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BioCop II – Monitoring chemical contaminants in foods

Guest Editors:
Chris Elliott and Doug Rutledge

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In vitro bioassays for the study of endocrine-disrupting food additives and contaminants

L. Connolly, E. Ropstad, S. Verhaegen

Endocrine-disrupting chemicals (EDCs) are capable of interfering with normal hormone homeostasis by acting on several targets and through a wide variety of mechanisms. Unwanted exposure to EDCs can lead to a wide spectrum of adverse health effects, especially when exposure is during critical windows of development. Feed and food are considered to be among the main routes of inadvertent exposure to EDCs, so there is an important need for efficient detection of EDCs in these matrices.

We describe in vitro bioassays that can complement current analytical chemistry in order to detect unwanted EDCs and describe their action, emphasizing assays that can measure effects on nuclear receptor signaling or hormone production. We outline both validated and unvalidated in vitro assays currently available in the scientific community for detecting and studying the effects of EDCs, and discuss their possible role in the food-safety context. We conclude by identifying gaps in the current battery of in vitro assays available for EDCs and suggest future possibilities for development and validation.

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Keywords: Endocrine disruptor; Food additive; Food contaminant; H295R; In vitro bioassay; Leydig; Mycotoxin; Nuclear receptor; Phytoestrogen; Reporter-gene assay

Abbreviations: 3β-HSD, 3β-Hydroxysteroid dehydrogenase; 17β-E2, 17β-Estradiol; 17β-HSD, 17β-Hydroxysteroid dehydrogenase; 17KSR, 17-Ketosteroid reductase; ACTH, Adrenocorticotropic hormone; AR, Androgen receptor; CALUX, Chemical-activated luciferase gene expression; CYP1A1, Cytochrome P450, family 1, subfamily A, polypeptide 1; CYP1B1, Cytochrome P450, family 1, subfamily B, polypeptide 1; CYP11A, Cytochrome P450, family 11, subfamily A; CYP11B1, Cytochrome P450, family 11, subfamily B, polypeptide 1; CYP11B2, Cytochrome P450, family 11, subfamily B, polypeptide 2; CYP17, Cytochrome P450, family 17; CYP17A1, Cytochrome P450, family 17, subfamily A, polypeptide 1; CYP19, Cytochrome P450, family 19; CYP19A1, Cytochrome P450, family 19, subfamily A, polypeptide 1; CYP21, Cytochrome P450, family 21; DHEAS, Dehydroepiandrosterone sulfate; DDT, Dichlorodiphenyltrichloroethane; EDC, Endocrine-disrupting chemical; EDSP, Endocrine Disruptor Screening Program; EPA, Environmental Protection Agency; ER, Estrogen receptor; GnRH, Gonadotropin-releasing hormone; hERα, Human estrogen receptor alpha; hERβ, Human estrogen receptor beta; HMGR, 3-hydroxy-3-methyl-glutaryl-coA reductase; HPA, Hypothalamus-pituitary-adrenal; HPG, Hypothalamus-pituitary-gonadal; HPT, Hypothalamus-pituitary-thyroid; LC-MS2, Liquid chromatography tandem mass spectrometry; LH, Luteinizing hormone; MC2R, Melanocortin 2 receptor (adrenocorticotrophic hormone); MeSO4 DDE, Methylsulfone-dichlorodiphenyldichloroethylene; P4, Progesterone; REP, Relative estrogenic potency; RGA, Reporter-gene assay; SERM, Selective estrogen receptor modulator; StAR, Steroidogenic acute regulatory protein

1. Introduction

Many chemicals are used for the improvements seen in modern day living. However, along with these improvements come problems. Some of these chemicals may enter our food chain and bodies. Food contaminants can have various acute biological effects (e.g., irritation, asphyxiation, narcotic, and sensitization). They can lead to genotoxicity, mutagenicity, or direct cytotoxicity at a cellular level [1]. In
addition, some contaminants lead to adverse systemic effects by interfering with normal hormone homeostasis. Such compounds are usually referred to as endocrine-disrupting chemicals (EDCs).

The term EDC was initially applied to chemicals that mimic or block the natural transcriptional activation of circulating steroid hormones by binding to their nuclear receptors. However, it is becoming increasingly clear that additional levels and modes of action exist [2]. EDCs have been linked with adverse effects on development, reproduction and fertility, and on the nervous and immune systems in human and wildlife populations. In addition, inadvertent exposure to EDCs during critical windows of development (e.g., perinatal and puberty) may be associated with chronic disease (e.g., metabolic syndrome or cardiovascular disease) and animal studies have indicated epigenetic alterations that can lead to potential transgenerational effects [3].

