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Evaluation of Six Personal Care Products for Estrogenic Activity in a Yeast Estrogen Screen.

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1. Introduction

Estrogenic compounds (EC) are chemical substances that bind to the estrogen receptors (ER) and mimic the effects of estrogens (Fuentes and Silveyra, 2019). Numerous publications pointed at the accumulation of endocrine disruptive chemicals and their adverse developmental effects in mammalian fetuses (Foster, 1998, Schönfelder et al., 2002, Brehm and Flaws, 2019). Some other consequences of human exposure to environmental EC are endocrine disruption and negative impact on reproductive health (Stewart et al., 2020). Several studies have shown that personal care products (PCPs) such as hair products and skin products may contain EC (Edwards et al., 2018, Myers et al., 2015).

The most common methods of environmental EC detection are bioassays based on cellular proliferation (Fang et al., 2000), *Drosophila melanogaster* endocrine disruption tests (Bovier et al, 2019), and ER binding assays in yeast *Saccharomyces cerevisiae* (YES assay) (Miller et al., 2010, Edwards et al., 2018). There are three known human receptors to estrogens. Two of them, ERα and ERβ, are nuclear receptors and the third one, G Protein-Coupled Estrogen Receptor GPER1, is a membrane receptor (Fuentes and Silveyra, 2019). In the YES assay, yeast cells carry a plasmid to express the human ERα or ERβ. The plasmid contains the β-galactosidase reporter gene (Miller et al., 2010). The assay is based on the spectrophotometric detection of colored products of the galactosidase enzymatic activity (Edwards et al., 2018). The synthetic enzyme substrates are *ortho*-nitrophenyl-β-galactopyranoside (ONPG) and chlorophenol red-β-D-galactopyranoside (CPRG). It was previously reported that CPRG was about 10-fold greater in sensitivity for the quantitation of galactosidase levels (Miller et al., 2010, Buller et al., 1991)).

In the present study, six personal care products used by the authors in everyday life were investigated for the presence of estrogenic compounds, and the limitations of the colorimetric method of detection were discussed.

2. Materials and methods

All chemicals for the estrogenic testing were purchased from Sigma-Aldrich except for galactose. D-galactose was acquired from Flinn. Estrogen (beta-estradiol) working stock solutions to create standard curves were 227.5 nM for yeast expressing ERα and 9.75 nM for yeast expressing ERβ in 50% ethanol.

One gram of each personal care product was combined with 10 ml of anhydrous ethanol. The mixtures were homogenized by vortexing for 30 minutes. The 40 μm cell strainers were used to collect supernatants into glass vials. The extracts were left to evaporate completely in a dark ventilated hood to protect samples from photobleaching. Dry extracts were reconstituted in 1 ml of 50% ethanol and looked completely transparent.

Glucose media, galactose media and LacZ buffers were prepared as described in (Edwards et al., 2018).

Yeast strains expressing either ERα or ERβ were received from Dr. Thea Edwards (Columbia Environmental Research Center, Missouri).

Absorbance of microplates was measured using Chromate microplate reader by Awareness Technology.

Absorbance spectra of PCP extracts were recorded with a wireless Pasco spectrometer.

The YES protocol adapted for undergraduate studies was used to assess estrogenic activity of PCP extracts (Edwards et al., 2018).

Ten-milliliter yeast cultures in glucose media were incubated for several days at 30^0C in sterile Erlenmeyer flasks. Two days before the YES test, 0.1 mL of active yeast cultures were added to 10 mL of filter-sterilized glucose media and incubated at 30°C for two nights.

On the 1st day of the assay yeast cultures were diluted to an optical density of 0.065 ±0.005 at 620 nm (OD_{620}) in filter-sterilized galactose media and pipetted into sterile 96 well plates using an eight-channel automatic pipette (320 μL per well). Five-μL samples of either estradiol control, 50% ethanol or PCP extracts were added to wells and incubated at 30°C for 17 hours.

