

## Additive Estrogenic Activities of the Binary Mixtures of Four Estrogenic Chemicals in Recombinant Yeast Expressing Human Estrogen Receptor

Kyung-Sun Kang, Sung-Dae Cho and Yong-Soon Lee\*

Department of Veterinary Public Health, College of Veterinary Medicine, School of Agricultural Biotechnology, Seoul National University, Seodun-Dong, Kwonson-Ku, Suwon 441-744, Korea.

### ABSTRACT

To evaluate the estrogenic activities of several chemicals such as 17 $\beta$ -estradiol (E2),  $\rho$ -nonylphenol, bisphenol A, butylparaben, and combinations of these chemicals, we used recombinant yeasts containing the human estrogen receptor [*Saccharomyces cerevisiae* ER + LYS 8127]. We evaluated E2 was most active in the recombinant yeast assay, followed by  $\rho$ -nonylphenol, bisphenol A, butylparaben. The combinations of some concentrations of 17-estradiol as a strong estrogen and bisphenol A or butylparaben as a weak estrogen showed additive estrogenic effects. Also, the combinations of some concentrations of nonylphenol and butylparaben and combination of butylparaben and bisphenol A showed additive effects in the estrogenic activity.

Therefore, the estrogenic activities of the combinations of two chemicals were additive, not synergistic.

**Key words :** Recombinant yeast, human estrogen receptor,  $\beta$ -galactosidase, combination, additive

### INTRODUCTION

There has been increasing public concern that chemicals in the environment are affecting human health by disrupting normal endocrine function, particularly through interaction directly with steroid hormone receptors. The exposure to these chemicals with steroid-like activity can disrupt normal endocrine function leading to alter reproductive capacity, infertility, endometriosis, and breast and uterine cancer [1, 2, 3, 4, 5, 6]. A wide variety of chemicals have steroid-like activity, including natural products such as coumestrol and genistein, pesticides and fungicides such as DDT and

commercial chemicals such as bisphenol A and  $\rho$ -nonylphenol [7, 8, 9, 10, 11, 12, 13]. Because of the widespread nature of these chemicals, a certain degree of exposure is unavoidable. Thus, it is necessary to determine whether exposure to these chemicals at environmentally relevant concentrations poses a threat to human health.

Bisphenol A is a monomer in polycarbonate plastics and constituent of epoxy resins that are used extensively in the food-packaging industry and in dentistry. Microgram amounts of bisphenol A have been detected in liquid from canned vegetables [21] and in the saliva of patients treated with dental sealants [22]. Estrogenic activity of bisphenol A has been shown in culture experiments where bisphenol A induced expression of estrogen-responsive genes and promoted proliferation in MCF-7, a breast cancer cell line [8]. In sewage sludge,  $\rho$ -nonylphenol is degraded from alkylphenols which are included in plastics such as polyvinylchloride (PVC) and polystyrene used in the food processing and packaging industries as plasticizers [23].  $\rho$ -nonylphenol may leak from plastics and contaminates water flowing through PVC tubing.  $\rho$ -nonylphenol is also used in the synthesis of surfactants such as nonoxyphenol, a compound present in intravaginal spermicides. Furthermore,  $\rho$ -nonylphenol is leaked from autoclaved plastic, which increases cell growth and progesterone receptor expression of mammary tumor cells [26], and modulates the estrogenic effect in fish hepatocytes [27].  $\rho$ -nonylphenol binds to isolated rat uterine estrogen receptor [24] and is weakly active in *in vitro* estrogen receptor transcription assay [25]. Alone or in combinations with other compounds, esters of 4-hydroxybenzoic acid, such as methyl, ethyl, propyl, and butyl 4-hydroxybenzoate commonly known as parabens are comprehensively used in preservation of cosmetics [30]. In an *in vitro* yeast-based estrogen assay and *in vivo* assay, the four most widely used parabens (namely methyl-, ethyl-, propyl-, and butylparaben) were all found to be weakly estrogenic [28, 29]. The low potencies of these compounds including  $\rho$ -nonylphenol, bisphenol A and butylparaben, when studied singly, suggest that they may have a little effect on biological systems. However, combinations of two weak environmental estrogens or combinations of a weak estrogen and a strong estrogen need to be

\* Corresponding author: Yong Soon Lee, D.V.M., Ph.D.  
Department of Veterinary Public Health College of Veterinary Medicine, Seoul National University 103 Seodun-Dong, Kwonson-Ku, Suwon 441-744, Korea  
Tel: +82-31-290-2742, Fax: +82-31-292-7610  
E-mail: leey@snu.ac.kr

evaluated. Furthermore, the activity of combinations of estrone, 17 $\beta$ -estradiol or 17 $\rho$ -estradiol in yeast strains expressing human estrogen receptor (hER) was synergistic at submaximal concentrations [14].