The origin and the fate of these contaminants can lead to transmission into the food chain [4]. It is widely accepted that food and diet are among the most important exposure routes for EDCs. There are many types of EDCs in food, ranging from natural compounds (e.g., hormones, phytoestrogens and mycotoxins) to synthetic compounds (e.g., pesticides, pharmaceuticals and industrial or process chemicals). New and unknown EDCs are being introduced to the market every day. Recent examples that have been of emerging concern include phthalates [5], parabens [6], and bisphenol A [7].

EDCs can be added to food deliberately or inadvertently. For example, bovine growth hormone (BGH) can be produced synthetically and may be legally administered to dairy cows in the USA to increase milk yield, resulting in concerns of side effects through drinking milk from these cows [8]. Alternatively, EDCs can inadvertently enter food by migration of chemicals from plastics packaging to food [9].

Detection of EDC food contaminants is a continual challenge, which traditionally has been carried out mainly by analytical chemistry [10]. However, despite the rapid improvements in analytical chemistry, these methods may not be able to deal with the ever-changing chemical structures of EDCs entering our food chain. Another concern is that, while some of these EDCs have been assessed as relatively safe to consume at low levels individually, they may combine with other low-level EDCs to create low-level cocktail or mixture effects [11–13].

Considering these points, the most appropriate way of detecting and studying the effects of EDCs and their mixtures may be through the use of bioassay systems that use their natural ligands and pathways. This can be achieved through animal bioassays or, preferably, in vitro bioassays that have the benefit of closely-related natural systems without use of animal testing and can detect compounds based on their effects. While in vitro bioassays cannot assess behavioral effects, they do give quick results, reduce animal testing and cost less than in vivo assays.

Recent advances in cell biology and biotechnology have allowed development of a new generation of bioassays focused on hormonal nuclear receptor signaling [14–18] and steroidogenesis pathways [11,19–25]. Some of these new bioassays are based on the ability to introduce specific properties and reporter genes into stable cellular systems. Others were developed through characterization of their ability to complete hormone-production pathways presenting the possibility of testing of compounds and their mixtures to reveal toxicogenomic effects. The inclusion of these new assays in modern test strategies will allow rapid screening and detection of both known and new or unknown EDC compounds [26]. These assays will also help to evaluate the possible health hazards involved with such compounds and their mixtures in the food chain [27].

The balance between benefits and threats in the risk assessment of foodstuffs is an ongoing concern for the scientific community and a permanent burden for policy-makers [28]. The debate about the cardiovascular protective effect of a fish diet and especially of its n-3 unsaturated long-chain acids in contrast with its contaminant contents is a clear example. This debate was fueled by two successive reports from Hites and co-workers of organic contaminants in salmon (Salmon salar) [29,30].

The use of substances having a hormonal action for growth promotion has been prohibited under European Union (EU) legislation [31]. According to EU national plan surveillance schemes, food products derived from animals must be analyzed for the presence of a number of specified compounds [32]. The chemical-activated luciferase gene expression (CALUX) bioassay was the first bioassay to be validated for food applications, the determination of dioxins and PCBs in bovine milk [15]. The new RIKILT yeast androgen bioassay (RAA) [18] is the first bioassay that has been fully validated according to the international criteria put forward in EC Decision 2002/657 [33]. Although a complete overview of assay validation and/or approval for food testing is outside the scope of this review, the interested reader can find up-to-date information about assay-validation status in the Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) [34].

This review describes in vitro bioassays that can supplement analytical-chemistry approaches to detecting EDCs in food matrices and to analyzing their effects, with special emphasis on their occurrence as complex mixtures. Given the wide scope of potential working mechanisms and sites of action of EDCs, the review focuses on those bioassays measuring interference of EDCs with nuclear receptor signaling (see sub-section 2.1) or...
Table 1. Summary of the in vitro assays discussed, including their advantages and disadvantages for measuring the effects of endocrine-disrupting compound (EDC) contaminants in food with examples of applications to food and validation details, where available

