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The Detection of Endocrine Disrupting Chemicals Leaching from Branded Bottled Water

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ABSTRACT

Any chemical, synthetic or endogenous, that has the capacity to disrupt normal hormonal signalling has the potential for endocrine disruption. Owing to the industrialised world in which we live and the reliance upon certain conveniences, exposure to these chemicals is inevitable and occurs through contact with household dust, prescription drugs, dermal lotions and clothes among others. Some of the most prolific man-made chemicals capable of upsetting normal hormonal balance are plasticisers, namely Bisphenol A and certain phthalates. Nowadays most food and beverages are packaged in some form of plastic and the exposure of these plastic containers to everyday stresses has the potential to cause undesirable compounds to leach into the food or beverage. The bottled water market over the last few years has been steadily growing however once purchased the bottled water is rarely stored as advised by the label and instead can be exposed to extremes of temperatures and UV light and at times bottles can be used repeatedly. All of these stresses increase the potential for leaching. This study aims to detect possible endocrine disrupting chemicals which are leaching from these containers and are in turn being ingested through the consumption of bottled water and is an initial step in the longer-term research of oestrogenic chemicals and their endocrine disrupting potential using analytical and biological based assays.

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INTRODUCTION

Within the past century, in excess of 80,000 new chemicals have been manufactured and utilised in ways that have resulted in extensive human exposure, with a percentage of these chemicals being identified as toxic due to their ability to cause endocrine disruption [1]. Many natural and man-made chemicals have been discovered to upset the normal and regular functioning of the endocrine system [2]. Any synthetic exogenous substance or mixture that imitates the actions of naturally occurring oestrogens [3] is considered to possess oestrogenic activity which can result in the interference of normal hormonal functioning and expression [4,5].Risk assessments of chemicals generally look at the effects of high doses of administered compounds to determine the lowest observed adverse effect levels (LOAELs) and no observed adverse effect levels (NOAELs). From these results reference doses which are considered safe for human exposure are then calculated taking into account a number of safety factors [6]. Therefore exposure of humans to thousands of environmental chemicals falls in the range of nonnegligible doses that are considered safe from a risk assessment perspective. However the ever-increasing data from epidemiological and human biomonitoring studies suggest otherwise. Low internal doses of EDCs present in typical human populations have been associated with obesity [7], infertility [8], immune dysfunction [9] and neurobehavioural disorders [10]. All endocrine disrupting chemicals (EDCs) have the capacity to stimulate negative effects on the endocrine systems of organisms [11]. The routes by which EDCscan enter the body can be varied [12], however it is now generally agreed that humans are exposed to the majority of EDCs through the consumption of foods and beverages which have been contained in plastic packaging. Much of this packaging is comprised of some form of plastic.Bottled water is generally packaged in either plastic or glass containers and is replacing tap water as the main source of drinking water with an increase in sales of 91% over the past number of years [13,14]. Numerous reports suggest that phthalates leach from polyethylene terephthalate (PET)and into the contents of the bottle. Some compounds which migrate from the plastic may be carcinogenic or mutagenic [14], and most certainly some will

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have some degree of oestrogenic activity [5]. Most plastic components in their primary form are harmless, it is the addition of additives, such as resins, or colourants, that increase the finished products potential to leach chemicals. This leaching of chemicals has the potential to increase when the product is exposed to common, every-day stresses such as heat or cold or ultra-violet (UV) light [5]. Although dietary exposure to hormonally active substances may ultimately be low through bottled mineral water sources, it can signify regular daily exposure for numerous individuals who consume, on a daily basis, bottled mineral water [3].

