FINAL REPORT

Detection of Estrogenic Activity in Plastic Cups and Plates

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I. EXECUTIVE SUMMARY

**Background:** Exposure to estrogenic chemicals (xenoestrogens) has been reported to produce a broad spectrum of biological and adverse health effects in a variety of species and tissues. Xenoestrogens include both natural and synthetic chemicals that can be found in environmental, biological and food samples, as well as in commercial and consumer products. Given the potential effects of xenoestrogens, their detection, identification, characterization and biological potency is both necessary and a challenge. The use of estrogen receptor (ER)-based bioassay systems provides one avenue in which to relatively rapidly identify chemicals and sample extracts which have estrogenic or antiestrogenic activity. The ER-based chemically-activated luciferase gene expression (ER-CALUX) bioassay is an established cell-based bioassay system that can detect such activity. It is currently an OECD and USEPA accepted method for detection of estrogenic activity of chemicals and extracts.

**Objectives:** Here we have determined whether extracts of children’s plastic cups and plates contains chemicals with estrogenic activity.

**Methods:** Extracts (100% ethanol, 10:90% (v/v) ethanol:water and/or 100% water) of 38 plastic products were examined for estrogenic activity in a recombinant human ovarian cell line (BG1Luc4E2) in which activation of the ER results in induction of firefly luciferase gene expression and a resulting increase in light production in a luciferase enzymatic assay. Increased light production in cells exposed to a given extract can quantitatively indicate the presence of an estrogenic chemical(s) in the extract.

**Results:** Positive estrogenic activity was detected in some, but not all, of the 100% ethanol sample extracts. Some cytotoxicity was observed with selected samples, but was eliminated with sample dilution to reveal positive estrogenic activity. Interestingly, for some products, use of a 10% ethanol extract resulted in a significant increase in luciferase activity compared to that of 100% ethanol. More surprisingly, was the presence of even higher levels of luciferase induction using 100% water extracts of these plastic products.

**Conclusions:** The results obtained in our analysis revealed the presence of a variety of estrogenic substances in solvent extracts of children’s plastic plates and cups. Additionally, the demonstration that incubation of selected products in 100% water can release estrogenic chemicals that can be detected in the cell bioassay is a significant finding and raises potential concerns as to the biological/toxicological significance of this xenoestrogen(s) in children. The identification and characterization of the responsible xenoestrogen is necessary.
II. TECHNICAL REPORT

1. Background, aim and scope

Endocrine disruptors are chemicals that may interfere with the body’s endocrine system through a wide variety of mechanisms to produce biological/adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife. In past decades, endocrine-disrupting chemicals (EDCs) have received considerable attention: the list of suspected EDCs has been steadily growing and the research in this field has made substantial progress (Hotchkiss et al., 2008). Currently the largest group of known endocrine disruptors is EDCs are those with estrogenic activities (EA), and include chemicals that act as direct or indirect hormone mimics via estrogen receptor-dependent mechanisms. Moreover, estrogenic chemicals have been implicated with a variety of reproductive and physiological abnormalities in exposed wildlife and human populations (WHO/IPCS, 2002). EDCs with EA can be isolated from a wide variety of natural and synthetic materials and as such, humans and animals are likely chronically exposed to these substances. Identification and characterization of these chemicals is a priority and numerous biologically-based screening methods have been used to identify such chemicals.

Until recently, food contact materials were considered an underestimated potential route of human exposure to EDCs: contaminants migrated from packaging materials into edibles (Lau and Wong, 2000; Muncke, 2011). In fact, Yang et al. (2011) have investigated the leaching of estrogenic activity (EA) from plastic packaging materials and concluded that most commercially available plastic products released chemicals having reliably detectable EA, and such chemicals have been shown to include bisphenol A, phthalates and others.

In the current study, we examined samples of plastic from plates and cups that are marketed for babies/children, given the fact that they are especially vulnerable populations and would be expected to be especially sensitive to low doses of chemicals with EA. As the specific chemical composition of a sample is often unknown and mixture interactions cannot always be inferred from the concentrations of the individual components, as such, the use of bioassays that can detect chemical contaminants based on their molecular mechanisms of action are now recognized as accepted monitoring tools for the identification and characterization of estrogenic chemicals present in complex mixtures and extracts (Leusch et al., 2006). To characterize the EA of extracts of these plastic product, we utilized an Estrogen Receptor-Chemically Activated Luciferase Expression (ER-CALUX) cell bioassay system, which is based on a recombinant human ovarian cell line (BG1Luc4E2) that contains an ER-responsive firefly luciferase reporter gene (Rogers and Denison, 2000). This assay (also referred to as the BG1LucER TA bioassay) has been officially accepted as part of the U.S. Environmental Protection Agency (USEPA) Tier 1 endocrine disruptor screening program and by the Organization of Economic Cooperation and Development (OECD) Validation Management Group for Non-Animal Testing as an international test guideline to detect estrogen receptor-dependent activity of chemicals in vitro.

