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# Screening of hormone-like activities in bottled waters available in Southern Spain using receptor-specific bioassays

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ABSTRACT

Bottled water consumption is a putative source of human exposure to endocrine-disrupting chemicals (EDCs). Research has been conducted on the presence of chemicals with estrogen-like activity in bottled waters and on their estrogenicity, but few data are available on the presence of hormonal activities associated with other nuclear receptors (NRs). The aim of this study was to determine the presence of endocrine activities dependent on the activation of human estrogen receptor alpha (hERa) and/or androgen receptor (hAR) in water in glass or plastic bottles sold to consumers in Southern Spain. Hormone-like activities were evaluated in 29 bottled waters using receptor-specific bioassays based on reporter gene expression in PALM cells [(anti-)androgenicity] and cell proliferation assessment in MCF-7 cells [(anti-)estrogenicity] after optimized solid phase extraction (SPE). All of the water samples analyzed showed hormonal activity. This was estrogenic in 79.3% and anti-estrogenic in 37.9% of samples and was androgenic in 27.5% and anti-androgenic in 41.3%, with mean concentrations per liter of 0.113 pM 17β-estradiol ($E_2$) equivalent units ($E_2$Eq), 11.01 pM anti-estrogen (ICI 182780) equivalent units (ICI 182780Eq), 0.33 pM methyltrienolone (R1881) equivalent units (R1881Eq), and 0.18 nM procymidone equivalent units (ProcEq). Bottled water consumption contributes to EDC exposure. Hormone-like activities observed in waters from both plastic and glass bottles suggest that plastic packaging is not the sole source of contamination and that the source of the water and bottling process may play a role, among other factors. Further research is warranted on the cumulative effects of long-term exposure to low doses of EDCs.



## 1. Introduction

Bottled water consumption has grown steadily worldwide over the past few decades, even in places where water of excellent quality is available from the tap. Mexico has the highest per capita consumption of bottled water (243 L/year), followed by Italy (187 L) and the United Arab Emirates (153 L), with Spain in eighth position (124 L) (Gleick et al., 2012). The increase in bottled water consumption has been attributed to concerns about the quality of tap water (Doria et al., 2009) and the presence of disinfection by-products in comparison to the quality of bottled water (Doria, 2010; Gopal et al., 2007). Its effective marketing and general changes in consumer habits have also been cited (Doria, 2006), but there are few scientific data on the reasons for the increased consumption of bottled water (Marcussen et al., 2013).

Some research has been conducted on the presence in bottled water of substances that interfere with the function of the endocrine system (Muncke, 2011; Yang et al., 2011), grouped under the generic name of endocrine-disrupting chemicals (EDCs). EDCs can interact with the endocrine system by mimicking or blocking natural hormones, and they may also influence the production, secretion, or metabolism of endogenous hormones and/or their nuclear receptors (Diamanti-Kandarakis et al., 2009). The diet is one of the main sources of exposure to these chemicals (Connolly, 2009), which are used as stabilizers, antioxidants, coupling agents, and pigments and can be a constituent of storage containers or added to optimize the physical and chemical properties of packaging materials (Lau and Wong, 2000). Bottled water can be

*Abbreviations:* Al, Aluminum; $Sb_2O_3$, Antimony trioxide; BP, Benzophenone; BPA, Bisphenol-A; Ce, Cerium; DCC, Dextran-coated charcoal; DIDP, Di-isodecylphthalate; DINP, Di-isononylphthalates; DMSO, Dimethyl sulfoxide; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide; 2,4-dtBP, 2,4-Di-tert-butylphenol; EDCs, Endocrine-disrupting chemicals; ESBO, Epoxidized soybean oil; $E_2$, 17β-estradiol; FBS, Fetal bovine serum; hAR, Human androgen receptor; hERα, Human estrogen receptor alpha; HDPE, High density polyethylene; Pb, Lead; R1881, Methyltrienolone; NP, 4-Nonylphenol; NOAEL, Non-observed adverse effects level; NRs, Nuclear receptors; OP, Octylphenol; PET, Polyethylene terephthalate; PP, Polypropylene; PTFE, Polytetrafluoroethylene; PE, Proliferative effect; SPE, Solid phase extraction; SRB, Sulforhodamine B; TCA, Trichloroacetic acid; Zr, Zirconium.

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contaminated by EDCs at different stages of the production process, from the supply of the waters to their handling, storage, and distribution. Thus, water can become contaminated at its source, during the bottling process in the plant, by migration from the bottle or cap material (monomers, catalysts, additives, or degradation products) or by the formation of organic compounds under deficient storage conditions (Nerin et al., 2013). Exposure to low doses of chemicals leaching into the water from plastic bottles is a cause of considerable concern to consumers, regulators, and manufacturers (Guart et al., 2011, 2014a).

Despite its toxicological relevance and the importance of the dietary exposure pathway, relatively little information is available on steroid hormone activities and hormone-like chemicals in food, beverages, or water (Connolly, 2009). Klinge et al. (2003) detected a maximum of 84 ng $E_2$Eq/L (308.3 pM $E_2$Eq/L) in red wine, and Promberger et al. (2001) reported a range from 23 to 41 ng $E_2$Eq/L (84–150 pM $E_2$Eq/L) in beer. These results were confirmed by Takamura-Enya et al. (2003) and Stanford et al. (2010), who reported a maximum activity of 140 ng $E_2$Eq/L (513.9 pM $E_2$Eq/L) in beer. Hartmann et al. (1998) suggested that dairy products are the main source of estrogens, calculating a total daily intake of 80–100 ng estrogens per day for adults. Pape-Zambito et al. (2012) reported concentrations of around 10 ng/L or Kg $E_2$ in dairy products and Courant et al. (2008) concentrations of 34 ng/L $E_2$ (0.12 nM $E_2$Eq/L) in milk. Schilirò et al. (2011) reported values of 0.04, 1.59, 1.08, and 0.41 μg $E_2$Eq100g in oranges, pineapples, kiwis, and tomatoes, respectively. Although $E_2$ and phytoestrogens in the foods and beverages may explain their estrogenicity, it has also been attributed to anthropogenic contamination. For example, our own group associated the estrogenic burden (range 5.44–720 nM $E_2$Eq/L) in the liquor of vegetables packed in lacquer-coated cans with the presence of bisphenol-A (BPA) from epoxy resins (Brotons et al., 1995) and related the estrogenicity of baby food products (range 0.25–70.13 pM $E_2$Eq/g) to the food packaging (Pandelova et al., 2011). In drinking waters, a maximum of 8.7 ng $E_2$Eq/L (31.94 pM $E_2$Eq/L) was detected in Brazil (Bergamasco et al., 2011) and a range of 0.19–0.72 ng $E_2$Eq/L (0.7–2.64 pM $E_2$Eq/L) in the USA (Stanford et al., 2010).

Although the characteristics of plastic materials in contact with food are tightly regulated by European regulations (EU, 2012), the frequent detection in food and water of contaminants released from plastics suggests that food packaging is a source of chemicals with hormonal activity (Muncke, 2011; Yang et al., 2011). In fact, Muncke (2009) compiled a list of 50 known EDCs that are authorized for the use in food contact materials in the EU and the US.

Bottled water is usually available in glass or plastic containers. Polyethylene terephthalate (PET), a polymer derived from crude oil (Royte, 2008), is used in around 80% of plastic bottles for water. Bottles made with PET are strong, light, impact-resistant, naturally transparent, and completely recyclable, and they impart no taste to the water (Gleick, 2010). Some authors have investigated the interaction of PET bottles with the drinking water they contain, yielding analytical data on PET components and additives (Keresztes et al., 2013; Sax, 2010; Welle and Franz, 2011; Westerhoff et al., 2008).

Despite the aforementioned regulation of food-contact packaging in Europe (EU, 2012), PET-bottled water has been found to contain phthalates and other inorganic species that may be present as residues from the catalysts or additives used to produce PET. This is the case with antimony trioxide ($Sb_2O_3$), the catalyst most widely used to synthesize this polyester (Sax, 2010). With regard to the use of glass for packaging, glass bottles have been found to leach lead (Shotyk and Krachler, 2007a), and the metal closures of glass jars have been described as a source of epoxidized soybean oil (ESBO), di-isodecylphthalate (DIDP), and/or di-isononylphthalate (DINP) (Pedersen et al., 2008).

