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Estrogenicity of paper and cardboard extracts used as food containers

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Abstract

Bisphenol-A (BPA), dibutyl phthalate (DBP), and di-2-ethylhexyl phthalate (DEHP), common chemical residues in food packaging materials, were investigated in paper and cardboard containers used for take-away food. The estrogenicity of aqueous extracts was tested in E-Screen bioassay and analysis carried out by HPLC and GC/MS. Estrogenicity was demonstrated in 90% of extracts (geometric mean [GM] = 11.97 pM estradiol equivalents/g). DEHP, DBP, and BPA (GM = 341.74, 37.59, and 2.38 ng/g of material) were present in 77.50, 67.50, and 47.50% of samples, respectively. In bivariate analyses, no significant association was found between levels of these chemicals and estrogenicity in cardboard/paper extracts. A close-to-significant association was found between estrogenicity and DBP ($\beta=1.25$; $p=0.06$) in paper extracts, which reached statistical significance in multivariate analysis ($\beta=1.61$; $p=0.03$). Paper and cardboard used in food packaging may contribute to the inadvertent exposure of consumers to endocrine-disrupting chemicals.

Keywords: Paper and cardboard, phthalates, bisphenol-A, estrogenicity

Introduction

Food and drink consumed outside the home represents a large and important part of the Western diet (World Cancer Research Fund and American Institute for Cancer Research, 1997). Take-away food is defined as food packaged immediately prior to sale and consumed shortly thereafter. Boxes, wrappings, cups, and trays used for take-away food are made of paper, cardboard, plastic (polystyrene), and aluminum, among other materials. The paper and cardboard can be new or recycled, grease-proof, printed, varnished, or glued. All of these materials are of particular interest because, although the contact period is usually short, the vast majority of these foods are often served hot and many are oily, favoring the migration of any contaminants in the packaging materials to the food.

Endocrine disrupting activity has been described for some food chemical contaminants such as bisphenol A and phthalates (Aurela et al., 1999). The presence of these hormonally active chemical residues in foods has been associated with food packaging materials and food processing (Soto et al., 1995; Castle et al., 1997; Song et al., 2000; Vinggaard et al., 2000; Summerfield and Cooper, 2001; Triantafyllou et al., 2002). Low levels of these chemicals have been found in baby food (Petersen and Breindahl, 2000), baby bottles (Brede et al., 2003), and recycled paper (Triantafyllou et al., 2002). Nevertheless, little attention has been paid to this contamination on the assumption that it carries negligible risk. More recently, there has been a demand for a more rigorous risk assessment, adopting the precautionary principle as a guide for preventive action (Kriebel et al., 2001). In this regard, Vinggaard et al. (Vinggaard et al., 2000) demonstrated the presence of multiple residues in recycled kitchen rolls, which showed a marked estrogenic response in an *in vitro* yeast estrogen screen. The possibility of