In the present studies, we examined whether there were synergistic or additive effect in the combinations of binary mixtures of four chemicals using the recombinant yeast assay.

## Materials and Methods

### Chemicals

$\rho$ -nonylphenol was obtained from Kanto Chemical Co. Inc. (Japan). 17 $\beta$ -estradiol, bisphenol A, and butylparaben were purchased from Sigma Chemical Co. (St. Louis, MO).

### Yeast strain

The *Saccharomyces cerevisiae* ER + LYS 8127 were obtained from Dr. Donald P. McDonnell (Duke University Medical Center, USA). This yeast strain was used for the estrogenicity assay.

### Growth of yeast for the estrogenicity assay

The *Saccharomyces cerevisiae* ER + LYS 8127 cells were grown in a shaking incubator at 30°C with 300 rpm in a selective growth medium containing yeast nitrogen base without amino acid (67 mg/ml), 1% dextrose, L-lysine (36  $\mu$ g/ml), L-histidine (24  $\mu$ g/ml). Following two days culture, the yeasts were then allowed to grow until OD values at 600 nm reached between 1.0 and 2.0.

### Treatment of chemicals

For the estrogenicity assay, the yeast cells were diluted to an OD<sub>600nm</sub> value of 0.03 in selective medium plus 50  $\mu$ M CuSO<sub>4</sub> to induce receptor production. The diluted yeasts were aliquoted into 50-ml conical tube (5 ml/tube) and 5  $\mu$ l of each test chemical or combination in DMSO (0.1%) were added. The cultures were incubated for 18 h in a shaking incubator at 30°C with 300 rpm.

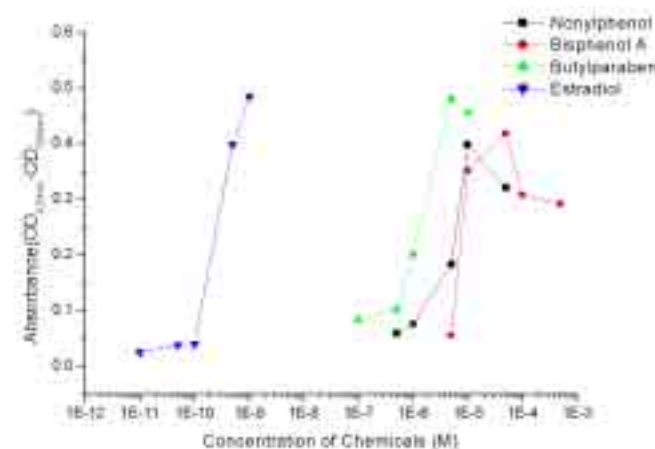
### $\beta$ -Galactosidase assay

After incubation the yeast culture samples were diluted in the appropriate selective medium to an OD<sub>600nm</sub> value of 0.25 and 100  $\mu$ l was added to each well of a 96-well microtiter plate. Each sample was assayed in quadruplicate.  $\beta$ -Galactosidase activity was induced by the addition of 100  $\mu$ l of a Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, pH 7.0) containing 2 mg/ml 0-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), 0.1% sodium dodecyl sulfate, 50 mM  $\beta$ -mercaptoethanol, and 200 U/ml oxalylase (Enzogenetics, Cornavillis, OR). The OD<sub>420nm</sub> and OD<sub>590nm</sub> values of each well were measured using Titertek Multiscan MCC/344 plate reader after allowing the tube to stand for 20 min. The OD<sub>420nm</sub> value of each well was corrected by subtracting the OD<sub>590nm</sub> value.