Various studies have detected hormone-like activities in commercially available water from PET and glass bottles. Thus, water stored in PET bottles has demonstrated *in vitro* estrogenic activity in MCF-7 human breast cancer cells (Wagner and Oehlmann, 2011), in recombinant cell lines (Plotan et al., 2013), and in recombinant yeast systems carrying the human estrogen receptor alpha (hERα) (Pinto and Reali, 2009; Wagner and Oehlmann, 2009), and it has shown *in vivo* estrogenic activity in a molluscan model (Wagner and Oehlmann, 2009). Anti-androgenic, progestogenic, and glucocorticoid-like activities were recently detected in bottled water, using a panel of reporter gene cell lines in a recombinant yeast system (Plotan et al., 2013), and a broader range of steroid receptor antagonists was found in bottled water by Wagner et al. (2013). However, most of the above studies on EDCs in bottled water have focused on the agonistic activity of xenoestrogens (estrogenicity).

In the present investigation, two bioassays were used to identify hormonal activity in extracts prepared from 29 waters in plastic or glass bottles commercially available in Southern Spain, after a solid phase extraction (SPE) step. The experimental approach focused on EDCs that are active *via* hERα binding and subsequent cell proliferation (E-screen bioassay, MCF-7 cells) and on those active *via* human androgen receptor (hAR) binding (PALM bioassay, human prostate cancer cells), determining the consequent gene expression.

## 2. Materials and methods

### 2.1. Chemicals, materials, and instrumentation

All reagents were analytical grade unless otherwise specified. Reference standards 17β-estradiol ($E_2$), methyltrienolone (R1881) and ICI 182780 (henceforth, ICI), puromycin, geneticin (G418), luciferin (sodium salt), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), sulforhodamine B (SRB), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich Inc. (St Louis, MO). Stock solutions (10 mM) of $E_2$, R1881, and ICI were prepared in ethanol, and successive dilutions were performed in culture medium. Stock solutions were kept at −20 °C, and dilution series were freshly prepared before each experiment. HPLC-grade solvents (methanol and acetone) were from Merck (Darmstadt, Germany) and dimethyl sulfoxide (DMSO) from Panreac (Barcelona, Spain). Culture medium and fetal bovine serum (FBS) came from Gibco (Invitrogen, Barcelona, Spain), and all cell culture plastics were supplied by Falcon (VWR International Eurolab, Barcelona, Spain).

Isolute ENV + (200 mg/3 ml) and C18 (500 mg/6 ml) SPE sorbent cartridges were supplied by Biotage (Uppsala,Sweden), while Supelclean ENVI-18 (500 mg/6 ml) and ENVI-Carb (500 mg/6 ml) SPE sorbent cartridges were obtained from Supelco (Madrid, Spain). A Supelco 12-column vacuum manifold connected to a Supelco vacuum tank and pump was used for the SPE.

For cell proliferation assays, the absorbance was read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm, while an infinite M200 luminometer (Tecan, Barcelona, Spain) was used to detect luciferase activity in intact cells.

### 2.2. Bottled water samples

Twenty-nine bottled water products were purchased at local shops in Granada (Southern Spain) between March and December 2012, obtaining all bottles marketed as “natural mineral water,” i.e., derived from natural springs and not processed or altered. The characteristics of the bottled waters are given in Table 1. Twenty-six of the bottles were plastic (products 1 to 26) and three were glass (products 27 to 29). The springs of the waters are located in different geographic regions, all being in Spain except for two in France and one in Portugal. A sufficient number of bottles of each type (to obtain at least 3 L) was purchased from the same batch and stored according to the manufacturer's guidelines (in a dry place and out of the direct sunlight) until testing commenced.

**Table 1**
Characteristics of the analyzed bottled water products.

| Product | Packaging | | | | | Waters | |
|---|---|---|---|---|---|---|---|
| | Material | Volume (L) | Weight (g) | Color | Caps | Spring location | Mineralization |
| 1 | PET | 2.50 | 68.18 | Blue | * | Lugo (Spain) | Weak |
| 2 | Plastic* | 1.50 | 24.18 | Blue | HDPE | Granada (Spain) | Weak |
| 3 | PET | 1.50 | 28.69 | Blue | HDPE | Guadalajara (Spain) | Weak |
| 4 | PET | 1.50 | 27.63 | Blue | HDPE | Segovia (Spain) | Weak |
| 5 | PET | 1.50 | 27.46 | Uncolored | HDPE | Valencia (Spain) | Weak |
| 6 | PET | 0.50 | 15.62 | Blue | * | Portalegre (Portugal) | Weak |
| 7 | PET | 1.50 | 30.61 | Uncolored | * | Granada (Spain) | Weak |
| 8 | Plastic* | 1.50 | 33.34 | Blue | HDPE | Valencia (Spain) | Medium |
| 9 | Plastic* | 2.00 | 31.39 | Blue | HDPE | Granada (Spain) | Weak |
| 10 | PET | 1.50 | 26.62 | Blue | HDPE | Lugo (Spain) | Weak |
| 11 | PET | 1.50 | 31.41 | Blue | * | Rhône-Alpes (France) | Medium |
| 12 | PET | 1.50 | 25.12 | Uncolored | * | Albacete (Spain) | Weak |
| 13 | Plastic* | 2.00 | 32.23 | Blue | HDPE | Teruel (Spain) | Very weak |
| 14 | PET | 5.00 | 93.02 | Blue | * | Girona (Spain) | Weak |
| 15 | PET | 1.50 | 27.49 | Blue | PE | Soria (Spain) | Weak |
| 16 | Plastic* | 0.50 | 16.05 | Blue | HDPE | Valencia (Spain) | Medium |
| 17 | PET | 0.33 | 17.83 | Uncolored | HDPE | Albacete (Spain) | Weak |
| 18 | Plastic* | 5.00 | 80.50 | Blue | * | Valencia (Spain) | Weak |
| 19 | Plastic* | 2.00 | 37.12 | Blue | * | Albacete (Spain) | Weak |
| 20 | PET | 1.50 | 26.27 | Uncolored | HDPE | Badajoz (Spain) | Very weak |
| 21 | PET | 1.50 | 51.70 | Blue | HDPE | Cuenca (Spain) | Weak |
| 22 | PET | 2.00 | 34.15 | Blue | HDPE | Cuenca (Spain) | Weak |
| 23 | PET | 1.50 | 27.94 | Blue | HDPE | Salamanca (Spain) | Weak |
| 24 | PET | 1.50 | 33.37 | Blue | * | Jaén (Spain) | Medium |
| 25 | PET | 0.50 | 13.68 | Blue | * | Toledo (Spain) | Weak |
| 26 | PET | 1.00 | 52.46 | Blue | HDPE | Albacete (Spain) | Weak |
| 27 | Glass | 1.00 | 549.09 | Uncolored | Metal | Cuenca (Spain) | Weak |
| 28 | Glass | 1.00 | 416.50 | Uncolored | Metal | Rhône-Alpes (France) | Medium |
| 29 | Glass | 1.00 | 622.20 | Uncolored | Metal | Girona (Spain) | Weak |

PET, polyethylene terephthalate; HDPE, high density polyethylene; *, not indicated; Very weak, <50 mg/L; Weak, 50–500 mg/L; Medium, 500–1500 mg/L; High, >1500 mg/L.

### 2.3. Sample treatment: Solid phase extraction (SPE)

Four different SPE sorbent cartridges, selected for their high capacity and selectivity for compounds covering a wide polarity range, were tested. Their efficacy to extract chemicals that could be active *via* hERα and/or hAR from bottled water was compared, using tap water as procedural blank and one water sample (product 3) in all cases. The cartridges were conditioned according to the manufacturer's recommendation, i.e., Isolute C18, Isolute ENVI+, and Supelclean ENVI 18 with 2 × 4 ml methanol and 2 × 4 ml tap water, and Supelclean ENVI-Carb with 2 × 4 ml acetone and 2 × 4 ml tap water. Then, 1.5 L or 3 L of tap water or water sample (for determination of (anti-)estrogenic or (anti-)androgenic activities, respectively) were loaded on each column at a flow rate of 12 ml/min. Columns were then dried under gentle nitrogen stream for 1 h and subsequently eluted with 4 ml acetone (ENVI-Carb) or 4 ml methanol (the other three sorbent cartridges). The resulting sample extracts (containing 50 μl DMSO as keeper) were concentrated under nitrogen (by factor of 30,000 for 1.5 L or 60,000 for 3 L) and kept in glass vials with polytetrafluoroethylene (PTFE) caps (−20 °C) until analysis in the different bioassays.

