxin, and dioxin-like chemicals, etc. These chemicals have been reported in most of the daily products used by man, including food, food cans, plastic bottles, toys, liners of metals, and some pesticides [1,2].

Influent and effluent waters from wastewater treatment plants (WWTPs) have also been shown to be major potential sources of estrogenic substances to both terrestrial and aquatic environments. Table I gives a summary of specific endocrine-disrupting compounds (EDCs) reported in influent and effluent waters from WWTPs in different parts of the world [3–12].
The concentrations of EDCs in the environment are poorly documented globally, and are, indeed, a difficult task to undertake. However, the overall presence of these compounds in the environment is worrisome due to an increase in hormone-related disorders, especially in industrialized countries, and their shown linkages with EDC exposure [13]. From the point of view of risk assessment, EDCs present a particular challenge because they may have non-monotonous dose–response curves not adequately covered by conventional toxicological experimentation, and because of their capability of causing untoward impacts at environmentally prevailing low concentrations [14]. Although limited scientific information is available on the potential adverse human health effects of EDC exposure, concern arises because EDCs present in the environment at very low concentrations have been shown to have adverse effects in wildlife species as well as in laboratory animals [15–17]. The difficulty of assessing public health effects is magnified by the fact that people are typically exposed to multiple endocrine disruptors simultaneously.

In fish, the impacts of EDCs include altered sexual development, appearance of intersex individuals, and changed mating behavior. High incidence of intersexuality has been reported in roach and wall-eye populations in rivers receiving effluents from municipal WWTPs that contain estrogenic hormones [15]. Chronic exposure of fathead minnows
to environmentally relevant concentrations of EDCs in an experimental lake area resulted in the feminization of males with induced vitellogenin production; this led to a near wiping out of the species in the lake. Also, early exposure to ethinyl estradiol at a concentration of 9.86 ng/L resulted in diminished courting behavior of female zebrafish and reduced female reproductive success [16]. More recently, Marmuqi et al. [17] reported that long-term exposure of mice to bisphenol A during adulthood leads to hyperglycemia and hypercholesterolemia. On the other hand, in humans the evidence is less solid. Studies have reported lowered sperm count, declining male reproductive health and elevated incidence of breast cancer as an aftermath of increased exposure to EDCs [18]. In Finland, the incidence of testicular germ cell cancer (TGCC) is on the increase and is attributed to environmental factors such as EDCs [19]. Also, the use of postmenopausal hormone therapy drugs in Finland between 1995 and 2007 has been associated with an increased risk of primary fallopian tube carcinoma [20].

With compelling or emerging evidence of the impact of EDCs in wildlife and humans, respectively, it is of utmost importance to continuously evaluate the possible sources of human exposure to these chemicals. The current study was aimed at determining the current situation in the most densely populated region in Finland with respect to potential exposure to xenoestrogens.

Materials and methods

Sampling and description of study site

Treated and untreated water samples, taken bi-monthly, were obtained from the WWTPs in Viikinmäki, Helsinki, Finland, over preceding 20-month periods, on two occasions, in 2011 and 2014. Viikinmäki is the largest WWTP plant in Finland, serving over one million inhabitants, and processing both household (85%) and industrial (15%) wastewater from five different districts (Helsinki, Kerava, Tuusula, Järvenpää, Sipoo, and from the central and eastern districts of Vantaa) in Finland. It receives approximately 270,000 m³ of wastewater per day, and an average 100 million m³ each year. The influent samples were taken before any treatment of the wastewater and the effluent samples at the end of the purification process, right before discharge into the tunnel that leads to the Baltic Sea. At each time-point, both represent 24-hour stream-weighted aggregate samples of the same day.

Equivalent samples from a household water purification plant (located in the same region) were also obtained in March, April, and June of 2014. Crude water for the household water plant in Viikinmäki, Helsinki, comes from Lake Päijänne (located in central Finland) through a 120-km long tunnel (Figure 1).