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3 similar compounds migrating from paper products used for food packaging has
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5 increased concerns about the health risks that may be involved.
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10 Phthalates used in the manufacture, stabilization, modification, and performance of
11 plastic polymers have been found in food (Petersen and Breindahl, 2000; Saito et al.,
12 2002). Food may be contaminated by ortho-phthalate esters such as dibutyl (DBP),
13 butylbenzyl phthalate (BBP), and di-2-ethylhexyl phthalate (DEHP), which are used in
14 polyvinylchloride as plasticizers and in adhesives (Song et al., 2000), printing inks
15 (Castle et al., 1989; Vinggaard et al., 2000; Summerfield and Cooper, 2001), and
16 colored laminated films (McNeal et al., 2000). Phthalates have also been detected in
17 virgin and recycled paper used for food packaging (Castle et al., 1997; Sipilainen-Malm
18 et al., 1997; Vinggaard et al., 2000; Summerfield and Cooper, 2001), most frequently
19 DBP and DEHP, whereas BBP was not found in paper and cardboard extracts
20 (Vinggaard et al., 2000). The presence of phthalates in some food has also been
21 associated with the use of PVC tubes in the production of baby food (Tsumura et al.,
22 2001), with cap-sealing resins in bottled foods (Hirayama et al., 2001), and with gloves
23 worn by food handlers (Tsumura et al., 2001). Finally, baby pacifiers (Sugita et al.,
24 2003) can also be a source of inadvertent exposure.
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48 Bisphenol-A (BPA), a starting material for the synthesis of polycarbonate and epoxy
49 resins, was detected in autoclavable flasks (Krishnan et al., 1993), baby bottles (Brede
50 et al., 2003), reusable water carboys (McNeal et al., 2000), and coffee and black tea
51 cans (Kang and Kondo, 2002), among other food materials and containers. The leaching
52 of BPA was related to the temperature, heating time, and type of food contained (Kang
53 et al., 2003). The estrogenicity of food preserved in cans was related to BPA and
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3 oligomers leaching from the inner surface of lacquer-coated cans (Brotons et al., 1995).
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5 The estrogenicity of kitchen roll extracts was attributed to BPA among other
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7 compounds (Vinggaard et al., 2000).
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12 Hence, bisphenols and phthalates may migrate to food packaged in paper products that
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14 contain these contaminants (Sipilainen-Malm et al., 1997; Vinggaard et al., 2000;
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16 Summerfield and Cooper, 2001). Whereas recycled paper used for kitchen rolls
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18 contained BPA, attributed to the office waste used, virgin paper did not appear to be an
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20 important source of bisphenol-A leaching (Vinggaard et al., 2000). In contrast,
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22 phthalates were found in both virgin and recycled paper, related to offset printing inks,
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24 adhesives, and lacquers (Vinggaard et al., 2000).
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32 The E-Screen assay, developed to measure estrogenic activity using cell proliferation as
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34 the end point (Soto et al., 1992; Aurela et al., 1999), is a common bioassay to identify
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36 estrogenic chemicals among environmental pollutants and is among the most sensitive
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38 and reproducible assays for estrogenic activity (Villalobos et al., 1995; Andersen et al.,
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40 1999; Fang et al., 2000). The present study aimed to investigate the estrogenicity of
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42 paper and cardboard used in take-away food packaging materials by testing their
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44 estrogenicity in the E-Screen bioassay. In addition, chemical analyses of the extracts
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46 were performed to identify some of the chemicals responsible for observed hormonal
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48 effects.
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55 **Material and Methods**

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Samples

Forty containers made of paper and cardboard were obtained from take-away food retail establishments in four European countries (Belgium, Italy, Portugal, and Spain). The containers were supplied by CONSEUR: VU-testACHATS-Belgium, DECO-Prostete-Portugal, ALTROconsumo-Italia, and OCU-Compramaestra-Spain. Eighteen containers were pizza boxes and cardboard triangles for individual pizza rations, sixteen were chipped-potato and chicken-finger containers, four were paper bags for sandwiches and fritters, one was for strawberries, and one for floury paste. Thirty-two of the containers were made of cardboard and eight of paper. The containers were wrapped by collecting agents after removal of the food and shipped to our Laboratory of Medical Investigation (Spain) for analysis. Only the side of the container in contact with the food was processed for analysis.

Chemicals and reagents

Chemicals used as standards for the analysis were bisphenol A (BPA), CAS n°: 3253-39-2 (Sigma-Aldrich Quimica S.A.); dibuthylphthalate (DBP), CAS n°: 84-74-2; and diethylhexylphthalate (DEHP), CAS n°: 117-81-7 (Dr Ehrenstorfer Lab., Ausburg, Germany). All solvents used were of high purity grade: acetonitrile, doubly distilled water, chloroform, ethanol, and hexane.

Apparatus

HPLC was performed with a Waters Model 501 Millipore apparatus (Marlborough, MA) equipped with two pumps and U6K injector of 500 µl load capacity. The ultraviolet/visible detector was a Waters Model 490 Millipore device with Millennium

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3 Chromatography Manager software. A Lichrocart Merck S5 ODS Column (20x 0.4 cm;
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5 Merck, Darmstadt, Germany) was used.
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10 Gas chromatography-Mass spectrometry was done using a Saturn 2000 ion-trap mass
11 spectrometer from Varian Instrument (Walnut Creek, CA) with automatic injector 8200,
12 SPI/1078; a DB5-MS capillary column (30 m x 0.25 mm) was used. The ion-trap mass
13 spectrometer was operated in SIS impact mode.
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20 21 22 *Sample processing* 23