For the main experiment, we selected the sorbent cartridges that repeatedly showed statistically significant estrogenic and/or anti-androgenic activity in extracts from bottled water and absence of activity in extracts from tap water. As a consequence, Isolute C18 sorbent cartridges and Supelclean ENVI-Carb sorbents cartridges were chosen for the extraction of chemicals that could be active *via* hERα and hAR, respectively (see Results section). This optimized SPE procedure was then applied to extract samples from the 29 different bottled water products.

### 2.4. Cell lines and culture conditions

Cell lines used in this study were cultured as previously reported (Molina-Molina et al., 2013). In brief, human breast cancer MCF-7 cells were cultured for routine maintenance in DMEM with phenol red supplemented with 10% FBS, while human prostate cancer PALM cells were cultured in Ham's F12 supplemented with 10% FBS, 1 mg/ml G418, and 1 μg/ml puromycin. Because of the hormonal activity of phenol red and FBS, experiments were performed in a test culture medium of, phenol red-free DMEM supplemented with 10% dextran-coated charcoal-FBS (10% DCC-FBS) for MCF-7 cells or of Ham's F12 supplemented with 6% DCC-FBS and 1% antibiotic for PALM cells, in a 5% $CO_2$ humidified atmosphere at 37 °C.

### 2.5. E-Screen bioassay

Assay procedure and data analysis were conducted as previously described (Molina-Molina et al., 2013) with some modifications for the testing of water samples. Briefly, MCF-7 cells were trypsinized and plated in 96-well culture plates at initial concentrations of $4 \times 10^3$ cells per well. On the second day, the seeding medium was removed and replaced with 150 μl test culture medium. Sample extracts (in DMSO) were serially diluted in the same medium (2–0.25%) and 50 μl were added per well, resulting in final solvent concentrations of 0.5–0.0625% (v/v). A dose–response curve (0.1–1000 pM) for $E_2$ and negative control (cell treated only with hormone-free medium) and solvent controls (blank and solvent) were included in each experiment. The bioassay was ended on day 6 (late exponential phase) by removing the media from wells, fixing the cells, and staining them with SRB. Finally, bound dye was solubilized and the absorbance was read at 492 nm.

Agonistic assays were performed with increasing concentrations (0.0625%, 0.125%, 0.25%, or 0.5%) of sample extracts. The ratio between the cell yield obtained and the proliferation of hormone-free control cells (negative control) was calculated for each concentration. Tests were done in triplicate and results were expressed as proliferative effect (PE) [MCF-7 cell proliferation (-fold over control)]. The antagonistic activities of extracts were determined by co-incubation with the agonist $E_2$ at 100 pM. Because the PE only provides information on the effect

of the sample extract in the E-Screen bioassay (0.0625%, 0.125%, 0.25% or 0.5% equivalent to 3.7, 7.5, 15 or 30 ml of bottled water, respectively), we derived $E_2$Eq and ICIEq (related to 1 L of bottled water) by reading from dose–response curves of these compounds in order to quantify the (anti-)estrogenic activity of the original water samples. Hence, the PE of each sample extract was referred to the maximal PE obtained with $E_2$ or ICI and transformed into $E_2$ or ICI equivalent units ($E_2$Eq or ICIEq). In particular, for each sample extract, $E_2$Eq or ICIEq was calculated by using the concentration obtaining the greatest induction or inhibition of cell proliferation, respectively. Given that the water samples were concentrated by a factor of 30,000 via SPE and tested at concentrations of 0.0625%, 0.125%, 0.25% and 0.5% in the E-Screen, the final concentration factor was 18.7, 37.5, 75, or 150 (respectively) for all samples. The $E_2$Eq and ICIEq values derived were corrected by the concentration factor and reported as $E_2$Eq/L or ICIEq/L of the original water sample.

### 2.6. PALM cell luciferase assay

Cells were seeded at a density of $5 \times 10^4$ cells per well in 96-well white opaque tissue culture plates in 150 μl test culture medium. Sample extracts were serially diluted (as described above for the E-Screen bioassay) and 50 μl were added per well at 8 h after seeding. On each plate, alongside the test samples, serial dilutions of the agonist R1881 (1–10,000 pM) were included as positive and negative controls (test culture medium alone or with solvent). PALM cells were incubated for 40 h at 37 °C, and the medium was then removed and replaced by test culture medium containing 0.3 mM luciferin. Next, the 96-well plate was introduced into a luminometer for 2 s to measure luminescence from intact living cells.

hAR-agonistic activities were tested with increasing concentrations of sample extracts, performing tests in quadruplicate for each concentration. Maximal luciferase activity (100%) was obtained in the presence of 10 nM R1881. The antagonistic activity of extracts was determined by coincubation with R1881 agonist (0.3 nM). Results were expressed as percentage of maximal luciferase activity.

Unlike in the E-screen bioassay, each water sample (3 L) was concentrated by a factor of 60,000 *via* SPE for determination of the (anti-) androgenic activity, based on previous experiments, and was tested at final concentrations of 0.0625%, 0.125%, 0.25%, or 0.5% in the well; therefore, the final concentration factor was 37.5, 75, 150, or 300, respectively. Accordingly, the luciferase activity reported here (referred to a volume of 200 μl per well) is equivalent to a sample volume of 7.5, 15, 30, or 60 ml, respectively, of the original water sample.

Finally, the luciferase activity in each sample extract was calculated as a percentage of the maximal luciferase activity obtained with R1881 or procymidone and transformed into R1881 or procymidone equivalent units (R1881Eq or ProcEq, respectively) by reading from dose–response curves of R1881 or procymidone (standard serial dilutions) included on each plate. R1881Eq or ProcEq were calculated from the concentration obtaining the greatest induction or inhibition of luciferase activity, respectively. Derived R1881Eq or ProcEq were corrected for the concentration factor and reported as R1881Eq/L or ProcEq/L of the original water sample.

### 2.7. MTT assay for evaluating cell toxicity

The effect of sample extracts on cell viability was assessed with the MTT test, using Denizot and Lang's (1986) modified technique. Briefly, cell lines (MCF-7 and PALM) were seeded at a density of $5 \times 10^4$ cells per well in 96-well culture plates for 8 h, followed by treatment with different concentrations (0.5–0.0625%) of each extract in the absence and presence of $E_2$ or R1881 (both at 10 nM concentrations) for a further 40 or 144 h (for PALM and MCF-7 cells, respectively). Cells were washed with PBS, and 100 μl of MTT solution (0.5 mg/ml) were then added to each well. After incubation (2 h), viable cells cleaved the MTT tetrazolium ring into a dark blue formazan reaction product, whereas dead cells remained colorless. The MTT-containing medium was gently removed, and 150 μl DMSO were added to each well. After shaking, the plates were read in absorbance at 540 nm. Cell viability of treated cells was calculated in reference to the untreated control cells using the following formula: viability (%) = 100 × (Sample Abs)/(Control Abs), where Abs is the absorbance value at 540 nm. Medium alone with no cells served as an additional control. Three independent experiments run in triplicate were performed, and data were expressed as the mean of three wells.

### 2.8. Data analysis

For all assays, each sample extract was tested at various concentrations in at least three independent experiments, and data were expressed as means ± SD. Individual dose–response curves for $E_2$, ICI, R1881, and procymidone were fitted using the sigmoid dose–response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA), expressing the results as $EC_{50}$ and $IC_{50}$ values. Data were analyzed for significant differences using one-way ANOVA followed by Dunnett's post-comparison test (vs. control). Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Efficacy of different SPE sorbent cartridges

No tap water extracts from any of the four SPE sorbents tested showed any significant (anti-)estrogenic activity in the E-Screen or (anti-)androgenic activity in the PALM bioassay, respectively, indicating that the extraction procedure itself did not contaminate. All four SPE sorbents yielded bottled water extracts that were significantly estrogenic in the E-Screen (Fig. 1A), obtaining the maximal increase in cell number (3.1-fold vs. control) in the proliferation test with the C18 sorbent. Only the ENVI-Carb sorbent yielded an extract that was significantly anti-androgenic in the PALM bioassay, with extracts from the other sorbents (C18, ENVI + and ENVI 18) proving unable to inhibit the luciferase activity induced by 0.3 nM of R1881 (Fig. 1B). Based on these results, C18 and ENVI-Carb cartridges were used to process water samples for the (anti-)estrogenic and (anti-)androgenic bioassays, respectively.