Tap water (hot and cold) samples were collected twice a month both from the premises of the University of Helsinki, Viikki campus, Helsinki, and a residential building in Vantaa, Finland, over a 3-month (March–May) period in 2014. Ten different brands each of bottled still and mineral waters (Supplementary Table I) were purchased from a local grocery store (Prisma, Viikki, Helsinki).

Chemicals and medium

Estradiol, progesterone, and testosterone were purchased from Sigma-Aldrich (Steinheim, Germany). D-Luciferin was obtained from Biotherma (Handen, Sweden). Yeast nitrogen base medium without amino acids was obtained from Becton Dickinson (New Jersey, USA).

Microorganisms

Two recombinant yeast strains Saccharomyces cerevisiae BMAERE/ERα and S. cerevisiae BMA64/luc [21] were used in this study. BMAERE/ERα served as a reporter strain, in which the ERα is expressed. Upon ligand binding, the dimerized receptor binds to the estrogen response elements in the promoter region of the luc reporter gene. In S. cerevisiae BMA64/luc, luciferase is expressed constitutively, and this strain was used for determination of cytotoxicity of the test samples. Both yeast strains were gifted by Dr Johanna Rajasärkkä of the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki, Finland. Yeasts were grown on Difco Yeast Nitrogen Base medium without amino acids, supplemented with 40% glucose and their respective amino acids.

Sample preparation

Five-hundred milliliters each of all samples were extracted by solid phase extraction method as described by Kopperi et al. [22] using Phenomenex strata-X 33u polymeric reversed phase 500 mg/5 mL (Phenomenex, Aschaffenburg, Germany).

Bioassay

The yeast bioluminescent assay was performed as previously described [23]. Estradiol served as the positive control, while progesterone and testosterone
served as negative controls. The yeast bio-reporter assay offers a number of advantages in its robustness and ability to determine receptor activation without interference by endogenous receptors. This assay type has been compared and reported to be as effective as liquid-chromatography tandem mass spectrometry (LC-MS) in its ability to detect anabolic steroids in food supplements [24]. However, it fails to detect compounds that require metabolism to gain estrogenic activity, and exhibits only a modest response to estrogen antagonists.

Data analysis

The fold induction, fold induction corrected (FIC), and limit of detection (LOD) were calculated as described previously [21]. The sigmoidal dose–response curves for increasing concentrations of estradiol were obtained using the software program Prisma 4.0 (GraphPad software Inc. San Diego, CA). The estradiol equivalents of food samples showing estrogenic activity were calculated from probit transformation of the curves.

Results and discussion

Environmental pollutants from WWTPs and plasticizers leaching from, e.g., plastic water bottles are subjects of continued debate on human exposure to EDCs, foremost xenoestrogens, globally. This is so because of recent evidence of a decline in male sperm count, an increased incidence of different cancer types amongst young men and women, and neurobehavioral diseases observed in the populations of different countries. These phenomena have epidemiologically been associated with EDC exposure, particularly during the intrauterine phase or during critical periods of postnatal development [13]. Thus, our study was aimed at evaluating the current situation in the most densely populated region in Finland with respect to existence of substances possessing estrogenic activity in both influent...
and effluent samples from water treatment plants as well as in tap and bottled water.

The mean values of the two influent wastewater samples with detectable estrogenic activities are shown month-wise for both years (2011 and 2014) in Table II. The estrogenic activity level remained low, at approximately 0.5 ng/L EEQ, over this period, except for two months in 2011, March and August, when it peaked to 14 and 7.8 ng/L, respectively. The reason(s) for these peaks are currently unknown, but the latter might be linked to exceptionally high rainfall in August 2011 [25]. However, in all treated (effluent) wastewater samples discharged into the Baltic Sea the estrogenic activity was below the detection limit of our assay (data not shown).