<table>
<thead>
<tr>
<th>Assay type</th>
<th>EDC effect</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Application to food safety (examples)</th>
<th>Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor binding</td>
<td>Binding to receptor</td>
<td>High throughput Easy to perform</td>
<td>Cannot determine activation of the receptor May suffer from cross-talk</td>
<td>Detection of illegal growth promoters in the meat-producing industry [37,38]</td>
<td>No</td>
</tr>
<tr>
<td>Mammalian reporter gene</td>
<td>Agonism/antagonism of receptors</td>
<td>Receptor activation can be determined Agonism/antagonism can be distinguished More sensitive than yeast-reporter-gene assays May identify compounds that require metabolism for identification</td>
<td>Less robust than yeast-reporter-gene assays May suffer from cross-talk</td>
<td>Detection of dioxins and PCBs in milk [15] Detection of glucocorticoids in bovine urine [26] Detection of dioxins and dioxin-like contaminants in milk, meat and fishery products [55]</td>
<td>[15] First bioassay to be validated for food and drink</td>
</tr>
<tr>
<td>Yeast-reporter gene</td>
<td>Agonism/antagonism of receptors</td>
<td>Receptor activation can be determined More robust than mammalian reporter-gene assays Do not have endogenous receptors, no cross-talk</td>
<td>Less sensitive than mammalian reporter-gene assays Cannot identify compounds that require metabolism for identification Modest responses to estrogen antagonism</td>
<td>Detection of androgenic activity in calf urine and animal feed [33] Detection of anabolic steroids in food supplements [60]</td>
<td>[33] Validated to international standards put forward in EC Decision 2002/657</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>Proliferation of cells</td>
<td>High throughput Simple read-outs</td>
<td>Expression of other receptors that can lead to non-specific induction of proliferation Test sample must not contain substances that can bind to other receptors Relatively slow Not the best bioassay of choice for food analysis</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Transcriptomics</td>
<td>Gene expression</td>
<td>Determination of transcriptomic fingerprint profiles Assessment of compounds with unknown modes of action for EDC-marker genes</td>
<td>Difference in gene expression does not necessarily translate into differences in protein quantity or activity</td>
<td>Phytoestrogens [67] Mycotoxins [68] Phytoestrogens in milk, cereals and baby food [69]</td>
<td>[69] Validated to international standards put forward in EC Decision 2002/657</td>
</tr>
<tr>
<td>Steroidogenesis – H295R</td>
<td>Hormone production</td>
<td>Human in vitro model Continuous cell line Capable of full steroidogenesis</td>
<td>Optimal responses during a limited number of passages only Not responsive to physiological stimulation with ACTH (stimulation can be mimicked with forskolin)</td>
<td>Has been applied to known food contaminants, either as single compound [20,23,24], or as mixtures [11,25] The authors have applied the assay to natural mixtures of POPs derived from fish liver (burbot, cod) [manuscripts in preparation] The model has also been used for proteome studies with selected EDCs [77] and mycotoxins</td>
<td>[34] for production of 17β-E2 and testosterone The authors also routinely measured P4, cortisol, and aldosterone and quantify expression of selected genes</td>
</tr>
</tbody>
</table>

(continued on next page)
hormone production (see sub-section 2.2). These mechanisms are very complementary and the bioassays and their use very well documented. We give attention to assays validated or being validated by control organizations. However, we also describe existing non-validated assays, or assays in use or being developed in experimental settings.

Table 1 summarizes the assay types discussed, as well as their advantages and disadvantages, together with their application to food and their validation status.

2. Discussion

As indicated, EDCs interfere with targets at different levels of the endocrine system through different modes of action. In this section, we emphasize in vitro assays that are capable of measuring agonistic or antagonistic effects of EDCs on nuclear receptor signaling (2.1) or hormone production (2.2).

2.1. Endocrine disruption at nuclear receptor signaling

Many different types of in vitro bioassays based on nuclear receptors have been produced to date, and they were extensively reviewed recently alongside in vivo bioassays [35,36]. As illustrated in Fig. 1, these in vitro bioassays vary from receptor-binding assays to receptor-dependent gene-expression assays and cell-proliferation assays. Each in vitro bioassay has advantages and disadvantages with regards to detection and study of EDCs or their mixtures. Many have been used in the detection and study of EDCs in food.

2.1.1. Receptor-binding assays. In vitro receptor-binding assays, including competitive binding assays, are suitable for detection or screening purposes because they are high throughput and easy to perform. However, they only determine binding to the receptor and not activation of the receptor. As a result, they cannot distinguish between receptor agonists and antagonists. Another problem is that competitive binding assays have mainly been performed using radioactive ligands and receptors tediously prepared and harvested from animal tissue [37]. Competitive binding assays that use cytosol preparations can suffer from cross-talk originating from the many other nuclear receptors and proteins present in the cellular homogenate. However, binding assays using recombinant receptor proteins have overcome both cross-talk and harvesting problems. For example, recombinant soluble receptors harvested from bacteria were recently reported for use in detection of illegal growth promoters with androgenic, estrogenic, progestagenic and glucocorticoid activity [38]. The desire to move these types of assays towards fluorescence-based systems remains largely unaccomplished.
2.1.2. Reporter-gene assays. Reporter-gene assays (RGAs) can be produced by transfecting cell lines with relevant receptors and incorporating a transactivation step with a signaling protein (e.g., luciferase). In contrast to receptor-binding assays, receptor activation can be measured through the signaling protein, so RGAs can distinguish between receptor agonists and receptor antagonists [14,39,40]. RGAs can provide one of the most specific, sensitive and biologically-relevant means to screen substances for their hormonal effects [41]. Several RGAs have been developed using both mammalian and yeast cells.