### 3.2. Cytotoxicity assessment

The MTT test was used to assess the cytotoxicity of sample extracts in a concentration range of 0.0625–0.5% for the two cell lines studied. When the viability of MCF-7 cells was analyzed after 144 h of incubation without $E_2$, the cell proliferation and therefore viability increased due to the presence of the estrogenic compounds in most of the sample extracts, indicating that the extracts were not cytotoxic. Moreover, studies employing a saturating concentration of $E_2$ (10 nM) indicated that MCF-7 cell viability or survival ranged from 96% to 100% with reference to the control cells. Finally, PALM cells showed no significant changes in cell viability after 40 h of incubation in the absence or presence of saturating concentrations (10 nM) of the agonist R1881, finding no cytotoxicity at any concentration (data not shown).

### 3.3. Effect of bottled water extracts in the E-Screen bioassay

In the E-screen bioassay, the bottled water samples extracted with the optimized method revealed a statistically significant estrogenic activity in 23 (79.3%) of the 29 products tested, with PE values ranging from 1.88 to 3.55 (products 22 and 4, respectively) and no significant estrogenic activity in the other six (products 11, 17, 19, 21, 26, and 28) (Fig. 2A). Estrogenicity was detected in 80.7% of the waters from plastic

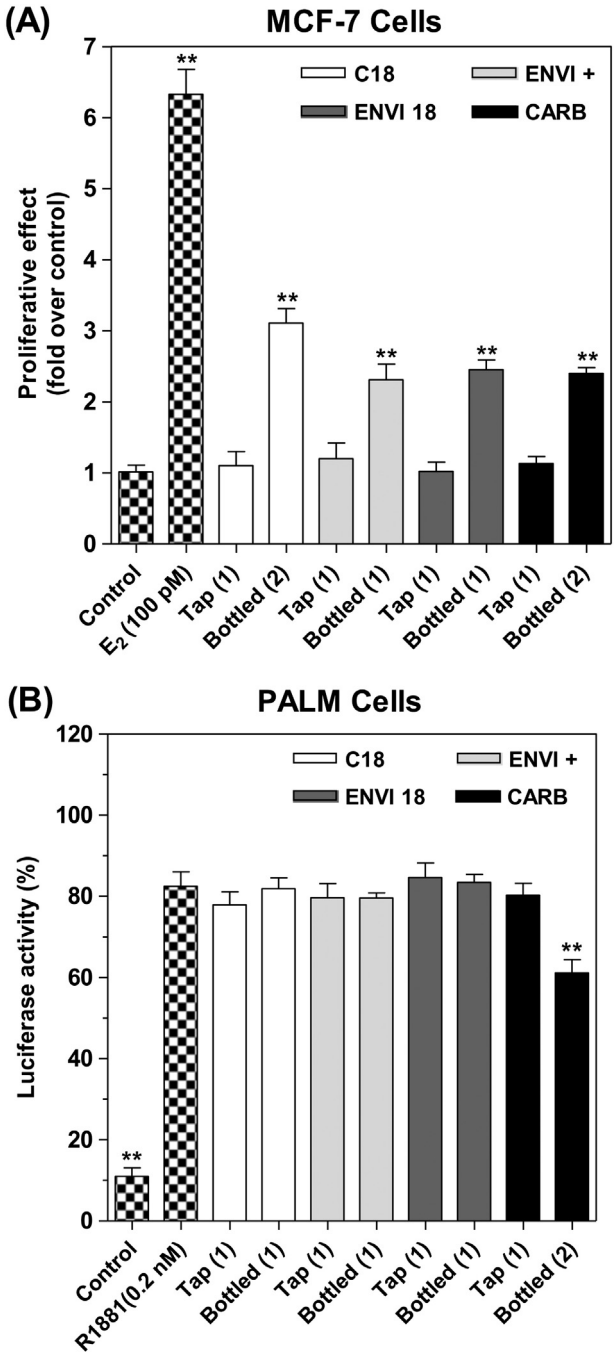


**Fig. 1.** Comparison of the extraction efficacy of different SPE methods to study the estrogenic and anti-androgenic activities of tap and bottled water. Panel (A): Estrogenic activity in MCF-7 cells. Results are expressed as proliferative effect (mean ± SD of three separate experiments). **p < 0.01 (versus hormone-free control). Panel (B): Anti-androgenic activity in PALM cells. Results are expressed as percentage of luciferase activity (mean ± SD of three separate experiments). **p < 0.01 (versus R1881 0.3 nM). The sample extract concentration producing the maximum effect/inhibition is reported in brackets: (1) 0.5% and (2) 0.25%.

bottles (21/26 bottles) *versus* 66.6% of the waters from glass bottles (2/3 bottles). It was then investigated whether these estrogenic effects were hER-mediated. As expected, co-incubation with pure anti-estrogen ICI (10 nM) abolished the estrogen response in all estrogen-positive samples, confirming that the cell proliferation observed by E-screen was mediated by hER (data not shown).

Significant hER antagonistic activity was detected in extracts from 11 of the 29 products (37.9%), inhibiting the proliferation induced by 100 pM of $E_2$ (Fig. 2B), whereas the remaining products showed no significant antagonistic activity. hER-antagonistic activity was observed in 38.4% of samples from plastic bottles (10/26 bottles) and in 33.3% of those from glass bottles (1/3 bottles). Interestingly, 63.6% of the extracts showing antagonistic activity also demonstrated agonist activity. Fig. 3 shows the dose–response curves of bottled water sample number 20 in the E-Screen bioassay.

As previously reported (Molina-Molina et al., 2013), the natural estrogen $E_2$ strongly induced significant proliferation of MCF-7 cells in a dose-dependent manner, with an $EC_{50}$ value of 0.018 nM (Fig. 4A), while the synthetic anti-estrogen ICI exhibited a marked hER antagonistic activity in this cell line, with an $IC_{50}$ value (concentration required for 50% of maximal inhibition of $E_2$-induced proliferation) of 0.97 nM (Fig. 4B). As shown in Table 2, the estrogenic potency of water extracts, expressed in $E_2$Eq/L, ranged from 0.041 pM (product 7) to 0.286 pM (product 25), with a mean value of 0.113 ± 0.07 pM $E_2$Eq/L for all estrogenic samples analyzed. Finally, the mean ICIEq/L was 11.01 ± 6.65 pM, and the anti-estrogenic activity ranged from 2.21 (product 3) to 19.12 pM (product 20).

### 3.4. PALM bioassay results for bottled water extracts

In the PALM bioassay, hAR was significantly activated by only eight (27.5%) of the bottled water extracts after 40 h of exposure (Fig. 5A). Luciferase activations ranged from 19.4% (product 3) to 24.4% (product 19), indicating the presence of androgen-like chemicals. Out of the 29 samples analyzed, 12 (41.3%) were hAR antagonists, moderately inhibiting the luciferase activity induced by 0.3 nM of R1881 (Fig. 5B); with luciferase inhibition values ranging from 17.5% (product 3) to 40.1% (product 25). Interestingly, anti-androgenic activities were only detected in samples of water from plastic bottles, whereas androgenic activities were detected in samples from both plastic and glass bottles (26.9% and 33.3% of the analyzed products, respectively). Fig. 6 shows an example of the dose–response curves of bottled water sample number 19 in the PALM assay.

In line with previous reports (Molina-Molina et al., 2013), the synthetic androgen R1881 exhibited hAR agonistic activity in PALM cells with an $EC_{50}$ value of 0.1 nM (Fig. 4C). By contrast, the well-known anti-androgen procymidone strongly inhibited the luciferase activity induced by 0.2 nM of R1881 in this cell line, with an $IC_{50}$ value of 1.98 μM (Fig. 4D). The mean androgenicity of the bottled waters was 0.033 ± 0.001 pM R1881Eq/L, ranging from 0.032 (product 3) to 0.036 (product 28) pM R1881Eq/L. When the anti-androgen potential of bottled waters was determined, activities ranged from 0.18 to 4.43 nM (ProcEq/L) (Table 2), with a mean value of 1.61 ± 1.07 nM ProcEq/L.