## Results

### 1) The estrogenicity of each chemicals

The *Saccharomyces cerevisiae* ER+ LYS yeast strain containing hER and an estrogen-specific reporter was used to examine the activity of the estrogens (17 $\beta$ -estradiol, bisphenol A, nonylphenol and butylparaben). Incubation of yeast with increasing concentrations of estradiol induced a dose-dependent increase in  $\beta$ -galactosidase activity (Fig. 1). The activity of 17 $\beta$ -estradiol was maximum at 1 nM. The activity of butylparaben, nonylphenol and bisphenol A was 1/5,000<sup>th</sup>, 1/10,000<sup>th</sup>, 1/20,000<sup>th</sup>, that of 17 $\beta$ -estradiol, respectively. The activity of the four estrogenic chemicals in yeast strain hER-ERE is consistent with the activity of these estrogens in *Saccharomyces cerevisiae* strain BJ3505 [15].

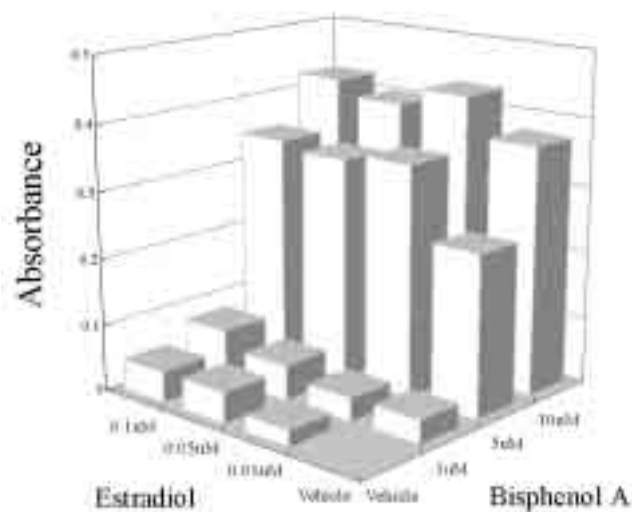


**Fig. 1.** Estrogenic activity of xenobiotics in Yeast recombinant assay. *Saccharomyces cerevisiae* ER+ LYS 8127 was grown for 18h in the presence of the estrogens at concentrations ranging from 5 X 10<sup>-4</sup>M to 1 X 10<sup>-11</sup>M. The induction of  $\beta$ -galactosidase was determined by OD<sub>420nm</sub>. □ :  $\rho$ -Nonylphenol; ● : Bisphenol A ; □ : Butylparaben; □ : 17 $\beta$ -Estradiol.

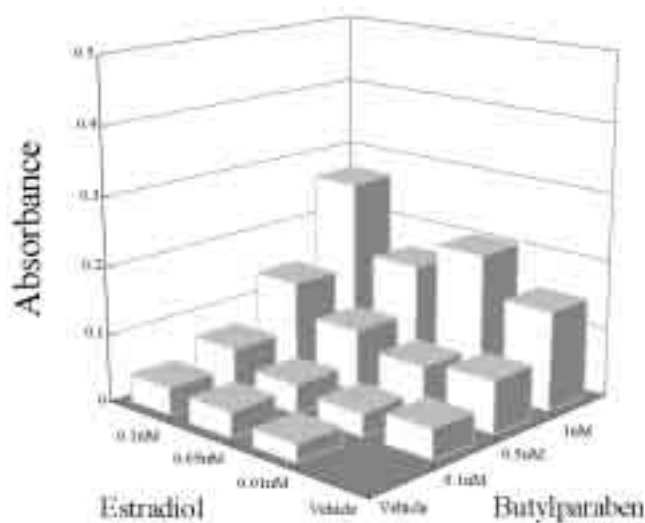
### 2) The estrogenicity in the combination of the strong estrogen and the weak estrogen

The activity of combinations of estrogenic compounds was investigated by generating dose-response relationships with one estrogen in the presence of a single dose of a second estrogen. Combinations of some concentrations of 17- $\beta$  estradiol as a strong estrogen and bisphenol A as a weak estrogen showed additive estrogenic effects (Fig. 2). For example, 0.1 nM 17- $\beta$  estradiol and 10  $\mu$ M bisphenol A showed 0.424 in OD<sub>420nm</sub>. A predicted OD<sub>420nm</sub> value of 0.421 would have been observed if the two estrogens were additive. Thus, the combination of 0.1 nM 17 $\beta$ -estradiol and 10  $\mu$ M bisphenol A produced a slight synergistic effect. Even though it implies that results produced effects greater than the sum of the parts, these effects are not synergistic. Also, additive effect was observed with the combinations of

17  $\beta$ -estradiol and butylparaben (Fig. 3).