24 In order to exclude the influence of the inks used on printed surfaces, chemical residues
25 were extracted from the side of the food container in contact with food. Internal parts
26 that were in contact with food were dissected using a scalpel and separated from the
27 internal structure of cardboards. A section weighing between 5 and 10 g was processed.
28
29 In brief, the sample was placed in a glass beaker, and 150 ml of distilled water were
30 added and heated for 2 h at 100°C, following one of the protocols recently proposed for
31 testing migration using food simulates, including water (Summerfield and Cooper,
32 2001; Triantafyllou et al., 2002). Next, the paper mass was mechanically pressed to
33 remove the absorbed water, which was then filtered through 110 nm Whatman® filter
34 papers. The extract obtained was concentrated at a reduced pressure to a volume of 2
35 ml. Then, 10 ml of water and 15 ml of chloroform were added through a decanting
36 funnel. The chloroform phase was filtered through a Na₂SO₄ anhydro-treated 110 nm
37 Whatman® filter. The eluted obtained was concentrated at reduced pressure to a volume
38 of 1 ml and dried under a stream of nitrogen. Two ml of ethanol were added to the dry
39 extract and the suspension was divided between two aliquots, 1ml for use in the E-
40 Screen bioassay and 1ml for chemical analysis. The aliquot for chemical analysis was
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3 processed by HPLC and confirmed by GC/MS after dilution in ethanol and hexane,
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10 *Bioassay for Measuring Estrogenicity*

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12 *MCF-7 cell line:* Cloned MCF-7 cancer cells were grown for routine maintenance in
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14 Dulbecco's modification of Eagle's medium (DME) supplemented with 10% fetal
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16 bovine serum (FBS) (BioWittaker, Walkersville, MD) in an atmosphere of 5% CO₂/95%
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18 air under saturating humidity at 37°C. The cells were subcultured at weekly intervals
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20 using a mixture of 0.05% trypsin and 0.01% EDTA.
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27 *Charcoal-dextran treatment of serum.* Sex steroids were removed from serum by
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29 charcoal-dextran stripping. Briefly, a suspension of 5% charcoal (Norit A, Sigma
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31 Chemical Co, St. Louis, MO) with 0.5% dextran T-70 (Pharmacia-LKB, Uppsala,
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33 Sweden) was prepared. Aliquots of the charcoal-dextran suspension of similar volume
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35 to the serum aliquot to be processed were centrifuged at 1000 g for 10 min.
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37 Supernatants were aspirated, and serum aliquots were mixed with the charcoal pellets.
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39 This charcoal-serum mixture was maintained in suspension by rolling at 6 cycles/min at
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41 37° C for 1 h. The suspension was centrifuged at 1000 g for 20 min, and the supernatant
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43 was then filtered through a 0.22 µm filter (Millipore). Charcoal dextran-treated bovine
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45 and human serum (CDFBS and CDHuS, respectively) were stored at -20° C until
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51 needed.
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53 *Cell proliferation experiments.* MCF7 cells were used in the estrogenicity test following
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55 a slight modification (Villalobos et al., 1995) of the original technique (Soto et al.,
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57 1992). Briefly, cells were trypsinized and plated in 24-well plates (Limbro, McLean,
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59 VA) at initial concentrations of 20 000 cells per well in 5% FBS in DME. Cells were
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3 allowed to attach for 24 h and the seeding medium was then replaced with 10% CDFBS
4 or CDHuS-supplemented phenol red-free DME. Different concentrations of extracts
5 were added, and the assay was stopped after 144 h by removing medium from wells,
6 fixing the cells, and staining them with sulforhodamine-B (SRB) as described elsewhere
7 (Olea et al., 1996; Perez et al., 1998). The linearity of the SRB assay with cell number
8 was verified prior to the cell growth experiments. The 100% proliferative effect (PE)
9 was calculated as the ratio between the highest cell yield obtained with 50 pM of
10 estradiol and the proliferation of hormone-free control cells.
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24 *Quantitative evaluation of estrogenicity of paper extracts.* One mL of ethanol
25 suspension of paper and cardboard extracts was dried, resuspended in 100 uL of ethanol
26 and then in supplemented phenol red-free medium, vigorously shaken, and left at rest
27 for 30 min; finally, it was passed through a 0.22 µm filter and tested in the estrogenicity
28 assay at dilutions ranging from 1:1 to 1:10.000. Each sample was run in triplicate with a
29 negative (vehicle) and positive (estradiol 50 pM) control in each plate. The proliferative
30 effect (PE) of the extracts was referred to the maximal proliferative effect obtained with
31 estradiol, transformed into estradiol equivalent units (Eeq), and expressed as pM
32 concentration by reading from a dose-response curve prepared using estradiol
33 (concentration range 0.1 pM to 10 nM). Mean cell numbers did not significantly differ
34 from those in steroid-free controls at concentrations below 1 pM estradiol, equivalent to
35 1 fmol in 1 ml of culture medium. Therefore, 1 fmol estradiol/well was considered the
36 lowest detectable amount of estrogen in this assay.
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58 *Chemical analysis*
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3 *High performance liquid chromatography.* The gradient was: at time= 0 min, 100%
4 phase A (acetonitrile-bidistilled water, 1:1) and 0% phase B (acetonitrile): at time = 15
5 min, 100% phase B and 0% phase A; and at time = 17 min, 100% phase A and 0%
6 phase B. The elution profile was monitored at 280 nm and 254 nm.
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12 *Gas chromatography and mass spectrometry.* The following working conditions were
13 used: initial column temperature, 50°C (3min), increased at 30°C/min to 200°C (5min),
14 increased at 30°C/min to 270°C (8min), and finally increased at 30°C/min to 310°C
15 (2min); injector temperature: 250°C split of 0.01; carrier gas, He (99.999%); injector
16 flow: 1ml/min; and injection volume 2 µl. BPA retention time: 8.16 min; m/z: 213, 228.
17 DBP retention time: 7.10 min; m/z: 149, 223. DEHP retention time: 10.12; m/z: 149,
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29 Recovery rates were determined before investigating the BPA, DEHP and DBP content
30 of extracts. Liquid from the paper mass was spiked with 10 - 70 µg/mL of BPA, DEHP
31 or DBP and then extracted according to the protocol described above under *Sample*
32 *processing*. Recovery rates ranged from 82.4 ± 6.1% to 86.2 ± 4.3%. Quantification was
33 performed by HPLC after determination of the limits of detection (LOD) for each
34 chemical (BPA=22.8 ng/mL; DBP=50µg/mL; DEHP=70µg/mL) and standard curves.
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Statistical analysis