## 4. Discussion

This study contributes, for the first time, quantitative data on the (anti-)estrogenic and (anti-)androgenic burden of commercially available bottled waters in Southern Spain. All samples tested evidenced at least one of the four hormonal activities measured, suggesting that bottled water consumption contributes to human exposure to EDCs. The hormone-like activities of the water samples were quantified in $E_2$Eq, ICIEq, R1881Eq, or ProcEq per liter of bottled water to facilitate comparison with other published results. Hormonal activities ranged from 27.5% (androgenicity) to 79.3% (estrogenicity) of the samples, with a mean concentration of 0.113 ± 0.07 pM $E_2$Eq/L, 11.01 ± 6.65 pM ICIEq/L, 0.033 ± 0.036 pM R1881Eq/L, and 1.61 ± 1.07 nM ProcEq/L. These findings suggest contamination during the processing and/or bottling of waters by hormone-like chemicals, because the sources of (anti-)estrogens and/or (anti-)androgens in water would always be exogenous, unlike the naturally-occurring hormones in foodstuff and beverages.

A large number of techniques are currently used for the extraction of EDCs in environmental matrices (Sosa-Ferrera et al., 2013). SPE, which is based on the partition equilibrium of analytes between sorbent and

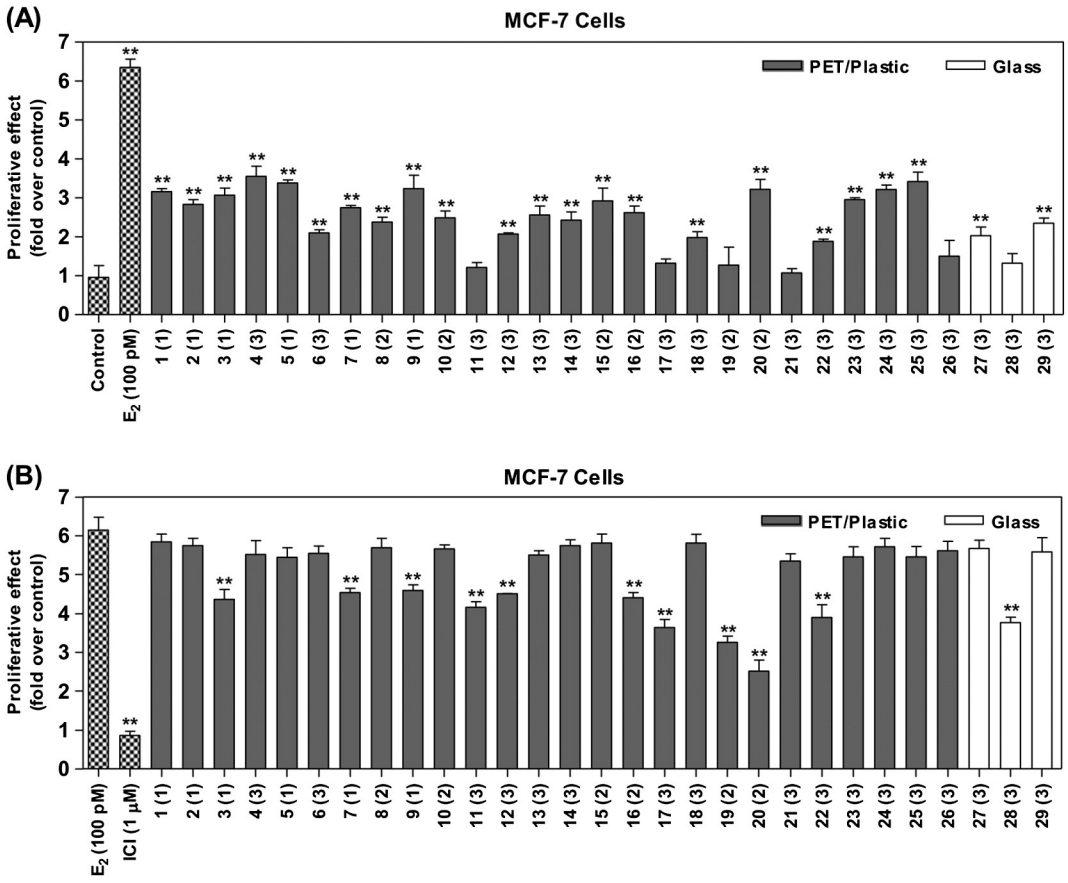


**Fig. 2.** Effect of bottled water samples in the E-Screen bioassay. Panel (A): Proliferative response of MCF-7 cells. Cells were incubated for 144 h at 37 °C with increasing concentrations (0.0625–0.5%) of water sample extracts of the studied products. Results are expressed as proliferative effect (mean ± SD of three separate experiments). **$p < 0.01$ (versus hormone-free control). Panel (B): Anti-proliferative response of MCF-7 cells. Cells were treated with 100 pM $E_2$ in the presence of the water sample extracts for 144 h at 37 °C. Results are expressed as proliferative effect (mean ± SD of three separate experiments). **$p < 0.01$ (versus $E_2$ 100 pM). The sample concentration producing the maximum (anti-)proliferative effect is reported in brackets: (1) 0.5%, (2) 0.25% and (3) 0.125%.

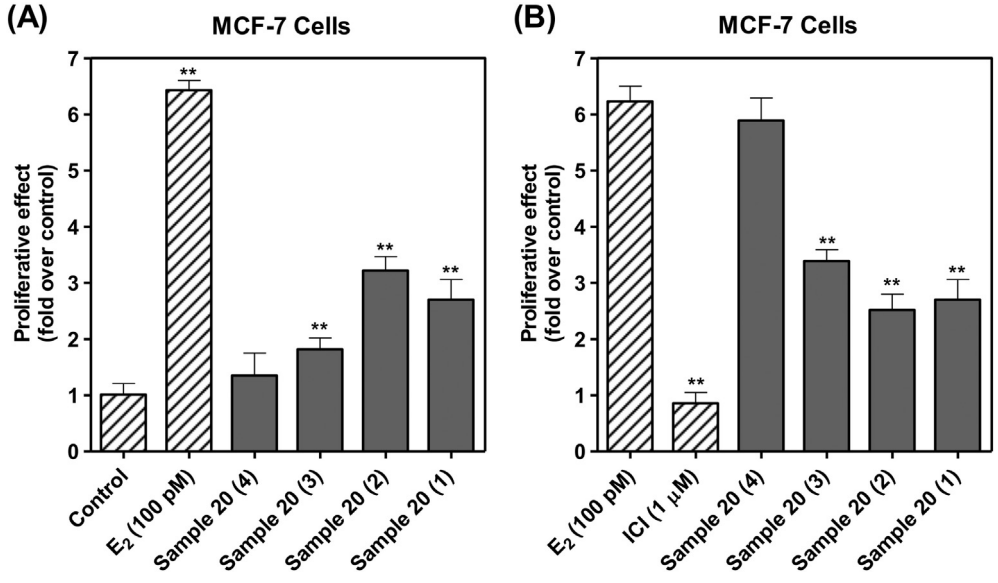


**Fig. 3.** Dose–response curves of bottled water sample number 20 in the E-Screen bioassay. Panel (A): Proliferative response of MCF-7 cells. Cells were incubated for 144 h at 37 °C with increasing concentrations (0.0625–0.5%) of this water sample extract. Results are expressed as proliferative effect (mean ± SD of three separate experiments). **$p < 0.01$ (versus hormone-free control). Panel (B): Anti-proliferative response of MCF-7 cells. Cells were treated with 100 pM $E_2$ in the presence of this water sample extract for 144 h at 37 °C. Results are expressed as proliferative effect (mean ± SD of three separate experiments). **$p < 0.01$ (versus $E_2$ 100 pM). The sample concentration producing the (anti-)proliferative effect is reported in brackets: (1) 0.5%, (2) 0.25%, (3) 0.125% and (4) 0.0625%.