2.1.2.1. Mammalian cell-line RGAs. Numerous transfected mammalian cell lines have been produced primarily to determine the hormonal mechanisms and activity of compounds [39–46]. Examples include cell lines with endogenous receptors that required only addition of a reporter-gene construct (e.g., the estrogen sensitive MMV-Luc assay based on the MCF-7 breast-carcinoma cell line [39] and the estrogen-sensitive estrogen-receptor (ER)-CALUX bioassay based on the T47-D human breast-carcinoma cell line [43]). An important disadvantage of this assay type is the potential presence of endogenous receptors that can interfere with the specific response of the cells. Other factors include the co-expression of human estrogen-receptor alpha (hERα) and human estrogen receptor beta (hERβ), which, when co-expressed, will have a great influence on how cells will respond to estrogens [17]. However, other cell lines (e.g., the U2-OS human osteosarcoma), which lack steroid receptors, have been transfected with the androgen receptor (AR) and ERα to produce newer forms of CALUX assays designed to overcome this cross-talk issue [46].

Mammalian RGAs can identify compounds that require metabolism for activation into their active state [40,43,47]. This ability to metabolize compounds has led to varying results in studies on the relative estrogenic potency (REP) of estrone and estriol in comparison to 17β-estradiol (17β-E2). However, conversion of estrone to 17β-E2 and vice versa was confirmed as the reason [47,48]. Differences have also been highlighted between the estrogenic and anti-estrogenic responses in the ER-CALUX assay and the YES recombinant yeast assay [49]. However, variability of agonistic and antagonistic effects of tamoxifen and other related compounds in different breast-cell lines has also been reported. Such studies have shown that compounds including tamoxifen are not pure anti-estrogenic compounds but selective estrogen-receptor modulators (SERMs) [50].
Overall mammalian-based RGAs have been shown to be more sensitive than yeast-based assays [49,51,52]. Consequently, a number of these assays have been utilized to detect EDCs in various matrices including water, sediment, biological samples and food [26,39,41,49]. The DR-CALUX assay, specific for dioxins and dioxin-like contaminants [53], has been used in food-safety applications to track contamination sources of the food chain [54] and screening food [55] and milk samples [15].

2.1.2.2. Yeast cell-line RGAs. There have been many yeast bioassays developed to date with the best-known ones being the YES screen [56] and the yeast-estrogen bioassay [57]. Both these well-known assays use yeast cells that express the hERβ upstream of a β-galactosidase-reporter gene. The major difference between these assays is that the hERβ is continuously expressed in the YES assay while hERβ expression must be induced in the yeast-estrogen bioassay. Yeast reporter-gene assays have also been developed to detect androgens and include the well-known yeast-androgen bioassay [58].

To date, most yeast bioassays have utilized β-galactosidase as the reporter protein. However, more recent improvements have been reported by the RIKILT yeast estrogen bioassay (REA), stably expressing the yeast-enhanced green fluorescent protein (yEGFP) and the human α receptor and β receptor [16,17]. These studies report yEGFP as an easier reporter gene to measure than the previously used β-galactosidase. The same group has also developed the new RIKILT yeast androgen bioassay (RAA) [18].

Yeast cells are unable to identify compounds that require metabolism for identification. Yeast RGAs also show modest responses upon exposure to anti-estrogens [59]. As stated in sub-section 2.1.2.1., yeast RGAs are also less sensitive than mammalian cells. However, yeast assays do have several other advantages for consideration, as they can be run relatively cheaply, are easy to handle, present a lack of endogenous receptors and do not require the preparation of steroid-free media. Probably the biggest advantage attributed to yeast cells is that they are extremely robust and can survive extracts from dirty sample matrices (e.g., urine and feed) [33]. A yeast-androgen RGA was recently compared with liquid-chromatography tandem mass spectrometry (LC-MS²) detection of anabolic steroids in food supplements [60]. The yeast bioassay was able to detect all positives detected by LC-MS² and two further positive samples that had been confirmed as negative by LC-MS². However, the two further positives contained EDCs that were not in the range of the LC-MS² method.