Bisphenol A [BPA, 2,2-bis(4-hydroxyphenyl)propane: CAS #80-05-7] is a man-made organic compound [15,16], which is comprised of two phenol rings which are linked by a methyl bridge, with two methyl functional groups connected to the bridge [17]. Over 2.7 billion kilograms of BPA are manufactured annually worldwide due to the increase in consumer usage of polycarbonate plastics and epoxy resins [18] making BPA one of the highest volume chemicals produced [19]. Polycarbonate is a thermoplastic polymer that has certain favourable characteristics such as low weight, high impact and heat resistance and high transparency [20] and as a result is used in many consumer products such as drinking bottles, mobile phones, household appliances, plastic food containers and as a replacement of glass in many products [17,20,21]. The molecular bonds created by BPA in polycarbonate is unstable [22] and if incomplete polymerisation occurs in the production process and under various conditions [12,23], any residual or unbound BPA may leach from the epoxy resin and therefore has the potential to contaminate food or drink products that come into contact with the resin [24,25] becoming a source for human exposure and as a result regular consumption of BPA is presumed [15].

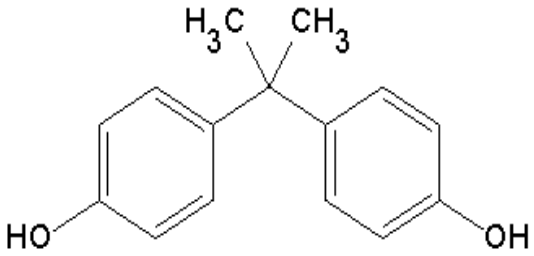

Fig.1: Chemical structure of BPA

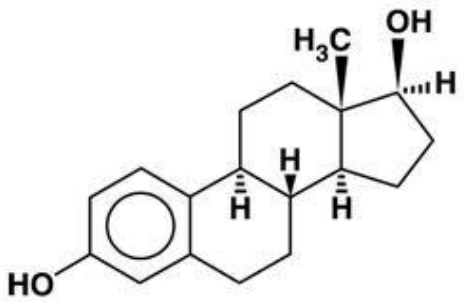

Fig. 2: Chemical structure of Estradiol

The BPA molecule has similarities in structural features, metabolism and mode of action to 17β-estradiol and other natural oestrogenic compounds [26,27] and it has also been suggested that once metabolised, the metabolites of BPA may in fact be more potent than the parent compound [15]. Bisphenol A disrupts thenormal activity of endogenous oestrogens byrestricting the proper function of the oestrogen nuclear hormone receptors in a variety of target tissues [28]. The two (4, 4')-OH side-chains and the two benzene rings allow BPA to fit into the oestrogen receptor binding pocket [25], and it has been shown to elicit adverse effects particularly at low doses [24]. The use of BPA as a food contact material has raised concerns and its toxicity has been widely studied since the 1970's and has shown a diverse range of adverse effects in laboratory animals [29]. Studies have also detected BPA in the environment and in human fluids and tissues. The development of strict laws governing the use of BPA in the manufacture of certain types of plastic has led to the development of alternative, heat stable bisphenol substitutes [29] currently marketed as "BPA Free".

Phthalates, which are esters of phthalic acid (PAE)[30], are the most abundant and widely distributed man-made environmental pollutants owing to their use over the last 50-60 years [23,31] and humans are exposed to them from a variety of sources throughout their lives [32].Within the bottling industry, bottles are produced from specific polymers depending on the volume of the container, with each bottle possessing unique characteristics such as type of dispenser and disposal, bottle strength and storage time [33]. Plastic bottles

manufactured from polyethylene, polycarbonate or polyvinyl chloride (PVC) are used for water in numerous countries [34], due to the advantages of both the physical and chemical properties such as light-weight, strength, transparency and easy recycling [23].Their physical properties and usage is dependent upon the branching and length of the dialkyl or alkyl/aryl side chains [35]. Phthalates are not chemically bound but physically bound to the plastic matrix [36,37], therefore increasing the potential of leaching, migration or evaporation into the environment or food products [34]. This process accelerates as the plastic products age and break down [38]. Certain phthalates, including their metabolites and degradation products can cause undesirable effects on human health [36]several of which have been recognised and classified as possessing endocrine disruptor activity [39], while some affect normal reproductive development and elicit carcinogenic effects [2].

The intricate biology of endocrine disruption means that no single assay or approach can be effectively employed to identify compounds that possess EDC characteristics [40].