After 17 hours 50 µL from each well were transferred to clean, non-sterile 96 well plates. Two hundred µL of LacZ buffer containing DTT and either ONPG or CPRG were added to all wells and OD_{620} of all wells was immediately measured using a plate spectrophotometer. Plates containing ERα yeast were incubated at 30 °C for 40 min for ONPG or 3 hours for CPRG. Plates containing ERβ yeast were incubated at 30 °C for 70 min for ONPG or 4 hours for CPRG. After incubating plates, 100 µL of sodium carbonate were added to each well to stop the β-galactosidase reaction and the OD_{405} for ONPG or OD_{570} for CPRG of all wells were measured using a plate spectrophotometer.

LacZ values were calculated using the following equations (Edwards et al., 2018) with two exceptions: OD for light scattering correction was measured at 620 nm (610 nm in the original study), and OD for CPRG was measured at 570 nm (574 nm in the original study) because of the microplate reader capabilities.

$$\text{LacZ value (for wells containing ONPG)} = \frac{\left(OD_{405} - \text{MeanOD}_{405\,vehicle\ control}\right) \bullet 1{,}000}{\left(OD_{620} - \text{MeanOD}_{620\,media\ control}\right) \bullet 50\ \mu l \bullet t}$$

$$\text{LacZ value (for wells containing CPRG)} = \frac{\left(OD_{570} - \text{MeanOD}_{570\,vehicle\ control}\right) \bullet 1{,}000}{\left(OD_{620} - \text{MeanOD}_{620\,media\ control}\right) \bullet 50\ \mu l \bullet t}$$

OD *vehicle control* is the optical density of wells containing 0.75% ethanol instead of estradiol or PCP extracts, OD *media control* is the optical density of wells containing galactose media, and *t* is the incubation time with the Lacz buffer in hours.

3. Results and discussion

Several skin products (Aveeno spf 50 sunscreen, Neutrogena oil-free acne moisturizer, Neutrogena oil-free acne wash and Liquid Neutrogena facial cleansing formula) and hair products (Pantene Frizz Fix and Frizz Ease) were assessed in the YES test. The results are shown on the Figure 1.