2.1.3. Cell-proliferation assays. An example of a cell proliferation assay (CPA) is the E-screen [61], which uses the ER-positive estrogen-responsive MCF-7 human breast-cancer cell line that shows increased proliferation upon exposure to estrogen agonists. However, MCF-7 cells also express other receptors, including the androgen, progesterone, glucocorticoid and retinoid receptors.

Substances that can bind to these exogenous receptors can antagonize estrogen-induced cell proliferation. As a result, the assay is unsuitable for testing compounds or extracts containing these other substances. The A-screen is an androgen-responsive CPA based on the MCF-7 cell line transfected with the human-androgen receptor [62]. The T-screen is another CPA used to detect binding and activation of the thyroid receptor (TR), thereby determining the ability of a compound to be a thyroid hormone receptor agonist or antagonist [20,63–65]. These assays are limited in that only effects related to binding can be detected but not effects linked to the disturbance of hormone-synthesis pathways or hormone transport. A further disadvantage of CPAs is that they are relatively slow in producing results, as responses occur only after a few days [66].

2.1.4. Transcriptomics. Recently, DNA micro-array technology has been utilized to profile estrogenic transcriptomic “fingerprints” generated by EDCs, including phytoestrogens [67] and mycotoxins [68]. The same DNA micro-array system has also been combined with LC-MS² to measure and to assess estrogenic activity of phytoestrogens in weaning foods [69]. hERα-positive Ishikawa plus and hERα-negative Ishikawa minus endometrial cancer cells have also been used to generate transcriptomic “fingerprints” of well-known EDCs [70]. The identified sub-sets of putative marker genes can be used for screening chemicals with unknown modes of action and assessing their potential for endocrine-disrupting effects.

2.2. Endocrine disruption at hormone-production level

EDCs can potentially interfere with each level of the hypothalamus-pituitary-gonadal (HPG) or hypothalamus-pituitary-thyroid (HPA) axis, and it is conceivable that exposure to mixtures of EDCs can lead to effects on multiple targets and with different modes of action. Thus, ideally, one could envisage a battery of in vitro models covering each of these levels. However, at present, a limited number of such models has been established, and, of these, a very small number has been validated for EDC screening by safety agencies.

Regarding models for steroidogenesis, EPA’s Endocrine Disruptor Screening Program considered the mouse Leydig cell-tumor line (MA-10), the rat Leydig cell-tumor line (R2C), and a human adrenocortical carcinoma-cell line (H295R), as well as an organotypic model based on sliced testes. However, development of the latter was discontinued at the prevalidation phase, due to concerns about the ability of the assay to discriminate between effects of cytotoxicity and direct effects of steroidogenesis.
The mouse and rat Leydig tumor-cell lines suffer by lacking a critical enzyme, 17-ketosteroid reductase (17KSR), so they can identify only substances that interfere with the first half of the steroid-synthesis pathway.

H295R offers advantages over the Leydig tumor-cell lines because it possesses all of the enzymes in the steroid-synthesis pathway. Hence, it can be used for identifying substances that modulate the entire steroidogenesis pathway from cholesterol recruitment by StAR protein to conversion of androgens to estrogens [34]. In addition, the human origin avoids interspecies extrapolation issues when used in the context of human risk assessment.

2.2.1. H295R adrenal steroidogenesis model. The H295R cells are human adrenocortical carcinoma cells that have characteristics of fetal adrenal stem cells. The cell line is capable of full steroidogenesis, and produces glucocorticoids, mineralocorticoids and steroid hormones (Fig. 2). The model has been characterized in detail and has been the subject of an interlaboratory validation under the Organization for Economic Cooperation and Development (OECD) and EPA guidance to assess effects of potential endocrine disrupters on 17β-E2 and testosterone production [34].

It was recently used to study the effects of nutrition-relevant mixtures of phytoestrogens. A mixture of all tested phytoestrogens (PEs) increased 17β-E2 production and decreased testosterone production, indicating increased aromatase activity [25]. In addition, it has been used to study androgenic effects of a mixture of three azole fungicides [11], and endocrine-disrupting effects of nitrate and nitrite [20] and parabens [63].

In addition, the H295R model was used to evaluate modulation of steroidogenic hormone production by single polychlorinated biphenyl (PCB) congeners [23], emerging pollutants (e.g., perfluorinated compounds) [24], and pharmaceuticals [19,71]. It was also deployed to look at effects of estrogenic mycotoxin, zearalenone, and the metabolites, α- and β-zearalenol. These are potential feed and food contaminants associated with estrogenic effects in farm animals and humans. The most striking observation was the increased progesterone production seen with β-zearalenol [72].