However, there is satisfactory proof that EDCs pose a risk to environmental and human health.Persistent exposure to EDCs, even at low concentrations is of toxicological concern, this then increases when humans are exposed tocombinations of similar acting EDCs [24]. Exposure to EDCs in adulthood can have adverse effects but foetal and exposure in early life appear to result in more severe effects that continue throughout life [41].

MATERIALS AND METHODS

Materials:

Potassium phosphate monobasic anhydrous, ammonium sulphate, potassium hydroxide pellets, magnesium sulphate anhydrous, iron (III) sulphate pentahydrate, L-leucine, L-histidine, adenine, L-arginine hydrochloride, L-methionine, L-tyrosine, L-isoleucine, L-lysine-HCL, L-phenylalanine, L-glutamic acid, L-valine, L-serine, D-glucose, L-aspartic acid, thiamine, Pyridoxine, pantothenic acid, iniositol, biotin, L-threonine, copper (II) sulphate, dichloromethane, ethyl acetate, molecular grade absolute ethanol and sodium chloride were all obtained from Sigma Aldrich (Ireland). Chlorophenol red-β-galactopyranoside was purchased from Roche (U.K). The recombinant yeast cells were a gift from Prof. J. P. Sumpter, Brunel University, Uxbridge, U.K. The 17β-oestradiol and Bisphenol A were purchased from Sigma Aldrich and used as received.

2.2 Preparation of samples for solvent extraction:

Plastic drinking bottles were treated to various conditions representing normal consumer usage. Liquid-liquid solvent extractions were performed as described by del Olmo *et al*. [42]with some modifications. All extracted samples were dried under a steady stream of nitrogen gas and reconstituted as needed. Extractions for analysis using the GC-FID were reconstituted in 100µl of DCM and extractions for analysis through the YES assay were reconstituted in 100µl sterile absolute molecular grade ethanol.

2.3 Yeast Oestrogen Screen (YES) Assay:

The yeast oestrogen screen, previously described by Routledge and Sumpter, 1996 [43] was used to assess the oestrogenic potency of the naturally occurring 17β-oestradiol and Bisphenol A. Stock solutions of both test chemicals were prepared by weighing each into sterile glass universals and diluting in sterile molecular grade ethanol. Serial dilutions were performed on the stock solutions in 96-well microtitre plates. All work was carried out in a type II laminar flow hood for sterility purposes.

2.3 Gas Chromatography:

All samples were analysed using a Shimadzu GC-9A (Mason, Dublin) with FID and a CR6A chromatopac. The column used was a non-polar BP1 capillary column (30m x 0.32mm i.d. x 0.25µm) coated with methyl silicon gum phase (SGE, Dublin). All injections were carried out manually with an injection volume of 0.2µl for standard solutions and 0.5µl for extracted samples. Two temperature courses were programmed into the machine and samples were analysed using both programmes which each had a total run time of 40 minutes. Programme 1 was an isothermal programme with an injector temperature of 320°C and a column temperature of 300°C. Programme 2 was a gradient temperature programme with an injector temperature of 260°C and a starting column temperature of 100°C holding for 4 minutes and an increase of 6°C per minute to a final temperature of 280°C.

RESULTS AND DISCUSSION

YES Assay:

The YES assay is used as a biomarker of oestrogenicity, it is an extremely efficient and reproducible method used for the detection of oestrogenic compounds. It is a colorimetric assay which is comprised of a yeast strain that has been stably transfected with the human oestrogen receptor alpha (hERα) gene and an expression plasmid which contains the oestrogen response element (ERE) that control the β-galactosidase-encoding