2. Samples and methods

Coded plastic samples were obtained from CEH and were logged in upon receipt. Each coded plastic sample (~1 g) was cut into small pieces (~3-5 mm) with ethanol- and water-washed wire cutters and placed in a glass tube that had been previously solvent washed and baked. For extraction, ethanol alone (100%), 10:90% ethanol:water or water alone (100%) was added into each plastic sample tube at a ratio of 1g plastic/2ml solvent (sample weight and volumes
described in Appendix 1, 3 and 6 for 100% ethanol, 10:90 ethanol:water and 100% water, respectively) and samples Teflon-capped and incubated in the dark at room temperature for 72 hours. Method and solvent blanks were also included to eliminate the possibility that estrogenic chemicals were obtained from the solvents and/or materials used in the extraction procedures. The resulting extract was transferred into another glass tube using a glass Pasteur pipette and evaporated to dryness under a stream of nitrogen. Sample residues were resuspended in DMSO (20 µl) and then tissue culture media (980 µl) added to each sample tube and mixed. An aliquot (100 µl) of each sample was added (in triplicate) into wells of a 96 well culture plate containing BG1Luc4E2 cells and culture media (100 µl). DMSO, 17β-estradiol (20 pM and 5 nM), and method blanks were added in triplicate wells as negative, weak positive, strong positive and method controls. Luciferase activity was determined after 24 hours of incubation as described in detail in the assay protocol (Appendix 2).

3. Results

The first screening of 100% ethanol extracts of 38 plastic samples revealed a significant increase in estrogenic activity in most all samples (Figure 1). Three sample extracts, SCO43, 49 and 57, at their original concentrations, caused visible cell toxicity and these results were not used. However, after 10x dilution of these samples with media, they no longer were cytotoxic and each induced significantly estrogenic activity (Figure 2). Overall, these results demonstrated the presence of EA chemicals in 100% ethanol extracts of a variety of children’s plastic products.

In order to determine whether the estrogenic chemicals are also released in a more aqueous solution, we determined the ability of 10 % ethanol (i.e. 10:90 ethanol:water) to extract the estrogenic chemicals. These analyses revealed that the decrease in ethanol from 100% to 10% resulted in a significant reduction in the estrogenic activity of the majority of the samples (only 14 out of 38 samples exhibited significant levels of EA (Figure 3)). However, these second round bioassay results were generally consistent with the previous 100% ethanol extract results (i.e., extracts of samples SCO43, 44, 48, 49, 51, 57, 62 and 2sf exhibited the greatest induction of luciferase activity in both experiments). Interestingly, the 10% ethanol sample extract of SCO47 had significantly higher luciferase activity than that obtained with the 100% ethanol extract, indicating this product might contain more water-soluble EA chemicals (Figure 3). Together, those values significantly greater than method blank control at p<0.05 as determined by Student’s t-test were: strong positive control, SC043, SC044, SC047-SC049, SC051, SC057-SC060, SC062, SC068, SC069 AND SCO2sf.

To assess the presence of water-soluble chemicals with EA in these plastics, we extracted SC047 and several other plastic samples that had high levels of EA with 100% water and then determined the luciferase activity of the water sample extracts as described in the methods. The results of these analyses (Figure 4) revealed that water extracts of all but one of the sample extracts tested (i.e. SCO51), exhibited EA activity higher or comparable to that of the positive control. These results further confirmed that the majority of these specific plastic samples contain water-soluble EA chemicals that can be readily released from the plastic.