In addition to hormone levels, expression levels can be monitored for about 15 genes coding for enzymes directly involved in steroidogenesis (e.g., STAR, CYP11A1, CYP19A1), nuclear transcription factors associated with these genes (NR5A1, NR0B1), hormone receptor genes (MC2R), and genes for CYP1-metabolizing enzymes (e.g., CYP1A1, CYP1B1).

Montaño et al. (manuscript in preparation) recently used the H295R model in exposure studies with POP extracts obtained from raw and processed commercially available cod-liver oil. Cod-liver oil contributes 33% of

![Steroidogenesis pathways in the human H295R adrenal model.](figure2.png)

Figure 2. Steroidogenesis pathways in the human H295R adrenal model. Hormones indicated by the dark boxes are routinely measured in the author’s laboratory using commercial radioimmunoassay (RIA) kits. Gene expression for the genes indicated is measured using quantitative reverse transcriptase PCR (qRT-PCR) (modified from [21]).
total dietary intake of very-long-chain n-3 fatty acids, and is an important source of vitamin A and D during the winter period in northern countries. Half the population in a Norwegian coastal municipality still consumes crude oil/liver \( \geq 2-3 \) times per month, while 59% of pregnant women take processed cod-liver-oil supplements daily [73]. Nonetheless, like salmon, cod (Gadus sp.) is also a carnivorous fish that feeds high in the food web and tends to bioaccumulate POPs capable of modulating or disrupting the endocrine system [74,75]. Liver-oil POP concentrations were at the same levels as those presented by Hites and co-workers, for both organochlorine contaminants [29] and polybrominated biphenyl ethers (PBDEs) [30]. Initial results suggest the system is sensitive enough to pick up potential endocrine-disrupting effects of food contaminants at environmentally-relevant concentrations.

In addition, the H295R model can be used to measure the effects of EDCs on the activity of enzymes involved in hormone production {e.g., Aromatase/CYP19 activity [22] or CYP17 activity [76]}. Moreover, the model can be exploited for proteome studies following EDC exposure [77,78].

One drawback of the H295R model is the loss of response of the cell line to natural adenocorticotropic hormone (ACTH), the physiological stimulator of adrenal steroidogenesis. Since the hormonal context of a system might affect its outcome under endocrine-disrupting conditions, the H295R model cannot be used to mimic conditions of steroidogenesis regulated by ACTH stimulation. One solution is to stimulate the cells with forskolin, a known cAMP agonist. This mimics hormone stimulation, albeit in the absence of hormone-receptor specificity. This version of the assay was recently used to

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**Figure 3.** Isolation and purification work-flow for primary porcine Leydig cells. In short, testis are obtained form routine castrations and transported to the laboratory on medium. The epydidimis is removed and, following decapsulation, the parenchyma is minced and enzymatically digested. The digested cellular material is sedimented at unit gravity to remove the bulk of tubule material. The supernatant is concentrated and put on a discontinous Percoll gradient (21/26/34/60%). After centrifugation, purified Leydig cells are obtained from the 34% layer, washed and plated [80]. These cultures can then be exposed to the test compounds. Estradiol and testosterone produced in the culture medium can be quantified. In addition, cells can be collected for RNA extraction, which is further submitted to cDNA conversion and qRT-PCR.
study effects of dietary chromium(III) picolinate on production of cortisol and dehydroepiandrosterone sulfate (DHEAS) [79]. In addition, adding exogenous hormones [e.g., 17β-E2 or progesterone (P4)] to the assay can be used to mimic different hormonal environments (e.g., different phases of the menstrual cycle or women using anti-conception drugs) [71].

2.2.2. Steroidogenesis in primary porcine Leydig cells. Recently, another model for steroid production was established by generating primary cultures of purified porcine Leydig cells. Testicles obtained from routine castrations of 8–10-day-old piglets were enzymatically digested, and Leydig cells isolated and purified using a discontinuous Percoll gradient [80]. Fig. 3 shows the isolation and purification workflow. These Leydig cells are then cultured in a standardized medium and can be exposed to various biochemical and physiological stimulators of hormone production (e.g., forskolin and luteinizing hormone (LH)). Recent experiments have shown excellent dose-response relationships for the production of testosterone, estradiol, and progesterone, under these stimulated conditions [81]. In addition, primers for steroidogenesis-related genes have been designed and validated for qRT-PCR on mRNA isolated from these exposures.