A univariate analysis was performed to explore the distribution of the outcome variables, and the potential correlation among the three residues was analyzed. In order to reduce the skewness and improve the adjustment in the linear regression between the estrogenicity and BPA, DBP, and DEHP levels in the extracts, all variables were

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3 transformed into their natural logarithms. Central tendency measurements (geometric
4 means, median, and standard deviation) were calculated for the proliferation test and
5 chemical analysis results, and the arithmetic mean was also reported for the purposes of
6 comparison with previous articles. A bivariate analysis was performed to estimate the
7 association between estrogenicity and BPA, DBP, and DEHP levels, and multivariate
8 models were developed to describe associations between each chemical and
9 estrogenicity in the extracts. Values below the LOD were replaced by the corresponding
10 random numbers from a table of randomly assorted digits (Snedecor and Cochran,
11 1980). All statistical analyses were performed using SPSS-11 (2006) and the STATA
12 Version 9 (2005) statistical software.
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29 **Results**

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33 Figure 1 shows the proliferative effect of estradiol, BPA, DBP and DEHP on MCF-7
34 breast cancer cells. The estrogenicity of these chemicals was assessed by determining
35 their relative proliferative potency (RPP) and their relative proliferative effect (RPE)
36 (Soto et al., 1995). RPP was 0.03% for BPA and <0.0001% for both phthalates; RPE
37 was 100% for BPA and 30% for both phthalates.
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49 Table I shows the arithmetic and geometric means, median, standard deviation, range,
50 and frequency of the estrogenicity values of all samples (total) and of cardboard and
51 paper samples separately. Values are expressed in pM estradiol equivalent (Eeq) per
52 weight (g) of material. The frequency of estrogenic response was similar in paper and
53 cardboard samples (87.50% and 90.00%, respectively). The geometric mean
54 estrogenicity was higher in cardboard (13.33 pM estradiol equivalents/g of cardboard)
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3 than in paper samples (7.78 pM estradiol equivalents/g of paper). Absence of toxicity in
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5 the extracts was demonstrated when any partial agonist effect was shown in the
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7 presence of 100 pM of estradiol at maximal dilution of the extract (data not shown).
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15 Table II shows the arithmetic and geometric means, median, standard deviation, range,
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17 and frequency of the content of BPA, DBP, and DEHP in total, cardboard, and paper
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19 samples. Values are expressed as ng of residue per g of material; 37.50-100% of
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21 analyzed samples showed detectable levels of the studied chemicals. The most
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23 frequently detected chemical was DBP, present in 100% of paper samples, followed by
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25 DEHP, present in 75.00% and 78.13% of paper and cardboard samples, respectively.
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27 BPA was present in half of cardboard samples but was less frequent and less abundant
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29 in paper extracts. DBP values were correlated with DEHP levels in the samples
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31 (p=0.040; $\rho=0.326$). The presence of BPA did not appear to be related to any of the
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33 phthalates studied.
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43 Table III shows the bivariate analysis between BPA, DBP, DEHP levels (ng/g of
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45 cardboard or paper) and estrogenicity values (pM estradiol equivalents/g cardboard or
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47 paper) for total, cardboard, and paper samples separately. No statistically significant