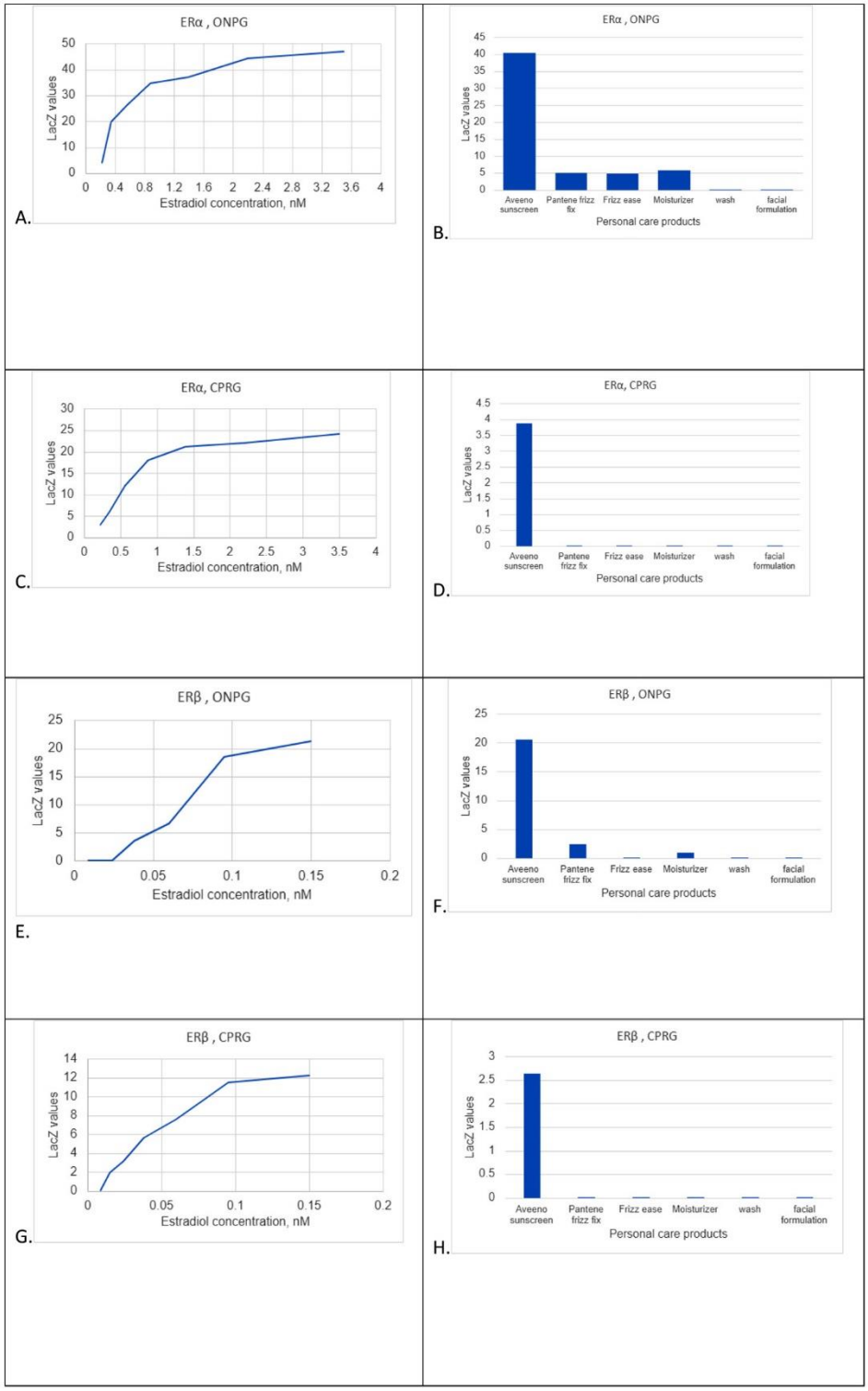
A.
ERα , ONPG
LacZ values
Estradiol concentration, nM
B.
ERα , ONPG
LacZ values
Aveeno sunscreen
Pantene frizz fix
Frizz ease
Moisturizer
wash
facial formulation
Personal care products
C.
ERα, CPRG
LacZ values
Estradiol concentration, nM
D.
ERα, CPRG
LacZ values
Aveeno sunscreen
Pantene frizz fix
Frizz ease
Moisturizer
wash
facial formulation
Personal care products
E.
ERβ , ONPG
LacZ values
Estradiol concentration, nM
F.
ERβ , ONPG
LacZ values
Aveeno sunscreen
Pantene frizz fix
Frizz ease
Moisturizer
wash
facial formulation
Personal care products
G.
ERβ , CPRG
LacZ values
Estradiol concentration, nM
H.
ERβ , CPRG
LacZ values
Aveeno sunscreen
Pantene frizz fix
Frizz ease
Moisturizer
wash
facial formulation
Personal care products

Figure 1. A, C, E and G are standard curves for the YES test with various concentrations of estradiol for yeast cells carrying either ERα (A and C) or ERβ (E and G). B, D, F and H are the Lac Z values of various personal care products in the YES test (B and D in yeast cells carrying ERα, F and H in yeast cells carrying ERβ). Lac Z values reflect the galactosidase cleavage of ONPG or CPRG substrates and the respective color change. All points are means of LacZ triplicates.

The standard curve for ERα yeast cells was obtained using concentrations 0.22-3.5 nM of estradiol (Figure 1A). Several samples of yeast cells carrying ERα incubated with PCP extracts and detected with the ONPG substrate showed a non-zero response (Figure 1B). ONPG was cleaved by the galactosidase enzyme to produce the yellow product nitrophenol (Miller et al., 2010). The absorbance of the product was measured at 405 nm. Aveeno sunscreen extract induced the highest response in ERα yeast cells corresponding to one of the highest concentrations of estradiol on the standard curve (Figure 1A). Three other extracts (skin product Neutrogena Acne Moisturizer and two hair products Pantene Frizz Fix and Frizz Ease) induced a minimal response in ERα yeast cells corresponding to one of the lowest concentrations of estradiol on the standard curve (Figure 1A and B), almost outside of the standard curve LacZ values. Since the absorbance of ONPG cleavage product was measured at 405 nm, the contribution of extracts to the total sample absorbance was assessed by recording the extract absorbances in 50% ethanol (the undiluted extract) and 0.75% ethanol (corresponding to the final dilution in yeast cell samples) (Figure 2).