This makes it an excellent model to investigate the direct effects of EDCs on steroidogenesis in Leydig cells, with respect to both production of hormones and quantitation of expression of genes coding for enzymes involved in steroidogenesis. This model was used to investigate the effect of natural marine POP mixtures, as described in sub-section 2.2.1 on hormone production and gene expression in both the unstimulated and LH-stimulated versions of the model [82]. In addition, it was employed to investigate DDT metabolites (o,p-DDD, MeSO4-DDE, and bis-MeSO4-DDE) [83,84]. This shows that the assay is responsive to potential food contaminants (e.g., POPs and their metabolites). Interestingly, opposite effects have been noted with certain compounds when tested in the unstimulated compared to the LH-stimulated version. This indicates that the hormonal environment present in the assay can modulate the outcome, and this should be taken into consideration in risk assessment. It is conceivable that the non-stimulated condition might model windows of low LH during development (e.g., pre-pubertal), whereas LH stimulation might reflect pubertal or adult stages.

2.2.3. In vitro models for other elements of the hormonal axes. Regarding the HPG axis, existing hypothalamic models include the gonadotropin-releasing hormone (GnRH)-producing GT1-7 cell line. This immortalized murine hypothalamic cell line has been widely used to investigate the regulatory mechanisms to GnRH expression, including steroid hormones involved in feedback loops. Recently, it has been shown that 17β-E2 directly regulates adrenergic receptors and the kisspeptin/GPR54 system in GT1-7 neurons [85]. In addition, a high-throughput GnRH-receptor RGA has been developed [86].

At the pituitary level, two models could be useful. LbetaT2 immortalized mouse-gonadotrope cells show basal and GnRH-stimulated levels of LH [87]. Thus, this model would allow testing of the effect on LH production in pituitary gonadotropes by EDC, under unstimulated or GnRH-stimulated conditions. Secondly, the murine AtT20 pituitary-corticotropin cells produce ACTH [88]. This model could allow investigation of the modulation of ACTH production by EDC. However, neither of these models has been validated or deployed in a food-safety context.

Gonadal models include the porcine Leydig cell model described above. Gregoraszczuk et al. has developed an equivalent in vitro model for the female-pig gonad, based on primary cultures of theca and granulosa cells derived from ovaries of slaughtered adult pigs. This model was used for, amongst others, mechanistic studies on natural POP mixtures derived from lake burbot and from Atlantic cod-liver oil [89,90].

3. Conclusion

3.1. Current state of the art and future challenges
Bio-activity-based assays have been used to detect and to study EDC activity in the environment and food chain. These bioassays include both mammalian and yeast-cell-based assays that vary from receptor-binding assays to receptor-dependent gene-expression assays, CPAs and hormone-production assays. Such bioassays present a very cost-effective tool for screening EDC activity in food and present the potential to detect low-level cocktails, and unknown and new compounds that may remain undetected by traditional analytical-chemistry screening. A disadvantage of bioassays, compared to analytical-chemistry methods, is that they cannot establish the identity of a compound without analytical-chemistry confirmation. However, in food-safety applications, most samples are expected to be compliant, so bioassays can be used effectively as screening tools where their purpose will be to identify the few samples that require additional chemical confirmation. Consequently, a number of these bioassays have been validated for food-analysis applications (see Table 1).

Table 1 summarizes the advantages and the limitations of in vitro bioassays, which can be considered in their application to food analysis. The choice of bioassay does not depend on food type but rather on the research purpose. Receptor-binding assays are suitable in high-throughput applications, where the search for
endocrine-disrupting food contaminants is limited to agonists (e.g., illegal growth promoters). CPAs are not usually the bioassay of choice for food analysis due to their relatively slow throughput. However, they are useful in experimental studies on effects of defined single compounds or mixtures. It has also become apparent that additional levels and modes of endocrine disruption exist. In this respect, steroidogenesis assays are important for looking at disruption effects on the hormone-synthesis pathways but will not uncover modes of action.

Transcriptomic assays examine the effects of compounds on the entire machinery of a cell. This holistic approach can uncover EDC-marker genes in screening foods for compounds with unknown modes of action. This emergent technology is gaining interest in the area of food safety but requires standardization and development of robust, user-friendly systems.