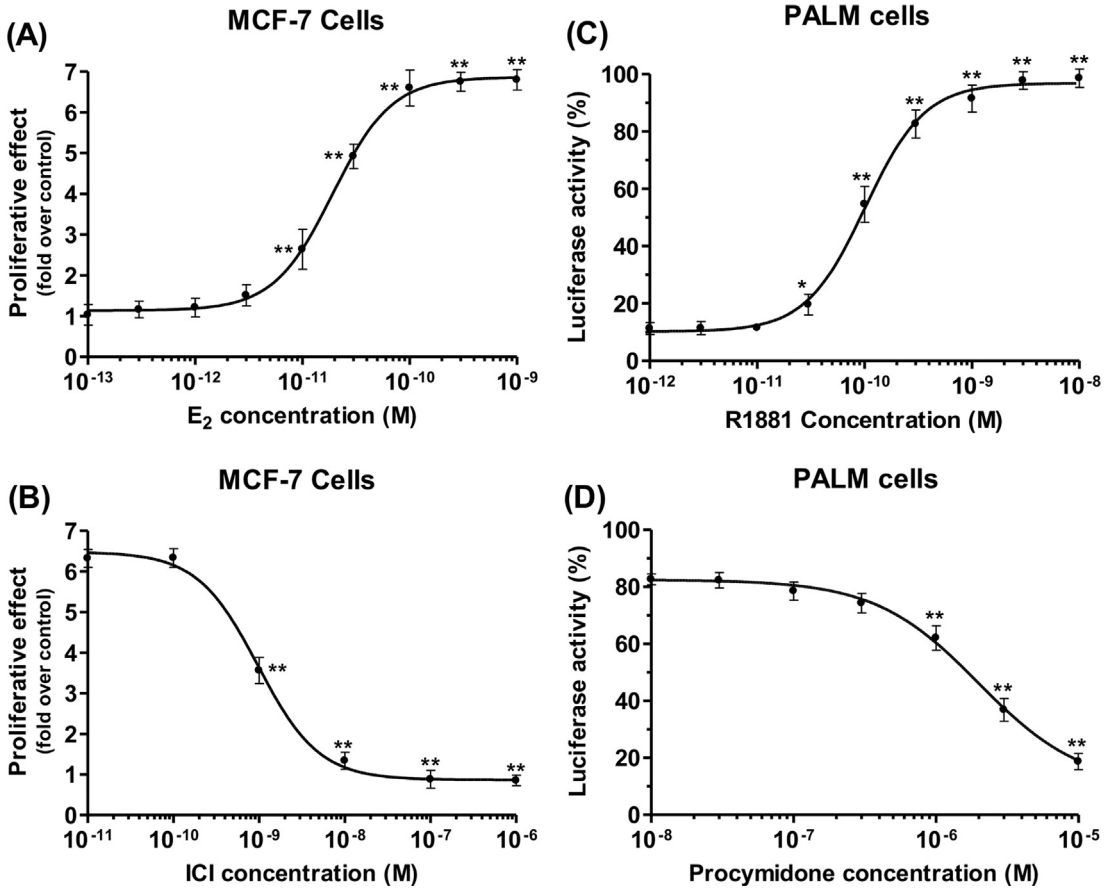


**Fig. 4.** Dose–response curves of $E_2$ and ICI on MCF-7 cells and R1881 and procymidone on PALM cells. MCF-7 cells were incubated for 144 h at 37 °C with $E_2$ (Panel A) or ICI in the presence of 100 pM $E_2$ (Panel B) at the indicated concentrations. Results are expressed as proliferative effect (mean ± SD of three separate experiments). *p < 0.05 and **p < 0.01 (versus hormone-free control or $E_2$ 100 pM). PALM cells treated with R1881 (Panel C) or procymidone in the presence of 0.3 nM R1881 (Panel D) at the indicated concentrations for 40 h at 37 °C. Results are expressed as percentage of maximal R1881 induction (mean ± SD of three separate experiments). *p < 0.05 and **p < 0.01 (versus R1881 10 or 0.3 nM).

samples, allows high enrichment and cleaning up of the samples. However, the selective interaction mechanism of most of the available SPE sorbents makes this a challenging task, because the analytes exhibit different physicochemical properties (D'Archivio et al., 2007). We therefore decided to test four different SPE sorbent cartridges in order to retain compounds with a wide polarity range. Both C18 and ENVI-18 are octadecyl-bonded silica sorbents, but ENVI-18 has a higher phase coverage and carbon content and is more resistant to pH extremes than is C18. They can be used to extract compounds such as parabens, phenols, phthalates, steroids, and non-polar to moderately polar pesticides and herbicides. In contrast, the polymeric sorbent ENV+ (hydroxylated polystyrene-divinylbenzene) has been used for the extraction of highly polar compounds from water (Majzik et al., 2006). Octadecyl-bonded silica sorbents have proven superior for low polarity compounds such as BPA (Maragou et al., 2006; Samaras et al., 2011). The other sorbent tested was ENVI-Carb, a graphitized carbon black sorbent that retains nonpolar compounds in addition to highly polar compounds such as alcohols, nitrophenols, perfluorinated carboxylic acids, and relatively polar herbicides (carbamates, phenylureas and phenoxy acids) with no need to adjust the pH of the sample (Seen et al., 2014). After comparing the four sorbents, we selected two that repeatedly showed statistically significant estrogenic and/or anti-androgenic activity in extracts from bottled water and absence of activity in extracts from tap water. C18 sorbent was selected for the extraction of (anti-)estrogenic substances, suggesting that non-polar and very low polar residues are the main contributors to these activities, and ENVI-Carb sorbent was selected for the extraction of (anti-)androgenic compounds, suggesting that, in addition to non-polar compounds, moderately or highly polar compounds may mediate these activities.

Three-quarters of the bottled waters were estrogenic, with an estrogenicity ranging from 0.041 to 0.286 pM $E_2$Eq/L. Furthermore, the bioassay showed a significant estrogenic signal in nearly 80% of the eighteen samples from PET bottles, in 86% of the eight samples from plastic bottles whose composition is not given on the label (see Table 1), and in 66.6% of the three samples from glass bottles.

Estrogenicity in bottled water was first reported by Wagner and Oehlmann (2009), using a recombinant yeast system carrying hERα to test 20 brands of water packaged in PET, glass, or coated paperboard. Estrogenicity was observed in a third of samples bottled in glass and almost 90% of brands in PET containers, suggesting that estrogenic chemicals responsible for hormonal activity were present regardless of the container type. Estrogenicity values ranged from below the quantification limit to a maximum of 75.2 ± 5.95 ng/L, corresponding to 270.0 ± 20.0 pM $E_2$Eq/L. Pinto and Reali (2009) used a yeast-based bioassay to investigate the estrogenicity of water samples from nine PET bottles available in Italy and reported estrogenicity values ranging from 0.9 to 23.1 ng $E_2$Eq/L (3.30 to 84.80 pM $E_2$Eq/L). Wagner and Oehlmann (2011), using an improved extraction procedure with the E-Screen bioassay, detected an estrogenic response in 61% of 18 samples analyzed, with values ranging from 1.9 to 12.2 pg/L $E_2$Eq (0.007–0.044 pM $E_2$Eq/L). In 2013, Plotan and coworkers confirmed the findings published by Wagner and Pinto using a bioassay based on reporter MCF-7 cells, reporting estrogenicity in 64.2% of 14 samples from PET or glass bottles, ranging between 1 and 34 ng $E_2$Eq/L (3.67 and 124.2 pM $E_2$Eq/L), with a mean value of 10 ng $E_2$Eq/L (36.71 pM $E_2$Eq/L).

**Table 2**
(Anti-)estrogenic and (anti-)androgenic potencies of the 29 bottled water products determined by E-Screen or PALM luciferase assay and expressed in concentrations equivalent to $E_2$, ICI, R1881, or Procymidone per liter.

| Products | E-Screen bioassay | | PALM luciferase assay | |
|---|---|---|---|---|
| | $E_2$Eq/L (pM) | ICIEq/L (pM) | R1881Eq/L (pM) | ProcEq/L ($10^3$ pM) |
| 1 | 0.048 | – | – | – |
| 2 | 0.042 | – | – | – |
| 3 | 0.046 | 2.21 | 0.032 | 0.18 |
| 4 | 0.214 | – | – | 1.11 |
| 5 | 0.053 | – | – | 2.01 |
| 6 | 0.108 | – | – | – |
| 7 | 0.041 | 2.65 | – | – |
| 8 | 0.241 | – | – | – |
| 9 | 0.054 | 3.10 | – | 1.88 |
| 10 | 0.061 | – | – | 0.88 |
| 11 | – | 15.93 | – | 1.45 |
| 12 | 0.047 | 10.71 | – | – |
| 13 | 0.141 | – | 0.033 | – |
| 14 | 0.124 | – | – | 1.55 |
| 15 | 0.098 | – | – | – |
| 16 | 0.081 | 6.21 | – | – |
| 17 | – | 17.76 | – | 2.10 |
| 18 | 0.088 | – | – | 2.05 |
| 19 | – | 9.86 | 0.035 | 0.74 |
| 20 | 0.098 | 19.09 | – | – |
| 21 | – | – | 0.034 | – |
| 22 | 0.086 | 14.82 | 0.033 | – |
| 23 | 0.190 | – | – | 0.85 |
| 24 | 0.253 | – | 0.033 | – |
| 25 | 0.286 | – | – | 4.43 |
| 26 | – | – | 0.034 | – |
| 27 | 0.098 | – | – | – |
| 28 | – | 18.71 | 0.036 | – |
| 29 | 0.161 | – | – | – |

(–) water samples without statistically significant hormonal activity.