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49 association was found between the levels of each chemical and the estrogenicity of the
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51 samples for the grouped data. When data for paper and cardboard were considered
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53 separately, the relationship of estrogenicity with DEHP concentrations in cardboard and
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55 DBP in paper was close to statistical significance ($\beta=0.21$; $p=0.17$ and $\beta=1.25$; $p=0.06$,
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8 Table IV shows the multivariate analysis for the association between BPA, DBP and
9 DEHP levels (ng/g of cardboard or paper) and estrogenicity values (pM estradiol
10 equivalents/g cardboard or paper) for total, cardboard, and paper samples. In the paper
11 samples (n=8), the multivariate model showed a statistically significant association with
12 DBP ($\beta=1.61$; $p=0.03$) and a close to significant association with DEHP ($\beta=-0.43$;
13 $p=0.07$). When a multivariate model with the two phthalates was constructed, both
14 showed a statistically significant association with estrogenicity (DBP: $\beta=1.68$, $p=0.01$;
15 DEHP: $\beta=0.44$, $p=0.04$) (Data not shown).
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32 Discussion

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34 Nine out ten aqueous extracts of paper and cardboard containers used in take-away food
35 showed a statistically significant proliferative effect on MCF7 human breast cancer
36 cells. The geometric mean estrogenicity of one gram of these materials was similar to
37 that of 11.97 pM of estradiol on MCF7 cells. In terms of surface area, extracts of each
38 square cm resulted in an estrogenicity of 0.60 pM of estradiol.
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50 In an attempt to identify the chemicals responsible for this hormonal effect, the presence
51 of bisphenol A and of phthalates DBP and DEHP was investigated. DBP (geometric
52 mean of 37.39 ng/g material) and DEHP (geometric mean of 341.74 ng/g material) was
53 detected in 67% and 77.5% of total samples, respectively. Several phthalates were
54 previously identified as components of virgin and recycled fibers (Sipilainen-Malm et
55 al., 1997; Vinggaard et al., 2000). Vinggaard et al. (2000) reported DBP in all extracts
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3 of kitchen rolls made from virgin (range, 0.1-28 $\mu\text{g/g}$) or recycled (range 2-22 $\mu\text{g/g}$)
4 paper at similar levels to those found in the present study (0.0001-10.77 $\mu\text{g/g}$). It has
5 been suggested that phthalates can derive from printing inks (Vinggaard et al., 2000;
6 Summerfield and Cooper, 2001) or adhesives (Song et al., 2000) used in food
7 containers, among other sources. In the present study, when walls of food containers
8 could be mechanically separated into layers, our protocol for sample processing only
9 used the internal part in direct contact with food, therefore the sample was not
10 contaminated by inks on printed surfaces. The levels of the two phthalates were
11 correlated ($p=0.040$; $\rho=0.326$), suggesting that they might have derived from the same
12 source. Both phthalates would contribute to the estrogenicity of the samples, as their
13 estrogenicity in the E-Screen assay is known (Aurela et al., 1999; Okubo et al., 2003).
14 Although the small sample size prevented statistically significant associations from
15 being reached between the estrogenicity and each chemical in the bivariate analysis,
16 when the total sample was divided between cardboard and paper, a clear tendency to
17 significance was found for these associations (See Table III). Given the small sample of
18 paper extracts ($n=8$), it is remarkable that this association was close to statistical
19 significance ($\beta=1.25$; 95% IC from -0.07 to 2.56), with the finding in the multivariate
20 analysis of a positive statistically significant association between estrogenicity and DBP
21 ($\beta=1.61$; $p=0.03$) and a negative association, close to significance, between
22 estrogenicity and DEHP ($\beta=-0.43$; $p=0.07$). Finally, when a multivariate model was
23 constructed with only the two phthalates, a statistically significant association was found
24 between chemical content and E-Screen estrogenicity in both cases (DBP: $\beta=1.68$,
25 $p=0.01$; DEHP: $\beta=0.44$, $p=0.04$).