**Fig. 2.** Estrogenic activity of combinations of xenobiotics in yeast recombinant assay. *Saccharomyces cerevisiae* ER+ LYS 8127 was grown for 18 h in the presence of 17- $\beta$  estradiol or bisphenol A alone and in combinations at increasing concentrations. The induction of  $\beta$ -galactosidase was determined by OD<sub>420nm</sub>.

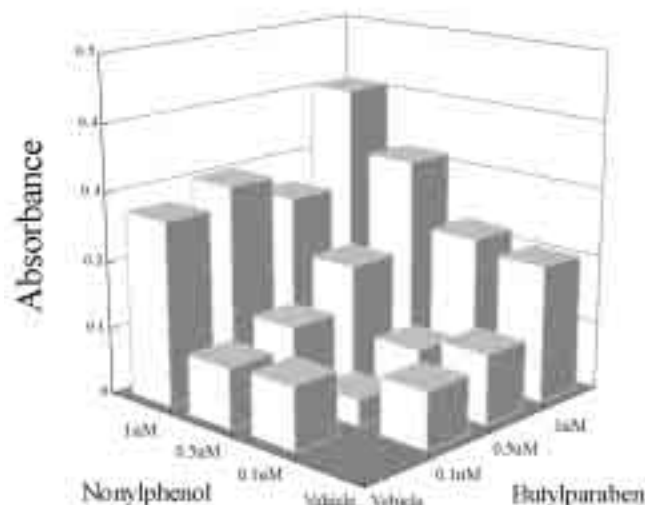


**Fig. 3.** Estrogenic activity of combinations of xenobiotics in yeast recombinant assay. *Saccharomyces cerevisiae* ER+ LYS 8127 was grown for 18 h in the presence of 17- $\beta$  estradiol or butylparaben alone and in combinations at increasing concentrations. The induction of  $\beta$ -galactosidase was determined by OD<sub>420nm</sub>.

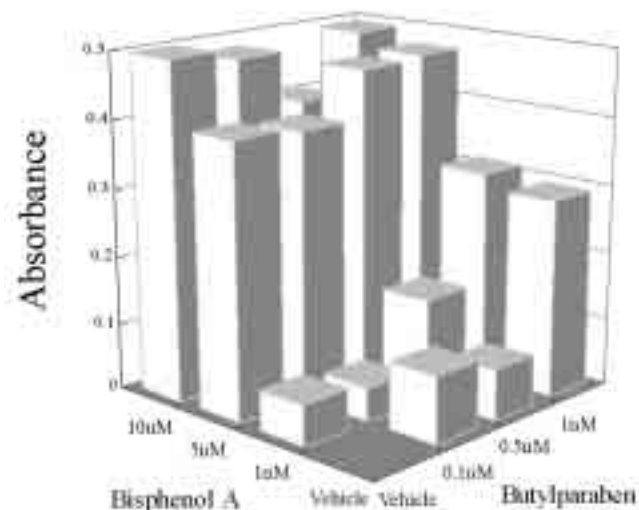
### 3) The estrogenicity in the combination of two weak estrogens.

The combinations of some concentrations of nonylphenol and butylparaben as a weak estrogen (Fig. 4) and combination of butylparaben and bisphenol A (Fig. 5) showed additive

effects in the estrogenic activity. However, the combinations of two estrogens at the high concentration produced lower estrogenic effects than that expected by summing the individual activities.



**Fig. 4.** Estrogenic activity of combinations of xenobiotics in yeast recombinant assay. *Saccharomyces cerevisiae* ER+ LYS 8127 was grown for 18 h in the presence of nonylphenol or butylparaben alone and in combinations at increasing concentrations. The induction of  $\beta$ -galactosidase was determined by OD<sub>420nm</sub>.



**Fig. 5.** Estrogenic activity of combinations of xenobiotics in yeast recombinant assay. *Saccharomyces cerevisiae* ER+ LYS 8127 was grown for 18 h in the presence of bisphenol A or butylparaben alone and in combinations at increasing concentrations. The induction of  $\beta$ -galactosidase was determined by OD<sub>420nm</sub>.

## Discussion

Previous studies have demonstrated that 17  $\beta$ -estradiol is strongly estrogenic and bisphenol A, nonylphenol and butylparaben are weakly estrogenic in the same assay system [16]. Similar results were observed in the present study at a single compound, respectively (Fig. 1).