4. Conclusions

The results of our analyses indicate that all of the plastic products tested contain estrogenic chemicals that can be readily released from the plastic by incubation in 100% ethanol. Interestingly, to make the extraction situation closer to “real world” conditions, samples were
also extracted with 10% ethanol and a large number of samples still released chemicals with EA and some plastics samples produced an even greater induction response when extracted with 10% ethanol that with 100% ethanol. The identification that water extracts of selected plastic products can induce luciferase to a greater extent than that of ethanol extracts indicates that these products contain a variety of substances with EA that have distinct chemicals characteristics. The CALUX bioassay allows identification of the ability of substances/extracts to stimulate ER-dependent gene expression, but they do not provide any indication of the identity of the responsible chemical or the chemical composition of the plastic product. Plastics are known to contain a wide variety of chemical components, including plasticizers, processing aids, additives, colorants and other chemicals. Employing the ER-CALUX cell bioassay method provides a mechanistically-based bioassay for detection of total EA of a given sample extract containing a complex mixture of chemicals. Our results demonstrate all commercially available plastic products we sampled contained ethanol-extractable EA chemicals, and the diversity in induction response with different plastic products indicates that each will leach different EA chemicals or leach amounts of EA chemicals (Figure 1). The results obtained here are in accordance with the results of Yang et al. (2011) who concluded that almost all commercially available plastic products release chemicals with EA.

To the best of our knowledge, this study provides first strong evidence that a water extract of a plastic product exhibited substantial estrogenic activity. Our study is consistent with a previous finding that suggested that chemicals with EA could migrate from packaging plastics into drinking water (Wagner and Oehlmann, 2011). It is significant to note that water extracts of several selected samples exhibited equal or greater induction of luciferase activity higher than that induced by a relatively high concentration of 17β-estradiol (5 nM), especially considering that this level of induction is produced by the chemicals extracted from only 0.5 g plastic into 1 ml of water (Figure 4). Overall, these findings suggest that plastic products might be a significant source of EA chemicals to which children and infants may be exposed.

References


Figure 1. Estrogenic activity of 100% ethanol extracts of plastic samples. Values represent the mean ± SD of triplicate determinations and were expressed as relative light units (RLUs). Values of all samples are significantly greater than method blank control as determined by the Student’s t-test.
Figure 2. Estrogenic activity of diluted (10X) 100% ethanol extracts of plastic samples that were cytotoxic in the initial round of screening (Figure 1). Values represent the mean ± SD of triplicate determinations and were expressed as relative light units (RLUs). Values of all plastic sample extracts are significantly greater than method blank control as determined by the Student’s t-test (p < 0.05).
Figure 3. Estrogenic activity of ethanol-water (10:90) extracts of plastic samples. Values represent the mean ± SD of triplicate determinations and were expressed as relative light units (RLUs). The values significantly greater than method blank control at p < 0.05 as determined by Student’s t-test are: strong positive control, SC043, SC044, SC047-SC049, SC051, SC057-SC060, SC062, SC068, SC069 AND SCO2sf.
Figure 4. Estrogenic activity of 100% water extracts of selected plastic samples. Values represent the mean ± SD of triplicate determinations and were expressed as relative light units (RLUs). Values of all plastic sample extracts (SCO43-SCO2SF) are significantly greater than method blank control as determined by the Student’s t-test (p < 0.05).


Protocols for ER-CALUX Bioassay for Detection of Estrogenic and Antiestrogenic Chemicals [Adapted from Rogers and Denison, 2000]

General maintenance, BG1Luc4E2 cells:

- Recombinant human ovarian carcinoma (BG1Luc4E2) cells are maintained in alpha-MEM (Invitrogen, Cat # 12000-063) containing 10% premium fetal bovine serum (Atlanta Biologicals, Cat # S11150).
- Cells should not exceed 90% confluency before passaging.
- BG1Luc4E2 cells may be reselected in media containing 400 mg G418 per liter media, if necessary.

Estrogen-stripping and BG1Luc4E2 cells:

Five days prior to plating of the BG1Luc4E2 cells into 96-well plates, the media that the cells are grown in should be changed from alpha-MEM containing 10% FBS serum to phenol red-free Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma, D2902-10L) containing 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Cat # S11650). Media must be changed daily until cells are plated out into 96-well plates. This provides sufficient time for background estrogenic activities to drop to very low levels [see Rogers and Denison, 2000]. Cells may be passaged into fresh plates (not 96-well) at day 3, 4, and 5, if necessary, with a double rinse of phosphate buffer saline before passaging and a continued daily media change. The only media BG1Luc4E2 cells should be exposed to during the estrogen-stripping period is phenol red-free DMEM.

Phenol Red-free DMEM with low glucose, contains 1 g/L glucose and L-glutamine and lacks phenol red and NaHCO₃.