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3 Chemical residues in paper and cardboard vary as a function of the primary processing
4 material and the use of recycled material. Thus, it was proposed that BPA was useful to
5 distinguish between virgin and recycled fibers (Vinggaard et al., 2000). The BPA levels
6 found in these take-away food packaging materials (0.00005-1.81 $\mu\text{g/g}$ material) were
7 lower than those reported by Vinggaard and coworkers (2000) in recycled kitchen rolls
8 (0.55-24.1 $\mu\text{g/g}$) but higher than those found in virgin rolls (0.03-0.1 $\mu\text{g/g}$). BPA levels
9 detected by Ozaki et al. (2004) in recycled materials (0.19-26 $\mu\text{g/g}$) were ten-fold-
10 higher than those found in virgin products (Ozaki et al., 2004). Disparities in levels and
11 frequency of BPA residues might be due to variability in the percentage of recycled
12 material and in the composition of the paper. We found the highest BPA concentration
13 in a pizza box made of recycled material. However, no association was found between
14 BPA levels and virgin or recycled types of paper or cardboard. It is worth noting that
15 only 6 out of 40 samples were described as containing recycled fiber according to
16 information printed on the containers. However, investigation by the container suppliers
17 revealed the presence of recycled fibers in all but one sample and a proportion of virgin
18 wood pulp that ranged from 36-98%. Interestingly, the sample with no recycled fibers
19 was negative for the presence of BPA. However, neither the classification of container
20 composition based on manufacturer's information nor our re-classification based on the
21 percentage of virgin fiber served to establish an association between recycled
22 composition and BPA levels. BPA is used in the production of epoxy resins and
23 polycarbonate, and epoxy resins are used in the formulation of printer inks (Ullmans
24 Encyklopadie of Technical Chemistry, 1984). Therefore, printed paper in waste from
25 offices is a possible source of BPA in recycled paper. The presence of BPA in half of
26 the studied samples suggests that this compound is a common contaminant of paper, as
27 reported by other authors (Vinggaard et al., 2000). Moreover, the estrogenic activity of