Figure 2A shows the absorbance spectrum of the undiluted Aveeno sunscreen extract containing 50 % ethanol. Aveeno sunscreen strongly absorbs light in the UVA region with a peak around 390 nm. The absorption extends into the visible part of the spectrum with a significant absorption at 405 nm. When a sample was diluted with water to a final concentration of ethanol 0.75% corresponding to the dilution of extracts in yeast cell suspensions, the absorbance peak increased and became broader, indicating the extract components' aggregation with the subsequent light scattering. Many sunscreens are based on inorganic UV filters – either zinc oxide or titanium oxide or both (Schneider and Lim, 2019). One of the main

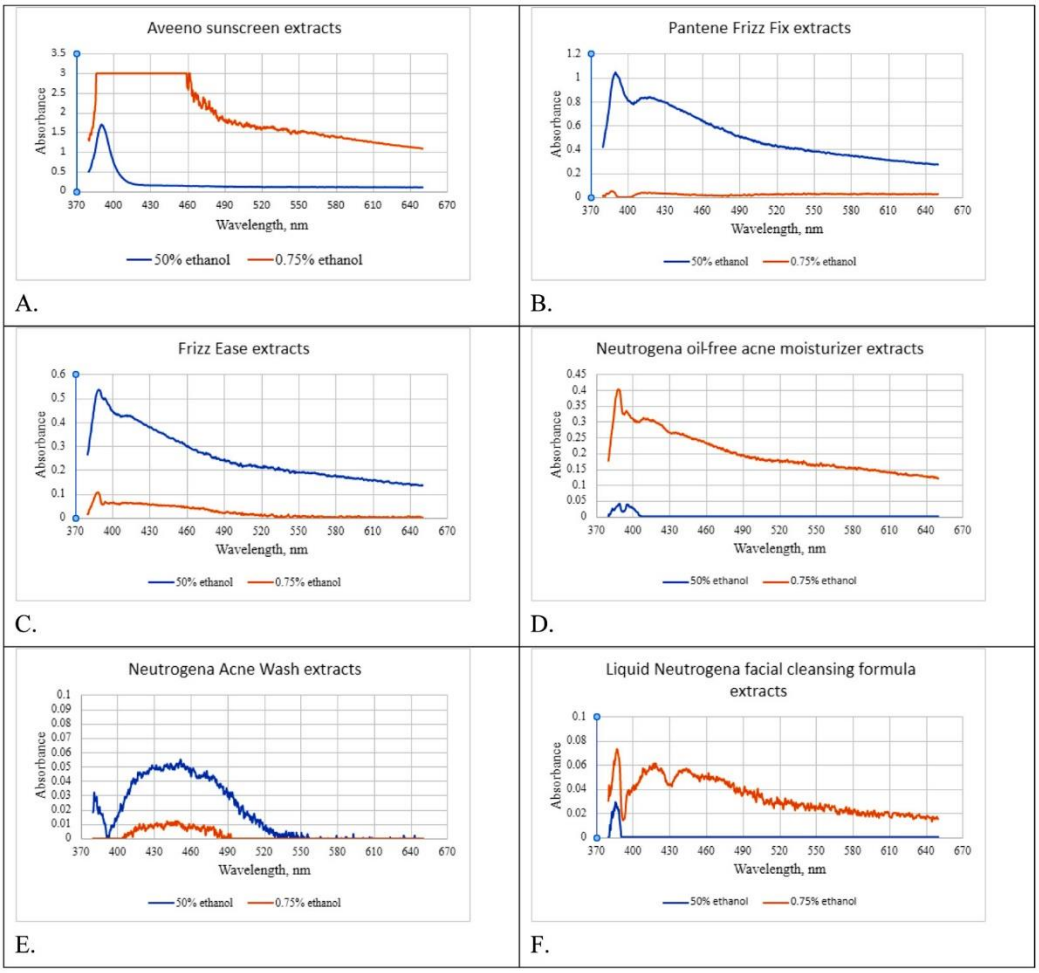

Figure 2. Absorbance spectra of personal care product extracts reconstituted in 50% ethanol and diluted extracts with 0.75% ethanol final concentration.

ingredients of the Aveeno sunscreen is zinc oxide (21.6%). Sunscreen formulations usually contain nanoparticles of inorganic UV filters that absorb, reflect, and refract UV light (Schneider and Lim, 2019). However, the photoprotection of sunscreens is mostly attributed to the absorption of UV light. The size of the zinc oxide particles varies from primary particles (less than 20 nm) to aggregates (30-150 nm) and agglomerates (> 1μm) (Schneider and Lim, 2019). Agglomerates reflect more visible light and have whitish appearance. It was shown previously that the size of zinc oxide particles in aqueous solutions depends on the pH, ionic species present, and the cell culture components (Meißner et al., 2014). The average size of zinc oxide particle was smaller in the presence of cell culture media (200-800 nm) compared to buffered solutions of zinc oxide (200-1200 nm) (Meißner et al., 2014).