Protocol for plating BG1Luc4E2 cells into 96-well plate:

Materials:  
- Trypsin (1X)  
- Phosphate buffered saline (PBS; 1X)  
- Glass Pasteur pipettes, autoclaved  
- Glass pipettes (10 mL, 25 mL), autoclaved  
- Sterile plastic tube (50 mL)  
- 10 μL pipette
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100 µL pipette tips (autoclaved)
Cell counter and slide
Cell trough
Multichannel pipette (200 µL) and tips (autoclaved)
Sterile 96-well white, clear bottom tissue culture microplate (Fisher, Cat #07-200-565)
Phenol Red-Free DMEM, 10% charcoal-stripped FBS

On the sixth day of DMEM media exposure
1) Double rinse BG1Luc4E2 cells with PBS (5 mL per plate per rinse); passage into 50 mL sterile tubes.
2) Fill all tubes to 50 mL mark with media (only phenol red-free DMEM, 10% charcoal-stripped serum should be during the plating procedure).
3) Cap and spin cells in centrifuge at room temperature for 5 minutes at 1,100 rpm.
4) In tissue culture hood, carefully aspirate media from centrifuged tubes. Add 10 mL media to tube and gently resuspend cells.
5) Remove 10 µL aliquot of resuspended cells for cell counting. For the bioassay, the optimal cell density for BG1Luc4E2 cells in the 96-well plate format is 750,000 cells/mL. Make appropriate dilutions to resuspended cells.
6) Plate cells into 96-well plate using cell trough and multichannel pipette (100 µL per well). Place cells in incubator at 37°C for 24 hours.

Protocol for treating BG1Luc4E2 cells with chemicals/sample extracts:

Materials: 7 mL Borosilicate glass tubes (autoclaved and baked)
10, 100, 1000 µL pipettes
100, 1000 µL pipette tips (autoclaved)
Samples and standards
Vortexer
Waste containers
Phenol red-free DMEM, 10% charcoal-stripped FBS

1) In tissue culture hood, prepare treatments with 1000 µL pipette and sterilized tips in 7 mL glass tubes using a 1:100 ratio of chemical (or sample extract) to phenol red-free
DMEM/FBS media (i.e. 10 µL chemical diluted in 1000 µL media). Vortex all treatments for several seconds. Treatment volumes should account for replicate (triplicate) wells.

2) Dump media from plated 96-well plate(s) into appropriate biological waste container; take care not to contaminate the cells during this procedure but to remove as much media as possible.

3) Apply treatments (chemicals or extracts) to appropriate wells using 100 µL pipette (mix treatments with pipette before application).

4) Place lid(s) on treated plate(s) and place in incubator at 37°C for 6-24 hours.

Protocol for lysing BG1Luc4E2 cells:

Materials: Cell culture lysis buffer (1X), prepared from Promega Luciferase Assay Lysis Buffer,
5X (Fisher, PR-E1351)
PBS (1X)
Cell troughs (2)
Multichannel pipette (200 µL) and tips (autoclaved)
Promega Luciferase Assay System (Fisher, PR-E1501)

1) Check every treated well in 96-well plate under microscope for cell health.
2) Dump media from 96-well plate(s) into appropriate biological waste container.
3) Wash wells twice with 150 µL PBS per well. Gently dump liquid into waste container.
4) Check cell health and confluency under the microscope after the PBS rinses to ensure that cells were not lost during washing. Firmly tap plate onto paper towels to remove any remaining PBS.
5) Add 50 µL of room temperature Promega lysis buffer (1X) to each well (1X lysis buffer is prepared by adding 30 mL 5X lysis buffer to 120 mL MilliQ water; store in glass bottle).
6) Transfer the microplate to a plate shaker and shake at a moderate speed for at least 20 minutes.
7) Prepare luminometer. Add 1 bottle of room temperature luciferase buffer to 1 bottle substrate (buffer and substrate from Promega Luciferase Assay System). Apply white backing tape to plate containing lysed cells. Read luminescence of treated wells in a microplate luminometer after automatic injection of Promega stabilized luciferase reagent.
References

APPENDICES

1. CEH plastic samples 100% ethanol extraction preparation chart.

2. Estrogenic activity of 100% ethanol extracts of plastic samples (two pages).

3. Estrogenic activity of diluted (10x) 100% ethanol extracts of plastic samples that were cytotoxic in the initial round of screening.

4. CEH plastic samples 10:90% ethanol:water (v/v) extraction preparation chart.

5. Estrogenic activity of ethanol-water (10:90) extracts of plastic samples (2 pages)

6. CEH plastic samples 100% water extraction preparation chart.

7. Estrogenic activity of 100% water extracts of selected plastic samples.
## Appendix 1. CEH plastic samples 100% Ethanol extraction preparation chart.