Yeast and mammalian RGAs have been the main assay of choice for food analysis to date, due to their high throughput and determination of receptor activation. In the case of assessing biological effects, yeast cells cannot be considered representative of higher vertebrates, because they lack the mammalian cell-interconnected hormonal-system networks that influence responses. However, in food-safety applications, yeast assays are an equally good alternative to mammalian-cell bioassays, due to their ease of use, lack of endogenous receptors and high-throughput potential. Despite their lower sensitivity, yeast cells generally are sufficiently effective for routine application in food-contaminant screening. The fact that yeast-based bioassays do not metabolize compounds may help avoid discrepancies in reported potencies, as in the E-screen, and their insensitivity to antagonist activity may lower the chance of false negatives. Mammalian-cell bioassays may be less robust in a dirty-matrix sample and suffer from cross-talk between different types of hormonal activity. However, mammalian-cell bioassays are much more sensitive than yeast-cell bioassays and improvements in their limiting issues (e.g., robustness and cross-talk) are progressing. Consequently, both yeast and mammalian bioassays have their merits and deserve further exploration for their potential applications in food safety.

There is a wide variety of food matrices in which EDCs may be present [4] and in vitro analysis greatly depends on the sample preparation. Many EDCs are found in food at very low concentrations and some foods contain interfering matrix elements that can mask or interfere with analysis of the sample of interest. There is therefore a requirement for development of a sample-preparation method that can extract, concentrate and clean up samples of interest. Common methods for sample preparation include organic-solvent extraction, centrifugation and solid-phase extraction or their variations [15,33,55,60,68,69]. The sample extracts must be prepared in solution for application to the in vitro bioassay. Direct water sample or extract reconstituted in organic solvents (e.g., methanol or dimethylsulfoxide) and diluted in bioassay media are the most commonly-used protocols. However, the solvent of choice needs to be compatible with the cell system, not itself causing any effect, but enabling distribution of the extracted compounds to the cells.

Measurements of cell viability and cytotoxicity are also essential in all bioassays. Extracts can be cytotoxic due to compounds co-extracted from the matrix. For example, feed and urine are notoriously considered dirty-matrix samples with toxic components and require the development of competent extraction methods. The fact that yeast cells are more robust than mammalian cells makes them potentially more suitable for the analysis of dirty-matrix samples. However, improved sample-preparation techniques have overcome many of these problems. Sample-preparation clean-up methods combined with analytical separation techniques have been used in mammalian RGAs, enabling detection of illegally administered growth hormones and their low-level cocktails in bovine urine [26].

Further improvements in bioassays are ongoing. More stable reporting proteins have been applied to yeast estrogen-based assays [16]. Mammalian cell lines lacking endogenous receptors have been transfected with single-receptor systems avoiding cross-talk [46]. In the context of detecting new and unknown compounds, a mixed approach involving bioassay screening and analytical confirmation has proved efficient for food-contaminant analysis in the case of androgen contamination of dietary supplements [60]. However, other problems remain to be overcome in the future. Agonists or antagonists that require metabolic activation for conversion to a bioactive state (prohormones) may not be detected. Bioassays that can detect such contaminants are essential in the fight against steroid contamination.

An interesting recent development in food-toxicity characterization is combination of these in vitro assays with analytical techniques in so-called effect-directed analysis (EDA). This approach is not recent in the EDC field and is already widely accepted in environmental research. Transfer and application of this concept to toxicity characterization in food matrices should be encouraged. The few experiences with EDA approaches in food toxicity were elegantly reviewed recently, and the examples indicate its application here should be strongly emphasized [27].

An important concern highlighted recently is that several estrogenic compounds have been reported to induce responses in vitro that are significantly higher than that of 17β-E2 itself. These so-called supra-maximal (SPMX) estrogenic effects do not occur consistently and seem to differ depending on the cellular models.
applied. A recent meta study analyzed the possible underlying causes, mechanisms and drivers for SPMX estrogenic effects in in vitro functional assays reported in the peer-reviewed literature [91]. The findings in this study advised that a number of specific assay characteristics could be responsible, so these variations should be considered when designing new assays. Another recent article reported that these effects may not be biologically relevant but may represent a post-transcriptional effect on luciferase-enzyme stability [92].

In conclusion, yeast and mammalian RGAs, which offer high throughput, adequate sensitivity, are free of cross talk and may be coupled to adequate clean-up methods, have been the main bioassays of choice for application and validation in food-safety applications to date. Assays looking at effects on steroidogenesis offer benefits in studying new levels of endocrine disruption, and can be developed into validated models capable of monitoring effects of environmentally-relevant EDC mixtures at environmentally-relevant levels. It is also clear there is a continued need to validate existing or to develop further in vitro models for the various levels of the endocrine axes. Further improvements in the current battery of bioassays, complemented by development of a new generation of bioassays, will be beneficial to the analysis of EDCs in food.

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