Figure 3 shows the absorption spectrum of the Aveeno sunscreen extract in the galactose culture media. The absorption peak is still broader compared to the 50% ethanolic solution of the Aveeno sunscreen extract (Figure 2A) with a significant absorbance at 405 nm used to assess the ONPG product presence. It makes it impossible to draw any conclusions about the estrogenic activity of the Aveeno sunscreen extract measured by ONPG cleavage product at 405 nm (Figure 1B) despite the correction for the light scattering at 620 nm calculating LacZ values (see materials and methods). Several authors have reported that detection of estrogenic activity using CPRG cleavage product at a longer wavelength (570 nm in the present study) is a more reliable and sensitive method especially in the presence of other chromophores (Pelisek et al., 2000, Buller et al., 2003, Eustice et al., 1991). Aveeno sunscreen extract is the only one that shows elevated LacZ value in ERα yeast cells based on the CPRG cleavage detected at 570 nm. Unfortunately, Aveeno sunscreen extract with 0.75% ethanol also scatters light at 570 nm and there is a significant difference in absorbance between 570 nm and 620 nm that is used for light scattering correction (Figure 2A and Figure 3). Other authors reported increased estrogenic activity of sunscreens, sometimes exceeding the range of the estradiol standard curve (LacZ values vs. estradiol concentration) (Edwards et al., 2018). It is unclear whether these sunscreens were based on zinc oxide or titanium oxide. And if they were, the contribution of aggregation/agglomeration and light scattering in

sunscreen extracts into the absorbance reading of yeast microplates in these studies are unknown.