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3 low concentrations of BPA in MCF-7 cells (Brotons et al., 1995; Soto et al., 1995; Olea
4 et al., 1996; Perez et al., 1998; Pulgar et al., 2000; Rivas et al., 2001) might suggest that
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6 this compound is a main cause of the activity observed in the E-Screen assay. However,
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9 we found no significant correlation between the presence or concentration of BPA in the
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11 samples and their estrogenicity. We cannot rule out the presence of other chemicals that
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13 may contribute to the estrogenicity of the extract. In addition, the estrogenicity of the
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15 extracts may be increased by additive or synergistic effects of the chemicals detected
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17 (BPA, DBP, DEHP) or by their interaction with any other hormonally active chemical
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19 that might be present in the extracts (Rajapakse et al., 2002).
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27 In Europe, the safety and quality of paper and cardboard used for food packaging are
28 regulated by the general directives for articles intended to come in contact with
29 foodstuffs (Directive 89/109/EEC), but there is no specific EC Directive for paper and
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31 cardboard. To our best knowledge, this is the first report of the estrogenicity of paper
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33 and cardboard that is in direct contact with take-away food. In-depth investigation is
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35 needed into the presence of hormonally active chemicals in these materials alongside a
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37 risk assessment evaluation. Our data highlight the usefulness of the E-Screen to estimate
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39 the total estrogen hormonal toxicity of paper/cardboard in direct contact with food and
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41 to perform an initial screening of suspected samples. In conclusion, the use of food
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43 containers made of paper or cardboard warrants closer scrutiny to determine whether
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45 chemicals that migrate to food from this type of packaging contribute to the inadvertent
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47 exposure of consumers to endocrine-disrupting chemicals.
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Appendices

Abbreviations: BPA, bisphenol A, CAS n°: 3253-39-2; CDFBS, Charcoal-dextran bovine serum; CDHuS, Charcoal-dextran human serum; DBP, dibutylphthalate, CAS n°: 84-74-2 ; DEHP, diethylhexylphthalate, CAS n°: 117-81-7 ; DME, Dulbecco's modification of Eagle's medium; Eeq, Estradiol equivalents; FBS, Fetal bovine serum; PE, Proliferative effect; RPP, relative proliferative potency; RPE, relative proliferative effect; SRB, Sulforhodamine-B.

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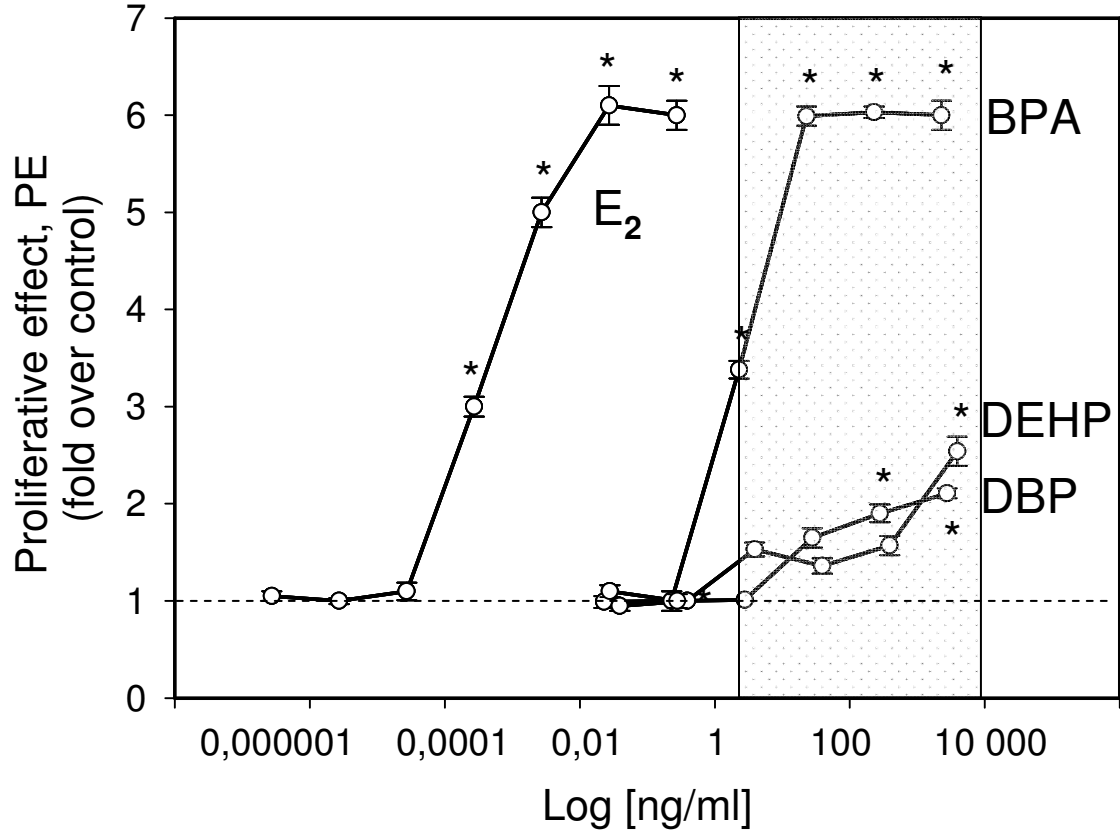
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5 **Figure 1.** Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-
6 treated human serum-supplemented medium were exposed for 144 h to different
7 amounts of estradiol-17 β , BPA, DBP and DEHP. Points represent quadruplicate
8 cultures; bars indicate standard deviations.
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Table I. Estimated values of estrogenicity (pM estradiol equivalents Eeq/g of cardboard or paper) and frequency of positive samples in the E-Screen assay of paper and cardboard extracts.