In light of the data presented in the literature (Table I), the concentration rates found in the current study appear to be lower than those reported in other parts of the world, albeit one should be cautious in comparing data generated by different methods. Also, the majority of these studies only screened effluent water samples for possible estrogenic activity. Using a similar in vitro bio-reporter assay to that of ours in studying influent wastewater samples, Bellet et al. [10] reported estrogenic activity ranging from below detection limit to 25 ng/L EEQ in France, while a recent report revealed strikingly high levels (1136 ± 269 ng/L EEQ) in China [6]. Interestingly, in the latter study the value increased (1417 ± 320 ng/L EEQ) after primary treatment. In Spain, Germany, and China, the concentrations measured by mass spectrometry were also far higher than those determined in our study (Table I). All the cited studies also reported varying levels of EDCs in effluent samples in contrast to the case here. Recently, 75 effluent samples from 16 European countries, including Finland, were screened for possible estrogenic activity. In accordance with our data, the effluent water from the Viikinmäki WWTP contained estrogenic activity below 0.5 ng/L EEQ [26]. Moreover, this was also the case for effluent waters from all other Finnish WWTPs (five) tested.

The overall outcome of the present study implies that the treatment method (activated sludge with mechanical, chemical and biological purification) currently employed in Helsinki, Finland is effective in removing estrogenic compounds from wastewater during treatment. Activated sludge and/or upflow anaerobic sludge blanket reactor followed by chlorination steps have also previously been found to be effective in removing EDCs from wastewater [27].

Tap and bottled water have been reported to represent potential sources of human exposure to EDCs. There are two recent studies carried out in Europe in which estrogenic activity in this type of water samples was assessed by a comparable in vitro yeast assay to that of ours. Pinto and Reali [1] analyzed mineral waters packed in polyethylene terephthalate (PET) bottles in Italy. The levels they detected varied from 0.03 through 23.1 ng/L (mode 9.5 ng/L) EEQs. Somewhat surprisingly, tap water made of either surface water or ground water contained approximately 15 ng/L EEQs. In another study, Wagner and Oehlmann [28] determined estrogenic activities in 20 major brands of bottled water in Germany. Twelve of these samples proved positive with the levels ranging from 2.64 to 75.2 ng EEQ/L (average 18.0 ng/L). Interestingly, in their material the highest estrogenic activities were recorded for waters packaged in either non-reusable poly(ethylen terephthalate) or reusable glass bottles, and even water packed in Tetra Pak™ bricks contained high levels (14–44 ng/L). More recently, five sachet-packed drinking water samples (a third of the samples analyzed) from Nigeria were reported to be estrogenic, with estradiol equivalents ranging from 0.79 to 44.0 ng/L [29], further reinforcing the contention that exposure to EDCs via drinking water can be of concern in some parts of the world. Interestingly, in the present study estrogenic activity was neither found in any brand of bottled still or mineral water, nor in tap water, testifying to a high chemical quality of these products in Finland.

The negative estrogenic activity obtained in this study stems from legislation, good administration, extensive research, and follow-up approaches aimed at sustainable use of clean water resources in Finland. Efficient measures for water protection in Finland date back to early 1970s, and are based on long-term goals and proactive strategies [30]. The outcome of such concerted effort is the provision of high-quality drinking water and protection of the environment from wastewater discharges.

In conclusion, the treatment methods employed in Finland appear to be effective in reducing estrogenic activity during wastewater treatment. Drinking water, whether bottled or tap, does not pose a problem with respect to its xenoestrogen contamination.

Table II. Influent samples from Viikinmäki WWTP harboring estrogenic potential in 2011 and 2014.

<table>
<thead>
<tr>
<th>Month</th>
<th>2011 EEQ (ng/L)</th>
<th>2014 EEQ (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>February</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>March</td>
<td>14</td>
<td>0.5</td>
</tr>
<tr>
<td>June</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>August</td>
<td>7.8</td>
<td>0.6</td>
</tr>
<tr>
<td>September</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*The values represent means of two samples taken two weeks apart. Only those months in which measurable estrogenic activity was detected are listed.
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Conflict of interest

None declared.

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