reporter gene lac-Z. Upon receptor activation, the lac-Z gene is expressed, this produces the enzyme β-galactosidase, which is secreted into the assay medium where it results in a colour change of the chromogenic substance chlorophenol red-β-galactopyranoside (CPRG) from yellow to pink. The assay is favoured for its high sensitivity and allows the detection of these compounds even which have low potency and may be biologically important through prolonged contact or high levels in the environment. The transfected yeast strain was exposed to increasing concentrations of BPA and E_2 standard solutions and reconstituted polycarbonate extraction for 32 hours. Absorbance readings were taken at 560nm (optimum wavelength for CPRG) and 620nm (optimum wavelength for yeast growth) and corrected values calculated ($A_{620nm} - A_{560nm}$). In the graph (Fig. 3) there is an increase in absorbance readings of the BPA and E_2 standards and the polycarbonate extraction sample in comparison to the control group suggesting some form of oestrogenic activity occurring. There is possible cytotoxicity of the yeast occurring at the higher range of E_2 concentrations which would explain the negligible difference in absorbance readings. The readings for BPA however are not showing cytotoxicity but instead there is an obvious increase in absorbance readings as the concentration range increases. The increase in absorbance readings of the extracted water sample from polycarbonate is also showing some oestrogenic activity in comparison to the control group with an increase in absorbance at the lower end of the concentration scale. The results obtained indicate that there are oestrogenic compounds present in the water which has been packaged in polycarbonate plastic bottles with further refining of the assay required to obtain a good dose response.

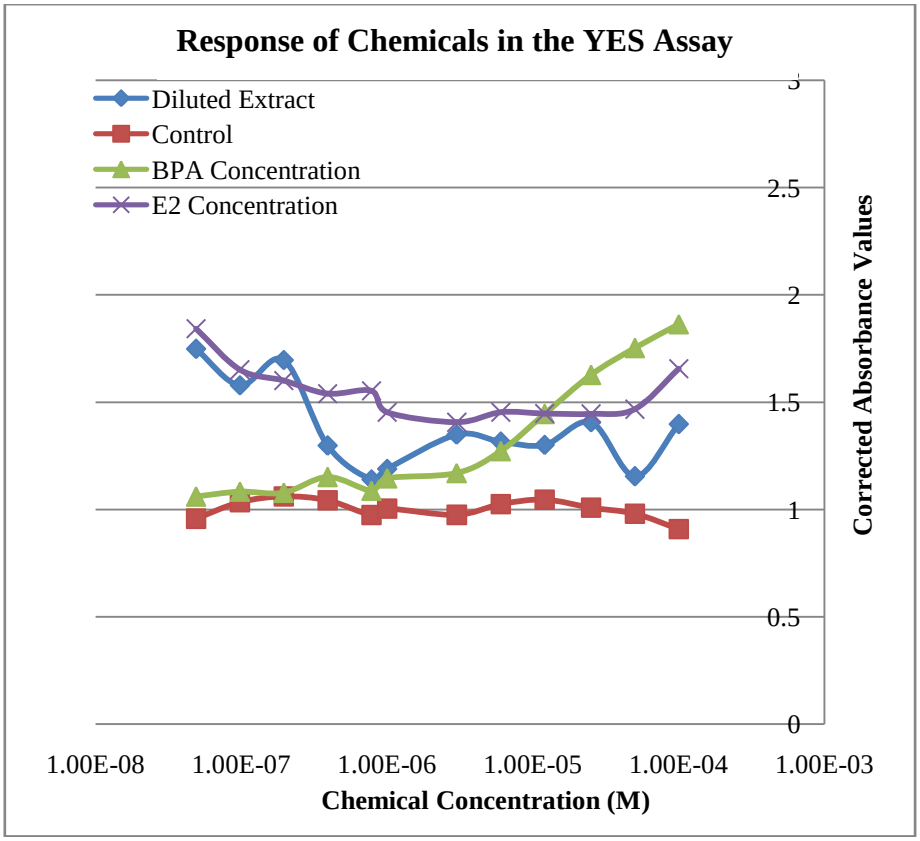

Fig. 3: Response of the Yeast Estrogen Screen to polycarbonate water bottle extraction, BPA and E_2

3.2 GC Analysis:

Gas chromatography analysis allows the identification and quantification of volatile compounds and was used to analyse water contained in polycarbonate plastic. A BPA standard of known concentration (120µg/ml) was manually injected onto the column and retention time and peak area recorded (Fig: 4). The same volume of extracted water sample was manually injected onto the column and retention time and peak area recorded (Fig: 5). From this an approximate concentration of BPA present in the extracted sample could be calculated using the following calculation:

$$\frac{\text{Peak area of standard}}{\text{Peak area of sample}} = \text{Ratio} \qquad \frac{\text{Concentration of standard}}{\text{Ratio}} = \text{Concentration of sample}$$