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### Appendix 2. Estrogenic activity of 100% ethanol extracts of plastic samples

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**Average treatment**

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**Blank values for t-test calculations**

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<tbody>
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**t-test**

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<th>RLU 3</th>
<th>Average</th>
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<tbody>
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<td>716</td>
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<td>882</td>
<td>121</td>
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</table>

---

**Graph:**

- **X-axis:** Plastic samples
- **Y-axis:** Luminescence activity (RLU)
- **Legend:**
  - Negative control
  - Weak positive control
  - Strong positive control
  - Method blank 1
  - Method blank 2
  - Method blank

---

**Legend:**

- SC042a
- SC043
- SC044
- SC045
- SC046
- SC047
- SC048
- SC049
- SC050
- SC051
- SC052
- SC053
- SC054
- SC055
- SC056
- SC057
- SC058
- SC059
- SC060
Appendix 2 (cont.). Estrogenic activity of 100% ethanol extracts of plastic samples

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<td>1608</td>
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</table>

Average, treatment, blank values for t-test calculations

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<td>1953</td>
<td>2186</td>
<td>2251</td>
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SD: Standard deviation
Appendix 3. Estrogenic activity of diluted (10x) 100% ethanol extracts of plastic samples that were cytotoxic in the initial round of screening.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RLU 1</th>
<th>RLU 2</th>
<th>RLU 3</th>
<th>Average SD</th>
<th>t-test Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (DMSO)</td>
<td>781</td>
<td>753</td>
<td>761</td>
<td>765 14.4221</td>
<td>0</td>
</tr>
<tr>
<td>Weak positive control</td>
<td>722</td>
<td>911</td>
<td>961</td>
<td>961 861.3333</td>
<td>122.3124 96.333333</td>
</tr>
<tr>
<td>Strong positive control</td>
<td>9584</td>
<td>8713</td>
<td>9109</td>
<td>9109 440.8412</td>
<td>8344</td>
</tr>
<tr>
<td>Method blank 1</td>
<td>540</td>
<td>459</td>
<td>639</td>
<td>546 90.14988</td>
<td>-219</td>
</tr>
<tr>
<td>Method blank 2</td>
<td>618</td>
<td>510</td>
<td>967</td>
<td>967 698.3333</td>
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<tr>
<td>SCD03 10x</td>
<td>3296</td>
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<td>4270 3780.333 487.0219</td>
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<td>SCD49 10x</td>
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<td>5079 4633.333 448.0182</td>
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<td>6236</td>
<td>6729</td>
<td>6729 6565.667 279.1224</td>
<td>5290 6667</td>
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</tbody>
</table>

Note: The table includes the average RLU values and the standard deviation (SD) for each sample, along with the t-test values for comparison. The bar chart visually represents the estrogenic activity levels for each treatment group.
Appendix 4. CEH plastic samples ethanol-water (10:90) extraction preparation chart.

<table>
<thead>
<tr>
<th>ID#</th>
<th>sample weight (g)</th>
<th>solvent added (ml)</th>
<th>sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO42b</td>
<td>1.02</td>
<td>2.04</td>
<td>clear rubber</td>
</tr>
<tr>
<td>SCO43</td>
<td>1.00</td>
<td>2</td>
<td>blue plastic</td>
</tr>
<tr>
<td>SCO44</td>
<td>1.00</td>
<td>2</td>
<td>blue plastic</td>
</tr>
<tr>
<td>SCO45</td>
<td>1.25</td>
<td>2.5</td>
<td>blue plastic</td>
</tr>
<tr>
<td>SCO46</td>
<td>1.02</td>
<td>2.04</td>
<td>purple plastic</td>
</tr>
<tr>
<td>SCO47</td>
<td>0.99</td>
<td>1.98</td>
<td>lime green plastic</td>
</tr>
<tr>
<td>SCO48</td>
<td>1.09</td>
<td>2.18</td>
<td>red/pink plastic</td>
</tr>
<tr>
<td>SCO49</td>
<td>0.97</td>
<td>1.94</td>
<td>pink/white plastic</td>
</tr>
<tr>
<td>SCO50</td>
<td>1.03</td>
<td>2.06</td>
<td>dark blue rubber</td>
</tr>
<tr>
<td>SCO51</td>
<td>1.13</td>
<td>2.26</td>
<td>yellow/white plastic</td>
</tr>
<tr>
<td>SCO52</td>
<td>1.06</td>
<td>2.12</td>
<td>light green rubber</td>
</tr>
<tr>
<td>SCO53</td>
<td>1</td>
<td>2</td>
<td>light blue rubber</td>
</tr>
<tr>
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<td>SCO57</td>
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<tr>
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<td>red rubber</td>
</tr>
<tr>
<td>SCO61</td>
<td>0.98</td>
<td>1.96</td>
<td>yellow rubber</td>
</tr>
<tr>
<td>SCO62</td>
<td>1.08</td>
<td>2.16</td>
<td>pink plastic</td>
</tr>
<tr>
<td>SCO63</td>
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<td>2.02</td>
<td>white plastic</td>
</tr>
<tr>
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<tr>
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<td>red plastic</td>
</tr>
<tr>
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</tr>
<tr>
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<td>yellow plastic</td>
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Appendix 5. Estrogenic activity of ethanol-water (10:90) extracts of plastic samples.