Arnold et al. has reported that the binary mixtures of 17  $\beta$ -estradiol and 17-estradiol interacted synergistically in yeast strains expressing hER. The activity of combinations of estrone was synergistic at submaximal concentrations (1nM) of 17  $\beta$ -estradiol [14]. It was hypothesized that the synergistic interactions may be due to two different binding sites on the Estrogen Receptor (ER) where binding to the second site enhances activity of the ER as a ligand-induced transcription factor [17]. Although the chemicals are different, Harris et al. [20] reported that the estrogenic activity of phthalate esters as weakly estrogenic compounds *in vitro* didn't produce synergistic effects but additive effects. Also, other studies have reported that synergistic interactions of the organochlorine pesticides as endocrine disruptors were not observed in several estrogen-responsive assays [18, 19]. Therefore, the concentration-dependent interactions of binary mixtures of four estrogens (17-  $\beta$ estradiol, bisphenol A, nonylphenol and butylparaben) as endocrine disruptors like some of organochlorine pesticides were investigated in the recombinant yeast assay. Combinations of some concentrations of a strong estrogen and a weak one produced a slight synergistic effects in reporter activity at the low concentrations (Fig. 2 and 3) and combinations of some concentrations of two weakly estrogenic compounds produced a slight synergistic effects in estrogenic activity at the low concentrations (Fig. 4 and 5). Even though some combinations of some low concentrations of estrogenic compounds produced greater effect than the sum of the parts in this recombinant yeast assay, synergistic interactions of the binary mixture were not observed. Also, some combinations of high concentrations of estrogenic compounds showed lower than additive effect in recombinant yeast assay.

Results from this study demonstrate that synergistic interactions of two weakly estrogenic compounds or weak estrogen and strong one are not observed in this recombinant yeast assay at low concentrations. ER expression may play an important role in the estrogenic activity of chemical mixtures. Because two estrogenic chemicals bind to ER competitively and in the limited ER expression of cells, ER-ligand binding will be saturated at the combination of some concentrations. However, before saturation of ER concentration, both two estrogenic chemicals combined at each low concentration can bind ER.

Thus, we have shown that some combinations of estrogens have additive effects, not synergistic effects in recombinant yeast system expressing hER.

## Acknowledgement

This work was supported by the G-7 project from the Korean Ministry of Environment and partly by the Brain Korea 21 project.

## References

1. **McLachlan, J.A.**, Functional toxicology: A new approach to detect biologically active xenobiotics. *Environ Health Perspect* 1993, **101**, 386-387
2. **Colborn, T.**, Environmental estrogens: Health implications for humans and wildlife. *Environ Health Perspect* 1995, **103**, 135-136.
3. **Jensen, T.K., Toppari, J., Keiding, N., Skakkebaek, N.E.**, Do environmental estrogens contribute to the decline in male reproductive health? *Clin Chem* 1995, **41**, 1896-1901.
4. **Safe, S.H.**, Do environmental estrogens play a role in development of breast cancer in women and male reproductive problems? *Human Ecol Risk Assess* 1995, **1**, 17-23.
5. **Vom Saal, F.S.**, Environmental estrogenic chemicals: Their impact on embryonic development. *Human Ecol Risk Assess* 1995, **1**, 3-15.
6. **Kang, K.S., Lee, G.X., Park, J.S., Lee, B.J., Che, J.H., Tai, J.H., Cho, J.J., Kim, S.H., Lee, D.S., Lee Y.S.**, Effect of green tea on prostate and seminal vesicle in rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Microbiol Biotechnol* 2000, **10**(3), 281-286.
7. **Soto, A.M., Chung, K.L., Sonnenschein, C.**, The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ Health Perspect* 1994, **102**, 380-383.
8. **Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., Feldman, D.**, Bisphenol A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 1993, **132**, 2279-2286.
9. **Soto, A.M., Lin, T.M., Justicia, H., Silvia, R.M., Sonnenschein, C.**, An *in culture* bioassay to assess the estrogenicity of xenobiotics (E-screen). In *chemically induced alterations in sexual and functional development: The wildlife/human connection* (T. Colburn and C. Clement, Eds.), 1992, 295-309.
10. **Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P.**, A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 1995, **103**, 582-587.
11. **Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen, J.A., Wilson, E.M.**, Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* 1995, **375**, 581-585.
12. **Vom Saal, F.S., Nagel, S.C., Palanza, P., Boechler, M., Parmigiani, S., Welshons, W.V.**, Estrogenic pesticides: Binding relative to estradiol in MCF-7 cells