From the chromatographs obtained (Fig. 4 & Fig. 5) the concentration of BPA detected in the polycarbonate water extraction can be estimated as 67.41µg/ml using the following calculation:

$$\frac{1569771}{880299} = 1.78 \text{ (ratio)} \qquad \frac{120}{1.78} = 67.41\mu g/ml$$

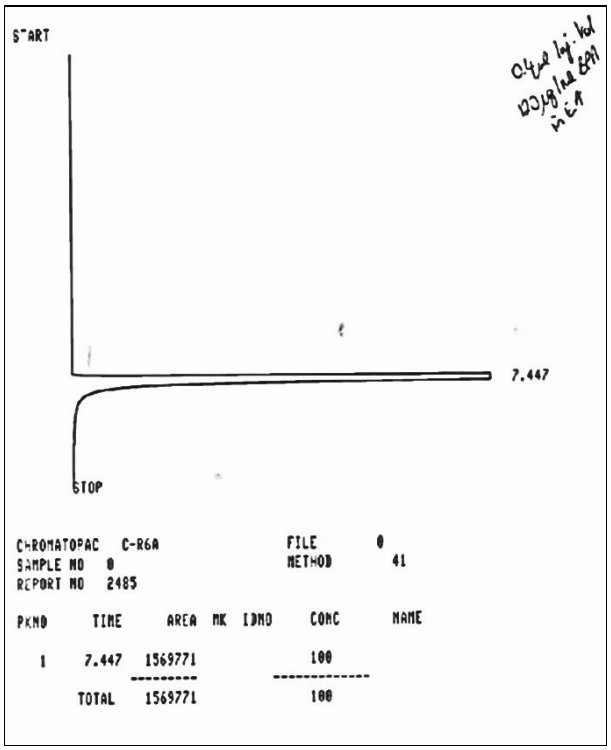

Fig. 4: Chromatograph of 120µg/ml BPA

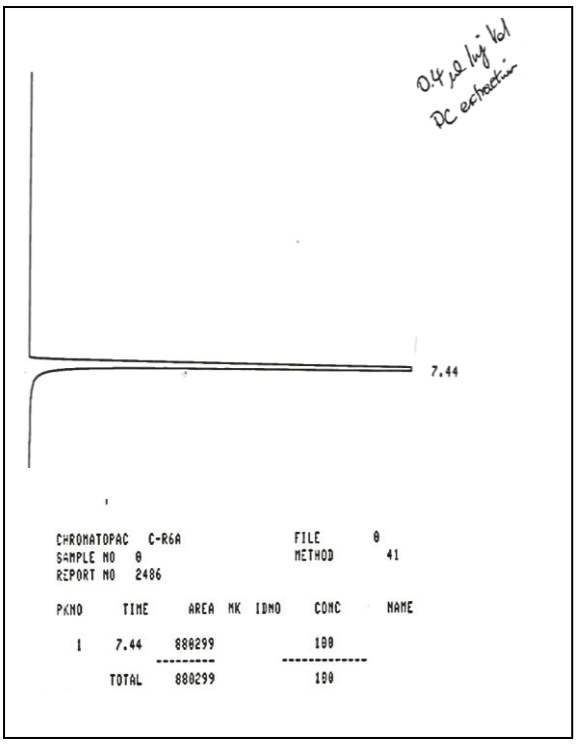

Fig. 5: Chromatograph of polycarbonate water bottle extract

Conclusion:

The safety concerns that surround BPA and other phthalates are increasingly controversial. Both BPA and phthalic acid esters are well known to possess oestrogenic activity albeit be it weak and have the potential for endocrine disruption due to their ability to interact with endogenous oestrogen hormone receptors. Exposure to these undesirable chemicals occurs numerous times throughout the day through the ingestion of food and beverages which are contained within plastic packaging.Further studies are needed in order to obtain a better understanding of the release of BPA and certain phthalates from water bottles which are exposed to real use conditions in order to more accurately identify and quantify leachates from the plastic matrix.

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