<table>
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<td>1179</td>
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<td>1208</td>
<td>4099</td>
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<td>5698</td>
<td>1274</td>
<td>1126</td>
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<td>4538</td>
<td>1468</td>
<td>1300</td>
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<td>996</td>
<td>1112</td>
<td>1073</td>
<td>6302</td>
<td>6498</td>
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</table>

**Negative control (NBED)**
- 1211 ± 1195 ± 6065 ± 2839 ± 1440 ± 1237 ± 3220 ± 1327 ± 1341 ± 5988 ± 1169 ± 1201 ± 1199

**Weak positive control**
- 1315 ± 1220 ± 4686 ± 1169 ± 1201 ± 1199 ± 1440 ± 1237 ± 3220 ± 1327 ± 1341 ± 5988 ± 1169 ± 1201 ± 1199

**Strong positive control**
- 6336 ± 4099 ± 1169 ± 1201 ± 1199 ± 1440 ± 1237 ± 3220 ± 1327 ± 1341 ± 5988 ± 1169 ± 1201 ± 1199

**Method blank 1**
- 1373 ± 1201 ± 1327 ± 1244 ± 1313 ± 1964 ± 8432 ± 1220 ± 1341 ± 5988 ± 1169 ± 1201 ± 1199

**Method blank 2**
- 1549 ± 1198 ± 1327 ± 1220 ± 1313 ± 1964 ± 8432 ± 1220 ± 1341 ± 5988 ± 1169 ± 1201 ± 1199

**Blanks for t-test calculations**
- 1525 ± 1169 ± 1244 ± 1337 ± 1201 ± 1199

**t-test**
- 0.689 ± 0.003

<table>
<thead>
<tr>
<th>RLU 1</th>
<th>RLU 2</th>
<th>RLU 3</th>
<th>Average</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1214</td>
<td>1314</td>
<td>6336</td>
<td>3220</td>
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</tr>
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<td>996</td>
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<td>1073</td>
<td>6302</td>
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</table>

**t-test**
- 0.689 ± 0.003
### Appendix 5 (cont.). Extragenic activity of ethanol-water (10:90) extracts of plastic samples

<table>
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<th>RLU 3</th>
<th>Average %</th>
<th>t-test values</th>
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<td>6011</td>
<td>1513</td>
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<tr>
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<td>986</td>
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<td>1587</td>
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</table>

**Blank values for t-test calculations:**

1618 1513 1407 1305 1249 1220 1567 1683 1421 1683

#### Graphs

- [Graph 1](#)
- [Graph 2](#)
Appendix 6. Water extraction preparation chart of selected samples

<table>
<thead>
<tr>
<th>ID#</th>
<th>sample weight (g)</th>
<th>solvent added (ml)</th>
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</tr>
<tr>
<td>SCO48</td>
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<td>2.28</td>
</tr>
<tr>
<td>SCO49</td>
<td>1.18</td>
<td>2.36</td>
</tr>
<tr>
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<td>2.26</td>
</tr>
<tr>
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<td>1.96</td>
</tr>
<tr>
<td>SCO62</td>
<td>1.41</td>
<td>2.82</td>
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<tr>
<td>SCO2SF</td>
<td>1.05</td>
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</tbody>
</table>
Appendix 7. Estrogenic activity of 100% water extracts of selected plastic samples

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|                |        |       |          |
| negative control | 4755 | 4269 | 3799 | 4274.3333 | 478.02231 |
| weak positive control | 5431 | 3786 | 3446 | 4221 | 1061.5908 |
| strong positive control | 9132 | 8078 | 7204 | 8138 | 965.3994 |