	Total samples	Cardboard	Paper
	n=40	n=32	n= 8
Arithmetic mean	60.52	64.24	45.61
Geometric mean	11.97	13.33	7.78
Median	18.69	21.51	16.80
SD	12.55	13.02	12.09
Range	0.01-355.50	0.01-355.50	0.08-280.90
Frequency (%)	90.00	90.63	87.50

Table II. Levels and frequency of BPA, DBP and DEHP (ng/g of cardboard or paper) in samples.

Residues	Total samples	Cardboard	Paper
	n=40	n=32	n= 8
BPA			
Arithmetic mean	97.34	115.32	25.43
Geometric mean	2.38	2.74	1.35
Median	0.52	0.52	0.49
SD	16.33	17.68	12.72
Range	0.05-1817.00	0.05-1817.00	0.08-188.00
Frequency (%)	45.00	46.88	37.50
DBP			
Arithmetic mean	713.17	706.32	740.49
Geometric mean	37.39	20.97	377.63
Median	121.84	75.62	548.55
SD	35.61	41.65	3.95
Range	0.10-10774.00	0.10-10774.00	29.10-3049.00
Frequency (%)	67.50	59.38	100
DEHP			
Arithmetic mean	3901.56	706.32	740.49
Geometric mean	341.74	302.11	559.54
Median	893.48	814.44	2751.84
SD	23.27	22.53	31.36
Range	0.52-61013.00	0.52-61013.00	1.53-10198.00
Frequency (%)	77.50	78.13	75.00

Table III. Bivariate analysis between BPA, DBP, DEHP levels (ng/g of cardboard or paper) and estrogenicity values (pM estradiol equivalents/g cardboard or paper) in total, cardboard, and paper samples.

Residues	Estrogenicity of total samples(n=40)			Estrogenicity of cardboard samples(n=32)			Estrogenicity of paper samples(n=8)			
	Ln scale	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
BPA		0.06	-0.24 to 0.35	0.70	-0.01	-0.33 to 0.33	0.99	0.34	-0.58 to 1.26	0.40
DBP		0.16	-0.07 to 0.38	0.17	0.16	-0.08 to 0.41	0.19	1.25	-0.07 to 2.56	0.06
DEHP		0.12	-0.14 to 0.38	0.37	0.21	-0.09 to 0.50	0.17	-0.17	-0.87 to 0.53	0.57

Table IV. Multivariate analysis between BPA, DBP, DEHP levels (ng/g of cardboard or paper) and estrogenicity values (pM estradiol equivalents/g cardboard or paper) in total, cardboard and paper samples.

Residues Ln scale	Estrogenicity of total samples(n=40)			Estrogenicity of cardboard samples(n=32)			Estrogenicity of paper samples(n=8)		
	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
BPA	0.05	-0.24 to 0.35	0.71	-0.02	-0.35 to 0.31	0.89	0.12	-0.52 to 0.76	0.63
DBP	0.14	-0.10 to 0.38	0.24	0.12	-0.13 to 0.39	0.32	1.61	0.32 to 2.91	0.03
DEHP	0.07	-0.20 to 0.35	0.60	0.17	-0.14 to 0.49	0.28	-0.43	-0.93 to 0.62	0.07
Constant	1.50	-0.30 to 3.31	0.10	1.24	0.76 to 3.24	0.22	-4.83	-11.97 to 2.31	0.13