None of the samples assayed for estrogenicity showed a maximal PE comparable to that observed with $E_2$. Furthermore, the quantification of estrogenic activity can be modified by the presence of compounds with antagonist activity competing with agonists for binding at the same site in the ligand-binding domain of the ER. In fact, almost one-third of the estrogenic samples assayed (7/23) showed antagonist activity in the presence of $E_2$. Hence, account should be taken of the possible presence of chemical residues with agonist and antagonist effects in the extracts. In this regard, Silva et al. (2002) combined eight xenoestrogens at levels equivalent to 50% of their individual no observed effect concentrations (NOECs) and obtained responses of up to 40% of the maximal estrogenic effect in a recombinant yeast estrogen screen (YES), suggesting that complex interactions may occur in mixtures.

Comparisons with the results of other studies are hampered by differences in sample preparation methods and bioassays. As mentioned above, because a specific sorbent is able to extract only a finite fraction of the broad spectrum of bioactive compounds from a complex mixture, the comparison of different methods of SPE and therefore the choice of sorbent determines the activity detected in the bioassay (Wagner and Oehlmann, 2011). Furthermore, the procedure used for the extraction of chemicals may depend on the different conditioning and eluting procedures used. In this context, various authors have concluded that all extraction protocols are not equally successful in extracting EDCs and that false negative results can be produced, which may explain the inability of some authors to detect hormonal activity in bottled water (Wagner et al., 2013). Interestingly, those using a similar approach and methodology, such as the E-Screen bioassay, found comparable results in terms of the frequency and activity of estrogenic samples, as in the case of the studies by Wagner and Oehlmann (2011) and Stanford et al. (2010). Bearing these limitations in mind, it is notable that studies from different institutions, performed with different bioassays and samples from different countries, come to the same conclusion: bottled water is widely contaminated with EDCs, mostly with estrogen-like properties.

In line with previous studies on anti-estrogenicity in bottled water, we found anti-estrogenic activity (11.01 ± 6.65 pM ICIEq/L) in 37.9% of the samples, more frequently in samples from plastic (38.4%) *versus* glass (33.3%) bottles. Wagner et al. (2013) reported moderate to potent anti-estrogenic activity (up to 60% of inhibition) in 72.2% of 18 bottled water samples analyzed with a yeast estrogen screen. These findings suggest that anti-estrogens are present in the majority of bottled water products.

Many environmental compounds with estrogenic activity can also have anti-androgenic activity (Sohoni and Sumpter, 1998), and we detected anti-androgenic activity in 41.4% (12/29) of the bottled waters tested, with percentage inhibition of hAR ranging from 17.5% to 40.1%. Weak agonistic activity was detected in around 30% of the samples. Wagner et al. (2013) found anti-androgenic activity in almost 90% of bottled water samples (16/18), with percentage inhibition ranging from 19% to 92%. In another recent study, Plotan et al. (2013) reported anti-androgenic activity in 42.8% (6/14) of bottled water samples.

The presence of hormone-like activities in bottled waters represents a complex problem, and the origin of the substances responsible remains controversial (Bach et al., 2012). The compounds that migrate from PET bottles comprise production residues and hydrolysis and thermal degradation products of PET itself as well as additives and, in the case of recycled or reused PET bottles, constituents of beverages previously contained in the bottles, such as flavor compounds or detergents. This migration depends on the chemical quality of the raw material, the molecular weight of the polymer, and the manufacturing technology used (Bach et al., 2013).

Phthalates have been frequently found in PET-bottled waters (Bošnir et al., 2007; Montuori et al., 2008) but are not used as additives in the manufacturing of PET bottles; therefore, their presence in bottled waters has been attributed to substances used for coloring the bottles (Kim et al., 1990). Furthermore, phthalate contamination in the bottling line (Higuchi et al., 2004), water treatment facilities (Leivadara et al., 2008), or cap-sealing resins (Hirayama et al., 2001) may also make a contribution. Research on leaching from PET has concluded that time is the main factor governing the release of organic substances (Criado et al., 2005).

Other chemical candidates for hormonal activity include alkylphenols (Amiridou and Voutsa, 2011; Li et al., 2010), which are of special interest in the context of PET recycling and re-utilization (Franz et al., 2004). Thus, Guart et al. (2011) found 4-nonylphenol (NP) and octylphenol (OP) in 2 out of 10 samples of PET-bottled water. Loyo-Rosales et al. (2004) concluded that the NP derived from the water itself or from washing steps during manufacture of the container. Another study attributed the presence of NP in glass-bottled water to the surfactants used to wash the bottles before filling (Bach et al., 2012).

Unlike polycarbonates, PET is believed to be BPA-free, but some authors have reported its presence in water from PET bottles (Amiridou and Voutsa, 2011; Maggioni et al., 2013). It has been suggested that the presence of BPA and other compounds in PET-bottled water may be due to the bottle caps (Guart et al., 2011), pollution of the water prior to its bottling (Li et al., 2010), or the use of recycled PET (Bach et al., 2012; Sax, 2010).

Finally, Guart et al. (2014b) detected benzophenone (BP) in water samples from PET bottles with high-density polyethylene (HDPE) caps. BP can be used as an additive or polymer production aid (EU, 2012) and as a photoinitiator catalyst for inks and lacquers cured with ultraviolet light. In comparison to glass bottles, PET-bottled waters were found to contain 2-fold higher levels of 2,4-di-tert-butylphenol (2,4-dtBP) (Bach et al., 2013), reported to be an androgen antagonist in CHO-K1 cells and rainbow trout (Tollefsen et al., 2008). The authors attributed this finding to the plastic material in the bottle caps rather than to PET. PE and polypropylene (PP) plastics are widely used for