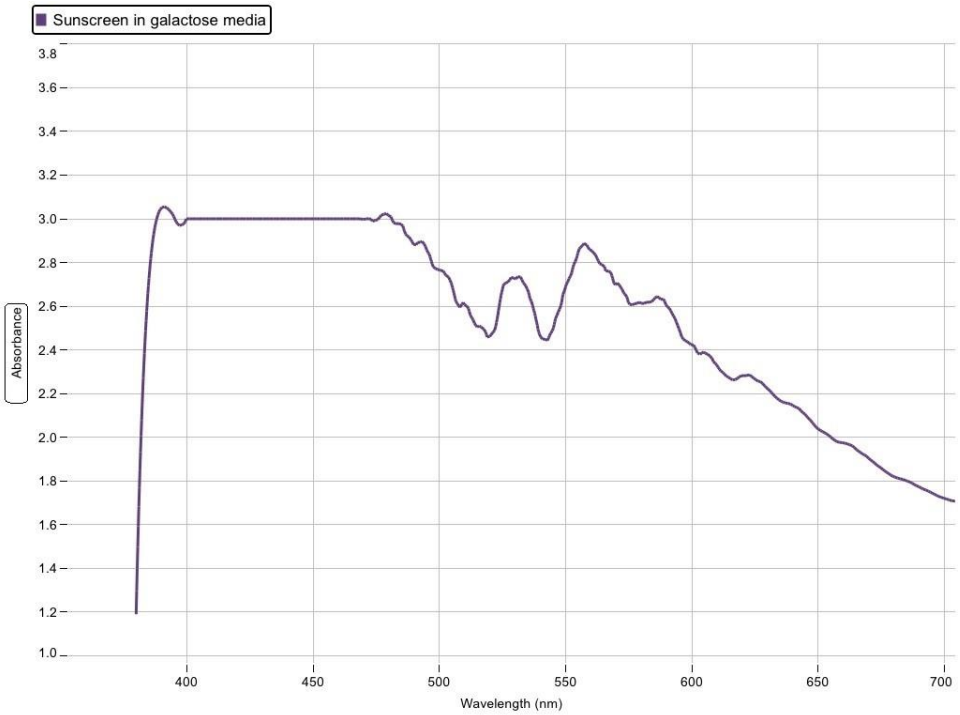

Figure 3. Absorbance spectrum of Aveeno sunscreen extract in the galactose culture media (0.75% ethanol).

Among the three other extracts (skin product Neutrogena Acne Moisturizer and two hair products Pantene Frizz Fix and Frizz Ease) with a minimal response in ERα yeast cells (Figure 1B), only the spectrum of the aqueous Neutrogena Acne Moisturizer (0.75% ethanol) shows any significant absorbance at 405 nm and can possibly contribute to the inflated absorbance readings in the YES test (Figure 2D). None of the extracts (except for Aveeno sunscreen) with 0.75% of ethanol showed any significant absorbance at 570 nm and did not interfere with the measurements of CPRG cleavage product at this wavelength (Figure 2B-F). Figures 1C and 1D show the standard curve and the response of ERα to extracts of PCPs. None of the extracts (except for Aveeno sunscreen) showed any elevated LacZ values.

ERβ yeast cells were reported to be more sensitive to estradiol, which is why smaller concentrations (0.009-0.15 nM) of the hormone were used for the standard curves (Figures 1E and 1G). Only one extract showed elevated LacZ values (Aveeno sunscreen extract) measured by both ONPG and CPRG galactosidase cleavage products at 405 nm and 570 nm, respectively. Pantene Frizz Fix and Neutrogena oil-free moisturizer showed slightly elevated LacZ values measured by the ONPG galactosidase cleavage product and no activity by the CPRG measurements. Since measurements of galactosidase activity are more sensitive by the detection of CPRG cleavage products, we can conclude that among the five extracts (Neutrogena oil-free acne moisturizer, Neutrogena oil-free acne wash and Liquid Neutrogena facial cleansing formula, Pantene frizz fix and Frizz Ease) none of them possess any estrogenic activity since the calculated LacZ values based on CPRG measurements are close to zero. In the case of the sixth extract (Aveeno sunscreen), the conclusion about the estrogenic activity cannot be reached because the spectrophotometry is probably an inadequate method of assessment when potentially estrogenic extract undergoes aggregation and agglomeration in aqueous solutions.

4. Conclusions

Our findings indicate that five PCPs (Neutrogena oil-free acne moisturizer, Neutrogena oil-free acne wash, Liquid Neutrogena facial cleansing formula, Pantene Frizz Fix and Frizz Ease) do not possess any significant estrogenic activity. The spectrophotometric method of detecting estrogenic activity in PCP extracts is limited to extracts that do not absorb light at the detection wavelength 405 nm and 570 nm and do not aggregate or agglomerate in aqueous solutions to scatter light as in the case of Aveeno sunscreen.

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