- and effects of exposure during fetal life on subsequent territorial behaviour in male mice. *Toxicol Lett* 1995, **77**, 343-350.
13. **Kang, K.S., Kim, H.S., Ryu, D.Y., Che, J.H., Lee, Y.S.**, Immature uterotrophic assay is more sensitive than ovariectomized uterotrophic assay for the detection of estrogenicity of -nonylphenol in Sprague-Dawley rats. *Toxicol Lett* 2000, **118**, 109-115.
  14. **Arnold, S.F., Bergeron, J.M., Tran, D.Q., Collins, B.M., Vonier, P.M., Crews, D., Toscano, W.A., McLachlan, J.A.**, Synergistic responses of steroidal estrogens in vitro (Yeast) and in vivo (Turtles) *Biochem Biophys Res Comm* 1997, **235**, 336-342.
  15. **Gaido, K.W., Leonard, L.S., Lovell, S., Gould, J.C., Babai, D., Portier, C.J., McDonnell, D.P.**, Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. *Toxicol Appl Pharmacol* 1997, **143**, 205-212.
  16. **Park, J.S., Lee, B.J., Kang, K.S., Tai, J.H., Cho, J.J., Cho, M.H., Inoue, T., Lee, Y.S.**, Hormonal effects of several chemicals in recombinant yeast, MCF-7 cells and uterotrophic assays in mice. *J Microbiol Biotechnol* 2000, **10**(3), 293-299.
  17. **McLachlan, J.A. , Arnold, S.F.**, Environmental estrogens. *Am Sci* 1996, **84**, 452-461.
  18. **Ashby, J., Lefebvre, P.B., Odum, J., Harris, C.A., Routledge, E.J., Sumpter, J.P.**, Synergy between synthetic estrogens. *Nature* 1997, **385**, 494.
  19. **Romamoorthy, K., Wang, F., Chen, I.C., Norris, J.D., McDonnell, D.P., Gaido, K.W., Bocchinfuso, W.P., Korach, K.S., Safe, S.**, Potency of combined estrogenic pesticides. *Science* 197, **275**, 405-406.
  20. **Harris, C.A., Henttu, P., Parker, M.G., Sumpter, J.P.**, The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 1997, **105**(8), 802-811.
  21. **Brotons, J.A., Olea-Serrano, M.F., Villalobos, M., Pedraza, V., Olea, N.**, Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect* 1995, **104**, 298-305.
  22. **Olea, N., Pulgar, R., Perez, P., Plea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A.M., Sonnenschein, C.**, Estrogenicity of resin-based composites and sealants used in dentistry. *Environ Health Perspect* 1996, **103**, 608-612.
  23. **Titow, W.V.**, *Technological Dictionary of Plastics Materials*. Pergamon Press, Oxford. 1998.
  24. **Shelby, M.D., Newbold, R.R., Tully, D.B., Chae, K., Davis, V.L.**, Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environ Health Perspect* 1996, **104**, 1296-1300.
  25. **Routledge, E.J., Sumpter, J.P.**, Structural features of alkylphenolic chemicals associated with estrogenic activity. *J Biol Chem* 1997, **272**, 3280-3288.
  26. **Abrutyn, D., McKenzie, B.E., Nadaskay, N.**, Teratology study of intravaginally administered nonoxynol-9-containing contraceptive cream in rats. *Fertil Steril* 1982, **37**, 113-117.
  27. **Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C.**, p-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ Health Perspect* 1991, **92**, 167-173.
  28. **Routledge, E.J., Parker, J., Odum, J., Ashby, J., Sumpter, J.P.**, Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol Appl Pharmacol* 1998, **153**(1), 12-19.
  29. **Pedersen, K.L., Pedersen, S.N., Christiansen, L.B., Korsgaard, B., Bjerregaard, P.**, The preservatives ethyl-, propyl- and butylparaben are oestrogenic in an in vivo fish assay *Pharmacol Toxicol* 2000, **86**(3), 110-113.
  30. **Labat, L., Kummer, E., Dallet, P., Dubost, J.P.**, Comparison of high-performance liquid chromatography and capillary zone electrophoresis for the determination of parabens in a cosmetic product. *J Pharm Biomed Anal* 2000, **23**(4), 763-769.