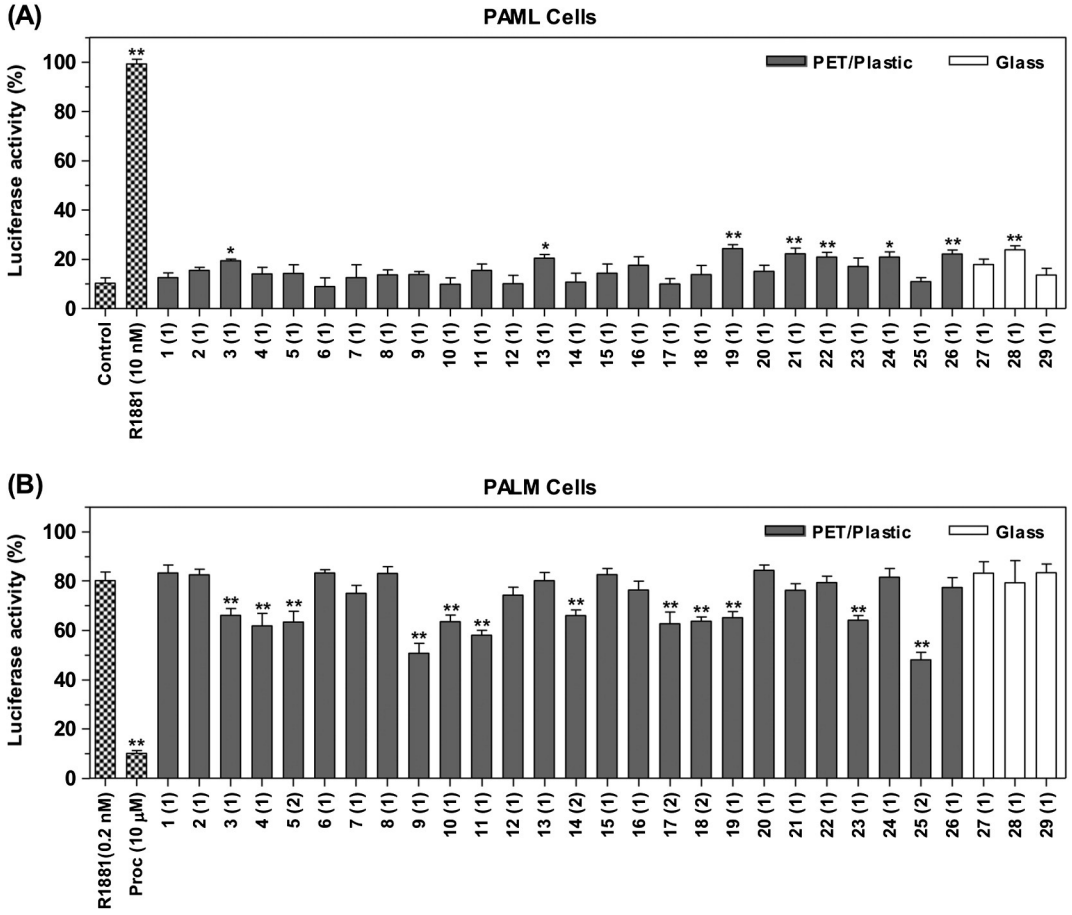


**Fig. 5.** Effect of bottled water samples on transcriptional activation *via* hAR. Panel (A): Induction of luciferase activity in PALM cells, which were incubated for 40 h at 37 °C with increasing concentrations (0.0625–0.5%) of the water sample extracts. Results are expressed as percentage of maximal R1881 induction (mean ± SD). *$p < 0.05$ and **$p < 0.01$ (versus versus R1881 10 nM). Panel (B): Inhibition of luciferase activity in PALM cells, which were treated with 0.3 nM R1881 in the presence of increasing concentrations of the water sample extracts for 40 h. Results are expressed as percentage of 0.3 nM R1881 induction. Values are mean ± SD of three separate experiments. **$p < 0.01$ (versus R1881 0.3 nM). The sample concentration producing the maximum induction/inhibition of luciferase activity is reported in brackets: (1) 0.5% and (2) 0.25%.

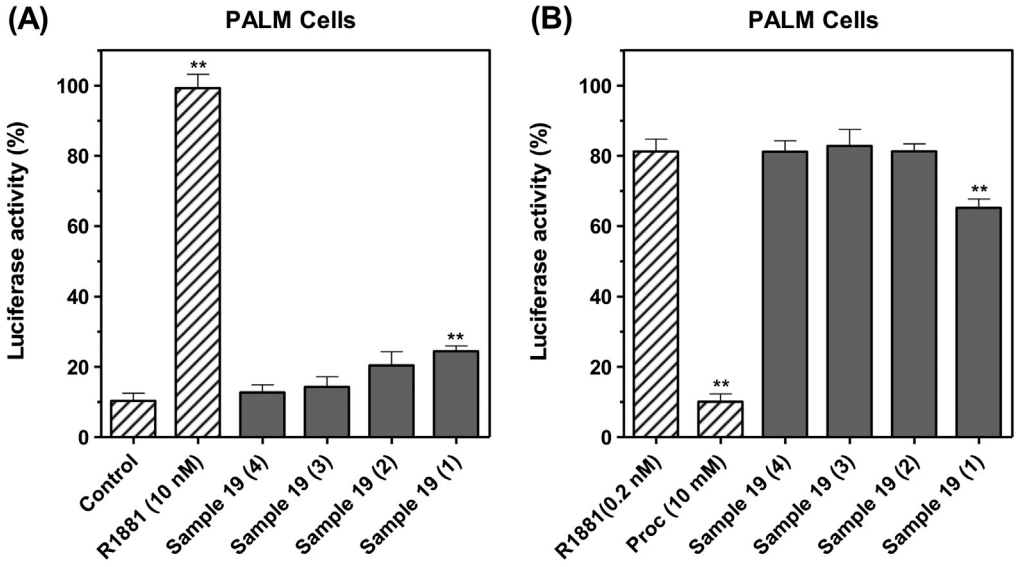


**Fig. 6.** Dose–response curves of bottled water sample number 19 in the PALM assay. Panel (A): Induction of luciferase activity in PALM cells, which were incubated for 40 h at 37 °C with increasing concentrations (0.0625–0.5%) of this water sample extract. Results are expressed as percentage of maximal R1881 induction (mean ± SD of three separate experiments). **$p < 0.01$ (*versus* R1881 10 nM). Panel (B): Inhibition of luciferase activity in PALM cells, which were treated with 0.3 nM R1881 in the presence of increasing concentrations of this water sample extract for 40 h. Results are expressed as percentage of 0.3 nM R1881 induction. Values are mean ± SD of three separate experiments. **$p < 0.01$ (versus R1881 0.3 nM). The sample concentration producing the induction/inhibition of luciferase activity is reported in brackets: (1) 0.5%, (2) 0.25%, (3) 0.125% and (4) 0.0625%.

bottle caps and can be a source of EDCs (Yang et al., 2011). Shotyk et al. (2006) reported up to 30-fold higher concentrations of Sb in water from PET bottles than in water from glass bottles manufactured using $Sb_2O_3$ as catalyst (Keresztes et al., 2009; Shotyk and Krachler, 2007b; Westerhoff et al., 2008). Sb concentrations detected in bottled water exhibited estrogenic activity *in vitro* (Sax, 2010). However, more metals (Ce, Pb, Al, and Zr) can leach into the water from glass than from PET bottles (Reimann et al., 2010).

The migration of EDCs into water from glass bottles (Shotyk and Krachler, 2007b) and plastic bottles or caps (Franz and Welle, 2009) is controversial. Franz and Welle (2009) studied the composition of plastic bottles and caps and concluded that the hormonal activity detected in bottled water cannot be explained by the migration of these chemical compounds, because their estrogenic potency is too weak. However, low concentrations of EDCs can have an additive or synergistic "cocktail" effect, which may account for the activity observed, and/or unknown compounds may play a role (Muncke, 2009). Expectations of the agonist and antagonistic interactions of chemicals present in bottled water extracts depend on the results of experimental studies of *ad hoc*-prepared chemical mixtures. First, the final hormonal effect seen in the bioassays can be described using the most accepted mathematical model (Concentration Addition), which indicates that there can be a mixture effect even when each component is present at a low and individually ineffective concentration (Christen et al., 2012; Kunz and Fent, 2009; Le Page et al., 2006; Orton et al., 2012; Rajapakse et al., 2002; Silva et al., 2002; van Meeuwen et al., 2007). When compounds interact additively, one compound can be replaced with a fraction of an equally effective concentration of another compound without changing the overall combined effect. Second, some deviations from additivity may be expected (Charles et al., 2002; Kjaerstad et al., 2010), either in a negative direction due to an increased metabolism of natural steroidal estrogens in the mixture (Silva et al., 2011) or in a positive direction because of synergistic interactions related to additional interactions with the AR at receptor sites other than the ligand-binding domain (Orton et al., 2012). Consequently, it should be taken into account that the presence of each individual chemical residue at a "no-observed-adverse-effect level" does not necessarily imply a zero effect and does not rule out a potential impact of the mixture (Kortenkamp et al., 2007; Scholze et al., 2014).

There have been calls for the integration of knowledge on the chemicals in food contact material and their migration into food and water in epidemiological studies (Muncke et al., 2014). The direct health consequences of EDC exposure from food contact materials are not known; however, given that the entire population is likely to be exposed, it is of utmost importance to reliably and rapidly fill the gaps in our knowledge. The present study was designed in this spirit.

## 5. Conclusions

Individuals consuming bottled water may be exposed to low doses of compounds that potentially deregulate the endocrine system. EDCs in the water may derive from plastic or glass bottles, bottle caps, transport pipelines, disinfection agents, the bottling process itself or even from environmental pollution of the water source. Further research is warranted on the compounds responsible for the hormone-like activities observed in bottled water. It would also be of interest to evaluate the effects of storage time on the levels of these compounds in the water.

## Competing financial interests

The authors declare they have no actual or potential competing financial interests.

## Acknowledgments

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